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Chemical Regulation of Glycosylation Processes

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Abstract

Glycans (monosaccharides and oligosaccharides) and their conjugates (glycoproteins, glycolipids, and proteoglycans) are structurally diverse biomolecules that are involved in many biological processes of health and disease. The structural diversity of glycans and glycoconjugates is owed to their monosaccharide composition, anomeric state, glycosidic linkage, modification (phosphorylation, sulfation, acetylation, etc.) and aglycone (protein, lipid, etc.). These diverse structures are controlled by complex glycosylation processes in cells, which are mediated by various glycosyltransferases and glycosidases. Glycosylation processes can be chemically regulated by inhibition of glycosyltransferases or glycosidases with natural and synthetic molecules. Treatment of cells with inhibitors of these enzymes results in the production of glycans or glycoconjugates containing missing or altered glycan chains. This approach is highly useful for examining the potential functional role(s) of glycans and glycoconjugates in cells or tissues, and in biological processes of health and disease. Eventually, it will provide novel mechanisms for disease treatment. This review highlights recent developments in chemical regulation of glycosylation processes with specific targets including: inhibition of (1) N-glycosylation, (2) O-glycosylation, (3) O-linked GlcNAc glycosylation, (4) proteoglycan biosynthesis, (5) glycolipid biosynthesis, and (6) terminal glycosylation. The goal of this review is to provide researchers with more competent choices in their research and lay a foundation on which continued advancements can be made to promote further explorations in glycoscience and biomedical research and applications.

A. Introduction

Glycoconjugates are diverse biomacromolecule of proteins and/or lipids modified with glycans in cells, which include glycoproteins, proteoglycans, GPI-anchored proteins, and glycolipids (Fig. 1A). The majority of glycoconjugates are expressed on the cell surface, where they form a thick layer known as glycocalyx. Glycoconjugates are also secreted inside of the cells, tissues and biofluids in the body. Glycoconjugates participate in many key biological processes including cellular adhesion, migration, growth, differentiation, signal transduction, receptor activation, immune response modulation, quality control of protein folding, and host-pathogen interactions (1). However, the molecular mechanisms in most events with the glycoconjugates involved are still unclear. Mostly, the role(s) of glycans on a specific biomolecule cannot be explained. Therefore, there is a high demand for studying glycan and glycoconjugate structures and functions for both basic biological sciences and biomedical applications.

Glycoconjugates are produced by the complex process of glycosylation, which is mediated by various glycosyltransferases and glycosidases (2). In mammalian cells, glycans are built up from ten monosaccharides—fucose (Fuc), galactose (Gal), N-acetylgalactosamine (GalNAc), glucose (Glc), N-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), iduronic acid (IdoA), mannose (Man), sialic acid (Neu5Ac as the major sialic acid) and xylose (Xyl) (Fig. 1B). Except for IdoA, which forms from epimerization of GlcA, the other nine monosaccharides are mainly generated by the de novo pathway ultimately from glucose, but also by the salvage pathway that utilizes sugars from recycled glycans or from extracellular origins. Most of these monosaccharides are converted to nucleotides for glycosylation processes generating corresponding glycans and glycoconjugates (Fig. 1B). The de novo pathway is highly conserved across species and accounts for most mammalian nucleotides. Glycosylation processes are comprised of sequential enzymatic reactions that occur in different cellular compartments, such as the cytosol, endoplasmic reticulum (ER), Golgi apparatus, nucleus, and the plasma membrane (3). The biochemical and enzymatic basis of glycosylation has recently been extensively reviewed elsewhere (4–6), and thus is discussed briefly from the point of regulation of glycosylation in this review. Overall, five classes of glycosylation, including N-linked, O-linked, O-GlcNAc-linked, GAG-linked, and glycolipid glycosylation are discussed for their biosynthesis and chemical regulation with inhibitors. Mostly, five major classes of glycosyltransferases that catalyze these glycosylation reactions are summarized in Table 1. The most common glycosyltransferases transfer sugar to an acceptor molecule using sugar nucleotide donors (e.g., UDP-Gal, GDP-Fuc, or CMP-Neu5Ac). Other types transfer sugar from a lipid-phosphoglycan donor, such as dolichol-phospho-mannose (or glucose) and dolichol-pyrophospho-oligosaccharide to acceptor molecules.

Regulation/manipulation of glycosylation processes in cells
and in vivo provides a powerful approach for studying glycan and glycoconjugate functions and mechanisms and discovering novel therapeutic targets as well (6, 7). Glycosyltransferases catalyze the synthesis of glycans and glycoconjugates. The expression and activity of these enzymes are controlled at the transcription, translation and post-translation levels. Therefore, glycosylation can be regulated at multiple levels (8), such as transcriptional (9), post-transcriptional (10), and post-translational (11) regulation. Gene silencing or knockout of a glycosylation processing enzyme is often used by molecular biologists to hinder glycan biosynthesis for examining the function of endogenous glycan chains. However, it may knock out all glycan biosynthesis and thus cause multiple effects, making it difficult to attribute a specific biological role to a specific glycan structure in a spatiotemporal manner. Glycosylation processes can be regulated by inhibition of glycosyltransferases or glycosidases that catalyze glycan assembly and synthesis (12, 13). Inhibitors of glycosyltransferases provide an important approach for studying glycosylation in cells, tissues, and whole organisms while avoiding some of the problems associated with studying protein mutants or gene silencing. Treatment of cells with inhibitors of glycosyltransferases results in the production of proteins containing missing or altered glycan chains, and thus allows us to examine the importance of endogenous glycans to specific cellular, tissue, or organ systems. Further, it provides an opportunity for designing drugs to treat diseases correlated with altered glycosylation.

