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REVIEW ARTICLE

Chemical tools to probe cellular *O*-GlcNAc signalling

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Protein *O*-GlcNAcylation is an abundant, dynamic and reversible type of protein post-translational modification in animals that has been implicated in signalling processes linked to innate immunity, stress response, growth factor response, transcription, translation and proteosomal degradation. Only two enzymes, *O*-GlcNAc (O-linked *N*-acetylglucosamine) transferase and *O*-GlcNAcase, catalyse the reversible addition of the *O*-GlcNAc residue to over 1000 target proteins in the human cell. Recent advances in our understanding of the structures and mechanisms of these enzymes have resulted in the development of potent and selective inhibitors. The present review gives an overview of these inhibitors and how they have been used on cell lines, primary cells and animals to modulate *O*-GlcNAc levels and study the effects on signal transduction.

Key words: inhibitor, *O*-GlcNAc transferase, *O*-GlcNAcase, O-GlcNAcylation, post-translational modification.

INTRODUCTION

Protein post-translational modification is a ubiquitous mechanism of regulating almost every cellular process. A relatively recently discovered modification, with an as yet poorly understood function, is glycosylation of serine and threonine residues on cytoplasmic proteins with a single *N*-acetylglucosamine sugar residue (O-GlcNAcylation; Figure 1) [1,2]. Protein O-GlcNAcylation was initially identified on nuclear pore complexes, but it soon became apparent that this modification is far more widespread [3,4]. To date, approximately 1000 proteins have been reported to be modified by *O*-GlcNAc (O-linked *N*-acetylglucosamine) in mammalian cells [5–8]. Furthermore, O-GlcNAcylation has been identified not only in mammals, but in many model eukaryotes, with the exception of yeast [5,9–14]. The occurrence of protein O-GlcNAcylation on serine and threonine residues raises the possibility of interplay with protein phosphorylation and global proteomic evidence of this has been reported [15,16] (Figure 1). Furthermore, protein O-GlcNAcylation and phosphorylation can occur on the same or neighbouring residues (reviewed in [5]); however, the exact function of the interactions between these two types of protein modifications remains to be explored. Unlike protein phosphorylation, where multiple kinases and phosphatases are involved in the targeted modification of proteins [17], the addition and removal of *O*-GlcNAc is cycled by only two enzymes: a uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase (*O*-GlcNAc transferase or *OGT*), which adds *O*-GlcNAc to the target polypeptide chain by transfer of the GlcNAc sugar residue from a UDP-GlcNAc substrate [18], and the glycoside hydrolase *O*-GlcNAcase [also known as OGA and *MGEA5* (meningioma expressed antigen 5)] that hydrolyses the glycosidic bond and returns the protein to its unmodified state [19] (Figure 1). The interplay between these two enzymes is highly dynamic and appears to be regulated (reviewed in [20,21]); however, owing to the large number of structurally diverse target proteins, the nature of the regulatory processes governing protein O-GlcNAcylation is not yet fully understood. The target specificity of OGT is most probably mediated by the N-terminus of OGT, which is composed of TPRs (tetratricopeptide repeats) [22,23]. TPRs are typically responsible for protein–protein interactions in various proteins [24]. There is also a growing body of evidence for accessory proteins involved in the recruitment of OGT to the target protein and therefore regulating the specificity of this enzyme at the subcellular scale [25–28].

In addition to its enzymatic activity, OGT was shown to possess an essential scaffolding activity [22]. This fact, in combination with the large number of OGT protein targets in the cell, causes significant obstacles in the study of OGT function in cell biology, as a simple knockdown of OGT will affect both the catalytic and scaffolding functions of the enzyme. Protein O-GlcNAcylation is essential for embryonic development [29], cell differentiation [30] and cell division *in vitro* [31], and therefore it is essential to develop precise and specific tools to manipulate the level of protein O-GlcNAcylation in the cell. To date, a number of inhibitors against the activity of OGT and OGA have been described and their biochemical potential has been reviewed on a number of occasions [21,32–34]. Additionally, in a recent review Cecioni and Vocadlo [35] have outlined the advances in identification of *O*-GlcNAc proteins and methods for probing of *O*-GlcNAc dynamics in living cells with analogues of OGT and OGA substrates. In the present review we will provide an overview of the OGT and OGA inhibitors available to date and their use to probe O-GlcNAcylation in living cells with the focus on broader

Abbreviations used: BADGP, benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside; DON, 6-diazo-5-oxonorleucine; GFAT, glutamine:fructose-6-phosphate amidotransferase; GLUT2, glucose transporter type 2; HEK, human embryonic kidney; Hex, hexosaminidase; MAPK, mitogen-activated protein kinase; NAG-thiazoline, 1,2-dideoxy-2′-methyl-*R*-D-glucopyranoso-[2,1-D]-Δ2′-thiazoline; NButGT, 1,2-dideoxy-2′-propyl-α-D-glucopyranoso-[2,1-D]-Δ2′-thiazoline; *O*-GlcNAc, O-linked *N*-acetylglucosamine; OGT, *O*-GlcNAc transferase; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenyl carbamate; 5S-GlcNAc, 2-acetamido-2-deoxy-5-thio-D-glucopyranose; STZ, streptozotocin; TGF-β, transforming growth factor β; TMG, Thiamet-G; TPR, tetra-tricopeptide repeat.

