Assigned Reading:

Chapter 55, Chemical Tools for Inhibiting Glycosylation
Essentials in Glycobiology, 3rd edition

Chapter 57, Glycans in Biotechnology and the Pharmaceutical Industry
Essentials in Glycobiology, 3rd edition

Chapter 55, Chemical Tools for Inhibiting Glycosylation
Essentials in Glycobiology, 3rd edition

Chapter 55

Chemical Tools for Inhibiting Glycosylation

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The use of chemical tools to inhibit glycosylation provides a powerful approach for studying glycan functions and serves as a starting point for drug discovery. This chapter discusses various types of inhibitors, including natural products, substrate-based tight-binding inhibitors, glycoside primers, inhibitors found through screening chemical libraries, and examples of rationally designed inhibitors based on three-dimensional structures of enzymes.

ADVANTAGES OF INHIBITORS

Chapters 44, 45, and 49 describe various natural and induced mutants with defects in glycosylation. These mutants have helped to define genes that encode various transferases and glycosidases, and in some cases alternate biosynthetic pathways have been uncovered. Mutants also provide insights into the function of glycosylation in cells and tissues and models for human inborn errors in metabolism and disease. However, one limitation of studying mutants is that the analyses are usually restricted to the cell or organism from which the mutant strain was isolated. Additionally, many mutations are lethal in animals, which makes the study of the gene in adult animals more difficult.

Inhibitors of glycosyltransferases and glycosidases provide another approach for studying glycosylation in cells, tissues, and whole organisms that avoids some of the problems associated with studying mutants. Many of these compounds are small molecules that are taken up readily by a variety of cell types and some can be absorbed through the gut, providing an opportunity for designing drugs to treat human diseases and disorders correlated with altered glycosylation (Chapter 57). Because the field is quite large, only a selection of inhibitors that act on specific enzymes or metabolic pathways and that illustrate certain basic concepts are discussed here (Table 55.1). Agents that block protein/carbohydrate interactions are surveyed in Chapter 29, and the aminoglycoside antibiotics are discussed briefly in Chapter 57.

METABOLIC INHIBITORS

A number of inhibitors have been described that block glycosylation by interfering with the metabolism of common precursors or intracellular transport activities. Some of these compounds act indirectly by impeding the transit of proteins between the endoplasmic reticulum (ER), Golgi, and *trans*-Golgi network. For example, the fungal metabolite brefeldin A causes retrograde transport of Golgi components located proximal to the *trans*-Golgi network back to the ER. Thus, treating cells with brefeldin A separates enzymes located in the *trans*-Golgi network from those found in the ER and Golgi and uncouples the assembly of the core structures of some glycans from later reactions, such as sialylation or sulfation. The drug can be used to examine if two pathways reside in the same compartment or share the same enzymes. Because the localization and array of the enzymes vary considerably in different cell types, extrapolating the effects of brefeldin A from one system to another is often difficult.

Some inhibitors act at key steps in intermediary metabolism where precursors involved in glycosylation are formed. For example, a glutamine analog, 6-diazo-5-oxo-L-norleucine (DON), blocks glutamine: fructose-6-phosphate amidotransferase, the enzyme of the hexosamine biosynthetic pathway that forms glucosamine from fructose and glutamine (Chapter 5). Depressing glucosamine production in this way has a pleiotropic effect on glycan assembly because all of the major families contain *N*-acetylglucosamine or *N*-acetylgalactosamine. DON also affects other glutamine utilizing enzymes and therefore, care should be taken to limit non-specific side effects. Chlorate is another type of general inhibitor that blocks sulfation. The chlorate anion (ClO_4^{2-}) is an analog of sulfate (SO_4^{2-}) and it forms an abortive complex with the sulfurylase involved in the formation of phosphoadenosine-5'-phosphosulfate (PAPS), the active sulfate donor for all known sulfation reactions. Thus, treating cells with chlorate (usually 10–30 mM) inhibits sulfation by more than 90%, but the effect is not specific for any particular class of glycan or sulfation reaction (e.g., tyrosine sulfation is also affected).

