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Glycan Antagonists and Inhibitors: A Fount for Drug Discovery

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ABSTRACT Glycans, the carbohydrate chains of glycoproteins, proteoglycans, and glycolipids, represent a relatively unexploited area for drug development compared with other macromolecules. This review describes the major classes of glycans synthesized by animal cells, their mode of assembly, and available inhibitors for blocking their biosynthesis and function. Many of these agents have proven useful for studying the biological activities of glycans in isolated cells, during embryological development, and in physiology. Some are being used to develop drugs for treating metabolic disorders, cancer, and infection, suggesting that glycans are excellent targets for future drug development.

KEYWORDS glycans, carbohydrates, glycosylation, inhibitors, therapeutics

INTRODUCTION

Animal cells elaborate a large array of glycoconjugates, which are composed of one or more glycans (carbohydrate chains) covalently bound to protein (glycoproteins and proteoglycans) or lipid (lipid-linked oligosaccharides, glycosphingolipids and glycosylphosphatidylinositols) backbones (Figure 1). At the cell surface, glycoconjugates form a thick layer (glycocalyx) through which all nutrients, hormones, growth factors, and soluble proteins must diffuse to gain access to plasma membrane receptors and transporters. Cells also deposit glycoproteins and proteoglycans along with various structural proteins in extracellular matrices, which provide support and organization to tissues and create barriers for regulating diffusion and filtration. For many years, glycans were thought to play merely structural roles, but we now know that they participate in fundamental properties of cells, including protein quality control, cell adhesion and motility, endocytosis, and signal transduction. Furthermore, they affect processes important in development, such as cell proliferation and differentiation, and morphogenesis. Microbes often exploit glycans as adhesin receptors for colonization and as portals of entry for infection. Essentially, all of these processes depend on binding events between small sets of sugar residues and specific carbohydrate-recognition domains in proteins. Glycosylation is not restricted to secreted and membrane proteins; many cytosolic and nuclear proteins undergo glycosylation as well, often at the same sites as phosphorylation. Thus, it is not surprising that organisms cannot survive in the absence of glycosylation. For comprehensive reviews of the field, see (Varki *et al.*, 1999; Brooks *et al.*, 2002; Taylor & Drickamer, 2003; Sansom & Markman, 2007).

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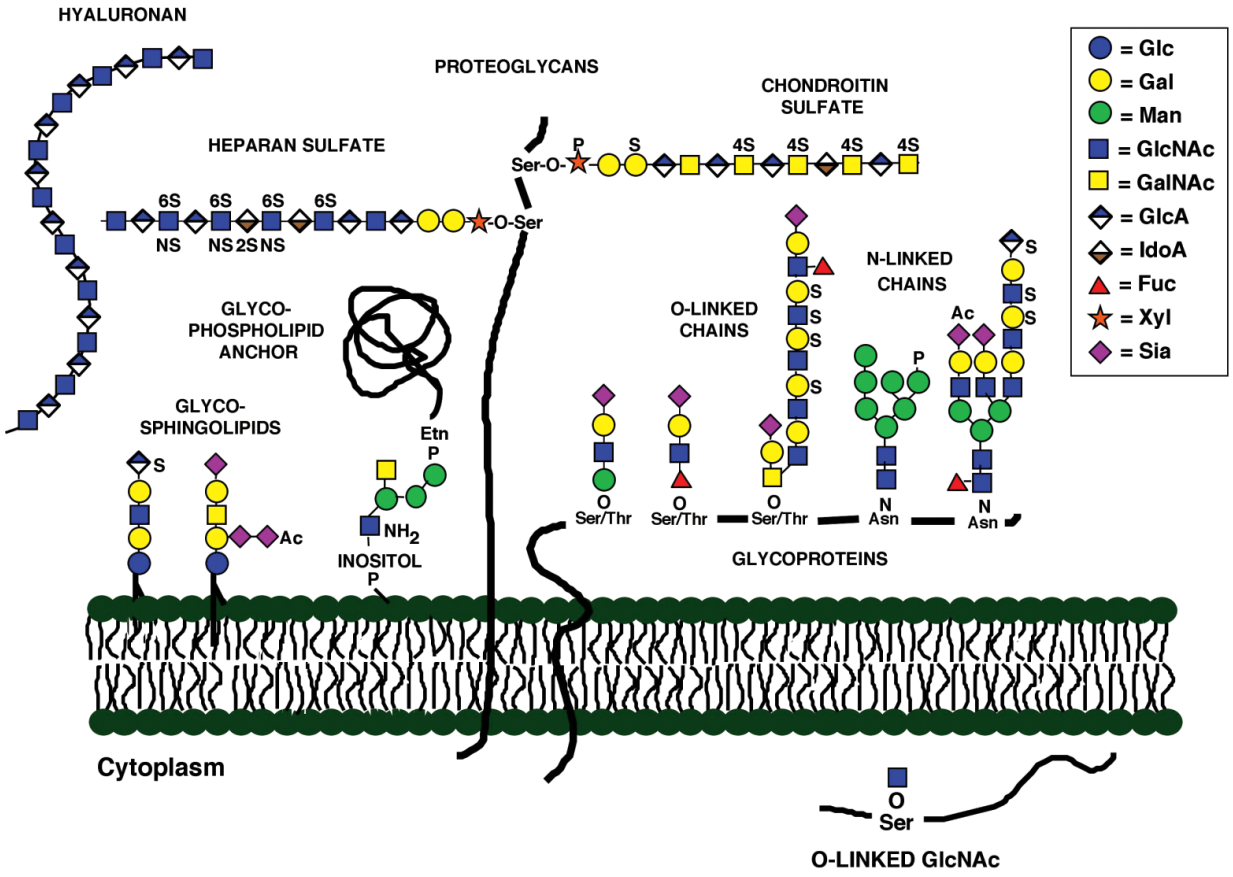

FIGURE 1 **Schematic representation of the glycocalyx on vertebrate cells showing the major classes of glycoconjugates. A symbol nomenclature of the various monosaccharides is described in the inset. The symbols used are based on the nomenclature in the book Essentials of Glycobiology (ISBN: 0-87969-560-9) and have been adopted by the Consortium for Functional Glycomics http://www.functionalglycomics.org/static/consortium/consortium.shtml). Glc, glucose; Gal, galactose; Man, mannose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; IdoA, L-iduronic acid; Fuc, L-fucose; Xyl, xylose; Sia, silic acid; Ac, acetyl, P, phosphate; S, sulfate; NS, *N*-sulfate; 2S, 3S, 6S indicate the position of *O*-sulfate groups; Etn, ethanolamine.**

Although glycans are essential, organisms can tolerate changes in their content and structure. Indeed, fluctuations in glycan composition may be a mechanism of ensuring a balance between positive and negative interactions with carbohydrate binding proteins needed by cells and expressed by infectious organisms (Bishop & Gagneux, 2007). Despite their plasticity, glycans often interact with proteins with great specificity and avidity. Thus, much effort has been devoted to the discovery or design of glycan-protein antagonists or agents that would modulate glycan metabolism. These agents have validated glycans and the enzymes involved in their metabolism as therapeutic targets. Some of the agents have proven therapeutic value and have provided leads for further drug development efforts (Table 1).

This review provides a primer on the structure, assembly, and metabolism of the major classes of glycans in vertebrates (Table 2), the principles by which available glycosylation inhibitors act, and a description of different classes of inhibitors and their development as drugs. These agents fall into four categories: (1) derivatives of monosaccharide precursors that alter glycan synthesis by their incorporation, (2) inhibitors that act on individual glycosyltransferase or glycosidases involved in glycan assembly and turnover, (3) agents that bind to glycans and antagonize their interaction with glycan-binding proteins, and (4) enzymatic approaches to partially remove glycans from cells or glycoconjugates.

Asparagine or N-Linked Glycans of Glycoproteins

Many glycoproteins contain glycans linked through a glycosylamine bond to asparagine residues. These N-linked glycans (or N-glycans) are classified into three subtypes depending on their degree of processing: high mannose, hybrid, and complex (Figure 2). They play a central role in protein quality control with the ER and

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TABLE 1 Glycan-based inhibitors and their therapeutic uses

Drug	Clinical status	Disease/Disorder	Mode of action
Targeting *N*-linked glycans			
Zanamivir (Relenza®)	On the market (Biota/Glaxosmithkline)	Influenza Type A and B infection	Enzyme inhibitor—inhibits neuraminidase
Oseltamivir (GS 4104, Tamiflu®)	On the market (Gilead/Roche)	Influenza Type A and B infection	Enzyme inhibitor—inhibits neuraminidase
6-Butanoyl castanospermine (Celgosivir)	Phase II (Migenix)	Chronic Hepatitis C and HIV infections	Enzyme inhibitor—Golgi α-glucosidase I and II
*GD0039, Hydrochloride salt of swainsonine	Phase II (GlycoDesign)	Metastatic renal cancer	Enzyme inhibitor - Golgi α-mannosidase II
*GCS-100	Phase II (GlycoGenesys)	Colon and pancreatic cancer	Carbohydrate derived from citrus pectin attaches to Galectin-3 and induces apoptosis
Targeting *O*-GalNAc glycans			
*CY 1503 (Cylexin®)	Phase III (Cytel)	Reperfusion injury	Carbohydrate-based inhibitor mimicking the natural ligand sLe^X
Bimosiamose (TBC1269)	Phase II (Revotar Biopharmaceuticals)	Asthma	Synthetic pan-selectin antagonist - small molecule dimer with minimal carbohydrate content
YSPSL	Phase II (Y's Therapeutics)	Ischemic reperfusion injury and delayed graft function (DGF)	P-selectin antagonist-recombinant P-selectin glycoprotein immunoglobulin (r-PSGL-Ig),
PSI-697	Phase I (Wyeth)	Scleritis	P-selectin inhibitor of leukocyte rolling in scleral blood vessels
HuLuc63	Phase I (PDL Biopharma)	Advanced refractory multiple myeloma	mAb to cell-surface glycoprotein CS1 exhibits anti-tumor effects through antibody-dependent cellular cytotoxicity activity on myeloma cells
ZP103	Preclinical (Zacharon Pharmaceuticals)	Metastatic carcinomas	Enzyme inhibitor—inhibits glycosyltransferases involved in sLe^X biosynthesis
Targeting glycosaminoglycans			
Heparin	On the market (multiple brands)	Anticoagulant; cancer	Inhibits antithrombin; Inhibits heparanase and blocks interactions between growth factors and heparan sulfate
Hyaluronan	On the market (multiple brands)	Ocular surgery; osteoarthritis; plastic surgery	Tissue space filler, anti-inflammatory agent
Laronidase (Aldurazyme®)	On the market (Genzyme)	MPSI, α-iduronidase deficiency	Enzyme replacement therapy (ERT)
Hyaluronidase (Cumulase®)	On the market (Halozyme)	In vitro fertilization; in development as an adjuvant for cancer chemotherapy	Degrades HA around oocytes improving fertilization; degrades HA in tumors to decrease intratumor pressure
Idursulfase (Elaprase®)	On the market (Shire)	MPS II (Hunter Syndrome) iduronate-2-sulfatase	Enzyme replacement therapy (ERT)
Tramiprosate (Alzhemed)	Phase III (Neurochem)	Amyloid diseases, Alzheimer disease and possibly other amyloidoses	Binds to amyloid plaque, blocks its formation

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TABLE 1 **Glycan-based inhibitors and their therapeutic uses (*Continued*)**

Drug	Clinical status	Disease/Disorder	Mode of action
Eprodisate (Kiacta®)	Phase II/III trial (Neurochem)	Amyloid A amyloidosis, renal	Interferes with glycosaminoglycan-amyloid interactions
PI88	Phase II (Progen Pharmaceuticals)	Lung, liver, prostate, multiple myeloma and melanoma	Inhibits heparanase and possibly other glycan-protein interactions
*GH9001	Phase I (GlycoDesign)	Anti-thrombotic	Mixture of medium molecular weight heparin and low molecular weight dermatan sulfate– inactivates thrombin and activated factor Xa
M118	Phase II (Momenta Pharmaceuticals)	Acute Coronary Syndrome (ACS)	Low molecular weight heparin—selectively binds to antithrombin III and thrombin
*Astenose	Preclinical (GlycoMed)	Restenosis	Chemically-modified heparin inhibits restenosis following angioplasty
Chondroitinase	Preclinical (Acorda Therapeutics)	Spinal cord injury	Degrades chondroitin sulfate that inhibits repair after spinal cord injury
Heparanase	Preclinical (Insight Biopharmaceuticals)	Wound angiogenesis and healing	Enzymatic glycan removal-degrades heparan sulfate side chains of proteoglycans
Targeting glycosphingolipids			
N-butyl-DNJ (Miglustat, Zavesca®)	On the market (Acetelion)	Type I Gaucher disease Niemann-Pick Type C, late onset Tay Sach, Type 3 Gauchers disease	Substrate reduction therapy; inhibits glucosylceramide synthase
Imiglucerase (Cerezyme®)	On the market (Genzyme Corporation)	Type 1 Gaucher disease, β-glucocerebrosidase deficiency	Enzyme replacement therapy (ERT)
β-agalsidase (Fabrazyme®)	On the market (Genzyme)	Fabry disease, for α-galactosidase A deficiency	Enzyme replacement therapy (ERT)
Genz-112638	Phase II (Genzyme)	Gaucher disease	Enzyme inhibitor – amino ceramide-like compound inhibits glucosylceramide synthase
*OGT 719	Phase I (Oxford GlycoSciences)	Liver cancer	Enzyme inhibitor – nucleoside analog with galactose attached to a fluorinated pyrimidine
Others			
Acarbose (Glucobay®)	On the market (Bayer)	Type 2 diabetes	Enzyme inhibitor – blocks intestinal α-glucosidases involved in digestion of dietary glycans
Alglucosidase alfa (Myozyme®)	On the market (Genzyme)	Pompe disease (glycogen storage disease) α-glucosidase A deficiency	Enzyme replacement therapy (ERT)
Allosamidin	Commercially available (Industrial Research Ltd)	Insecticide	Chitinase inhibitor

Compounds targeted to microbial glycans, such as the aminoglycoside antibiotics or other inhibitors of cell wall assembly have not been included.
*To the best of our knowledge, these agents are no longer under development.

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TABLE 2 **Major classes of glycans and glycoconjugates present in animal cells**

Glycan Class	Diseases of Glycans
Asparagine(*N*)-linked glycans of glycoproteins	• Tumor growth, invasion, angiogenesis and metastasis
Serine/threonine(*O*)-linked glycans of mucins and glycoproteins	• Lysosomal storage disorders
Hyaluronan, and sulfated glycosaminoglycans of proteoglycans	• Diabetes
Glycosphingolipids (gangliosides and cerebroside)	• Acute and chronic inflammation
Glycosylphosphatidylinositol(GPI)-anchors on membrane proteins	• Gain of function disorders caused by neo glycosylation sites
O-GlcNAc on cytosolic and nuclear proteins	

Golgi lectins (glycan-binding proteins) calnexin, calreticulin, ER-Golgi intermediate compartment (ERGIC)-53, and vesicular integral membrane protein (VIP36) (Schrag *et al.*, 2003; Moremen & Molinari, 2006). The nonreducing ends of N-linked glycans can contain sialic acids, which are recognized by families of sialic acid binding proteins (*e.g.*, siglecs (Varki & Angata, 2006; Crocker *et al.*, 2007), selectins (Kelly *et al.*, 2007), microbial adhesins and viral hemagglutinins (Sharon, 2006)). Other terminal sugars such as galactose, mannose, and 4-*O*-sulfated *N*-acetylgalactosamine can facilitate clearance of glycoproteins by hepatic and macrophage receptors (Woodworth & Baenziger, 2001; Weigel & Yik, 2002; Allavena *et al.*, 2004). Fucosylation and sulfation also can occur, giving rise to several of the blood group antigens (*e.g.*, ABO and Lewis, Marionneau *et al.*, 2001).