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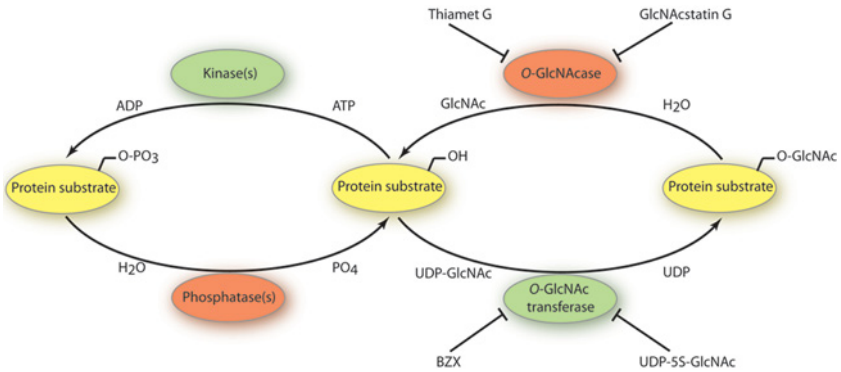


Figure 1 ***O*-GlcNAc cycling enzymes and their inhibitors**

Serine and threonine residues on cellular protein substrates can be reversibly phosphorylated by kinases or O-GlcNAcylated by *O*-GlcNAc transferase. The subsequent modification can be hydrolysed by phosphatases or *O*-GlcNAcase respectively, returning the target protein to its original state. The inhibitors of the *O*-GlcNAc transferase and *O*-GlcNAcase currently recommended as cell biological tools are indicated by T-bars.

aspects of cell biochemistry, as well as their specificity and cell toxicity.

O-GlcNAc TRANSFERASE INHIBITORS

OGT is a metal-independent glycosyl transferase enzyme and was classified by CAZy [36] to the GT41 glycosyl transferase family (http://www.cazy.org). hOGT (human OGT) comprises an N-terminal domain, which is essential for substrate recognition but not for the catalytic activity, and the C-terminal catalytic domain [22,37]. The crystal structure of the catalytic domain was solved independently of the N-terminal domain, initially for a bacterial OGT homologue [38,39] and subsequently for the human enzyme [40]. Enzyme kinetics and structural studies of the OGT suggest an ordered sequential Bi Bi catalytic mechanism, where UDP-GlcNAc binds to the active site prior to the polypeptide substrate [40,41]. The sugar–nucleotide substrate of OGT, UDP-GlcNAc, is an obvious template for the development of small molecule inhibitors owing to its size and the mode of binding to the enzyme. Indeed, the reaction product UDP is capable of inhibiting OGT *in vitro* ($IC_{50} = 1.8\ \mu M$) and paradoxically is the most potent OGT inhibitor known to date [34]. However, UDP is used in a wide variety of biochemical processes and is not cell-penetrant, and is thus unsuitable for use in cell biological studies. Nonetheless, on the basis of the structure of the OGT substrate, a number of compounds have been designed which can be taken up by cells and inhibit the enzyme.

Alloxan, BADGP (benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside) and other non-specific inhibitors

Prior to the discovery of OGT-specific inhibitors, whose application is discussed later in the present review, several chemicals were found to affect the activity of OGT. The first widely used OGT inhibitor was 2,4,5,6-tetraoxypyrimidine, commonly known as alloxan (Table 1). Alloxan is not only an analogue of uracil, which allows for inhibition of OGT ($IC_{50} = 100\ \mu M$) [34,42], but is also a glucose mimetic [43–45]. Owing to the latter property, alloxan can be imported across cell membranes by glucose transporters and its uptake by pancreatic β-cells was shown to be dependent on GLUT2 [glucose transporter type 2; also known as SLC2A2 (solute carrier family 2, member 2)] transporters [46]. Treatment of isolated rat cardiomyocytes [47,48], mouse neuroepithelial cells [49], primary embryonic fibroblasts [50], *Xenopus* oocytes [51] or rat hippocampal slices [52] with 0.3–5 mM alloxan (Table 1) resulted in a significant decrease in protein O-GlcNAcylation over time. The crystal structure of the bacterial OGT homologue from *Xanthomonas campestris* (*Xc*OGT) in complex with alloxan confirmed its binding in the UDP pocket [34], but not the previous prediction that alloxan formed a covalent bond with one of the exposed SH groups in the active site [42,53]. However, the high level of similarity of alloxan to uracil makes it highly non-specific, and inhibition of other enzymes by alloxan, including OGA [54], has been shown. Furthermore, alloxan is a highly unstable molecule with a half-life at physiological pH of less than 1.5 min [55], which makes it unsuitable for cell biological applications. Nonetheless, owing to its extensive use in the protein O-GlcNAcylation field, alloxan has proved useful in revealing the potential involvement of OGT in various processes, of which examples include protection against ischaemia [47], embryonic cell proliferation [49], development of hippocampal synapses [52] and regulation of the circadian clock [50]. However, the known cytotoxic effects of alloxan complicate the interpretation of the data obtained from studies with this inhibitor. Comparison of these results with an analogous experiment with a more specific inhibitor is needed to verify these studies [56], as exemplified by the paper by Dehennaut et al. [51] where results obtained using alloxan and the 'compound 5' inhibitor [57] were compared proving the requirement of protein O-GlcNAcylation for entry into the M-phase of the cell cycle.