B. Chemical Regulation of Glycosylation Processes

A number of inhibitors have been identified that interfere with glycoconjugates biosynthesis (6, 12–14). Because the field is quite large, only a selection of inhibitors that act on specific enzymes or metabolic pathways and that illustrate certain basic concepts are discussed here. Specifically, this review summarizes recent developments in chemical regulation of glycosylation processes by inhibiting glycosyltransferases and glycosidases that are responsible for assembly, processing, and turnover of glycans in glycoproteins, proteoglycans, and glycolipids biosynthesis (Table 2).

B-1. Inhibition of N-Glycosylation

N-Glycosylation is one kind of common protein post-translational modification (PTM), in which the glycan is attached to an asparagine (N) located within a canonical sequence N-X-S/T (X, any amino acid except proline) (15). N-Glycosylation begins in the ER and is continued throughout the Golgi apparatus (16).
Initially, the dolichol phosphate-dependent N-acetylglucosamine 1-phospho-transferase (DPAGT) catalyzes the transfer of GlcNAc from UDP-GlcNAc to dolichol phosphate to produce dolichol-PP-GlcNAc. Then, another GlcNAc and five mannose residues are enzymatically added to the dolichol phosphate. Next, this dolichol-PP-oligosaccharide "flips" to the lumen side of the ER, where various enzymes add more mannoses and three glucoses to the existing glycan (15, 16). Further, the oligosaccharide precursor is transferred onto the asparagine residue of the polypeptide chain. Next is the quality control "checkpoint", where three glucose residues are removed from the oligosaccharide by α-glucosidase I and II before leaving the ER. After entering the Golgi apparatus, the glycan is further modified to form different N-glycans, which are usually terminated with sialic acid and fucose moieties. All N-glycans contain a common sugar core comprising two GlcNAc and three mannose residues.

### Table 2. Classic inhibitors for major glycosylation pathways

<table>
<thead>
<tr>
<th>Glycosylation</th>
<th>Major inhibitor/modulator</th>
<th>Target/mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Glycosylation</td>
<td>a) Antibiotics</td>
<td>Inhibition of the initial dolichol-PP-GlcNAc formation by inhibiting DPAGT1 in N-glycosylation pathway</td>
</tr>
<tr>
<td></td>
<td>b) Plant alkaloids</td>
<td>Inhibition of glycan processing glycosidases (α-glucosidase I, II; α-mannosidase I, II) in N-glycosylation processing steps</td>
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<td>O-Glycosylation</td>
<td>a) Nucleotide sugar analogs</td>
<td>Inhibition of the initial attachment O-GalNAc of Mucin-type glycans</td>
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<td></td>
<td>c) Substrate analogs</td>
<td>Diverting the assembly of glycosaminoglycans from endogenous acceptors to exogenous primers in proteoglycan biosynthesis</td>
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<td>d) Phenyl-2-decanoylamino-3-morpholino-1-propanols</td>
<td>Inhibition of glycosyltransferases or glycosidases in O-glycosylation pathway</td>
</tr>
<tr>
<td></td>
<td>e) N-Alkylated imino sugars</td>
<td>Inhibition of the initial GlcCer formation in ganglioside biosynthesis by inhibiting GlcCer synthase</td>
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<tr>
<td></td>
<td>d) Others</td>
<td>Inhibition of terminal glycosylation, e.g. fucosylation, poly-N-acetyllatosamine, and sialylation</td>
</tr>
</tbody>
</table>

1 Modified from Ref. (13, 14).

![Fig. 2. Partial (simplified) N-glycosylation process showing known targets for inhibition.](image)

N-Glycosylation is initiated in the ER, where DPAGT catalyzes the formation of dolichol-PP-GlcNAc, which is followed to produce a lipid-linked oligosaccharide precursor, which is transferred co-translationally to newly synthesized protein. Then, the glycoproteins transit to the Golgi, where N-glycans are further modified, giving rise to mature N-glycosylated proteins decorated with oligosaccharides ranging from high mannose and complex to hybrid N-glycans. Known targets for N-glycosylation process inhibition with inhibitors: (a) tunicamycin, amphomycin, flavomycin, tsushimycin and diumycin inhibiting DPAGT, (b) castanopermine, 1-deoxynojirimycin and australine inhibiting α-glucosidase I and II, (c) 1-deoxymannojirimycin inhibiting α-mannosidase I, and (d) swansonine inhibiting α-mannosidase II. Referred from Ref. (5, 6).
entirely and (ii) inhibition of glycan trimming reactions, which inhibits specific N-glycosylation processing steps.