Biosynthesis of N-Linked Glycans

The initial phase of N-linked glycan synthesis involves the assembly of a 14-residue oligosaccharide precursor attached to the isoprenoid lipid carrier dolichol ($Glc_3Man_9GlcNAc_2$-dolichol). Its assembly occurs in a sequential manner starting with the addition of *N*-acetylglucosamine-P to dolichol-P followed by the addition of other monosaccharides to the non-reducing end of the nascent chain (Figure 2). The process occurs in two topologically distinct phases on opposite sides of the ER membrane, with the final product facing the lumen. After completion of the mature lipid-linked precursor, an oligosaccharyltransferase (OST) complex transfers the large precursor *en bloc* from dolichol to specific asparagine residues as the protein emerges from membrane-bound ribosomes and folds (Kelleher & Gilmore, 2006). The hallmark of all N-linked glycan attachment sites is the tripeptide "sequon" Asp-X-Ser/Thr, where X is any amino acid except proline. As the protein folds, the glycan undergoes processing by α-glucosidases and α-mannosidases to remove the glucose residues and several mannose residues, respectively. After demannosylation in the Golgi complex by Golgi mannosidase I,

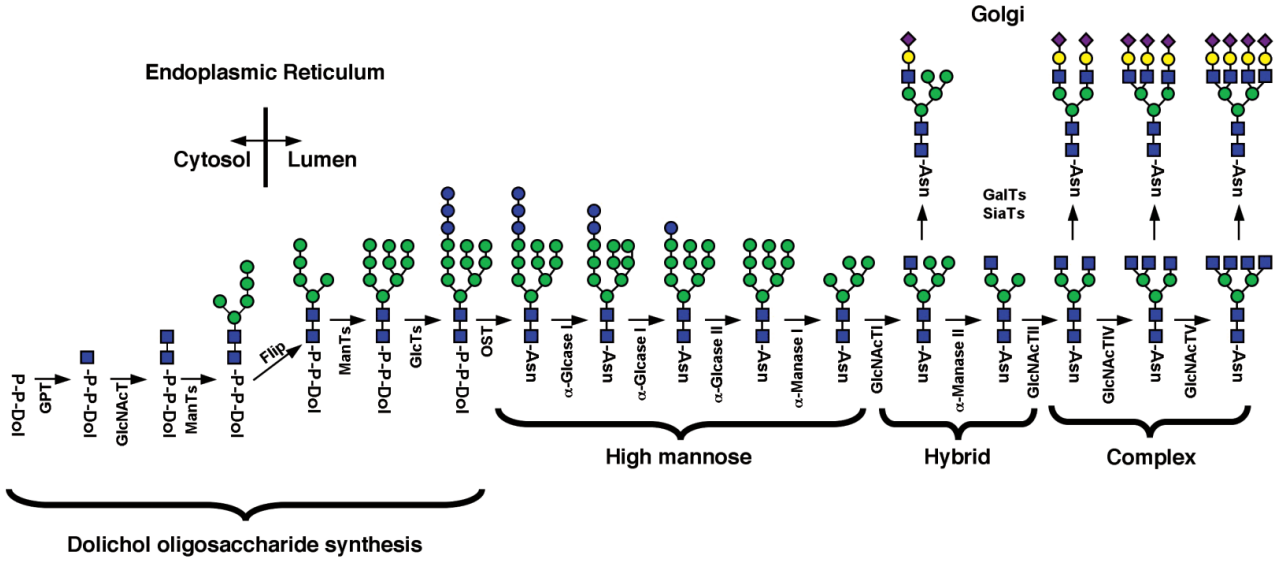

FIGURE 2 **Biosynthesis of N-linked glycans. GPT, GlcNAc phosphotransferase; GlcNAcT, GlcNAc transferase; ManT, mannosyltransferase; GlcT, glucosyltransferase; OST, oligosaccharyltransferase; Glcase, glucosidase; Manase, mannosidase; GalT, galactosyltransferase; SiaT, sialyltransferase. Symbols are described in Figure 1.**

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N-acetylglucosaminyltransferase-I (GlcNAcTI) adds the first *N*-acetylglucosamine unit to the mannose core, producing the acceptor for α-mannosidase II, and subsequently a series of other *N*-acetylglucosaminyltransferases (GlcNAcTII, TIV, and TV) initiate specific branches of bi-, tri-, and tetra-antennary chains. The final structures and abundance of N-linked glycans produced can vary dramatically in different cells and tissues, since they can undergo further modification, such as fucosylation, sialylation and sulfation. Genetic studies show that altering GlcNAcTI has profound effects on N-glycans and survival, whereas deletion of α-mannosidase II, GlcNAcTII, TIII, TIV, or TV, sialyltransferases, fucosyltransferases, and sulfotransferases leads to milder, albeit profound phenotypes often mimicking human disorders (Lowe & Marth, 2003).

Inhibitors of N-Linked Glycans

A large number of inhibitors that target N-linked glycans have been described. Most of the available agents target very early steps in the biosynthetic pathway and therefore affect the assembly of all types of N-linked chains. The available inhibitors described below have been extremely useful in probing the biological functions of N-glycans both in cell free systems and in cells or tissues (Tables 3 and 4).

Monosaccharide Inhibitors

One class of inhibitors consists of modified monosaccharides that resemble naturally occurring precursors and therefore are incorporated into the nucleotide sugar pools within cells and eventually into the nascent glycans (Table 3). By selectively altering specific functional groups, their incorporation can affect further processing of the chains. For example, several deoxygenated sugars can be converted into their corresponding uridine nucleotide analogs in cells and subsequently incorporated into the glycan by glycosyltransferases. If a crucial hydroxyl group is removed, its incorporation can terminate further extension (Schwarz & Datema, 1982; Elbein, 1987). Fluorinated derivatives of *N*-acetylglucosamine have been made as well (Dimitroff *et al.*, 2003). The major limitation of this approach is that the analogs lack specificity since they can be incorporated into other glycan subclasses and they can be converted into other precursors through intermediary metabolism. Thus, associating changes in growth or other cellular properties with alterations in a specific class of glycans is difficult.

Inhibitors of Dolichol Precursor Assembly

Several antibiotics have been described that block the biosynthesis of the dolichol oligosaccharide precursor (Table 3). Tunicamycin is a nucleoside analog isolated from *Streptomyces lysosuperificus* that inhibits the formation of GlcNAc-PP-dolichol by blocking the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to dolichyl-P catalyzed by GlcNAc phosphotransferase (GPT, Figure 2) (Elbein, 1987). Tunicamycin acts as a tight binding competitive inhibitor (Ki for tunicamycin $\sim 5 \times 10^{-8}$ M) because it resembles the donor nucleotide sugar (Km value for UDP-GlcNAc $\sim 3 \times 10^{-6}$ M) (Figure 3). Other fungal antibiotics that alter N-linked glycosylation include amphomycin, showdomycin, and diumycin (Table 3). Amphomycin inhibits the production of GlcNAc-PP-dolichol by binding to dolichol-P, whereas the other antibiotics primarily reduce the production of $(GlcNAc)_2$-PP-dolichol (Kean & Wei, 1998). Conflicting data exists regarding the effects of these compounds in different experimental settings, which could reflect variable uptake and culture conditions or differences in the level of expression of the transferases.

Inhibitors of Processing Enzymes

Maturation of nascent N-linked chains transferred to glycoprotein substrates requires the action of α-glucosidases and α-mannosidases that trim glucose and mannose residues from $Glc_3Man_9GlcNAc_2$ oligosaccharide (Figure 2). Several plant alkaloids have been described that inhibit these enzymes. All have in common polyhydroxylated ring systems that mimic the orientation of hydroxyl groups in the natural substrates (Figure 3), but the stereochemistry of the compound does not always correlate with the specificity of the enzyme target (α-glucosidase *vs.* α-mannosidase). The compounds contain nitrogen, usually in place of the ring oxygen, which when protonated may mimic the positive charge on the ring oxygen of sugars that arises during hydrolysis (Asano *et al.*, 2000).

The most widely used inhibitors in this class include castanospermine and deoxynojirimycin (Elbein, 1987; Elbein, 1991) (Figure 3). The α-glucosidase inhibitors differ in specificity towards α-glucosidase I and II and therefore alter N-linked biosynthesis in different ways. Castanospermine inhibits both α-glucosidase I and II and causes the accumulation of fully glucosylated chains. In contrast, other inhibitors selectively

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TABLE 3 Examples of inhibitors that block N-glycan biosynthesis in cells or tissues

Inhibitor	Target
Monosaccharide Inhibitors	
2-deoxy-Glc	Man($GlcNAc_2$)-PP-dolichol formation by 2-deoxyglucose-($GlcNAc)_2$-PP-dolichol
2-fluoro-Glc, 4-fluoro-Glc, 2-fluoro-Man	Man($GlcNAc_2$)-PP-dolichol formation
4-fluoro-Man	$Man_2(GlcNAc)_2$-PP-dolichol formation
Inhibitors of dolichol precursor assembly	
Tunicamycin	GlcNAc-PP-dolichol
Amphomycin	GlcNAc-PP-dolichol
Showdomycin	$GlcNAc_2$-PP-dolichol
Diumycin	$GlcNAc_2$-PP-dolichol
Processing inhibitors	
Castanospermine	α-glucosidase I and II
Australine	α-glucosidase I
Deoxynojirimycin	α-glucosidase II
Kifunensine	α-mannosidase I
Deoxymannojirimycin	α-mannosidase I and II
α-D-mannopyranosyl-methyl-p-nitrophenyltriazene	α-mannosidase I
1,4-Dideoxy-1,4-imino-D-mannitol	α-mannosidase I
Swainsonine	α-mannosidase II
Mannostatin	α-mannosidase II

inhibit α-glucosidase I or II leading to the accumulation of N-linked chains with one or two glucose residues (Table 3). Because this class of inhibitors acts after the oligosaccharide precursor is transferred to proteins, they block further processing of the chain resulting in global loss of complex chains. After removal of glucose residues, further trimming occurs by α-mannosidases. Deoxymannojirimycin, 1,4-dideoxy-1,4-imino-D-mannitol, α-D-mannopyranosylmethyl-p-nitrophenyltriazene inhibit ER α-mannosidase I and cause accumulation of $Man_{7-9}GlcNAc_2$ oligosaccharides on glycoproteins (Elbein, 1991). Swainsonine and mannostatin A block Golgi α-mannosidase II, causing accumulation of $Man_5GlcNAc_2$ glycans and an accumulation of hybrid and complex type glycans.

TABLE 4 Examples of inhibitors that block specific enzymes in N-linked glycan biosynthesis in cell free systems

Nucleotide sugar analogs	
UDP-2-fluoro-Gal	α4GalT and β4GalT
GDP-2-fluoro-1-Fuc	α3FucTV
GDP-carba-Fuc	α3FucTV
GDP-2-flouro-Fuc	FucTIII, TV, TVI and TVII
GDP-6-flouro-Fuc	FucTIII, V, VI and VII
CMP-3-fluoro-Neu5Ac	α6SiaT
Acceptor analogs	
Manα6(Manα3)Man-octyl	β2GlcNAcTI
GlcNAcβ2Manα3Man-octyl	β2GlcNAcTII
2-deoxy-Manα6 (GlcNAcβ2Manα3) Man-octyl	β2GlcNAcTII
GlcNAcα2(6-deoxy) Manβ6Man-*O*−R	β6GlcNAcTV
GlcNAcα2(6-deoxy) Manβ6Glc-*O*−R	β6GlcNAcTV

Nucleotide Sugar and Acceptor Analogs

Most of the enzymes involved in glycosylation can be assayed *in vitro* using synthetic acceptors composed of one or more sugar residues conjugated to an aglycone and a nucleotide sugar donor. This encouraged a number of investigators to synthesize nucleotide sugar

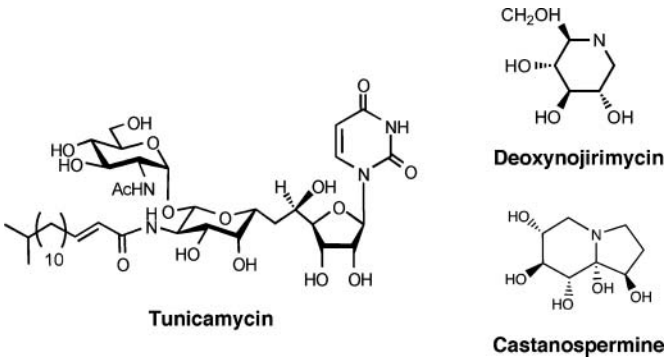

FIGURE 3 Structures of tunicamycin and two alkaloids, deoxynojirimycin and castanospermine.

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derivatives to probe the enzymes or inhibit their activity (Murray *et al.*, 1996; Hayashi *et al.*, 1997; Takayama *et al.*, 1999; Burkart *et al.*, 2000; Norris *et al.*, 2004). These agents have been useful for studying reaction mechanisms, but they lack activity in cells and tissues presumably due to poor uptake caused by the charged phosphate groups.

Glycoside acceptors usually consist of 1 to 4 sugars and mimic the acceptor ends of natural macromolecular substrates. Some have sufficiently low Km values that they compete with glycoprotein substrates and act as competitive inhibitors with Ki values in the micromolar to millimolar range. Specific modifications to the sugar residues can preclude their function as an acceptor, but some continue to bind at the active site of the target enzyme and block its activity (Palcic *et al.*, 1990; Hindsgaul *et al.*, 1991; Kajihara *et al.*, 1992, 1993; Khan *et al.*, 1993; Lowary & Hindsgaul, 1993, 1994; Lowary *et al.*, 1994; Reck *et al.*, 1994; Helland *et al.*, 1995; Paulsen *et al.*, 1995; Reck *et al.*, 1995; Lu *et al.*, 1997). In theory, these compounds could act in intact cells, by flooding the Golgi with alternate substrates. However, the hydrophilicity of disaccharides and the detergent properties of some of the compounds have limited their usefulness in cells and tissues. As discussed in the section on *O*-GalNAc linked glycans, derivatives can be made that can enter cells, act as substrates, and divert the synthesis of glycans from the endogenous glycoprotein acceptors. Thus, this class of potential inhibitors of N-linked glycans should be further developed.