The enzymatic activity of OGT can also be reduced by modulating the cellular concentration of UDP-GlcNAc (as reviewed in [58]). Two widely used inhibitors, BADGP and DON (6-diazo-5-oxonorleucine), are able to reduce levels of protein O-GlcNAcylation in cells by affecting UDP-GlcNAc biosynthesis (for examples see [31,51,59–65]) and were used in the studies listed in Table 1. Instead of specific inhibition of OGT, these two compounds affect general UDP-GlcNAc-dependent glycosylation mechanisms in cells [66–68]. Not unexpectedly, BADGP was shown to also affect the glycosylation and synthesis of mucins [69], highlighting the non-specific nature of this inhibitor. Similarly, DON is an inhibitor of GFAT (glutamine:fructose-6-phosphate amidotransferase), which is the rate-limiting enzyme of the hexosamine biosynthetic pathway [66]. Overexpression or inhibition of GFAT affects the level of cellular protein O-GlcNAcylation by modifying the levels of available

Table 1 Inhibitors of *O*-GlcNAc transferase

Inhibitors recommended for cell biological studies are indicated in bold. CHO, Chinese-hamster ovary.

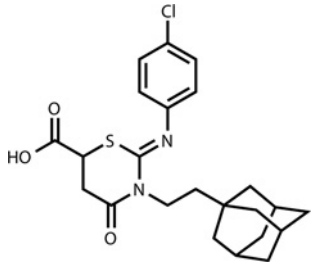
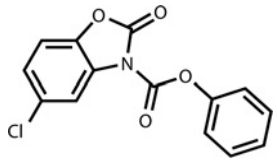
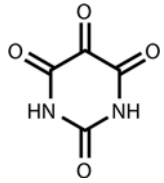
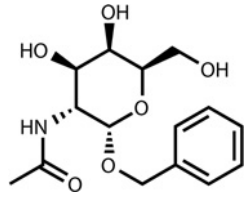
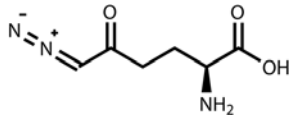
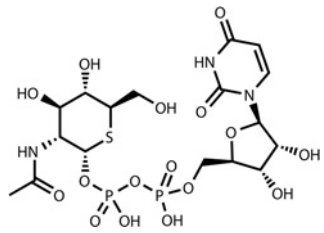
Inhibitor	Structure	Used in tissues/cells	Applied concentration (μM)	Reference(s)	Known non-specific effects	Commercial availability
3-(2-Adamantanylethyl)-2-[(4-chlorophenyl)azamethylene]-4-oxo-1,3-thiazaperhydroine-6-carboxylic acid, ST045849 or compound 4		Whole isolated pancreas (rat)	20	[73]	None	Yes
4-Methoxyphenyl 6-acetyl-2-oxobenzo[*d*]oxazole-3(2*H*)-carboxylate ST060266, compound 5 or BZX		LNCaP VCap Oocytes (*Xenopus*)	10 500	[72] [51]	None	Yes
Alloxan		Cardiomyocytes (rat) Neuroepithelial cells (mouse) Primary embryonic fibroblast (mouse) Hippocampal slices (rat) Oocytes (*Xenopus*) Pancreatic β-cells (mouse) Breast cancer cells (human)	2500 500–4000 1000 5000 5000 300 500	[47,48] [49] [50] [52] [51] [42,43,61] [103]	Insulin desensitizing and generation of reactive oxygen species	Yes
BADGP		Pancreatic β-cells (human) Whole pancreatic islets (rat) Isolated murine zygotes Embryonic stem cells (mouse)	500–7500 5000 2000 5000	[61,62] [73] [60] [59]	Inhibition of multiple glycosylation mechanisms [67–69]	Yes

Table 1 Continued

Inhibitor	Structure	Used in tissues/cells	Applied concentration (μM)	Reference(s)	Known non-specific effects	Commercial availability
DON		MDA468	40	[64]	Inhibits GFAT and reduces cellular UDP-GlcNAc concentration [66]	Yes
		Oocytes (*Xenopus*)	100	[51]		
		Pancreatic β-cells (rat)	100	[82]		
		HEK-293	50	[65]		
UDP-5S-GlcNAc administered as 4Ac-5S-GlcNAc		COS-7	50 (4Ac-5S-GlcNAc precursor)	[74]	Can cause delayed cell proliferation due to downstream effects [75]	No
		CHO				
		EMEG32				
		SK-N-SH				
		HepG2				
		PC12				
		Hybridoma				
		Pancreatic duct epithelial cells (human)	250	[77]		
		HeLa	100	[75]		
		HepG2	100	[76]		

UDP-GlcNAc [70]. Given the likelihood of pleiotropic effects, modulators of UDP-GlcNAc biosynthesis should be used with caution in experiments aiming to assess protein O-GlcNAcylation.