B-1-1. Inhibition of Dolichol-PP-GlcNAc Assembly

N-Glycan biosynthesis can be terminated by inhibition of the first committed enzyme, DPAGT, which catalyzes the transformation from UDP-GlcNAc to GlcNAc-pyrophosphoryl-dolichol (Fig. 2). Several DPAGT1 inhibitors have been identified to date. Tunicamycin (1) was the first inhibitor of N-glycosylation discovered by Alan Elbein 40 years ago (18). Tunicamycin (1) prevents protein N-glycosylation by inhibiting the formation of GlcNAc-pyrophosphoryl-dolichol, which often leads to protein misfolding. Nowadays, tunicamycin (1) is commonly used to induce the “unfolded protein response” or ER stress (19). Amphomycin (2) is another antibiotic that inhibits the lipid-linked saccharide pathway by blocking the formation of dolichyl-phosphoryl-mannose. Other antibiotics that act at the lipid-level are flavomycin (3), tsushimycin (4), and diumycin (5) (Fig. 3). Overall, these antibiotics have been widely used in a number of functional studies of N-glycosylation (6, 13, 17).

B-1-2. Inhibition of Glycan Trimming Reactions

Removing monosaccharides from the nascent N-linked oligosaccharide by glycosidases, also called glycan trimming, is a part of the quality control “checkpoint” of the ER in N-glycosylation pathways. Compounds inhibiting this glycan trimming process can prevent an intact N-glycan formation, and thus N-glycosylation. Several compounds have been shown to inhibit the glycan trimming process. For example, castanospermine (6), australine (7), deoxynojirimycin (8), 1,1-deoxymannojirimycin (9), and swainsonine (10) are plant alkaloids that block N-linked glycosylation by inhibiting the processing glycosidases (α-glucosidases and α-mannosidases) involved in trimming nascent glycan chains (Fig. 3) (6). These alkaloids inhibit the trimming reactions that occur after the Glc3Man9GlcNAc2 oligosaccharide is attached to a glycoprotein. Specifically, castanospermine (6) inhibits α-glucosidases I and II, australine (7) preferentially inhibits α-glucosidase I, and deoxynojirimycin (8) preferentially inhibits α-glucosidase II (Fig. 2). All of these α-glucosidase inhibitors are involved in the initial processing of N-glycans and in quality control of protein folding. On the other hand, 1,1-deoxymannojirimycin (9) inhibits α-mannosidase I, a key enzyme for N-glycan processing in the Golgi (20). By inhibiting α-mannosidase I activity, the target protein will not generate N-linked oligosaccharides with complex and hybrid man-
nose contents. Swainsonine (10) inhibits mannosidase II, which removes \(\alpha-1,3\) and \(\alpha-1,6\) mannose residues from the GlcNAc-Man5GlcNAc2-peptide. By inhibiting \(\alpha\)-mannosidase II activity, the target protein will not generate \(N\)-linked oligosaccharides with complex mannose contents. In addition, alkylated and acylated analogs of the alkaloids have been explored as potent glycan trimming inhibitors. Alkylation and acylation facilitate their easy across the plasma and Golgi membranes and thus can improve the inhibition potency of the compounds (14). Some of these compounds have shown positive effects for treating diabetes, lysosomal storage diseases, cancer, and HIV infection, but may also induce male sterility (6).

B-2. Inhibition of \(O\)-Glycosylation

\(O\)-Glycosylation is another type of common PTM, in which the glycan is attached to the protein via an oxygen atom on serine (Ser) or threonine (Thr) (4, 21). \(O\)-Glycosylation occurs at a later stage during protein processing in the Golgi apparatus. Mostly, \(O\)-glycosylation begins with the attachment of a \(\alpha\)-GalNAc on Thr or Ser residues of target proteins catalyzed by the enzyme polypeptide \(N\)-acetylgalactosaminyltransferase (ppGalNAcTs) (2). The attached \(O\)-GalNAc glycan is further modified by several glycosyltransferases acting in a sequential manner in order to extend the glycan chain according to substrate specificity. Mucin-type \(O\)-glycosylation is initiated by GalNAc-Ts forming the Tn structure, which is elongated by the core 1 synthase, C1Gal-T1, or the core 3 synthase, \(\beta3\)GnT1, and further branched by the core 2 synthases, C2GnT1-3 and core 4 synthases, C4GnT2, respectively. The different core structures can be further elongated and branched by \(N\)-acytllactosamine chains and/or terminated by blood group AH-related structures, fucose and sialic acids. Adding sialic acid (sialylation) may terminate chain elongation and branching as indicated by the action of ST3Gal-I on core 1, which produces the ST structure. Premature sialylation of the first GalNAc by ST6GalNAc-I leads to the cancer-associated structure STn (Fig. 4) (4, 21).

Fewer inhibitors are available for inhibition of \(O\)-linked glycan biosynthesis compared with \(N\)-linked glycan biosynthesis. Basically, two approaches are mainly used: (i) inhibition of \(O\)-GalNAc initiation of Mucin-type glycans, which blocks glycosylation of glycoproteins entirely and (ii) glycoside primers, which is an indirect inhibition of glycosyltransferase by metabolic interference.