Blocking N-Glycan-Protein Interactions

In theory, any carbohydrate-binding protein that recognizes N-linked glycans can be used to block their function. Agents that have proven useful include plant lectins (Rüdiger, 1998), anti-carbohydrate antibodies (Pazur, 1998), soluble animal lectins, soluble domains of membrane receptors that bind carbohydrate (Gabius *et al.*, 2002; Kilpatrick, 2002), as well as many carbohydrate-binding proteins derived from bacteria and viruses (Bovin *et al.*, 2004). Since many of these agents are multivalent, they often exhibit high avidity for cell surfaces and have cell-agglutinating activity. Many of these agents also can stimulate or diminish cell adhesion and some plant lectins are cytotoxic either by interfering with protein synthesis or by ligating cell surface receptors. These agents are attractive as candidates for anti-adhesion therapy.

Enzymatic N-Glycan Removal

The function of N-glycans can also be assessed by their enzymatic removal (Table 5). Some enzymes will cleave the N-linked glycans from proteins, irrespective of its structure, whereas others are quite specific. For example, PNGase F hydrolyzes nearly all types of N-glycans from glycoproteins, whereas endoglycosidase F1 cleaves N-linked glycans consisting of high mannose and hybrid chains, but not complex glycans. In contrast, endoglycosidase F3 cleaves N-linked biantennary and triantennary complex N-linked glycans with specific fucosylation patterns (Maley *et al.*, 1989; Tarentino & Plummer, 1994). In addition to endoglycosidases a variety of exoglycosidases exist that can remove sialic acids, fucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine residues. These enzymes remove the target residues regardless of glycan class.

Applications for N-Linked Glycan Inhibitors

While many N-linked glycan inhibitors are known, few have progressed as drugs. Two examples provided in Table 1 describe the use of the alkaloids 6-butanoyl castanospermine and swainsonine, which target α-glucosidases and α-mannosidases responsible for the removal of glucose and mannose residues. Since these inhibitors act early in the biosynthetic pathway, they reduce the complexity of the chains and alter N-linked glycan structure on all glycoproteins. Thus, their lack of specificity may limit further development and/or limit their application to only the most life-threatening diseases. No inhibitors of the dolichol-linked oligosaccharide biosynthetic enzymes have been advanced, perhaps because of similar reservations.

Inhibitors specific for the terminal modifications might prove useful since changes to the outer antennae and sugar residues are tolerated based on genetic experiments in mice (Lowe and Marth, 2003). Furthermore, some structures appear to correlate with disease, *e.g.*, formation of the β1,6 antenna initiated by GlcNAcTV is greatly elevated in certain carcinomas and has been correlated with tumor growth (Granovsky *et al.*, 2000).

Two drugs currently on the market target the neuraminidase expressed by influenza, which removes sialic acid residues and aids in viral spread (Tamiflu® and Relenza®) (Table 1). The first inhibitor for neuraminidase was deduced by assuming that the

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TABLE 5 Examples of enzymes that can remove or deglycosylate N-glycans

Enzyme (source)	Cleavage activity
Endoglycosidase F1 (*Chryseobacterium meningsepticum*)	Cleaves between chitobiose core of hybrid biantennary chains, but not complex type. Core fucosylation reduces activity.
Endoglycosidase F2 (*Chryseobacterium meningsepticum)*	Cleaves between chitobiose core of complex biantennary chains
Endoglycosidase F3 *(Chryseobacterium/Flavobacterium)*	Biantennary and triantennary complex chains depending on the state of core fucosylation
Endoglycosidase H (*Streptomyces plicatus)*	Cleaves between chitobiose core of oligomannose and hybrid, but not complex chains
PNGase A *(almonds)*	Cleaves between GlcNAc and asparagine residue of high mannose, hybrid and complex glycans deaminating the asparagine to aspartic acid
PNGase F (*Chryseobacterium/Flavobacterium*)	Cleaves between GlcNAc and asparagine residue of high mannose, hybrid and complex glycans, but not glycans containing core α1-3fucose deaminating the asparagine to aspartic acid
Neuraminidase (Newcastle disease virus, *Arthrobacter ureafaciens*)	Removes sialic acid residues from glycans
Fucosidase (Multiple sources)	Removes fucose residues from glycans

hydrolysis reaction probably involved a transition state with a carbocation intermediate at C2 of sialic acids, which would result in C2 and C3 adopting a trigonal planar configuration. Thus, compounds that mimic this configuration could block the enzyme. Neu5Ac-2-ene (DANA) has a micromolar Ki value and an analog containing a positively charged guanidinium group instead of O4 (4-guanidino-DANA) has a Ki value of 10^{-11} M, presumably due to an additional salt bridge formed between the charged guanidinium group and the carboxylates lining the active site (Figure 4). These agents block activity and diminish further spread of the virus. Interestingly, these agents are highly selective for influenza neuraminidase and do not affect the activity of mammalian sialidases.

Finally, it should be pointed out that glycan-based inhibitors that bind to pharmacologically relevant proteins could also be useful (Sharon, 2006). In theory, any oligosaccharide or glycan mimetic can be used to interfere with normal glycan function in cells or tissues. Thus, an oligosaccharide derived from citrus pectin binds to galectin-3 and induces apoptosis of cancer cells (Chauhan *et al.*, 2005). An especially appealing aspect of this approach is that competitive glycans could act as adjuvants with conventional therapeutic agents, thus allowing reduction of their concentration and undesirable side effects. Other examples of blocking glycan function by exogenous administration of oligosaccharides are discussed in the next section.

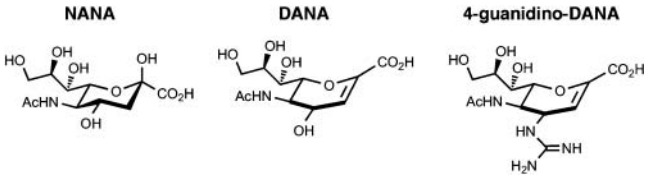

FIGURE 4 Three examples of influenza neuraminidase inhibitors. NANA, N-acetyl-neuraminic acid (Neu5Ac); DANA, 2-deoxy 2,3-dehydro-*N*-acetyl neuraminic acid; and 4-guanidino-DANA (Relenza, zanamivir).

Serine/Threonine O-Linked Mucins and Glycoproteins

Several classes of O-linked glycans exist, the classical type containing a glycan attached to proteins *via* α-*N*-acetylgalactosamine (GalNAc) to the hydroxyl group of serine/threonine side chains and others that contain *O*-fucose, *O*-glucose, or *O*-mannose. The *O*-GalNAc-linked glycans are found on many membrane and secreted glycoproteins and in great abundance on mucins made by epithelial cells that line ductal tissue (*e.g.*, alimentary and urogenital tracts and glandular tissues such as the salivary and mammary glands), leukocytes, and endothelial cells (Hanisch, 2001). The high capacity of mucins to bind water ensures adequate hydration of

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epithelial surfaces provides a barrier function and aids in clearance of foreign material from tissue (*e.g.*, in the pulmonary tract). Membrane bound mucins on leukocytes and endothelial cells serve as ligands for adhesion receptors (Varki, 1997; Rosen, 2004).

O-linked fucose-containing glycans have been described on proteins containing EGF repeats, such the Notch family of receptors and Notch ligands (Delta, Serrate/Jagged), and on proteins containing thrombospondin type 1 repeats (Haltiwanger & Lowe, 2004; Lu & Stanley, 2006). As discussed below, the *O*-GalNAc linked glycans vary in size and complexity, whereas *O*-fucose linked glycans appear to be more homogeneous (*e.g.*, Siaα3/6Galβ4GlcNAcβ3Fucα-*O*-Ser/Thr). *O*-glucose containing glycans have not been characterized in great detail (Shao *et al.*, 2002). *O*-mannose glycans are prevalent in brain and a major substituent on α-dystroglycan, an essential component of the dystrophin-glycoprotein complex, which links the actin cytoskeleton to extracellular matrix in muscle and nervous tissues (Endo & Manya, 2006). Defects in formation of the glycan chain on dystroglycan can result in congenital muscular dystrophy (Barresi & Campbell, 2006).

Biosynthesis of O-Linked Glycans

The formation of GalNAcα-*O*-Ser/Thr is catalyzed by a family of polypeptide α-*N*-acetylgalactosaminyltransferases (ppGalNAcTs) numbering 24 in vertebrate genomes (Figure 5) (Ten Hagen *et al.*, 2003). The enzymes display overlapping substrate specificities *in vitro* although some have unique properties. For example, ppGalNAcT3 glycosylates the HIV-V3 peptide, whereas ppGalNAcT1 and -T2 cannot (Van den Steen *et al.*, 1998). Two of the enzymes act selectively on proteins that already contain *N*-acetylgalactosamine residues. GalNAcα-Ser/Thr (termed the "Tn" antigen) is further elaborated by a β3galactosyltransferase (β3GalT) to form the "T" antigen, the building block for Core 1 O-glycans. The importance of Core 1 O-linked glycans has been demonstrated in mutant mice, which die during embryonic development with severe angiogenic defects (Xia *et al.*, 2004). Core 2 glycans arise from the action of one or more β1,6 *N*-acetylglucosaminyltransferases (β6GlcNAcT) (Fukuda, 2002). Knockout mice revealed that Core 2 O-linked glycans are not required for development but play an important role in inflammation and myeloid homeostasis (Ellies *et al.*, 1998; Snapp *et al.*, 2001). Thus, reactions downstream from Core 1 synthesis could be favorable pharmaceutical targets. Although, other types of *O*-GalNAc cores exist (Cores 3-8), these are less common and their expression is more limited (Brockhausen, 2006).

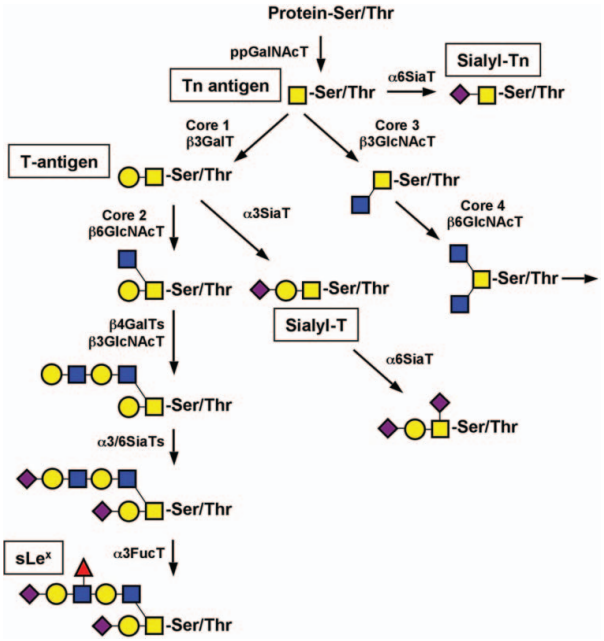

FIGURE 5 **Biosynthesis of *O*-GalNAc linked glycans. ppGalNAcT, polypeptide GalNAc transferase; GalT, galactosyltransferase; GlcNAcT, GlcNAc transferase; SiaT, sialyltransferase; FucT, fucosyltransferase. Symbols are described in Figure 1.**

O-linked and N-linked glycans are often elongated by adding poly-*N*-acetyllactosamine (Galβ4GlcNAc or Galβ3GlcNAc) units. Their biosynthesis is catalyzed by alternating action of i-extension enzyme (iGlcNAcT) and β1,4 galactosyltransferase IV (β4GalTIV). These structures can be further modified by adding α2,3 or α2,6 sialic acid and/or α1,3 or α1,4 fucose. The Lewis antigens (Le^X, sLe^X, Le^Y), type-1 blood group antigens (Le^a and Le^b) and blood group antigens (A, B, H) are some of the better characterized structures found on poly-*N*-acetyllactosamine chains.

Formation of *O*-fucose and *O*-mannose linked glycans depends on specific polypeptide *O*-fucosyltransferase (POFUT) and *O*-mannosyltransferases (POMT) (Haltiwanger & Lowe, 2004). Enzymes that add the *N*-acetylglucosamine units have been described as well (Fringe enzymes and POMGnT1, respectively). β1,4galactosyltransferase and α2,3sialyltransferases then complete the chains.

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Inhibitors of O-Linked Glycans

In contrast to N-linked glycans (Tables 3 and 4), fewer inhibitors of O-linked glycan biosynthesis have been described (Table 6). In part this may reflect lack of emphasis in the field, which historically focused on N-linked chains perhaps due to their utility for studying the secretory pathway in cells and greater complexity (lipid linked precursors, processing steps, and topological constraints). However, as discussed below, inhibitors of O-linked glycan formation have great promise as therapeutics for treating cancer and inflammation.

Monosaccharide inhibitors

Since *O*-GalNAc glycans on leukocyte mucins act as ligands for selectin adhesion receptors and mediate leukocyte trafficking during inflammation and tumor metastasis (Varki, 1994), considerable interest exists in finding inhibitors to block these interactions. 4-fluoro-GlcNAc has been used to alter selectin ligand expression on T-cells (Dimitroff *et al.*, 2003; Dimitroff *et al.*, 2003). Apparently, the cells activate and incorporate the derivative into nascent poly-*N*-acetyllactosamine chains, preventing further polymerization by blocking the attachment site for galactose. The compound attenuates lymphocyte E-selectin ligand expression in skin-draining lymph nodes and decreases the capacity of effector T cells to enter antigen-challenged skin, thus preventing allergen-induced contact dermatitis (Descheny *et al.*, 2006). Its use in preventing tumor formation and metastasis was shown several years ago, but further studies have not been reported (Woynarowska *et al.*, 1996; Dimitroff *et al.*, 1998). In general, this compound must be added to culture medium at millimolar concentrations to compete with glucose and other sugars for

TABLE 6 **Examples of *O*-linked glycan inhibitors**

Inhibitor	Target	Cellular Activity
Monosaccharide inhibitors		
4-fluoro-GlcNAc	Poly-*N*-acetyllactosamine	Yes
Enzyme inhibitors		
Uridine analog 1-68A	ppGalNAcTs	Yes
Uridine analog 1-143	UDP-Glc/GlcNAc C_4-epimerase	No
Acceptor analogs		
GalNAcα-*O*-benzyl	β3GalT (Core 1), all *O*-GalNAc glycans	Yes
Acetylated Galβ4GlcNAcβ-*O*-napthalenemethanol	Glycosyltransferases involved in sLe^X biosynthesis	Yes
Acetylated GlcNAcβ3Galβ-*O*-naphthalenemethanol	Glycosyltransferases involved in sLe^X biosynthesis	Yes
Acetylated 4-deoxy-GlcNAcβ3Galβ-*O*-napthalenemethanol	β4GalTI	Yes
1-thio-N-butyryl-N-GlcNAcβ-*O*-2-naphthol	β4GalTI	No
4-deoxy-Galβ3GlcNAc$-$*O*-8-methoxycarbonyloctyl	α3/4FucT	No
Fucα2(3-deoxy)Galβ-*O*-octyl	α3GalNAcTA	No
Fucα2(3-fluoro)Galβ-*O*-octyl	α3GalT	No
2-deoxy-Galβ3GlcNAcβ-*O*-methoxycarbonyloctyl	α2FucT	No
Galβ3(4-deoxy)GlcNAcβ-*O*-methoxycarbonyloctyl	α4FucT	No
6′-Deoxy N-acetyllactosamineβ-*O*-methyl	α6SiaT	No
Blocking glycan-protein interactions		
sLe^X analog GSC-150	Selectins	Yes
1-deoxy-sLe^X analogs	Selectins	Yes
1-deoxy-3′-*O*-sulfo Le^X analogs	Selectins	Yes
1-deoxy-3′-*O*-phoshono Le^X analogs	Selectins	Yes
Biomosiamose	Selectins	Yes
OJ-R9188	Selectins	Yes
Benzoic acid derivatives	Selectins	Yes
Enzymatic glycan removal		
O-sialoglycoprotease	Highly sialylated mucins	

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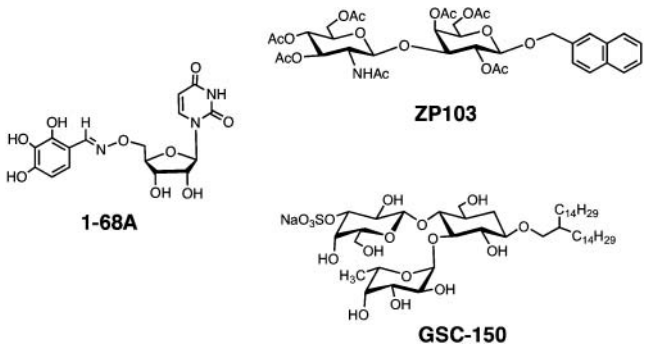

FIGURE 6 **Three examples of O-GalNAc glycan inhibitors.**

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uptake. Thus, its use *in vivo* might be limited by undesirable side reactions or multiple effects on other glycans containing *N*-acetylglucosamine.