Inhibitors specific to OGT

OGT initially resisted crystallization and structure determination, which made the design of specific and effective inhibitors challenging. However, development of an enzyme activity assay for OGT allowed for the design of a high-throughput screen to search for new small molecule inhibitors [57]. As a result, two new compounds were isolated: 'compound 4' {3-(2-adamantanylethyl)-2-[(4-chlorophenyl)azamethylene]-4-oxo-1,3-thiazaperhydroine-6-carboxylic acid, available under commercial designation ST045849} and benzoxazolinone-based 'compound 5' [4-methoxyphenyl 6-acetyl-2-oxobenzo[d]oxazole-3(2*H*)-carboxylate, also known as BZX or ST060266] (Table 1). A later crystallographic study identified the mechanism of the BZX family inhibitors as an irreversible cross-linking of the active site residues Lys^{842} and Cys^{917} [71]. To date, BZX has been used in several studies involving live cells and was found to be cell-permeable. A concentration of 500 μM BZX, which is over 15-fold higher than the IC_{50} value of this compound *in vitro*, was found to inhibit OGT glycosyltransferase activity in *Xenopus* oocytes [51]. However, owing to the chemical reactivity of BZX, there is a significant risk of non-specific toxicity of this compound.

Itkonen et al. [72] have recently reported satisfactory inhibition of OGT in LNCaP and VCap cell lines derived from human prostate cancer with compound 4 at concentrations as low as 100 μM (Table 1) and identified OGT as a regulator of c-Myc stability. Compound 4 was also applied during a study of rat pancreatic β-cells and helped with the initial characterization of *O*-GlcNAc involvement in pancreatic cell development [73]. Both of these compounds have a high affinity towards human OGT and although no adverse effects have been observed in cell culture thus far, their reactivity in combination with the high concentrations used may preclude their use in animal models.

UDP-5S-GlcNAc (2-acetamido-2-deoxy-5-thio-D-glucopyranose), an OGT substrate-mimicking inhibitor

Inhibitors of OGT can be obtained by derivatization of the donor substrate UDP-GlcNAc; however, such sugar–nucleotide derivatives are not cell penetrant. A unique solution to this problem was recently reported by Gloster et al. [74] where the biosynthetic pathway of UDP-GlcNAc was 'hijacked' to synthesize an intracellular OGT inhibitor from an extracellularly applied precursor. The sugar–nucleotide substrate for OGT is synthesized in the hexosamine biosynthetic pathway from glucose-derived fructose-6-phosphate, glutamine, acetyl-CoA and UTP. The authors decided to utilize this pathway to synthesize a potent OGT inhibitor, UDP-5S-GlcNAc, from a cell-penetrable GlcNAc analogue precursor 5S-GlcNAc. UDP-5S-GlcNAc can be turned over by OGT at a much slower rate than UDP-GlcNAc and, surprisingly, was shown to be selective towards OGT over other GlcNAc transferases although the basis of this selectivity is yet to be understood. To further increase cell permeability, the precursor was acetylated to 4Ac-5S-GlcNAc and the EC_{50} value of this compound was measured to be 5 μM for COS-7 cells [74]. The authors have tested 4Ac-5S-GlcNAc on a range of cell lines (Table 1) and no toxic effects could be observed. Since the original publication, 4Ac-5S-GlcNAc was also adopted in other studies utilizing HeLa [75], HepG2 [76] and pancreatic duct epithelial [77] cells with similar success (Table 1). Together these studies have provided proof for the requirement of functional protein O-GlcNAcylation for cell survival and proliferation control.

Protein O-GlcNAcylation was shown to be essential for entry into the cell cycle [75] as well as for control of apoptosis where hyper-O-GlcNAcylation reduces caspase-3-dependent apoptosis. Inhibition of OGT decreases levels of anti-apoptotic Bcl-xL and increases cleavage of caspase 3 and 9 [77]. In the light of their results, the authors proposed that increased O-GlcNAcylation might be a universal marker of a cancer cell. Furthermore, nucleocytoplasmic OGT was shown to localize to lipid microdomains in the cell membrane where it plays a role in insulin sensing as part of a self-enhancing feedback loop that activates PI3K (phosphoinositide 3-kinase) [76]. Most importantly, in all three studies the experimental results were comparable whether the expression of OGT was knocked down or the enzymatic activity inhibited with UDP-5S-GlcNAc, the effective inhibitor derived from 4Ac-5S-GlcNAc precursor [33]. This highlights that the enzymatic activity of OGT, not simply the presence of the protein, is required for regulation of these cellular processes.