B-2-1. Inhibition of \(O\)-GalNAc Initiation of Mucin-Type Glycans

Mucin-type \(O\)-linked glycan biosynthesis is initiated by ppGalNAcTs that uses UDP-GalNAc as a common donor and various glycoprotein acceptors. Inhibition of ppGalNAcTs consequently prevents the entire \(O\)-glycosylation. Hatanaka and coworkers proposed UDP-GalNAc-based inhibitors using the model of tunicamycin, a well-known inhibitor of \(N\)-glycosylation. They synthesized compounds substituting UMP with different length fatty acid chains (11-13; Fig. 5), which resulted in the 16 carbons-substituted UMP derivative having a slight inhibition of ppGalNAcT (24). A C-glycosidic UDP-GalNAc analog (14) was reported as a ppGalNAcTs inhibitor (23). Later, 3-, 4-, and 6-methylated UDP-GalNAc compounds (15–17; Fig. 5) were reported as they showed an inhibition pattern similar to UDP-GlcNAc (22). The most promising ppGalNAcTs inhibitor is compound 18 (Fig. 5), which shows inhibition activities against a series of

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Fig. 4. Partial \(O\)-glycosylation pathways showing known target for inhibition. The common mucin-type \(O\)-glycosylation core 1–4 biosynthetic pathways. Known target for \(O\)-glycosylation inhibition with inhibitors: (a) UDP-GalNAc analogs inhibiting ppGalNAcTs.
ppGalNAcTs (ppGalNAcT-1 to T5, T7, T10, and T11) in the micromolar range, which was obtained by screening a uridine-based library designed to target enzymes that utilize UDP-sugar substrates (25, 26). The selectivity against this enzyme family is promising since inverting and retaining GalTs or other UDP-sugar utilizing enzymes were not inhibited. It was confirmed that compound 18 inhibited O-glycosylation but not N-glycosylation and induced apoptosis in two different cell types (Jurkat, a lymphoma cell line; and HEK293T). Therefore, compound 18 is a useful compound for O-glycosylation inhibition and O-glycosylation function studies.

B-2-2. Glycoside Primers

Glycoside primers are unnatural analogues of the acceptors that compete with the native acceptors in glycosylation and have been used as tools to regulate O-glycosylation. GalNAc-α-O-benzyl (19) was initially reported to selectively inhibit O-glycosylation through its ability to compete with GalNAc-α-O-Ser/Thr for the β1-3galactosyltransferases involved in the biosynthesis of O-glycans and thus suppressing the elongation of O-glycans on target proteins (27). As a result, cells treated with glycoside primers may express truncated mucin-type O-linked glycans. However, later studies confirmed that short exposure (24 h) of mucus-secreting HT-29 cells to 5 mM GalNAc-α-O-benzyl (19) resulted in a 13-fold decrease in the levels of mucin-associated sialic acid and an increase of T antigen (26). This indicates that GalNAc-α-O-benzyl (19) inhibited sialylation rather than the transferring of Gal to GalNAc-α-O-Ser/Thr. These effects were associated with the metabolism of GalNAc-α-O-benzyl (19) to Gal/β1-3GalNAc-α-O-benzyl, which is a potent competitor of the α2,3-sialyltransferase activity present in HT-29 cell extracts (28, 29). It was further confirmed that GalNAc-α-O-benzyl (19) selectively inhibits sialylation of apical glycoproteins and perturbs lysosomal enzyme processing and may be reminiscent of the alterations found in sialic acid storage diseases (30, 31).

B-3. Inhibition of O-GlcNAc Modification

O-GlcNAc modification is another important PTM, which is characterized by the linkage of a single β-GlcNAc to the hydroxyl group of Thr or Ser residues found in nuclear, cytoplasmic, and mitochondrial proteins (32). The addition of O-GlcNAc to proteins is catalyzed by O-GlcNAc transferase (OGT), and its removal is catalyzed by O-GlcNAcase (OGA). This dynamic O-GlcNAc glycosylation is analogous to phosphorylation and more than 1000 proteins have been described to be O-GlcNAcylated to date (33).

The importance of O-GlcNAc addition to many cytoplasmic and nuclear proteins has stimulated great interest in developing agents to inhibit its addition by OGT or its removal by GlcNAcase (34). Alloxan (20) is the first reported human OGT inhibitor, which is thought to inhibit OGT by binding to the uracil binding pocket or alternatively has been proposed to act through a covalent modification of cysteine residues (35). Besides, GalNAc-α-O-benzyl (19), a glycoside primer for O-glycosylation, was reported to perturb O-GlcNAc in cells as well (36). Later, 3-(2-adamantanylethyl)-2-[(4-chlorophenyl)azamethylene]-4-oxo-1,3-thiazaperhydroine-6-carboxylic acid, (ST045849, 21) and 4-methoxyphenyl 6-acetyl-2-oxobenzo[d]oxazole-3(2H)-carboxylate (BZX, 22) were developed as OGT inhibitors by high-throughput screening against a large library of drug-like compounds (37). The inhibition efficiency of the two molecules was tested in vitro against full-length OGT constructs showing IC_{50} of 53 µM for compound 21 and 10 µM for compound 22. In addition, a substrate mimetic inhibitor, Ac4-5S-GlcNAc (23), dramatically reduced global O-GlcNAcylation in cells, in part because the active form of the inhibitor, UDP-5S-GlcNAc (24), accumulates in cells.
Ac4-5S-GlcNAc is a useful cellular inhibitor of OGT nowadays. Another small cell-permeable molecule inhibitor, OSMI-1 (25), was designed based on the high-throughput screening mentioned above (39). OSMI-1 (25) reduced global O-GlcNAc levels in different mammalian cell lines with an IC\textsubscript{50} of 2.7 µM (39). Recently, a bi-substrate OGT inhibitor, goblin (26), with an acceptor peptide linked to UDP was reported as a new specific OGT inhibitor (40). Goblin (26) exhibited low micromolar affinity for OGT and inhibited O-GlcNAcylation of peptides and protein substrates \textit{in vitro} (40).