A number of sugar analogs have been made with the hope that they might exhibit selective inhibition of glycosylation. 2-Deoxyglucose and fluorinated analogs of sugars (3-deoxy-3-fluoroglucosamine, 4-deoxy-4-fluoroglucosamine, 6-deoxy-6-fluoro-*N*-acetylglucosamine, 2-deoxy-2-fluoroglucose, 2-deoxy-2-fluoromannose, 2-deoxy-2-fluorofucose, and 3-fluorosialic acid) inhibit glycoprotein biosynthesis, but the mechanism underlying the inhibitory effect is unclear in many cases. Early studies of 2-deoxyglucose showed that the analog was converted to UDP-2-deoxyglucose as well as to GDP-2-deoxyglucose and dolichol-P-2-deoxyglucose. Inhibition of glycoprotein formation apparently occurs as a result of accumulation of various dolichol oligosaccharides containing 2-deoxyglucose, which cannot be elongated or transferred to glycoproteins normally. Similarly, 4-deoxy-N-acetylglucosamine is converted to UDP-4-deoxy-*N*-acetylglucosamine, resulting in inhibition of heparan sulfate formation without incorporation of the analog. 4-

deoxy-xylose also inhibits glycosaminoglycan assembly presumably by competing with naturally occurring xylosylated substrates. Care must be taken in interpreting the results of experiments employing these compounds because they may have pleiotropic effects on glycan assembly due to overlap of nucleotide precursors.

TUNICAMYCIN: INHIBITION OF DOLICHOL-PP-GlcNAc ASSEMBLY

A number of natural products have been found to alter glycosylation. Tunicamycin belongs to a class of nucleoside antibiotics composed of uridine, an 11-carbon disaccharide called aminodeoxydialdose (tunicamine), and a fatty acid of variable length (13–17 carbons), branching, and unsaturation (Figure 55.1). Tunicamycin was first identified in *Streptomyces lysosuperificus*, and related compounds were found later in other microorganisms. It derives its name from its antiviral activity, which occurs by inhibiting viral coat (or "tunica") formation.

Tunicamycin inhibits N-glycosylation in eukaryotes by blocking the transfer of *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) from UDP-GlcNAc to dolichol-P (catalyzed by GlcNAc phosphotransferase; GPT), thereby decreasing the formation of dolichol-PP-GlcNAc (Chapter 9). Other GlcNAc transferase reactions are not inhibited (e.g., GlcNAcTI–V), but the transfer of GlcNAc-1-P to undecaprenyl-P and the formation of undecaprenyl-PP-MurNAc pentapeptide (which is involved in bacterial peptidoglycan biosynthesis) are sensitive to tunicamycin (Chapter 21). Tunicamycin acts as a tight-binding competitive inhibitor, presumably because it resembles the donor nucleotide sugar. The K_i value for tunicamycin is about 5×10^{-8} M, whereas the K_m value for UDP-GlcNAc is approximately 3×10^{-6} M. The actual amount of tunicamycin needed to inhibit glycosylation varies in different cells (0.1–10 μg/ml), possibly because of variable uptake and culture conditions or differences in the level of expression of the phosphotransferase. Given the key role of N-glycosylation in protein folding and quality control in the ER (Chapter 39), it is not suprising that tunicamycin is cytotoxic to cells, and that resistant mutants overproduce GPT. Similarly, transfection of cells with the cloned GPT confers resistance, suggesting that the variable dose of inhibitor required in different cells may reflect variation in enzyme levels.

Tunicamycin has been used extensively for studying the role of N-glycans in glycoprotein maturation, secretion, and function, since its first discovery in 1973. The drug induces apoptosis preferentially in cancer cells, presumably because of alterations in glycosylation of various cell-surface receptors and signaling molecules and by inducing ER stress (Chapter 39). Thus, inhibition of N-glycan formation could be useful for treating cancer patients. Other potential applications include substrate reduction therapy for treatment of lysosomal storage disorders (Chapter 44), congenital disorders of glycosylation (Chapter 45), or naturally occurring mutations that create N-glycosylation sites in cell-surface receptors (gain-of-glycosylation mutants; Chapter 45).

Amphomycin, a lipopeptide, inhibits dolichol-P-mannose synthesis by apparently forming complexes with the carrier lipid dolichol-P. Other lipophilic compounds that bind lipid intermediates in bacterial cell wall synthesis also have also been studied (Chapter 21).