O-GlcNAcase INHIBITORS

OGA is a member of the glycoside hydrolase family 84 (GH84) (http://www.cazy.org), hydrolysing the *O*-GlcNAc glycosidic bond and thus returning proteins modified by OGT to their unmodified state [19,78]. OGA was initially isolated from the rat spleen and was characterized as a hydrolase with specific activity towards a single *O*-GlcNAc moiety on serine or threonine residues of a polypeptide chain [19]. The enzymatic mechanism of OGA, which involves substrate-assisted catalysis [79,80], is similar to that of the lysosomal Hex (hexosaminidase) A and HexB belonging to glycoside hydrolase family 20 (GH20) [79]. Furthermore, both these enzyme classes are readily inhibited by inhibitors like PUGNAc [*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenyl carbamate] [19] or STZ (streptozotocin) [81,82]. Both PUGNAc and STZ show good inhibition towards OGA (K_i = 50 nM [83] and 64 μM [84] respectively) *in vitro*, which has led to their frequent application in the study of the biological function of OGA at the cell line and animal levels.

STZ and PUGNAc are non-specific inhibitors of OGA

STZ is an antimicrobial agent that is routinely used to induce diabetes in rodents owing to its selective killing of insulin-producing β-cells in the pancreas [85]. Since OGT is highly expressed in human pancreatic β-cell islets [86] and STZ is a GlcNAc analogue (Table 2) taken up by the GLUT2 glucose transporter selectively expressed in these cells [87], the pancreas was one of the first systems probed for effects of protein O-GlcNAcylation in cell biology. One of the early observations made by Liu et al. [70], after treatment of the whole β-cell islets with STZ, was elevation of *O*-GlcNAc levels in the cells in association with reduced cell viability. This led to the conclusion that insulin resistance of pancreatic β-cells, induced into a diabetic state, is tightly linked with cellular *O*-GlcNAc levels and causes cell death [70]. Simultaneously it was shown that significantly higher levels of STZ are required for OGA inhibition in isolated homogenized rat brain [88] (Table 2). The differences in effective concentration between these two tissue types can be explained by the differences in abundance of the glucose transporters required for the uptake

Table 2 Inhibitors of *O*-GlcNAcase

The inhibitors of the highest selectivity are indicated in bold. AHP, adult hippocampal progenitor; CHO, Chinese-hamster ovary.

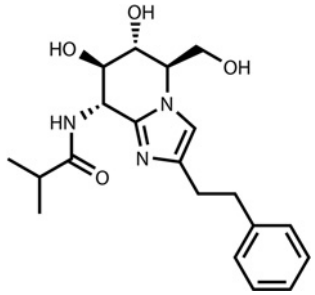
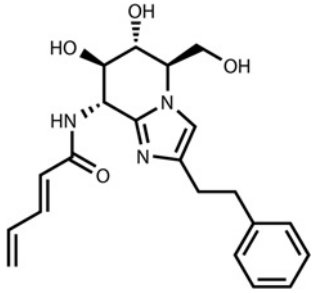
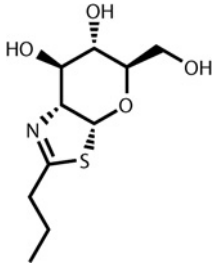
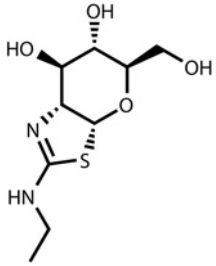
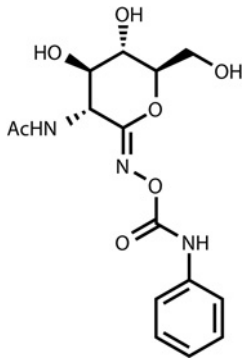
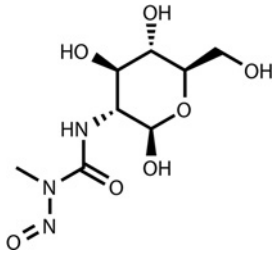
Inhibitor	Structure	Used in tissues/cells	Applied concentration (μM)	Reference(s)	Known non-specific effects	Commercial availability
GlcNAcstatin C		HEK-293 SH-SY5Y HeLa	0.001–0.1	[108]	None, 190-fold selectivity for OGA over HexA/B [83]	Yes
GlcNAcstatin G		HEK-293	0.1–20	[6,83,109]	None, over 900 000-fold selectivity for OGA over HexA/B [83]	Yes
NButGT		3T3-1L Primary neonatal ventricular myocytes (rat) C57BL6/J mice Breast cancer cells (human) CHO Pancreatic duct epithelial cells (human)	100 100 75 mg/kg 100 100 50	[95] [102] [52] [103] [97] [77]	None, 3100-fold selectivity for OGA over OGA	No
TMG		PC-12 Sprague–Dawley rats Lung carcinoma (human) Mesangial cells (rat) Mice (unspecified line) AHP cells Embryonic stem cells (mouse)	0.2 200 mg/kg 5 0.025 175 μg/mouse 0.02 0.005	[104] [107] [106] [105] [59]	None, 35 000-fold selectivity for OGA over HexA/B [104]	Yes