On the other hand, several O-GlcNAcase inhibitors were designed based on GlcNAc structure. The first compound in this class is PUGNAC (27) (O-[2-acetamido-2-deoxy-D-glucopyranosylidene]-amino-N-phenylcarbamate; Fig. 6B) that inhibits O-GlcNAcase at nanomolar concentrations (41). The drawback of PUGNAC is that it also inhibits lysosomal β-hexosaminidases (HexA and HexB). Later, Thiamet-G (28) and the related GlcNAc-thiazoline (NAG-thiazoline, 29) were developed as they are more specific and potent than PUGNAC (42). Also, a rationally designed glucimidazole, GlcNAcstatin (30), was reported that inhibited O-GlcNAcase with a \( K_i \) of 4.6 pM and shows 10\(^5\)-fold selectivity over HexA and HexB (43). Overall, these compounds inhibit the enzyme in cells and tissues, providing useful tools to study the function of O-GlcNAc, and are potential lead compounds for drug development. Interestingly, Okuda reported that PUGNAC and Thiamet-G increased globotetraosylceramide (Gb4Cer) levels in human umbilical vein cells (HUVEC) (44). It was found that both of PUGNAC and Thiamet-G treatment up-regulated the expression levels of α-1,4-galactosyltransferase/Gb3Cer synthase gene which encodes a key enzyme in Gb4Cer synthesis. These results indicate that protein-O-GlcNAcylation can regulate the expression levels of cellular Gb4Cer.

**B-4. Inhibition of the Assembly of Glycosaminoglycan (GAG) on Proteoglycan**

Proteoglycans are composed of a core protein and one or more GAGs (45, 46). The major classes of GAG chains are chondroitin sulfate (CS)/dermatan sulfate (DS) and heparan sulfate (HS)/heparin. These GAGs are all connected to the protein core \textit{via} a linker tetrasaccharide (GlcAβ(1→3)Galα(1→3)Galβ(1→4)Xylβ(1→O-Ser), extending from Ser-Gly sites in the protein. The linkage tetrasaccharide is synthesized by sequential transfer of xylose, galactose, galactose, and glucuronic acid residues from their corresponding sugar nucleotides catalyzed by glycosyltransferases: xylosyltransferase-1/2, galactosyltransferase-1, galactosyltransferase-2, and so on. Inhibiting these enzymes can inhibit the assembly of GAG on proteoglycans.
ase-2, and glucuronyltransferase-1, respectively. From this linker tetrasaccharide, the sugar chains are extended by the addition of two alternating monosaccharides, an aminosugar and GlcA, catalyzed by glycosyltransferases: GalNAcT-I, GlcNAcT-I and GlcAT-II, respectively. In CS/DS, the aminosugar is GalNAc and in HS and heparin it is GlcNAc. The sequential glycosylation, epimerization and sulfation occur in the Golgi apparatus catalyzed by specific glycosyltransferases, epimerases, and sulfotransferases (Fig. 7). The extent of epimerization of GlcA to iduronic acid (IdoA) and the sulfation pattern of the disaccharide units distinguish CS from DS and HS from heparin.

The biological activities of proteoglycans often depend on the GAG side chain’s interaction with other proteins (46, 47). The ability to manipulate or inhibit GAG biosynthesis would allow for a better understanding of the functions of endogenous GAGs and proteoglycans as well (48, 49). Several different biochemical approaches have been utilized to manipulate GAG biosynthesis (47). Among them are two chemical approaches: (i) Xyloside primers, targeting the linkage region as a starting place and also known as decoys for GAG biosynthesis, and (ii) GAG precursor utilization inhibitors, preventing GAG extension.

**B-4-1. Xyloside Primers**

Okayama et al. first found that p-nitrophenol β-O-xyloside inhibited the assembly of GAGs on proteoglycans 43 years ago (50). It was found that the xyloside mimics the natural substrate, xylosylated serine residues in proteoglycan core proteins, and thus acts as a substrate, which diverts the assembly process from the endogenous core proteins and causes inhibition of proteoglycan formation, known as “priming”. In general, cells incubated with xylosides secrete large amounts of individual GAG chains and accumulate proteoglycans containing truncated chains. Xylβ-O-estradiol (31), 4-keto-Xylβ-O-benzyl (32), and per-acetylated Galβ1,4Xylβ-O-naphthol (33) represent common glycoside primers for preventing synthesis of GAG on growing proteoglycans (Fig. 8) (46). In addition, 4-methyl-umbelliferone (34) is often used to block hyaluronan biosynthesis (51). In general, priming by glycosides occurs in a concentration-dependent manner and the efficiency varies widely among different compounds and cell types.