Enzyme Inhibitors

One strategy for the design of enzyme-based inhibitors has been to synthesize nucleotide sugar donor analogs of UDP-GalNAc (Table 6). A UDP-Glc/GlcNAc:C4-epimerase inhibitor with a Ki of 11 μM was identified from a uridine-based library (Winans & Bertozzi, 2002) (Table 6). The same library yielded two inhibitors of the ppGalNAcTs with Ki values of ~8 μM (Table 6, Figure 6) (Hang *et al.*, 2004). Incubation of cells with these inhibitors results in a marked decrease in cell surface *O*-GalNAc glycans without affecting N-linked glycans. These compounds rapidly induced apoptosis in cultured cells and in glandular tissue (Tian *et al.*, 2004), which may reflect the ability of the compounds to inhibit multiple isozymes. New derivatives that target individual ppGalNAcTs might prove more selective. Inhibitors of other enzymes unique to O-linked glycan synthesis have not been described.

Acceptor Analogs

As in N-glycan synthesis, acceptor analogs represent another starting point for designing inhibitors. Hindsgaul and others have synthesized a number of compounds that target specific glycosyltransferases *in vitro* (Hindsgaul, 1991; Hindsgaul *et al.*, 1991; Kajihara *et al.*, 1992; Kajihara *et al.*, 1993; Khan *et al.*, 1993; Lowary & Hindsgaul, 1993; Lowary & Hindsgaul, 1994; Lowary *et al.*, 1994; Helland *et al.*, 1995; Paulsen *et al.*, 1995; Lu *et al.*, 1997; Chung *et al.*, 1998; Laferte *et al.*, 2000; Mukherjee *et al.*, 2000; Brockhausen *et al.*, 2005; Westerlind *et al.*, 2005; Brockhausen *et al.*, 2006). Bisubstrate analogs have also been prepared consisting of the nucleotide donor covalently attached to the acceptor substrate by way of a neutral bridging group (Palcic *et al.*, 1989; Hashimoto *et al.*, 1997; Takayama *et al.*, 1999; Mitchell *et al.*, 2002; Schwörer & Schmidt, 2002; Hinou *et al.*, 2003; Skropeta *et al.*, 2003; Izumi *et al.*, 2005; Izumi *et al.*, 2006). Most of these types of compounds block glycosyltransferases *in vitro*, with Ki values in the range of the binding constant for the natural substrate (see Jung & Schmidt, 2003). However, they do not exhibit inhibitory activity in cells due to poor membrane permeability. The large number of polar hydroxyl groups and the lack of membrane transporters for oligosaccharides in most cells prevent their uptake.

In contrast to disaccharide and larger oligosaccharide glycosides, monosaccharide glycosides can passively diffuse across the plasma and Golgi membranes and serve as substrates for O-glycan formation. For example, *N*-acetylgalactosaminides (*e.g.*, GalNAcα-*O*-benzyl) are taken up and utilized as a substrate for *O*-linked oligosaccharides similar to those found on mucins (Kuan *et al.*, 1989; Zhuang *et al.*, 1991; Kojima *et al.*, 1992; Chen *et al.*, 2006). Assembly of glycans on the glycoside diverts its synthesis from endogenous glycoprotein substrates, thus inhibiting the formation of mature glycoconjugates. GalNAcα–*O*-benzyl has been used to alter expression of Lewis blood group antigens on the surface of cells, which in turn inhibited adhesion of treated cells to activated endothelial cells (Kojima *et al.*, 1992), Similarly, *N*-acetylglucosaminides also act as substrates for poly-*N*-acetyllactosamine chains with and without terminal sialic acid and alter the formation of chains on glycoconjugates. Interestingly, the amount as well as the structure of the primed oligosaccharide products are strongly influenced by the aglycone, which could provide a way to improve their efficacy as inhibitors (Neville *et al.*, 1995; Miura *et al.*, 1999).

Only a few monosaccharides have been reported to be primers/inhibitors, presumably because some of the enzymes require more elaborate structures as substrates. Others may have not yet been tested (*e.g.*, fucosides). Disaccharides are also active and have a distinct advantage over monosaccharides in that they more closely resemble natural intermediates and therefore will better target specific enzymes in the biosynthetic pathway. The large number of polar hydroxyl groups makes them relatively impermeable (Sarkar *et al.*, 1995). This problem can be circumvented by covering the hydroxyl groups with biologically reversible blocking groups, such as short chain acyl esters or acetoxymethyl

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esters (Dennis *et al.*, 1993; Schultz *et al.*, 1993). Cells possess carboxyesterases for removing esters, and apparently this occurs in a way that makes the deblocked compounds available to the biosynthetic apparatus in the Golgi (Sarkar *et al.*, 1995).

Several peracetylated disaccharide compounds (*e.g.*, per-*O*-acetylated Galβ1-4GlcNAcβ-*O*-naphthalenemethanol (NM), per-*O*-acetylated GlcNAcβ1-3Galβ-*O*-NM, and per-*O*-acetylated Galβ1-3GalNAcβ-*O*-NM) are deacetylated to form primers of oligosaccharide synthesis, generating products related to *O*-GalNAc linked glycans (Brown *et al.*, 2003). These compounds divert the assembly of the O-linked chains from endogenous glycoproteins, much like GalNAcβ-*O*-benzyl, resulting in inhibition of expression of terminal Lewis antigens that are recognized by selectins (Sarkar & Esko, 1995; Sarkar *et al.*, 1995, 1997, 2000; Brown *et al.*, 2003). Inhibition occurs at a much lower dose than that for monosaccharide glycosides ($\sim$25 μM *versus* 1–2 mM, respectively). Activity also depends in part on the structure of the aglycone (Miura *et al.*, 1999; Mong *et al.*, 2003). Brown and coworkers have recently identified a peracetylated 4-deoxy-modified disaccharide that inhibits β1,4galactosyltransferase TI involved in sLe^X biosynthesis and blocks experimental lung metastasis in mice (Brown *et al.*, 2008).

Blocking O-glycan-Protein Interactions

Several strategies have been developed for blocking O-glycan-protein interactions, specifically focused on selectin-binding glycans. These include (i) competition by glycolipids and soluble recombinant forms of selectins and glycoprotein ligands, (ii) peptides based on the primary sequence of the carbohydrate binding site, (iii) anti-selectin antibodies, (iv) oligosaccharides related to Le^A and Le^X, (v) inositol polyanions and sulfated sugars, (vi) heparin, and (vii) molecular mimics of sLe^X, including oligonucleotides (for a review, see (Chhabra *et al.*, 2003). This approach has much appeal since pharmacological blockade of protein-carbohydrate interactions can be initiated quickly by intravenous injection of the inhibitor. In contrast, the glycoside primers and monosaccharide inhibitors require metabolism and turnover of existing glycans. Some of the agents that block glycan-protein interactions require high concentrations, due to the low affinity of most protein-carbohydrate binding interactions (micromolar to millimolar).

Enzymatic O-Glycan Removal

O-glycans can be removed by O-Glycanase (Endo-α-*N*-acetylgalactosaminidase) isolated from *Streptococcus pneumoniae* or by recombinant enzyme expressed in *E. coli*. Many of the exoglycosidases that act on N-linked glycans also work on O-linked glycans since they share terminal structures (*e.g.*, galactose, *N*-acetylglucosamine, sialic acids, and fucose. A mucin-specific endopeptidase called *O*-sialoglycoprotease can also be used to selectively remove sialylated mucins from the surface of cells (Kim *et al.*, 1999). Endoglycosidases that act on *O*-glycan chains have not been described.

Applications for O-Linked Glycan Inhibitors

O-linked glycans have a number of important biological functions. For example, sialyl Lewis X (sLe^X; NeuAcα2,3Galβ1,4(Fucβ1,3)GlcNAc) on *O*-GalNAc linked mucin-type glycans on leukocytes plays a crucial role in inflammation by facilitating leukocyte rolling. Tumor cells also express mucins containing related carbohydrate ligands. Binding of platelets to tumor cells mediated through selectin-mucin interactions results in tumor cell protection against cytolytic elements of the immune system and permits aggregates to form, which may facilitate seeding in the microvasculature during blood-borne metastasis (Kim *et al.*, 1998, 1999; Fuster *et al.*, 2003). Attachment of tumor cells to endothelial selectins also may facilitate metastatic seeding. Clinical data supports this model, wherein patients that type positive for sLe^X have poor prognosis and survival due to metastatic tumor spread (Hoff *et al.*, 1989, 1990; Nakagoe *et al.*, 1993; Nakamori *et al.*, 1993, 1997). Tumor mucins shed from tumor cells into the circulation also can cause Trousseau syndrome, a spontaneous, superficial migratory thrombophlebitis that correlates with platelet-rich clots in small blood vessels (Wahrenbrock *et al.*, 2003). Thus, O-linked glycan structures together with their biosynthetic enzymes are important therapeutic targets for anti-inflammatory and anti-metastatic treatment.

Per-*O*-acetylated GlcNAcβ3Galβ-*O*-naphthalenemethanol (ZP103), has been tested *in vitro* and *in vivo* as an antimetastatic agent (Tables 1 and 6). This compound reduces sLe^X expression on tumor cells *in vitro* and blocks selectin-dependent tumor cell adhesion to recombinant selectins, activated platelets, and activated endothelial cells (Sarkar *et al.*, 1997; Fuster

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et al., 2003). Treated cells show reduced tumor burden in experimental metastasis models. Importantly, ZP103 only reduces the level of reduction of sLe^X expression on tumor cells by two- to three-fold, yet this effect was sufficient to reduce metastasis presumably because tumor cell adhesion to platelets and endothelial cells is a multivalent process. Subcutaneous infusion of ZP103 also reduced spontaneous metastasis of tumor cells in mice (Brown *et al.*, 2006).

Shirota and coworkers developed GSC-150, a sLe^X analog, and showed suppression of inflammation and reduced liver metastasis when administered to mice (Shirota *et al.*, 2001) (Figure 6). Ulbrich and coworkers described dibenzoic acid-based pan-selectin inhibitors that block rolling and E-selectin adhesion in an induced peritonitis model of acute inflammation in mice (Table 6) (Ulbrich *et al.*, 2006). Biomosiamose (Retovar Biopharmaceuticals) is an effective pan-selectin inhibitor that attenuates late asthmatic reactions (Beeh *et al.*, 2006) (Table 1). The synthetic selectin blocker ([*N*-(2-tetradecylhexadecanoyl)-*O*-(L-α-fucofucosyl)-D-seryl]-L-glutamic acid 1-methylamide 5-L-arginine salt, OJ-R9188) inhibits infiltration of leukocytes in an allergic dermatitis model *in vivo* (Ikegami-Kuzuhara *et al.*, 2001).

Interestingly, few enzyme-based inhibitors have been described in the system. Genetic studies demonstrate several suitable enzymatic targets for inhibitor design. For example, genetic inactivation of ST3Gal-IV demonstrated its role in the biosynthesis of selectin ligands in leukocytes, whereas other sialyltransferases contribute to their synthesis in tumor cells (Ellies *et al.*, 2002). FucTVII and to a lesser extent FucTIV are required for sLe^x biosynthesis in leukocytes (Maly *et al.*, 1996; Homeister *et al.*, 2001). It should be possible to adapt high-throughput screening methods to target these enzymes for discovery of new inhibitors (Winans & Bertozzi, 2002; Best *et al.*, 2004; Bryan *et al.*, 2004).

Malignancy can result in altered expression of O-linked glycans, especially on mucins. Incomplete glycosylation and elevated expression of Tn and T antigens often occurs (Figure 5). Normal epithelial cells do not express these truncated glycans frequently, and a correlation exists between the expression of these antigens, the presence of anti-Tn and anti-T serum antibodies, and the prognosis of patients with carcinomas. Thus, O-linked glycans present on mucins and the mucin polypeptide backbone itself have received attention as potential targets for vaccine development. One approach is to induce immune responses by injecting patients with muc-1 peptides or synthetic peptide antigens bearing Tn, sialyl-Tn, or polysialic acid (Holmberg & Sandmaier, 2004; Krug *et al.*, 2004; Acres & Limacher, 2005; Gilewski *et al.*, 2007). Potentially, agents that inhibit O-linked glycosylation at early steps in the pathway could result in the appearance of some of these determinants as well and provoke an immune response.

Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) are linear glycans that contain alternating amino sugars (*N*-acetylglucosamine or *N*-acetylgalactosamine) and uronic acids (glucuronic acid and L-iduronic acid) or galactose. The six major types of GAGs are heparan sulfate (HS) and heparin, chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronan (HA) and keratan sulfate (KS). HS, heparin, CS, DS, and KS are assembled on core proteins (proteoglycans), whereas HA is made as a free glycan. Each type of GAG has unique physical and biological properties. Altering their composition could provide ways to modulate a number of pathophysiological conditions, *e.g.*, amyloid plaque formation, inflammation, tumor growth, angiogenesis and metastasis, excessive scarring, and spinal cord and corneal repair. Thus, there is much interest in developing inhibitors as drug leads.

Biosynthesis of Glycosaminoglycans

HS, heparin, CS and DS biosynthesis initiate with the transfer of xylose from UDP-xylose to specific serine residues of proteoglycan core proteins (Figures 7 and 8). Specificity exists with respect to sites of attachment, which always contain a glycine residue to the C-terminal side of the serine residue and usually one or more acidic residues (Zhang & Esko, 1994; Wang *et al.*, 2007). Thereafter, two residues of galactose and one of glucuronic acid are added to form the tetrasaccharide linkage region, GlcAβ3Galβ3Galβ4Xylβ-*O*-L-Ser. The subsequent addition of *N*-acetylglucosamine initiates HS synthesis, whereas the addition of *N*-acetylgalactosamine initiates chondroitin synthesis (Figures 7 and 8).