Table 2 Continued

Inhibitor	Structure	Used in tissues/cells	Applied concentration (μM)	Reference(s)	Known non-specific effects	Commercial availability
PUGNAc		HT49 3T3-1L Min6 Pancreatic β-cells (rat) Isolated mouse embryos Neuroepithelial cells (mouse) CHO Primary embryonic fibroblasts (mouse)	40 0.3–300 100 10 100 10–100 100 100	[94] [95] [90] [73] [60] [49] [97] [50]	Inhibits HexA/B, causes insulin desensitizing and causes accumulation of unusual oligosaccharides [97]	Yes
STZ		Whole isolated pancreatic β-islets (rat) Min6 Neuroepithelial cells (mouse)	500–1000 5000–10000 100–1000	[70,88] [84,90] [49]	Increases cellular concentration of reactive NO species and directly alkylates DNA [91,92]	Yes

of STZ [87]. STZ was also proposed to inhibit OGA by the formation of covalent bonds with the enzyme's catalytic core or by the formation of a tightly binding inhibitor through an enzyme-catalysed reaction [88,89]. The correlation between the increase in protein O-GlcNAcylation upon treatment with STZ and diabetes-like effects suggested that STZ could be a very promising inhibitor of OGA. However, comparative studies of OGA inhibition by STZ and PUGNAc have clearly shown that the cell toxicity of STZ is not linked to OGA inhibition, since PUGNAc treatment resulted in similar O-GlcNAc accumulation without toxic effects, even after prolonged exposure [84,90]. Furthermore, it was shown that in the insulinoma cell line Min6 a *galacto*-configured stereoisomer of STZ, Gal-STZ, did not increase cellular levels of O-GlcNAcylated proteins, but had the same cytotoxic effects as STZ, including DNA fragmentation and induction of apoptosis [84,91,92]. Thus STZ is a β-cell-specific toxin and should not be used as a tool to probe the cell biology of *O*-GlcNAc.

PUGNAc is a lysosomal hexaminidase inhibitor [93], which was also shown to potently inhibit OGA [94] (Table 2), and was the first inhibitor used to study the activity of OGA when the enzyme was first isolated [19]. PUGNAc was shown to inhibit OGA in the HT-49, NIH 3T3, HeLa and CV-1 cell lines, with maximal inhibition in HT-49 cells at concentrations as low as 40 μM [94,95]. However, similarly to STZ, PUGNAc inhibits HexA/B equally as well as it inhibits OGA [19,93,96]. The non-specific effects of PUGNAc activity are well exemplified in the study by Mehdy et al. [97]. The authors described unusual free oligosaccharides containing 2–5 consecutive residues of HexNAc in CHO (Chinese-hamster ovary) cells treated with PUGNAc. Formation of these oligosaccharides was attributed to inhibition of HexA and HexB by PUGNAc. Therefore, despite significant accumulation of *O*-GlcNAc proteins in cells treated with PUGNAc reported on multiple occasions, the physiological effects of PUGNAc may not be solely due to OGA inhibition. An example of this was the report by Macauley et al. [95] where the authors showed that the desensitizing of cells to insulin by PUGNAc is not a result of OGA inhibition. This result was apparent upon comparison of the response of cells treated with PUGNAc and the OGA-specific inhibitor NButGT {(1,2-dideoxy-2′-propyl-α-D-glucopyranoso-[2,1-D]-Δ2′-thiazoline)} ([79] and see below). Thus the main caveat of PUGNAc application is possible non-specific effects on the additional cellular processes. Attempts to further modify PUGNAc with the aim of increasing its specificity towards OGA have led to significant loss of inhibitory potency [98].

OGA inhibitors based on rational design

The publication of two crystal structures of bacterial homologues of the human OGA [80,99] and a mechanistic enzymology study [79] have recently uncovered the molecular mechanism of OGA function. A key feature of this 'substrate-assisted' mechanism is the presence of an oxazoline-like reaction intermediate. Based on this postulate, the authors synthesized several small NAG-thiazoline (1,2-dideoxy-2′-methyl-*R*-D-glucopyranoso-[2,1-D]-Δ2′-thiazoline)-derived molecules [100] mimicking the reaction intermediate, which were then tested for inhibition of OGA. The compound '9c', later renamed as NButGT [79], was found to be a low micromolar inhibitor of OGA *in vitro* and to be active in the green monkey kidney COS-7 cell line at a concentration of 50 μM [79] (Table 2). NButGT is selective towards OGA, showing a significantly lower inhibition of HexA/B (1500-fold selectivity) [79]. It was also shown not to cause insulin resistance, unlike the non-specific STZ and PUGNAc OGA inhibitors [95,101]. Since its initial description, NButGT has been used in a number of *in vivo* studies showing inhibition of OGA. For instance, Champattanachai et al. [102] noted attenuation of necrosis in NButGT-treated cells following ischaemia/reperfusion, but no effect of NButGT-induced hyper-O-GlcNAcylation on the level of mitochondrial Bcl-2, suggesting a relation between protein O-GlcNAcylation and apoptosis.