**B-4-2. GAG Precursor Utilization Inhibitors**

Heparan sulfate (HS) and chondroitin sulfate (CS) are synthesized by a number of glycosyltransferases following linkage tetrasaccharide formation appending the protein core. Hascall et al. reported a fluorinated analogue of glucosamine (4-fluoro-N-acetylglucosamine (4-F-GlcNAc, 35)) that reduced the content of HS and CS side chains in cultured smooth muscle cells (Fig. 8) (52). Treatment of the cells with 4-F-GlcNAc (35) (100 µM) reduced the quantity (by 64–76%) and decreased the size of HS/CS GAGs associated with the cell layer. They proposed that 4-F-GlcNAc (35) inhibits CS synthesis by inhibiting 4-epimerization of UDP-GlcNAc to UDP-GalNAc, thereby depleting one of the substrates required, whereas HS elongation is inhibited by truncation when the nonreducing terminus of the growing chain is capped with 4-F-GlcNAc, which has a fluorine group at the 4-position instead of a hydroxyl group. Later, per-acetylated 4-F-GlcNAc (36) was proposed to increase penetration across the cell membranes. Once entered into the cytoplasm, the acetate groups are hydrolyzed by esterases, releasing the 4-F-GlcNAc (35), which could be ultimately converted to the 4-epimerase inhibitor: UDP-4-F-GlcNAc. Yong et al. therefore tested whether per-acetylated 4-F-GlcNAc (36) could reduce synthesis of CS on proteoglycans from cultured astrocytes.
They also found that acetylated 1-propanoic fluorosamine (37) was effective at reducing CS synthesis in cultured astrocytes.

### B-5. Inhibition of Glycolipid Biosynthesis

Glycolipids are glycosyl derivatives of lipids such as acylglycerols, ceramides and prenols. They are a larger family of glycoconjugates with complex structures. In this review, sialoglycosphingolipids (gangliosides containing one or more sialic acid residues) are discussed. Ganglioside biosynthesis starts from the formation of ceramide at the cytoplasmic leaflet of the ER membrane (54, 55), followed by the stepwise transfer of nucleotide-activated monosaccharide units onto ceramide and they are then continually modified in the Golgi apparatus by sequential addition of additional sugars to an existing acceptor lipid molecule (54).

Briefly, ganglioside synthesis starts from the addition of Glc to ceramide (Cer) to form GlcCer catalyzed by ceramide β-D-glucosyltransferase. Extension of GlcCer occurs through the addition of Gal catalyzed by GlcCer β1,4-galactosyltransferase to make lactosylceramide (LacCer). Except GM4, which is derived from galactosylceramide (GalCer), most gangliosides are synthesized from LacCer. The simple ganglioside GM3 is synthesized by addition of a sialic acid to LacCer by LacCer α2,3sialyltransferase (GM3 synthase or ST-I). Further, GD3 and GT3 are synthesized from GM3 by sequential addition of sialic acid catalyzed by specific glycosyltransferases, respectively. Based on the number of sialic acids connected to the “inner” galactosyl residue, gangliosides are classified into members of the 0-, a-, b-, and c-series (Fig. 9) (56, 57).

Inhibition of ganglioside synthesis has been well studied for the treatment of glycosphingolipid storage diseases in the past 40 years as an alternative approach to enzyme replacement therapy (58, 59). (R,R)-(α-threo)-Isomer of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, 38) (Fig. 10) is the first class of inhibitors for the inhibition of glucosylceramide formation, which is the first step in ganglioside synthesis (60). PDMP potently inhibits ceramide β-D-glucosyltransferase and causes the reversible depletion of cellular gangliosides. This compound has been used extensively to study the metabolism and function of gangliosides in vitro (61–64). Later, several inhibitors have been developed using PDMP as the lead compound. Among them, α-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4, 39) showed more potent inhibitory activity against GlcCer synthase than PDMP, and did not increase ceramide levels or inhibit cell growth (65). The P4 analogs, α-threo-1-(3′,4′-ethylenedioxy) phenyl-2-palmitoylaminol-3-pyrrolidino-1-propanol (EtDO-P4, 40) and α-threo-4′-hydroxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (pOH-P4, 41) have similar properties to inhibit ceramide β-D-glucosyltransferase (Fig. 10). In addition, N-alkylated imino sugar N-butyldeoxynojirimycin (NB-DNJ, 42) was found as an inhibitor for ganglioside synthesis (Fig. 10). Originally, NB-DNJ (42) was a potent inhibitor (micromolar concentrations) of N-linked oligosaccharide processing enzymes α-glucosidase I and II (66, 67). However, in vivo, NB-DNJ (42) does not significantly impact N-glycoprotein processing because a sufficient concentration of the inhibitor could not be attained in the lumen of the endoplasmic reticulum, where the glucosidases exist. In contrast, concentrations of NB-DNJ (42) in the cytosol are sufficient to inhibit GlcCer synthesis, which takes place on the cytosolic surface of the Golgi apparatus (68, 69). Therefore, NB-DNJ (42) is a useful inhibitor for ganglioside synthesis. Interestingly, a galactose analogue, N-butyldeoxygalactonojirimycin (NB-DOJ, 43) (Fig. 10), also inhibits GlcCer synthesis but shows no inhibition of α-glucosidase I and
In vitro (70). NB-DNJ (42) and NB-DGJ (43) would be useful compounds for inhibiting gangliosides synthesis and studying their function.