After addition of the initiating *N*-acetylglucosamine residue, HS biosynthesis occurs by the alternating addition of GlcAβ1,4 and GlcNAcα1,4 units. A series of enzymes modify the polymer by *N*-deacetylation

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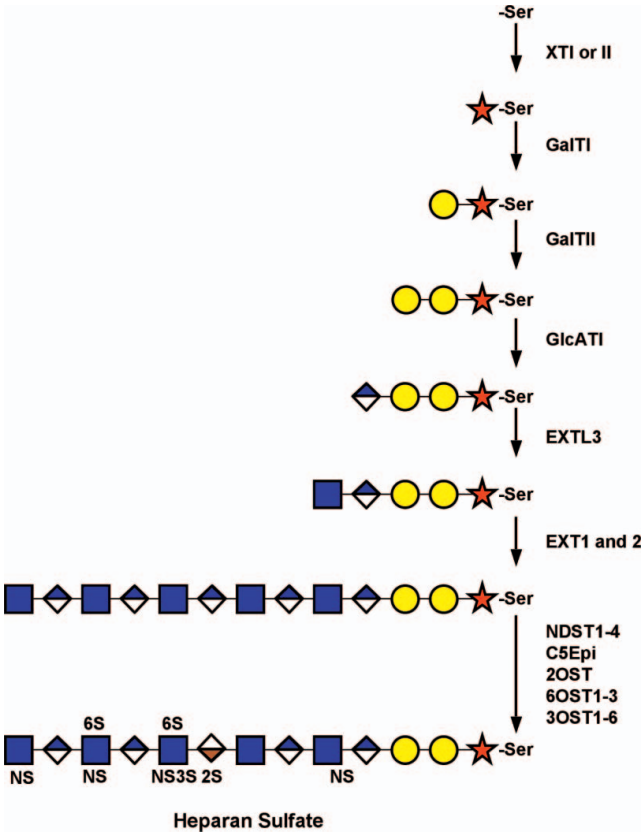

FIGURE 7 **Biosynthesis of heparan sulfate. XT, xylosyltransferase, GalT, galactosyltransferase; GlcATI, glucuronosyltransferase; EXTL3, GlcNAc transferase I; EXT1/EXT2, copolymerase complex (GlcNAc transferase/Glucuronosyltransferase); NDST, GlcNAc N-deacetylase/N-sulfotransferase; C5epi, uronosyl C5 epimerase; 2OST, uronyl-2-O-sulfotransferase; 6OST, glucosamine 6-O-sulfotransferase; 3OST, glucosamine 3-O-sulfotransferases. Symbols are described in Figure 1.**

and *N*-sulfation of *N*-acetylglucosamine residues, C5 epimerization of adjacent glucuronic residues to iduronic acid, 2-O sulfation of iduronic acid and less frequently of glucuronic acids, and 3 and 6-O sulfation of glucosamine units. These modifications occur substoichiometrically in a template independent fashion, giving rise to enormous structural heterogeneity. However bias exists in the system since some of the reactions depend on prior reactions and the modifications tend to occur in restricted regions of the chain interspersed by segments containing few or no modifications (Esko & Lindahl, 2001).

CS synthesis is initiated from the same core tetrasaccharide by the addition of an *N*-acetylgalactosamine residue (Figure 8). This intermediate is extended by copolymerization of glucuronic and *N*-acetylgalactosamine units, GalNAcβ1,4GlcAβ1,3. The polymer can be modified by 4-O and/or 6-O sulfation of the *N*-acetylgalactosamine units to produce CS. In dermatan sulfate, a portion of glucuronic acids undergo C5 epimerization to iduronic acid catalyzed by an epimerase that is distinct from the one involved in HS synthesis. The iduronic acids also can be sulfated at C2 and more rarely at C3 (Kinoshita-Toyoda *et al.*, 2004). Like HS, CS modifications are incomplete and not template driven which generates a complex final product.

The specific arrangement of sulfate groups and uronic acid epimers generates binding sites for proteins. The best-studied examples are the binding of antithrombin (AT) and fibroblast growth factor 2 (FGF2) to HS. High affinity AT binding occurs to a pentasaccharide sequence, GlcNAc6S-GlcA-GlcNS3S-IdoA2S-GlcNS6S (Esko & Lindahl, 2001). In contrast, binding and signaling by FGF2 requires *N*-sulfation, 6-O sulfation, and 2-O-sulfation, but a specific linear sequence of modified sugars does not appear to be essential (Kamimura *et al.*, 2006). Instead, the ligand prefers a certain spatial arrangement of charged groups, which can be accommodated by more than one linear sequence of sulfated sugars (Kreuger *et al.*, 2006). The relevant binding/activation motifs have not been determined for most other ligands (Conrad, 1998).

Genetic studies have shown that HS is required for the normal development (Lin *et al.*, 2000; Ringvall *et al.*, 2000; Inatani *et al.*, 2003; Stickens *et al.*, 2005). However, mutations induced in specific tissues or in adult animals do not cause lethality or gross dysfunction, suggesting that small changes in HS structure can be tolerated (Fuster *et al.*, 2007; MacArthur *et al.*, 2007). Mice deficient in specific CS core proteins display severe chondrodysplasia (Arikawa-Hirasawa *et al.*, 1999; Watanabe & Yamada, 2002), whereas mice lacking a chondroitin sulfotransferase develop normally but display reduced numbers of naïve T lymphocytes in the spleen (Uchimura *et al.*, 2002). Mutants blocked in the polymerization of CS or any of the enzymes involved in DS synthesis have not yet been described.

KS is found on a limited subset of core proteins (Funderburgh, 2000, 2002), including several members of the small leucine rich proteins (lumican, keratocan, mimecan, and decorin). Its synthesis follows the pathways described for N-linked (KSI) and *O*-GalNAc linked (KSII) glycans and involves two sulfotransferases, one that adds sulfate to the C6 of galactose and another that adds to C6 of glucosamine residues of the poly-*N*-acetyllactosamine chains (Funderburgh, 2000)

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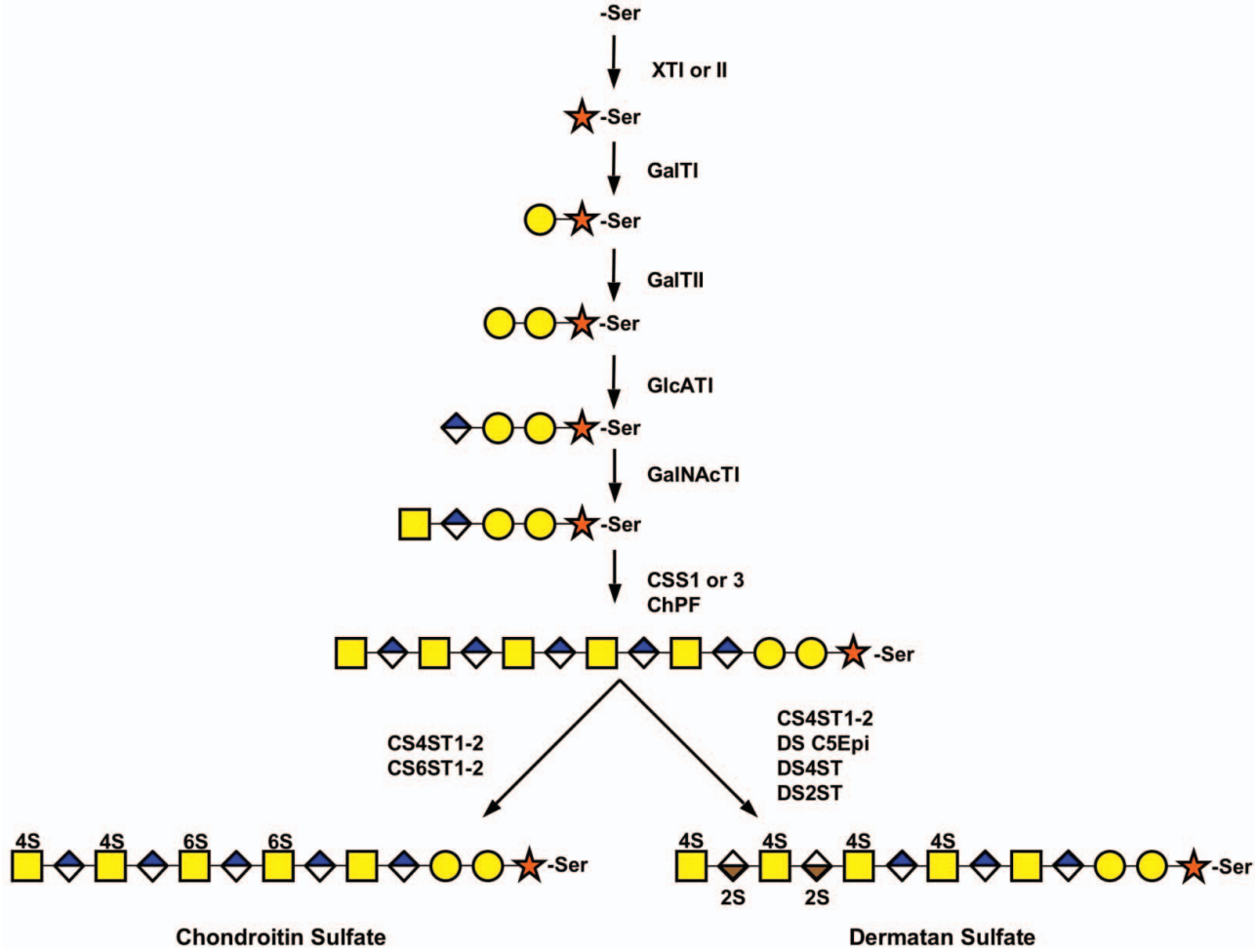

FIGURE 8 **Biosynthesis of chondroitin and dermatan sulfate. XT, xylosyltransferase, GalT, galactosyltransferase; GlcATI, glucuronosyltransferase; GalNAcTI, GlcNAc transferase I; CSS, chondroitin synthase; ChPF, chondroitin polymerizing factor; CS4ST, chondroitin sulfate GalNAc 4-O-sulfotransferase; CS6ST, chondroitin sulfate GalNAc 6-O-sulfotransferase; DSEpi, dermatan sulfate glucuronosyl C5 epimerase; DS4ST, dermatan sulfate GalNAc 4-O-sulfotransferase; DS2ST; dermatan sulfate uronyl 2-O-sulfotransferase. Symbols are described in Figure 1.**

(Figure 9). Macular corneal dystrophies (MCD) have been linked to defects in KS metabolism (Volpi, 2006). In humans, cartilage is the main tissue containing KS and KS levels in serum and urine might be a marker for osteoarthritis and other forms of cartilage damage.

HA is the simplest of GAGS, consisting of GlcNAcβ1,4GlcAβ1,3-units (Figure 10). HA synthesis occurs without a core protein via one of three HA synthases (HAS) located in the plasma membrane (Itano & Kimata, 2002). Assembly occurs from the reducing end of the chain, and the growing polymer extrudes from the cell as it polymerizes. This process contrasts the assembly of sulfated GAGs, which always occurs while attached to core protein, from the non-reducing end, and only in the Golgi. HA also is not modified by sulfation or epimerization. Due to its large size, HA has viscoelastic properties that render it an excellent lubricant and space filling molecule in tissues. HA has signaling properties mediated through specific HA-binding proteins (*e.g.*, hyalectins and TLR2) (Toole, 2004). Interestingly, HA size determines some of its signaling properties, suggesting that altering its degradation could have selective effects (Stern *et al.*, 2006).

Glycosaminoglycan Inhibitors

Metabolic Inhibitors

Inhibitors of glycosaminoglycans include agents that block the assembly of common intermediates. For example, deoxygenated and fluorinated analogs of *N*-acetylglucosamine inhibit sulfated GAG biosynthesis, presumably via activation to their nucleotide sugar analogs, which would terminate polymer extension if they were incorporated into the growing chain (Berkin

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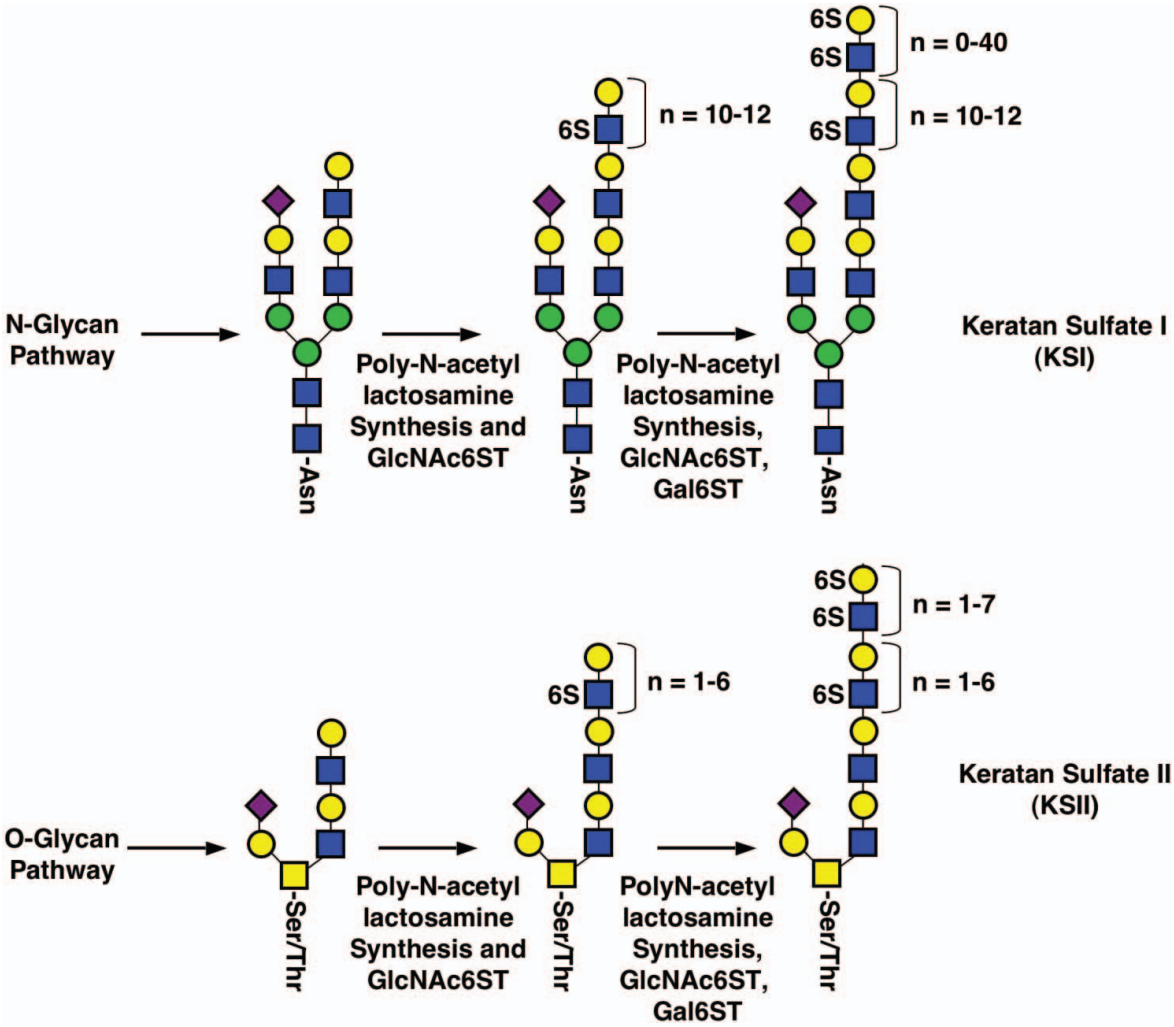

FIGURE 9 **Biosynthesis of keratan sulfate I and II. Symbols are described in Figure 1. GlcNAcT, GlcNAc transferase; GalT, Gal transferase; ST, sulfotransferase.**

et al., 2005). Since *N*-acetylglucosamine is a common component of other glycans, it is not surprising that these analogs have pleiotropic effects on glycan assembly (see Table 6). A similar caveat applies to compounds like 6-diazo-5-oxo-L-norleucine, a glutamine analog that inhibits GAG biosynthesis by reducing the availability of the hexosamine building blocks (Clark *et al.*, 1987; Iozzo & Clark, 1987). This inhibitor has found limited use due to its general mechanism of reducing UDP-*N*-acetylhexosamine concentrations that would affect a broad range of glycans.