In the same study, a similar compound, '9d', was described, which is characterized by an additional methylene group in the side chain, a 3100-fold selectivity towards OGA and a significantly higher K_i value [79]. This compound was shown to efficiently inhibit OGA in the mouse brain [52]. By injecting mice with compound 9d the authors implicated protein O-GlcNAcylation in the regulation of synaptic transmission and plasticity. Furthermore, compound 9d was used in a study of cultured human breast cancer cells [103] in which the authors have elegantly demonstrated enhancement of cancer cell growth mediated by protein hyper-O-GlcNAcylation. Retrospectively this paper provides an argument in favour of the hypothesis by Ma et al. [77], suggesting that protein hyper-O-GlcNAcylation is the universal feature of cancer cells. Furthermore, the report by Caldwell et al. [103] was the first to link the dynamics of growth and level of invasiveness of cancer cells with OGT activity and protein O-GlcNAcylation.

Further work on NAG-thiazolines culminated in the discovery of TMG (Thiamet-G) [104] that showed a 37 000-fold selectivity against OGA over HexA/B. It is water-soluble and is assumed to penetrate both the cell and blood–brain barriers as the systemic application of TMG increases O-GlcNAcylation levels in the brain (Table 2 and [104,105]). Cell culture-based experiments have shown the maximal inhibition of OGA in neuron-like PC-12 cells at over 200 nM TMG [104]. Injection of a TMG solution into the lateral ventricles of mouse brains resulted in a 5-fold increase in the total abundance of O-GlcNAcylated proteins after 4.5 h and a 10-fold increase after 24 h [105]. In that study, the authors also demonstrated a significant decrease in phosphorylation of the neuronal microtubule-associated protein tau as a result of OGA inhibition and accumulation of O-GlcNAcylated tau in the brain. However, to achieve OGA inhibition to the presented degree, the authors injected into the mouse brain 175 μg of TMG, which translates to an approximate concentration of 700 μM, assuming an average volume of a mouse brain being 900 μl [105]. Nonetheless, together these two reports provide very interesting information on the effects of O-GlcNAcylation on the phosphorylation of the neuronal protein tau, further suggesting involvement of O-GlcNAcylation in the development and progression of Alzheimer's disease.

Owing to its high stability, solubility and selectivity, TMG has been used in multiple studies in cell cultures as well as in animals (Table 2). Inhibition of OGA with TMG allowed for the identification of a link between O-GlcNAcylation and phosphorylation of profibrotic p38 MAPK (mitogen-activated protein kinase) in response to cellular high glucose concentrations in mesangial cells [106]. As a result, protein O-GlcNAcylation is indirectly activating expression of genes encoding mesangial matrix proteins. In another report Mi et al. [107] demonstrated the presence of hyper-O-GlcNAcylation in lung and colon cancer tissues. The authors also demonstrated that elevated levels of O-GlcNAcylation allow for enhanced anchorage-independent and invasiveness of cancer cells. These findings add to the growing pool of evidence for increased protein O-GlcNAcylation as a universal feature of cancer cells as proposed by Ma et al. [77].

In parallel with the thiazoline-based inhibitors, another group exploited the structural similarity of glucoimidazoles to the OGA transition state and rationally designed the GlcNAcstatins, a family of OGA inhibitors [96]. These inhibitors have been

shown to inhibit OGA even in the picomolar range *in vitro* [96] and to be cell-penetrant [83]. The individual GlcNAcstatins differ in the inhibition potential of OGA and HexA/B enzymes [83,96,108]. The high specificity, cell penetrability and visible effects within as little as 1.5 h after administration [83] make these inhibitors state-of-the-art tools for the modulation of cellular O-GlcNAc levels. Probably the best characterized is GlcNAcstatin C, which shows moderate selectivity towards OGA over HexA/B and activity in the nanomolar range in HEK (human embryonic kidney)-293, SH-SY5Y and HeLa cells (Table 2 and [83,108]). The recently reported GlcNAcstatin G shows a 900000-fold selectivity towards OGA HexA/B [83] and is the most selective OGA inhibitor reported to date. GlcNAcstatin G was used to show that O-GlcNAcylation of TAB1 [TGF-β (transforming growth factor β)-activated kinase 1/MAP3K7 (MAPK kinase kinase 7)-binding protein 1] on Ser^{395} was shown to be essential for activation of TAK1 (TGF-β-activated kinase 1) kinase and therefore activation of NF-κB (nuclear factor κB), linking *O*-GlcNAc to innate immunity signalling [109]. GlcNAcstatin G has also been used in the enrichment of the fraction of O-GlcNAcylated proteins in the proteomic analysis of HEK-293 cells [6], thus validating GlcNAcstatin G as a valuable tool for the proteome-wide study of protein O-GlcNAcylation. The biggest drawback of GlcNAcstatins is their low water solubility and complicated synthesis [96,110]. Owing to the high potency of GlcNAcstatin G and TMG, as well as the cell penetrance of these compounds, significantly lower concentrations are sufficient to achieve the same inhibitory effects as with other inhibitors (Table 2).