B-6. Inhibition of Specific Glycosyltransferases of the Terminal Glycosylation Processes

Glycans are often terminated with specific sugars, like fucose, poly-N-acetyll lactosamine and sialic acid. Due to their terminal locations, these sugars are closely involved in recognition processes of glycoconjugates in either physiological or pathological pathways. Therefore, inhibition of the terminal glycan formation will provide a powerful approach for studying glycan and glycoconjugate function and may provide new therapeutic targets and mechanisms. In this review, the recent approaches for regulation of terminal fucose, poly-N-acetyllactosamine and sialic acid attachment on either N-glycans or O-glycans are discussed briefly. There are several comprehensive reviews for each category reported recently (71–73). The readers are recommended to these reviews for more detailed information and biomedical interests.

B-6-1. Inhibition of Fucosylation

L-Fucose often exists as a terminal sugar in glycoconjugate glycans that are essential for either physiological or pathological activities, such as inflammation, bacterial and viral infections, tumor metastasis, and genetic disorders (72). Fucosyltransferases (FUTs) catalyze the incorporation of L-fucose residues, a process referred as fucosylation. Several GDP-fucose analogs have been reported to inhibit FUTs (71, 72). However, the charged groups on the GDP portion prevent their uptake into cells and limit their use in biological systems. A fluorinated analog of fucose, 2-fluoro-L-
Fucose (2F-Fuc, 44, Fig. 11A), has been reported to easily enter cells via passive diffusion wherein it is metabolized into a GDP-Fuc analog, GDP-2F-Fuc, via the salvage pathway (75). It was found that GDP-2F-Fuc accumulated in cells and also led to a shutdown of the de novo pathway that synthesizes natural GDP-Fuc. It was confirmed that the addition of 2F-Fuc efficiently suppressed the endogenous production of GDP-Fuc, which dramatically inhibited the formation of fucosylation in both cancer and plant cells (76). Recently, Gu et al. reported that inhibition of fucosylation by 2F-Fuc suppresses human liver cancer HepG2 cell proliferation and migration as well as tumor formation (77). On the other hand, Paulson et al. reported that peracetylated 2F-Fuc (2F-Fuc-per-Ac (45)) is readily taken up by cultured cells and converted to GDP-2F-Fuc by the endogenous salvage pathway. It then inhibits the synthesis of the natural GDP-Fuc substrate (78). It is clear that 2F-Fuc-per-Ac (45) dramatically reduced fucosylation of cell surface glycans in cells and can serve as a global inhibitor of FUTs. In addition, peracetylated 6-alkynyl-fucose (6-Alk-Fuc, 46) (73) and peracetylated 5-thiofucose (5T-Fuc, 47) (79) have been developed as they can be converted in cells by the salvage pathway into GDP analogs of fucose, which are inhibitors primarily of FUTs (80). These compounds resulted in broad inhibition of fucosylation in cells. In addition, 6-Alk-Fuc (46) has been used as a tool to label glycans (81) or modify glycan function (82).

B-6-2. Inhibition of Poly-N-Acetyllactosamine

Poly-N-acetyllactosamines (Poly-LacNAc) is a unique glycan composed of repeating units of the common disaccharide (Galβ1,4-GlcNAcβ1,3)n found both in N- and O-glycans. The
control of chain length of Poly-LacNAc depends primarily on β 4 galactosyltransferase (β4GalT). There are seven β4GalTs characterized to date, among which β4GalT-I and β4GalT-III variants are the most widely expressed (81). The others are expressed tissue-specifically to a lesser extent (83). Increasing evidence demonstrates the association of poly-LacNAc chains found both in O- and N-glycans with cancer. Therefore, inhibiting enzymes involved in the LacNAc biosynthesis is an attractive approach for cancer treatment. A variety of β4GalT inhibitors have been developed so far, including glycosyphosphate, a bi-substrate analogue acceptor substrate analogue (84). Chung and coworkers developed selective β4GalT inhibitors based on the acceptor substrate by attaching GlcNAc to aromatic aglcone moieties (48, Fig. 11B), which exhibited Ki of 3.5–9.5 μM (85). Brockhausen and coworkers reported that 1-thio-N-butyrylGlcNAcβ-(2-naphthyl) (49) inhibits 68–95% of human β4GalT activity in a series of tumor cell line lysates, without compromising the activity of other enzymes (86). In addition, the specificity of 49 against β4GalT was confirmed by testing the enzyme activity of recombinant glycosyltransferases. Later, Takaya and coworkers have substituted C2 or C6 of Gal in UDP-Gal with a flexible trioxadecanyl group linked to a terminal naphthyl group in order to enhance the stacking interaction with Trp314 and to block the acceptor substrate entrance (87). They found that modification at the C6 position (50) was more effective than the C2 position of Gal residue, yielding a more potent inhibitor (Ki of 1.86 μM) against UDP-Gal (Km of 4.91 μM) (Fig. 11B) (87).