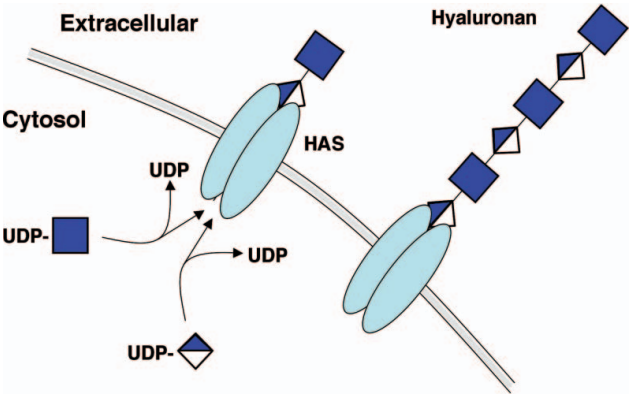

FIGURE 10 **Biosynthesis of hyaluronan. Symbols are described in Figure 1. HAS, hyaluronan synthase.**

Other types of compounds have been shown to inhibit GAG synthesis, for example, diethylcarbamazine, monensin, and brefeldin A (Stevens *et al.*, 1985; Yanagishita & Hascall, 1985; Harper *et al.*, 1986; Spiro *et al.*, 1986; Spiro *et al.*, 1991; Uhlin-Hansen & Yanagishita, 1993). These compounds alter HS, CS and DS biosynthesis by disrupting the organization of the endoplasmic reticulum and Golgi. Ammonium chloride, chloroquine, and other lysosomotropic amines have been shown to interfere with lysosomal degra-

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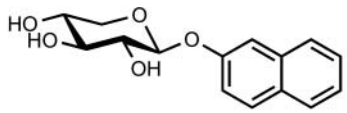
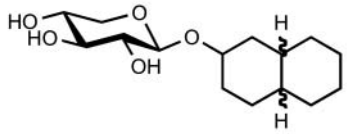


Xyl-O-naphthol



Xyl-O-*cis/trans* decahydro-2-naphthol

FIGURE 11 **Two examples of xylosides.**

dation of GAGs (Yanagishita and Hascall, 1984; Locci *et al.*, 1996). The effect that this has on the biosynthesis and function of GAGs has not been well characterized.

Enzyme Inhibitors

Selenate and sodium chlorate will decrease GAG sulfation by blocking the sulfurylase required for the formation of the universal sulfate donor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Baeuerle & Huttner, 1986). Reducing PAPS levels in this way also affects the sulfation of other glycans and tyrosine residues. By grading the concentration of chlorate, it is possible to affect the distribution of sulfate groups on the glucosamine and uronic acid units (Safaiyan *et al.*, 1999). Since chlorate competes with sulfate, the concentration of inhibitor must be adjusted according to the incubation conditions (Dietrich *et al.*, 1988; Humphries & Silbert, 1988; Keller *et al.*, 1989; Safaiyan *et al.*, 1999). However, like other metabolic inhibitors, chlorate affects multiple glycans and macromolecules, requiring some caution in interpreting its effects.

Glycosides of D-xylose (β-D-xylosides, Figure 11) resemble xylosylated core protein intermediates and thereby compete with endogenous xylosylated core proteins for galactosyltransferase I, the second enzyme in the biosynthesis of the tetrasaccharide linker region (Okayama *et al.*, 1973; Schwartz *et al.*, 1974). Adding galactose to the xyloside generates the substrate for the next reaction, and so forth, resulting in the formation of free glycosaminoglycan chains, which cells secrete. Generating free GAG chains in this way results in the accumulation of proteoglycan core proteins containing truncated GAG chains. Most xylosides prime CS and DS efficiently, but HS poorly. In part, this reflects the specificity of the first α-glucosaminyltransferase (ExtL3, Figure 7), which shows preference for the aglycone located four residues away (Fritz *et al.*, 1997). Thus, Xylβ-*O*-2-naphthol will prime HS chains as well as CS chains, whereas the closely related analog, Xylβ-*O*-decahydro-2-naphthol will not (Fritz *et al.*, 1994)(Figure 11). In parallel, Xylβ-*O*-2-naphthol is a better inhibitor of HS proteoglycan synthesis than Xylβ-*O*-decahydro-2-naphthol. The relative amount of HS primed by Xylβ-*O*-2-naphthol depends on concentration, suggesting that ExtL3 selects preferred substrates based on affinity (Zhang & Esko, 1994; Zhang *et al.*, 1995). Together, these inhibitors can be used to inhibit heparan and/or CS biosynthesis in cells and tissues (Miao *et al.*, 1995; Kantor *et al.*, 2004). They also show similar properties when administered to mice (Belting *et al.*, 2002).

Despite their wide use, xylosides have some limitations. They tend to be weak inhibitors (10 μM to 1 mM), they generate free GAG chains in addition to producing truncated chains on proteoglycans, they can also affect glycolipid biosynthesis, and they can induce the production of unusual glycans (Freeze *et al.*, 1993; Izumi *et al.*, 1994; Nakamura *et al.*, 1994; Etchison *et al.*, 1995; Manzi *et al.*, 1995; Salimath *et al.*, 1995; Shibata *et al.*, 1995). Despite these limitations, xylosides are currently regarded as the best inhibitors to block the assembly of HS, CS, and DS.

Surprisingly, few other specific inhibitors of glycosyltransferases involved in GAG synthesis have been described (Table 7). Gem-diamine 1-*N*-iminosugars related to L-iduronic acid have been identified as inhibitors of HS uronyl-2-*O*-sulfotransferase *in vitro* (Brown *et al.*, 2006). These compounds lack activity in cells, most likely due to their inability to access the 2-*O*-sulfotransferase in the Golgi of intact cells. Bertozzi and coworkers screened purine derivatives and found compounds with high selectivity towards individual sulfotransferases, suggesting that subtle differences in the PAPS binding sites can be exploited (Armstrong *et al.*, 2000; Kehoe *et al.*, 2002; Verdugo & Bertozzi, 2002). Inhibitors of HA or KS synthesis have not been described. A recent report has shown that rapamycin (Sirolimus, Rapamune®) can reduce HA biosynthesis in cultured cells by inhibiting the mTOR pathway (Table 1). However, mTOR is a central regulator of many signaling pathways, making it difficult to use it as a tool to study specifically the roles of HA.

Blocking Glycan-Protein Interactions

GAG-protein interactions can also be probed by the addition of soluble GAGs or GAG mimetics, *e.g.*, su-

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TABLE 7 Examples of glycosaminoglycan inhibitors

Inhibitor	Target
Monosaccharide inhibitors	
2-acetamido-2,4-dideoxy-Xylβ-O-methyl	Heparan sulfate
Enzyme inhibitors	
6-diazo-5-oxo-L-norleucine	Glutamine:Fructose amidotransferase (UDP-N-acetylhexosamines)
Chlorate, selenate	ATP Sulfurylase (PAPS)
Gem-diamine 1-N-iminosugars	HS uronyl 2-O-sulfotransferase
Acceptor analogs	
Xylβ-*O*-2-naphthol	β4GalTI, heparan and chondroitin sulfate
Xylβ-*O*-cis/trans decahydro-2-naphthol	β4GalTI, chondroitin sulfate
Enzymatic glycan removal	
K5 Heparan Lyase	Non-sulfated segments
Heparinase I (heparin lyase I)	Fully sulfated segments
Heparinase II (heparin lyase II)	All segments
Heparinase III (heparitinase)	Non-sulfated segments
Chondroitinase ABC	All chondroitins
Chondroitinase AC	Chondroitin-4- and chondroitin-6-sulfate
Chondroitinase B	Dermatan sulfate
Keratanase	Keratan sulfate
Hyaluronidase	Hyaluronan
Blocking glycan protein interactions	
Cationic peptides and proteins (lactoferrin, protamine, polylysine)	Heparan sulfate and other glycosaminoglycans
Anionic glycans and mimetics (suramin, sucrose octasulfate, dextran sulfates)	Heparan sulfate and other glycosaminoglycans
Guanidinylated aminoglycosides	Heparan sulfate
Aminoquinurides	Heparan sulfate
Peptidic foldamers	Heparin

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crose octasulfate, suramin, pentosan polysulfate and dextran sulfates, which presumably occupy the GAG-binding sites in proteins (Zhu *et al.*, 1993; Botta *et al.*, 2000). Another approach is to use other proteins or polypeptides containing clusters of positively charged amino acids that bind to the negatively charged sulfate and carboxyl groups, *e.g.*, protamine (Portmann & Holden, 1949), lactoferrin (Hekman, 1971), as well as synthetic peptides containing lysine and arginine (Morad *et al.*, 1984; Fuchs & Raines, 2004; Schick *et al.*, 2004; Wang & Rabenstein, 2006). The most common application of this approach is the use of heparin to interfere with HS-protein interactions, but heparin will often block other GAG-protein interactions (*e.g.*, selectins (Borsig *et al.*, 2001; Wang *et al.*, 2002; Ludwig *et al.*, 2006). Chemically modified GAGs (*e.g.*, 6-O desulfated heparin) can be used to reveal the structural requirements of a specific interaction. Other low molecular weight compounds have been discovered that bind specifically to HS and block HS-protein interactions (*e.g.*, guanidinylated-neomycin, surfen, peptidic foldamers) (Choi *et al.*, 2005; Elson-Schwab *et al.*, 2007; Schuksz *et al.*, 2007). These agents provide a simple organic scaffold that can be further modified to explore whether other more specific inhibitors can be obtained.

Enzymatic GAG Removal

The enzymatic removal of GAGs is another approach for understanding GAG function. Bacterial heparan lyases, chondroitinases, keratanases, and hyaluronidases are commercially available. These enzymes degrade the GAG chains into component disaccharide units. Different isozymes exist which cleave HS chains in regions devoid of sulfate (*e.g.*, heparin lyase III, also known as heparitinase, and an enzyme called K5 lyase (Robinson *et al.*, 2006)) or regions rich in sulfate (*e.g.*, heparinases, such as heparin lyases I and II) (Linhardt *et al.*, 1990). A number of chondroitinases exist as well that cleave in different regions (*e.g.*, chondroitinases A, B, C, and ACII) (Linhardt *et al.*, 2006). Because GAG degradation generates disaccharides and some preparations contain proteases, care must be used in interpreting the results

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of experiments employing these reagents.

Applications for Glycosaminoglycan Inhibitors

Inhibitors of GAG biosynthesis have many potential therapeutic uses, including inhibition of tumor growth and angiogenesis, repair of spinal cord injuries, and diminution of lysosomal storage of GAGs. HS is a well validated anti-cancer target that is essential for tumor growth (Esko *et al.*, 1988; Kleeff *et al.*, 1998; Sharma *et al.*, 1998; Kleeff *et al.*, 1999; Matsuda *et al.*, 2001; Lai *et al.*, 2003). Recent data show that HS is selectively required for tumor angiogenesis but not physiological angiogenesis (Fuster *et al.*, 2007). Based on these data, effective small molecule inhibitors of HS might have potential as anti-cancer drug candidates. Heparin and low molecular weight heparins also appear to extend life expectancy of patients, but the exact mechanism underlying its mode of action is unclear (Volpi, 2006; Yip *et al.*, 2006; Niers *et al.*, 2007). Its most likely activity is to block metastasis, but it may also interfere with growth factor and chemokine activation of cells. Inhibitors of HS biosynthesis are not yet in clinical use, but these agents could be used for substrate reduction therapy in lysosomal storage diseases, as antiviral agents, and in oncology applications (Roberts *et al.*, 2006; Tiwari *et al.*, 2007).

CS inhibitors would have direct application in the treatment of spinal cord injury. CS is an inhibitor of neural repair following spinal cord injury (Fawcett, 2006). Enzymatic digestion of CS with chondroitinase has shown promising results in animal models (Caggiano *et al.*, 2005). A complementary approach might employ an inhibitor of CS biosynthesis, but this class of agents has not been described.

HA has been in use clinically for nearly three decades, as a supportive matrix for intraocular surgery and for treatment of osteoarthritis. Increased HA expression is a consistent feature of a wide range of human cancers and has been linked to aggressive tumor progression (Llaneza *et al.*, 2000; Toole *et al.*, 2002; Yabushita *et al.*, 2004; Adamia *et al.*, 2005). While the mechanism by which HA increases tumor progression is unclear, HA inhibitors have potential as anti-cancer agents (Toole, 2004). Tumors also contain substantial amounts of HA and due to its capacity to hold water increases intratumor pressure. Hyaluronidase reduces the interstitial fluid pressure in solid tumors, and thereby can increase the permeation of chemotherapeutic agents (Brekken *et al.*, 2000; Heldin *et al.*, 2004). HA also plays significant roles in leukocyte adhesion and inflammation (Stuhlmeier, 2006), suggesting that decreasing its synthesis or enhancing its degradation could have anti-inflammatory effects.

Glycosphingolipids

Glycosphingolipids (GSLs) consist of ceramide (*N*-acylsphingosine) linked to a glycan composed of one or more sugars. Their assembly takes place in the Golgi and the final products reside in the outer leaflet of the plasma membrane. GSLs are distinguished by their sugar composition and linkages: ganglio-, lacto-, and neolacto-series, globo-, isoglobo-, and muco-series are all derived from lactosylceramide (Figure 12). Gangliosides contain one or more sialic acid residues. GSLs form aggregates with glycosylphosphatidylinositol (GPI) anchored proteins in cholesterol-rich microdomains called lipid rafts (Degroote *et al.*, 2004). These sites may represent centers where growth factor dependent signaling reactions occur. GSLs are especially rich in the brain, where they represent >80% of the total glycan (Schnaar, 2000). They aid in cell adhesion, for example during axon outgrowth, and in the formation of the myelin sheath.