CONCLUSION

Investigation of complex cellular signalling pathways is challenging and careful selection of chemical biology tools is essential. Inhibitors showing great promise in the test tube may well display pleiotropic effects in a cellular context, hampering accurate interpretation of results. Since these problems are common to a wider audience interested in biochemical processes, a set of guideline criteria for the publication of studies using enzyme inhibitors in intact cells was published [56]. Although these guidelines were aimed at the use of protein kinase inhibitors [111–113], most of them are universal and can be applied to any type of biochemical inhibitor used *in vivo*. The key guidelines are that: (i) the specificity of the chemical inhibitor must be tested against a large panel of protein targets; (ii) the cellular effects of the chemical inhibitor should be observed with at least two structurally unrelated inhibitors and at concentrations that inhibit modification of a characterized target by the investigated enzyme; and (iii) the cellular effects of the compound should not be observed in the cells in which the expression of the inhibited enzyme and closely related isoforms have been ablated [56]. The field of protein O-GlcNAcylation is a perfect example where a range of broad-spectrum inhibitors have been used to study the effects of protein-modifying enzymes, OGT and OGA, on cellular processes, but it only recently became possible to distinguish the true impact of protein modification from non-specific inhibition and unrelated cell toxicity. Despite their well-described effects on mechanisms not related to the activity of OGT and OGA, alloxan, STZ and PUGNAc are unfortunately still widely used to assess the effects of protein O-GlcNAcylation (see Tables 1 and 2). In the present review we have described a set of small molecule inhibitors available in the literature that have been shown to effectively modulate the level of protein O-GlcNAcylation. We also highlight a range of non-specific effects that have been attributed to some of these molecules with the hope of providing a guide for the selection of appropriate tools for use in future studies of this important mechanism.

Inhibitors: strong or selective

A significant obstacle in the design process of a strong and selective inhibitor of O-GlcNAcylation was the initial lack of full understanding of the OGA/OGT reaction mechanism. This led to the application of mechanistically uncharacterized inhibitors that clearly affected the level of protein O-GlcNAcylation with potentially spectacular effects on cell biology. A predominant example is the link made between deregulation of protein O-GlcNAcylation by treatment with alloxan or STZ and insulin desensitizing observed in the treated cells [42,70]. Indeed the level of protein O-GlcNAcylation changes in response to insulin (exemplified in [62,114]). However, this effect is likely to be mediated by insulin's influence on the cellular UDP-GlcNAc pool and not directly on O-GlcNAc cycling enzymes (reviewed in more detail in [20]); whereas, the cytotoxic effects of these two inhibitors can be attributed to the general cytotoxicity of STZ [84,90] and the potential of alloxan to generate reactive oxygen species [46]. Only in recent years has the development of new inhibitors been reported, such as the OGT inhibitors reported by Gross et al. [57] or UDP-5S-GlcNAc [74]. These inhibitors have a high potential to inhibit OGT in cell culture and do not display any adverse effects (Table 1); however, this is surprising given their mode of action, involving either covalent active-site modification or mimicry of a sugar–nucleotide, which is widely used in other cellular processes.

Similarly to OGT inhibitors, the design of selective inhibitors against OGA was greatly facilitated by the publication of the structures of the bacterial homologues of OGA [80,115]. This allowed the identification of the essential differences between OGA and HexA/B, which use very similar mechanisms of glycoside hydrolysis. GlcNAcstatin G [83] and Thiamet-G [104] are two of the strongest, and at the same time most selective, inhibitors of OGA with minimal activity against HexA/B (Table 2). Both of these compounds were shown to be cell-penetrable and not to cause non-specific toxic effects in the cell culture. Simultaneously, the biochemical crystallography-based mechanistic analysis of these two compounds provided proof of non-covalent binding to the enzyme's active site making TMG and GlcNAcstatins a good starting point for the understanding of the biochemical processes driving protein O-GlcNAcylation in the absence of drug-like inhibitors.

Development of new classes of inhibitors

The majority of OGT and OGA inhibitors available in the literature were constructed by derivatization of known compounds, as was the case with LOGNAc (lactone oxime), a PUGNAc derivative [98], or the non-hydrolysable OGT inhibitors UDP-S-GlcNAc and UDP-C-GlcNAc [34,38]. Unfortunately, these compounds have proved to be less effective than their template molecules, highlighting the difficulties in the design of new inhibitors. A noteworthy recent addition to the OGT inhibitor family are the OGT bisubstrate-linked inhibitors (goblins) (V.S. Brodokin, M. Schimpl, M. Gundogdu, D.A. Robinson and D.M.F. van Aalten, unpublished work). This novel inhibitor scaffold comprises a short peptide with high affinity to OGT covalently bound to UDP via a short alkyl spacer. These molecules are the first OGT inhibitors combining the selectivity of a specific peptide target with the high inhibitory capability of UDP, although

cell permeability has yet to be achieved. Further OGA inhibitor development is also a subject of ongoing research, such as the recently reported application of 'click chemistry' for the rapid generation of potential OGA inhibitor libraries [116]. Similarly the high-throughput screening approach is a powerful method of identifying new small molecules with inhibitory properties, which has already yielded two of the inhibitors discussed in the present review, BZX and compound 4 [57]. Perhaps this approach will allow for development of novel drug-like classes of OGA/OGT inhibitors in the near future.

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