### B-6-3. Inhibition of Sialylation

Sialic acids, existing as terminal sugars of glycoconjugates, play important roles in various physiological and pathological processes, such as cell–cell adhesion, immune defense, tumor cell metastasis, and inflammation (88, 89). In eukaryotic cells, Neu5Ac is synthesized in the cytosol and then transferred to the nucleus and activated by cytosine 5′-monophosphate N-acetylneuraminic acid (CMP-Neu5Ac) synthetase to form CMP-Neu5Ac that then goes to the Golgi to be transferred to glycoconjugates by sialyltransferases, which are subsequently secreted or delivered to the cell surface. So far, twenty sialyltransferases have been identified for catalyzing the addition of sialic acids to terminal non-reducing position of the oligosaccharides of different sugar acceptors in different linkages on proteins and lipids (90). Each sialyltransferase presents high selectivity toward its acceptor substrate to create α2,3-, α2,6-, α2,8-linkages, respectively. In general, sialyltransferases can be classified into four families depending on their linkage specificities and acceptor substrates: (i) the ST3Gal family, catalyzing the addition of sialic acids to a terminal galactose of N- or O-linked glycans and glycolipids in α2,3-linkage; (ii) the ST6Gal family, adding α2,6-linking sialic acids to galactose residues of N-glycans; (iii) the ST6GalNAc family, adding sialic acids to terminal N-acetylgalactosamine (GalNAc) residues of glycoproteins and glycolipids in α2,6-linkage; and finally, (iv) the ST8Sia family, the only known sialyltransferases that promote the linkage to another sialic acid residue in N- or O-glycans in α2,8-linkage. Overall, the levels and linkages of sialic acids, named as sialylation status, are controlled by the levels and activities of sialyltransferases, which vary upon cell activation related to both physiological and pathological processes.

Compounds that inhibit sialyltransferase have important applications in both biochemical and biomedical research. First, sialyltransferase inhibitors are useful tool to study sialyltransferase function and related mechanisms. In addition, inhibitors of sialyltransferases are of medicinal interest, especially for the cancer therapy as elevated sialyltransferase activity leads to overexpression of cell surface sialic acids and contributes to many disease developments, such as cancer and inflammation. We have highlighted recent development of inhibitors of sialyltransferases reported since 2004 (73). The inhibitors are summarized in eight groups: 1) sialic acid analogs (51), 2) CMP-sialic acid analogs (52), 3) cytidine analogs (53), 4) oligosaccharide derivatives (54), 5) aromatic compounds (55), 6) flavonoids (56), 7) lithocholic acid analogs (57), and 8) spirocyclic drimane (58) (Fig. 11C). Recently, Szabo and Skropeta published another review of the latest developments in ST inhibitors from design and high-throughput screening, addressing both the challenges and opportunities in targeting cell surface sialylation (74). The review features an overview of the biological evaluation methods, computational and imaging tools, inhibitor molecular diversity, and selectivity toward ST subtypes, along with the emerging role of ST inhibitors as diagnostic tools for disease imaging (74). Therefore, readers are recommended to refer these two comprehensive reviews (73, 74) for more detailed information.

### C. Summary and Future Perspective

Glycoconjugates are ubiquitous biomolecules found in all kingdoms of life. The diverse structures of glycoconjugates are controlled by complex glycosylation process and are metabolically responsive and occur in a cell- and protein-specific manner, conferring tissue type-specific properties. They have essential roles in diverse physiological and pathological processes, including intercellular signaling, inflammation, protein quality control, and cellular adhesion as well as cell differentiation and proliferation. However, uncovering the physiological roles of various glycoconjugates remains as an obstacle limiting basic biological sciences and biomedical applications. Subtle and selective manipulation of biosyn-
thesis of glycans and glycoconjugates both in cells and in vivo will contribute to understanding their functions and the underline mechanisms of biological pathways. Selective small-molecule inhibitors of glycosylation processing enzymes hold great potential for such manipulation as well as for determining the function of glycans and glycoconjugates. In this review, recent advances and existing inhibitors of glycosylation enzymes were discussed, especially on the prospects for glycan assembly on the glycoconjugates. While several inhibitors are currently available and are very potent cellular inhibitors of glycosylation enzymes, cautions must be taken when using them. Especially, prospects for overcoming off-target effects are highly needed, particularly if they require enzymatic processing in order to become active. From the reports to date, it is clear that metabolic inhibitors of glycosyltransferases have the potential to modulate glycosylation in cultured cells and animals, and that significant selectivity needs to be achieved for enzymes of a single family. In addition, there remains a pressing need for cell-permeable small molecule inhibitors that are amenable to chemical modification. Finally, the coordinated efforts of chemists, biochemists and biologists will be crucial for creating, characterizing and validating inhibitors that are useful tools both for advancing a basic understanding of glycobiology as well as for discovering new potential therapeutic targets and mechanisms for disease prevention and treatment.

References

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