Biosynthesis of Glycosphingolipids

GSL biosynthesis initiates by formation of ceramide in the endoplasmic reticulum by condensation of palmitate with serine, followed by acylation of the free amino group. The first glycosylation step, UDP-glucose:ceramide glucosyltransferase (GlcCer synthase), occurs in the Golgi or a pre-Golgi compartment on the cytoplasmic side of the membrane. The subsequent reactions occur within the Golgi, indicating a membrane translocation step must exist. Lactosylceramide is the precursor of most of the GSLs found in vertebrates (Figure 12). Its formation is catalyzed by lactosylceramide synthase. A number of branched pathways exist to generate a large diversity of structures (Tifft & Proia, 2000; Kolter *et al.*, 2002). Sialic acid addition generates hematosides, GM3, GD3, and GT3, which then serve as precursors for even more complex gangliosides (not shown). Some GSLs contain galactose linked to ceramide, but this pathway is less prominent in vertebrates (Figure 12).

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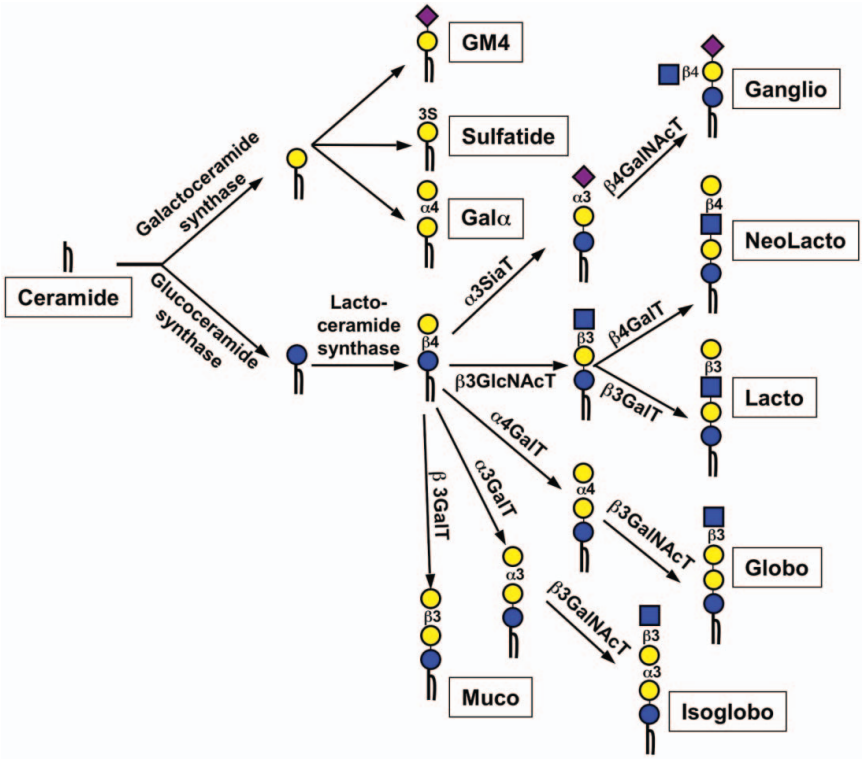

FIGURE 12 **Biosynthesis of glycosphingolipids. Different glycolipids serve as intermediates in the assembly of families of glycosphingolipids indicated in the boxes. Symbols are described in Figure 1.**

Inhibitors of GSLs

Like other glycans, GSLs have been the target of intense research to understand their structural diversity, assembly and function. However, inhibitor design and drug development efforts have not developed at the same pace.

Enzyme Inhibitors

The GSL inhibitor, *N*-butyldeoxynojirimycin (NB-DNJ, OGT918, Figure 13) inhibits glucosylceramide synthase in cultured cells (Tables 1 and 8) (Platt *et al.*, 1994). A series of imino-sugar analogs have since been identified and characterized as potential inhibitors of GSL synthesis (reviewed in (Compain & Martin, 2001; Dwek *et al.*, 2002; Asano, 2003)). NB-DNJ was originally tested in humans as an anti-viral agent due to its capacity to inhibit HIV replication *in vitro*. This activity was due to the inhibition of the N-linked glycan processing enzymes, α-glucosidase I and II. However, a clinical trial to test the potential of using NB-DNJ in patients with HIV-1 found no efficacy and resulted in serious gastrointestinal side effects (Fischl *et al.*, 1994). NB-DNJ can be delivered orally at 2.4 g/kg/day to mice, which causes a global reduction in GSL levels in mice by 40 to 70% (Platt *et al.*, 1997; Platt *et al.*, 1997). However, when tested in humans the compound had serious side effects including lymphoid depletion, weight loss, diarrhea, and peripheral neuropathy (Tifft & Proia, 2000). At lower doses, NB-DNJ induces reversible male sterility, possibly due to loss of seminolipid (a sulfated galactoglycerolipid) in spermatozoa or other GSLs in the testes and epididymides (Suganuma *et al.*, 2005; Bone *et al.*, 2007).

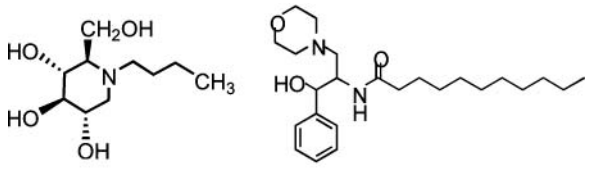

FIGURE 13 **Two examples of glycosphingolipid inhibitors.**

Another class of GSL inhibitors consists of glucosylceramide inhibitors, such as 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PDMP) (Figure 13). PDMP and closely related analogs (*e.g.*, *p*OH-P4) inhibit GlcCer synthase and produce the reversible depletion of cellular GSLs (Abe *et al.*, 1992; Chatterjee *et al.*, 1996). Although PDMP is a more potent inhibitor of GlcCer synthase than NB-DNJ, it

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was found to be toxic from an increase in intracellular ceramide levels (Abe *et al.*, 1992; Abe & Shayman, 1998; Lee *et al.*, 1999). *p*OH-P4 was found to have greater specificity for the glucosylceramide synthase, did not increase intracellular ceramide levels in cultured cells, and thus is noncytotoxic (Lee *et al.*, 1999). Another example of a synthetic inhibitor is the exocyclic epoxide derivative of glucosylceramide, which inhibited glycosphingolipid biosynthesis in cultured neurons (Zacharias *et al.*, 1994).

Acceptor Analogs

As described above, β-D-xylosides will serve as an intermediate for the formation of HS and CS chains (Table 7). Freeze and coworkers showed that Xyl-β-*O*-4-methylumbelliferol will generate GSL-like products in human melanoma and Chinese hamster ovary (CHO) cells (Freeze *et al.*, 1993) (Table 8). Several hydrophobic glycosides of N-acetylglucosamine (*e.g.*, GlcNAc-*O*-benzyl) and lactosides were shown to act as primers of polylactosamine synthesis and affect glycolipid synthesis in cells as well (Neville *et al.*, 1995; Nakajima *et al.*, 1998) (Table 8). These observations have not been exploited to study GSL function, presumably due to their lack of specificity.

Enzymatic glycan Removal

Endoglycoceramidases have been described that hydrolyze the linkage between the glycan and the ceramide moiety (Ito *et al.*, 1993; Ito & Komori, 1996; Ishibashi *et al.*, 2007). These reagents are useful for characterizing the glycan moiety, but their use *in vivo* is limited due to the simultaneous production of ceramide and loss of the glycan. Many of the exoglycosidases that act on N-linked and O-linked chains also will remove terminal monosaccharides from the non-reducing end of the carbohydrate (*e.g.*, neuraminidase, β1,3 galactosidase, $\beta-N$-acetyl-galactosaminidase and α-fucosidase).

TABLE 8 **Examples of inhibitors that block glycosphingolipid biosynthesis**

Inhibitor	Target
Enzyme inhibitors	
NB-DNJ	GlcCer synthase
NB-DGJ	GlcCer synthase
PDMP	GlcCer synthase
pOH-P4	GlcCer synthase
Acceptor analogs	
GlcNAcα-*O*-benzyl	lacto-series glycolipid GM_3
GlcNAcβ-*O*-phenyl	lacto-series glycolipid GM_3
Xylβ-*O*-R	glycosphingolipids
Galα4-lactosides	glycosphingolipids
Galα3-lactosides	glycosphingolipids

R = different hydrophobic aglycones.

Applications for Glycosphingolipid Inhibitors

GSLs are not essential in cultured cells but complete ablation of their biosynthesis in mice results in early lethality indicating they play a crucial role in embryonic development (Yamashita *et al.*, 1999). However, disruption of the genes required for diversification of the chains results in viable mice with relatively mild neurological defects (Takamiya *et al.*, 1996; Sheikh *et al.*, 1999; Chiavegatto *et al.*, 2000; Yamashita *et al.*, 2003). These findings suggest inhibition of individual GSLs might be tolerated and therefore could be of therapeutic use.

Gangliosides are thought to play a role in the growth of neuroblastoma and melanoma (reviewed in Ledeen, 1984; Valentino *et al.*, 1990; Ledeen *et al.*, 1998; Fredman *et al.*, 2003). Chimeric human/murine anti-GD_2 monoclonal antibody can induce lysis of neuroblastoma cells by antibody-dependent and complement-dependent cytotoxicity (Yu *et al.*, 1998; Batova *et al.*, 1999). A novel monoclonal antibody raised by immunization of mice with colorectal tumor cell lines recognizes a sialyltetraosylceramide and can directly induce tumor cell death without immune effector cells or complement (Durrant *et al.*, 2006). The growth and spread of melanoma and neuroblastoma can be inhibited when glucosylceramide synthase inhibitors are fed to mice (Deng *et al.*, 2000; Ranes *et al.*, 2001), even after the tumor is established (Weiss *et al.*, 2003). Thus, tumor glycolipids represent an excellent target for chemotherapy.

Inherited metabolic disorders of GSL metabolism are caused by mutations in lysosomal degradative enzymes. Afflicted individuals exhibit extensive storage of GSL in multiple organs and often exhibit neurodegenerative disorders. Enzyme replacement therapy has advanced considerably over the last decade, with two therapeutics now available, Imiglucerase (Cerezyme®) for treatment of Type I Gaucher disease (glucocerebrosidase deficiency) and β-agalsidase (Fabrazyme®) for treatment of Fabry disease (α-galactosidase deficiency). An alternative approach called "substrate deprivation" at-

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TABLE 9 **Examples of GPI anchor inhibitors**

Inhibitor	Target	Cellular Activity
Monosaccharide inhibitors		
2-deoxy-2-fluoro-D-Glc	Dolichol-P-Man and GPI mannosylation	Yes
$ManNH_2$	α2ManT	Yes, in trypanosomes
Enzyme inhibitors		
YW3548 terpenoid lactone	Mannosyltransferases	Yes
Phenanthroline	Phosphoethanolaminetransferase	Yes
Natural product candidate	UDP-Glc 4′-epimerase	No
GlcN-PI analogs	Phosphoethanolaminetransferase	No

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tempts to subdue production of GSLs, *e.g.*, by treatment with *N*-alkylated imino sugars. Although NB-DNJ has moderate activity as a glucosylceramide synthase inhibitor (IC_{50} ~20 μM), treatment of mouse models of Tay-Sachs and Sandhoff disease (β-hexosaminidase A deficiency) showed delayed accumulation of ganglioside GM_2 and delayed onset of neurologic symptoms (Platt *et al.*, 1997; Jeyakumar *et al.*, 1999). NB-DNJ (miglustat, Zavesca®) is now approved for treating type I Gaucher disease. *p*OH-P4 works in a similar manner by depletion of globotriosylceramide (Gb3) from Fabry disease lymphocytes (Abe *et al.*, 2000). Whether substrate deprivation therapy will prove useful for other types of lysosomal storage diseases (*e.g.*, mucopolysaccharidoses) remains an unexplored area. Imino sugars also have molecular chaperone activity, assisting protein folding and stability of mutant enzymes so that low levels of enzyme activity are restored (Lieberman *et al.*, 2007; Steet *et al.*, 2007; Yu *et al.*, 2007).

Many cancer patients have circulating antibodies to gangliosides, consistent with the observation that some tumors overexpress subsets of GSLs. Thus, GSLs have received considerable attention as potential antigens for anti-tumor vaccination. Both passive immunotherapy by infusion of monoclonal antibodies and active immunization with purified glycolipid preparations have been attempted. Relevant GSL targets include GM1, GM2, N-glycolyl GM3, GD2, GD3, and Globo-H(Gilewski *et al.*, 2001; Krug *et al.*, 2004; Ragupathi *et al.*, 2005; Guthmann *et al.*, 2006; Sabbatini *et al.*, 2007). Because glycans tend to induce IgM responses and low IgG titers, the trend is to prepare multivalent antigens conjugated to adjuvants to produce a strong IgG response. Fully synthetic vaccine candidates have been generated to overcome the poor immunogenicity of tumor-associated glycans, for example using a three-component vaccine composed of a TLR2 agonist, a promiscuous peptide T-helper epitope and a tumor-associated glycopeptide expressed by the tumor (Ingale *et al.*, 2007). These approaches hold great promise for treating a variety of tumors.

Glycosylphosphatidylinositol Anchors

Glycosylphosphatidylinositol (GPI) is a glycolipid composed of a glycan conjugated to phosphatidylinositol. It acts as a membrane anchor for as much as 20% of membrane proteins, tethering them to the outer leaflet of the plasma membrane (Figure 14). In mammals, the GPIs consist of a conserved core glycan (Manα1,2Manα1,6Manα1,4GlcNH_2) linked to the 6-position of D-myo-inositol of phosphatidylinositol (PI) (Ferguson, 1999). In addition, one or more ethanolamine-phosphate groups are attached to the mannose residues. GPI-linked proteins are functionally diverse, but many have hydrolytic activity, serve as receptors, or play roles in cell adhesion (Orlean & Menon, 2007).

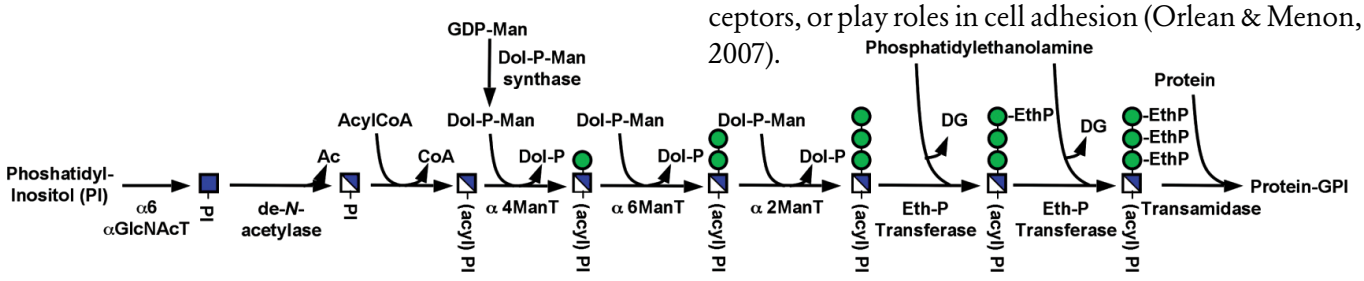

FIGURE 14 **Biosynthesis of glycosylphosphatidylinositol anchors. Symbols are described in Figure 1. Dol-P, dolichol-phosphate.**

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Biosynthesis of GPI Anchors

The first step in GPI biosynthetic pathway involves the transfer of N-acetylglucosamine from UDP-GlcNAc to endogenous phosphatidylinositol (PI) to form GlcNAc-PI, which is rapidly de-*N*-acetylated to give glucosaminyl-PI (GlcN-PI). Inositol acylation of GlcN-PI is prerequisite for GPI mannosylation in vertebrates. A series of glycosyltransferases encoded by the *PIG* genes then assemble the core glycan by transfer of individual mannose residues from dolichol-P-mannose to the $GlcNH_2$-PI core and mannosylated intermediates. The last step in the formation of the conserved core glycan is the transfer of ethanolamine phosphate to position 6 of the third mannose residue. The first mannose residue and to a lesser extent the second mannose can be modified by additional ethanolamine phosphate moieties. The completed GPI precursor is attached to nascent proteins via a transamidase-like reaction where a C-terminal GPI attachment signal peptide is released (Ferguson *et al.*, 1999; Ferguson, 1999). The abundance of GPI-anchored proteins in *Trypanosoma brucei (T. brucei)*, has made this organism extremely useful for the study of GPI anchor biosynthesis. Differences exist in substrate specificity between the biosynthetic enzymes of trypanosomal and mammalian GPI anchor biosynthesis, which validate the GPI pathway as a drug target and for the development of anti-parasite therapies (Ferguson, 2000; Smith *et al.*, 2004).

Inhibitors of GPI Anchors

Monosaccharide Inhibitors

Dolichol-P-mannose, the mannose donor for all GPI mannosyltransferase reactions, is synthesized from dolichol-phosphate and GDP-Man by dolichol phosphate mannose synthase. Mannosamine (2-deoxy-2-amino-mannose, $ManNH_2$) was shown to inhibit α1,2 mannosyltransferase when fed to HeLa cells (Sevlever & Rosenberry, 1993) (Figure 15). However, *T. brucei* actually incorporates $ManNH_2$ into the GPI anchor biosynthetic pathway, forming $ManNH_2$-Man-$GlcNH_2$-PI. This is a dead-end intermediate, resulting in significant impairment of the parasites ability to synthesize GPI anchor intermediates beyond Man_2-$GlcNH_2$-PI (Ralton *et al.*, 1993). Glucose analogs (*e.g.*, 2-deoxy-2-fluoro-D-glucose) inhibit the formation of dolichol-P-mannose *in vivo* and block GPI formation (Takami *et al.*, 1992).

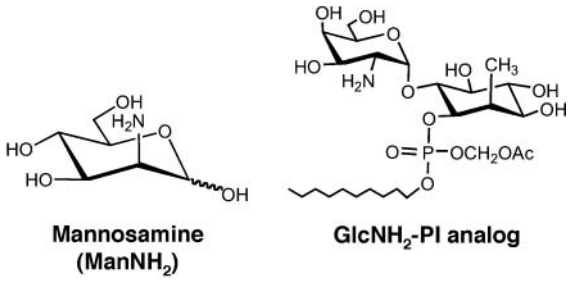

FIGURE 15 Two examples of GPI inhibitors.

Enzyme Inhibitors

A variety of substrate analogs based on $GlcNH_2$-PI have also been synthesized and tested as inhibitors of GlcNAc de-*N*-acetylase in human cells (Smith *et al.*, 1997; Sharma *et al.*, 1999) (Figure 15). A natural terpenoid lactone, YW3548, was isolated from *Codinea simplex*, which causes accumulation of the Man_2-GlcN-(acyl)-PI in mammalian lymphoma cells, although not in parasitic protozoa (Sütterlin *et al.*, 1997). It appears to prevent the addition of the third mannose residue in the biosynthetic pathway. A similar effect was seen in mammalian cells treated with the chelator, phenanthroline (Mann & Sevlever, 2001; Sevlever *et al.*, 2001). Mammalian cells treated with this compound accumulate GPI intermediates that are substrates for ethanolamine phosphate transfer reaction. Thus, both YW3548 and phenanthroline are most likely inhibitors of GPI-phosphoethanolamine transferases. Two $GlcNH_2$-PI analogs, *e.g.*, $GlcNMe_2$-PI, $GlcNCONH_2$-PI, and others, were found to inhibit both parasite and human GlcNAc de-*N*-acetylase (Smith *et al.*, 2001; Smith *et al.*, 2002).

In addition to the mammalian GPI inhibitors, a number of parasite-specific inhibitors have been described. $GlcNH_2$-PI and GlcNAc-PI analogs containing acetoxymethyl esters of the phosphate and with variations in their alkyl chain length are *T. brucei* specific suicide substrate inhibitors (Crossman *et al.*, 1999; Smith *et al.*, 1999; Smith *et al.*, 2001; Smith *et al.*, 2004). Another class of inhibitors is based on fatty acid analogs that only trypanosomes incorporate into their GPI anchors. Trypanosomes, unlike the mammalian hosts, can incorporate myristic acid into their GPI anchor by exchanging myristic acid for other fatty acids in the PI moiety. Myristic acid analogs such as 10-(propoxy)decanoic acid have shown toxicity towards *T. brucei* but not to mammalian cells (Doering *et al.*, 1991; Doering *et al.*, 1994).

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Enzymatic Glycan Removal

Phosphatidylinositol-specific phospholipase C (PI-PLC) is an established reagent for the identification and release of GPI-anchored proteins. This enzyme is commercially available and can be used to reduce or eliminate GPI anchors from isolated proteins and intact cells.

Applications for GPI Anchor Inhibitors

Disruption of GPI anchor biosynthesis greatly impairs trypanosome survival in the mammalian host and thus the various biosynthetic enzymes are targets for the development of parasite-specific therapeutic agents (Ferguson *et al.*, 1999). Many fungi also produce GPI anchors as well. Cultured mammalian cells can survive without GPI anchors, but they are essential *in vivo* (Kawagoe *et al.*, 1996). Mutations in *PIG-A* gene occur spontaneously in hematopoietic cells in humans and can cause a hemolytic disease known as paroxysmal nocturnal hemoglobinuria (PNH) in which red blood cell lysis occurs due to diminished levels of GPI-linked proteins on the plasma membrane (Brodsky & Hu, 2006). Thus, any drug candidates would have to exploit differences in GPI anchor assembly in fungi or trypanosomes compared to mammals. However, few of the available compounds appear to be under development, possibly because the major markets are in third world countries and alternative treatments are available for fungal infection.

O-GlcNAc on Cytosolic and Nuclear Proteins

In contrast to other forms of glycosylation, O-linked *N*-acetylglucosamine (*O*-GlcNAc) involves addition of a single monosaccharide residue to serine and threonine residues of intracellular proteins. *O*-GlcNAc is found on as many as 500 proteins including transcription or translation factors, nuclear pore components, proteins involved in stress responses and energy metabolism, cytoskeletal elements and proteins involved in cytoskeletal regulation, kinases, and enzymes of intermediary metabolism (Zachara & Hart, 2006). All multicellular animals have *O*-GlcNAc modified proteins, whereas bacteria and yeast do not. This form of glycosylation has diverse effects on the modified proteins, by altering their phosphorylation, stability, localization, and activity (Zachara & Hart, 2006).

TABLE 10 **Examples of inhibitors of *O*-GlcNAc**

Inhibitor	Target
6-diazo-5-oxo-L-norleucine	Glutamine: Fructose amidotransferase
Azaserine	Glutamine: Fructose amidotransferase
Alloxan	*O*-GlcNAc Transferase
Streptozotocin	*O*-GlcNAc Transferase
PUGNAc	*O*-GlcNAcase and lysosomal β-hexosaminidases
NAG-thiazoline	*O*-GlcNAcase
GlcNAcstatin	*O*-GlcNAcase

Biosynthesis of O-GlcNAc

In animal cells, *O*-GlcNAc modification of proteins is catalyzed by a single *O*-GlcNAc transferase (OGT) (Kreppel *et al.*, 1997). OGT is a soluble protein that has primarily a nuclear localization. Genetic deletion of OGT is lethal in isolated cells and in mice, with embryos succumbing at the single cell stage (Shafi *et al.*, 2000; O'Donnell *et al.*, 2004). A single *O*-GlcNAc specific β-hexosaminidase removes *O*-GlcNAc residues. The dynamic addition and removal of *O*-GlcNAc, which may be the counterpart of phosphorylation and dephosphorylation, is regulated by the balance of OGT and *O*-GlcNAcase activity (Gao *et al.*, 2001). Cycling of *O*-GlcNAc residues is thought to serve as a nutrient and stress sensor in cells (Yang *et al.*, 2002; Iyer *et al.*, 2003; Iyer & Hart, 2003).

Inhibitors of O-GlcNAc

Enzyme Inhibitors

O-GlcNAc levels in cells are closely linked to their physiological status. OGT activity can be modulated by altering the intracellular levels of UDP-GlcNAc by feeding exogenous glucosamine or by modulating the hexosamine biosynthetic pathway. Azaserine (*O*-diazoacetyl-L-serine) and 6-diazo-5-oxonorleucine (DON) inhibit the rate-limiting step catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFAT), thus reducing *O*-GlcNAc levels (Marshall *et al.*, 1991).

Effective inhibitors of both the OGT and the *O*-GlcNAcase have provided a powerful system to up- and down-regulate intracellular *O*-GlcNAc levels. *O*-GlcNAc addition can be inhibited using the uracil analog, alloxan (Konrad *et al.*, 2002) and streptozotocin but these compounds may have nonspecific effects on other

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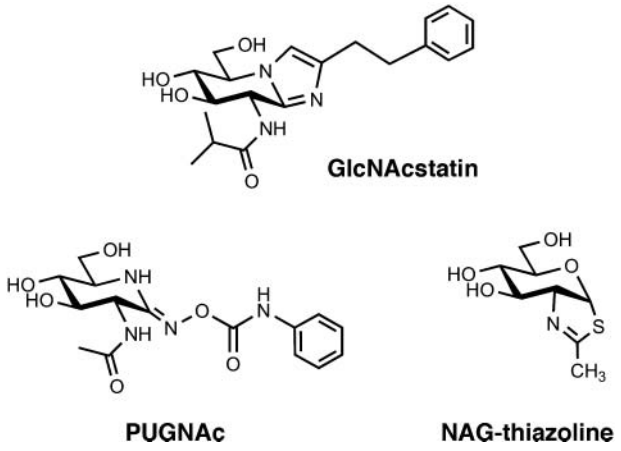

FIGURE 16 **Three examples of O-GlcNAcase inhibitors.**

enzymes that recognize uracil in addition to the generation of superoxide radicals (Szkudelski, 2001). Three OGT inhibitors have been discovered using an enzyme-based high throughput screen. These noncarbohydrate small molecule inhibitors were found to compete with UDP-GlcNAc binding to OGT, but not to a bacterial GlcNAc transferase (Gross *et al.*, 2005).

Several inhibitors of the *O*-GlcNAcase have been devised based on the structure of *N*-acetylglucosamine (Figure 16). The first compound in this class, PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate) inhibits *O*-GlcNAcase at nanomolar concentrations, but also inhibits lysosomal β-hexosaminidases (Dong & Hart, 1994; Haltiwanger *et al.*, 1998). Recently, a more specific *O*-GlcNAcase inhibitor, NAG-thiazoline, was shown to have more selectivity for the *O*-GlcNAcase over the lysosomal enzymes (Macauley *et al.*, 2005; Whitworth *et al.*, 2007). A new rationally designed glucoimidazole, GlcNAcstatin, inhibits *O*-GlcNAcase with a Ki of 4.6 picomolar and exhibits 10^5-fold selectivity over lysosomal β-hexosaminidases (Dorfmueller *et al.*, 2006). These compounds inhibit the enzyme in cultured cells, providing new tools to study the function of *O*-GlcNAc and potential candidates for drug therapy.

Enzymatic Glycan Modification

Two approaches have been used to alter the extent of *O*-GlcNAc modification enzymatically: overexpression of OGT, *O*-GlcNAcase, or a soluble galactosyltransferase to modify the *O*-GlcNAc residues so that the *O*-GlcNAcase would be unable to remove them (Holt & Hart, 1986; Fang & Miller, 2001). More recently, an enzymatically inactive form of the *O*-GlcNAcase has been shown to act as a dominant negative inhibitor through its ability to form complexes that exclude the endogenous active *O*-GlcNAcase (Whisenhunt *et al.*, 2006). Additionally, siRNA mediated silencing of OGT has been reported (Dauphinee *et al.*, 2005; Andrali *et al.*, 2007; Robinson *et al.*, 2007).

Applications for O-GlcNAc Inhibitors

N-acetylglucosamine plays a central role in many glycosylation reactions, and evidence has been presented that the hexosamine biosynthesis pathway regulates aspects of glucose uptake, glycogen synthesis, and glycolysis (Hebert *et al.*, 1996). Interestingly, increasing hexosamine levels can cause insulin resistance in cultured cells and animals. Elevation of *O*-GlcNAc levels by PUGNAc impairs insulin-stimulated glucose uptake in cells and tissues possibly by decreasing trafficking of GLUT4 transporters, insulin-dependent signaling, and decreased glycogen synthesis (Vosseller *et al.*, 2002; Arias *et al.*, 2004). *O*-GlcNAc levels can also affect insulin secretion directly through modification of PDX-1, a transcription factor required for insulin expression (Akimoto *et al.*, 2007).

Another area of interest concerns *O*-GlcNAc addition to cytoskeletal proteins, especially in the brain. Many proteins involved in bridging actin and regulating microtubule assembly, cytokeratins, and neurofilaments are extensively *O*-GlcNAc modified. The microtubule-associated protein tau normally undergoes *O*-GlcNAc modification (Arnold *et al.*, 1996; Liu *et al.*, 2004). OGT deletion results in decreased *O*-GlcNAc and hyperphosphorylation of tau in neurons and subsequent cell death, suggesting the possibility that formation of neurofibrillary bundles in Alzheimer's disease brain may be related to *O*-GlcNAc levels. These findings suggest that modulating *O*-GlcNAc addition or removal might provide a way to modulate abnormal secretion and deposition of amyloid proteins.

Coda

What should be apparent from this overview is that the major classes of glycans elaborated by vertebrates play many roles in human physiology. Indeed, 1% to 2% of the human genome encodes enzymes that assemble and degrade glycans and various glycan-binding proteins. Thus, several hundred targets exist for the development of enzyme and lectin receptor inhibitors. The repertoire of available compounds, although extensive,

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contains few agents that have the affinity and specificity required for converting a laboratory reagent into a drug. However, the few drugs that have been developed (Table 1) have already proven their value as therapeutic agents. These success stories represent only the beginning of what we hope will be a new chapter in glycobiology research and in the development of novel drugs for treating human disease.

ACKNOWLEDGMENTS

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