PLANT ALKALOIDS: NATURAL INHIBITORS OF GLYCOSIDASES

Plant alkaloids block N-linked glycosylation by inhibiting the processing glycosidases (α-glucosidases and α-mannosidases) involved in trimming nascent chains (Table 55.2). Unlike tunicamycin, which blocks glycosylation of glycoproteins entirely, the alkaloids inhibit the trimming reactions that occur after the $Glc_3Man_9GlcNAc_2$ oligosaccharide is attached to a glycoprotein (Chapter 9), resulting in the appearance of glycoproteins on the cell surface lacking the characteristic termini found on mature N-glycans (Chapter 14). α-Glucosidase inhibitors involved in the initial processing of N-glycans and in quality control of protein folding (Chapters 32 and 39) include castanospermine (from the seed of the Australian chestnut tree, *Castanosperum australe*), which inhibits α-glucosidases I and II, australine (also from *C. australe*), which preferentially inhibits α-glucosidase I, and deoxynojirimycin (from *Streptomyces* species), which preferentially inhibits α-glucosidase II (Table 55.2). Castanospermine and australine cause accumulation of fully glucosylated chains, whereas deoxynojirimycin results in chains containing one to two glucose residues. Unexpectedly, treating cells with these inhibitors revealed that some trimming of the mannose residues could occur independently of removal of the glucose residues (Chapter 9).

Swainsonine was first discovered in plants from the western United States (*Astragalus* species, also known as locoweed) and Australia (*Swainsona canescens*), and was later found in the fungus *Rhizoctonia leguminocola* that infects red clover. Consumption of these plants in animals causes a severe abnormality called locoism and accumulation of glycoproteins in the lymph nodes. Swainsonine inhibits α-mannosidase II, causing the accumulation of high-mannose oligosaccharides ($Man_4GlcNAc_2$ and $Man_5GlcNAc_2$) and hybrid-type chains at the expense of complex oligosaccharides. In addition, swainsonine also inhibits the lysosomal α-mannosidase. Mannostatin A works in a similar way, but differs significantly in structure from swainsonine (Table 55.2). Other mannosidase inhibitors include deoxymannojirimycin and kifunensin, which selectively inhibit α-mannosidase I. These agents cause the accumulation of $Man_{7-9}GlcNAc_2$ oligosaccharides on glycoproteins.

All of the above listed inhibitors have in common polyhydroxylated ring systems that mimic the orientation of hydroxyl groups in the natural substrates, but a strict correlation between stereochemistry and enzyme target (α-glucosidase vs. α-mannosidase) does not exist. The compounds contain nitrogen, usually in place of

the ring oxygen. One idea is that the nitrogen in the protonated state may mimic the positive charge on the ring oxygen that arises from delocalization of charge from the tentative carbocation at C-1 generated during the hydrolysis reaction. Crystal structures for the α-mannosidase are available with bound inhibitors.

Alkylated and acylated analogs of the alkaloids have interesting and useful properties. *N*-Butylation of deoxynojirimycin actually converts the glucosidase inhibitor into an inhibitor of glycolipid biosynthesis. Alkylation of the amino group or acylation of the hydroxyl groups can improve the potency of the compound, presumably by facilitating uptake across the plasma and Golgi membranes. Some of these compounds have shown positive effects for treating diabetes, lysosomal storage diseases, cancer, and HIV infection, but also induces male sterility (see Chapter 57).

INHIBITION OF *O*-GalNAc INITIATION OF MUCIN-TYPE GLYCANS

Few inhibitors are available that block O-linked glycans compared to N-linked glycan biosynthesis. Mucin-type O-linked glycan biosynthesis is initiated by polypeptidyl *N*-acetylgalactosaminyltransferases (ppGalNAcTs), a large family of enzymes that use UDP-GalNAc as a common donor and various glycoprotein acceptors (Chapter 10). Screening a synthetic library of uridine analogs against members of the ppGalNAcT family yielded two compounds that disrupt *O*-GalNAc addition (Figure 55.2). These compounds have K_i values of approximately 8 μM with respect to UDP-GalNAc. Like tunicamycin, these inhibitors suppress glycosylation without selectivity for different glycoprotein targets. These first generation enzyme inhibitors work on O-linked glycans, and raise the possibility that inhibitors of specific ppGalNAcT isoforms as well as other types of O-linked glycans, such as *O*-xylose (Chapter 17), *O*-glucose (Chapter 18), and *O*-GlcNAc (Chapter 19) may be developed eventually.

INHIBITION OF O-GlcNAc MODIFICATION

The importance of *O*-GlcNAc addition to many cytoplasmic and nuclear proteins (Chapter 19) has stimulated great interest in developing agents to inhibit its addition by *O*-GlcNAc transferase (OGT) or its removal by *O*-GlcNAc-specific β-hexosaminidase (*O*-GlcNAcase). Alloxan and streptozotocin affect *O*-GlcNAc addition, but these compounds lack specificity. The first potentially useful OGT inhibitors were obtained by screening chemical libraries for compounds that displaced a fluorescent derivative of the donor sugar, UDP-GlcNAc. The active compounds do not block other *N*-acetylglucosamine addition reactions, for example one involved in formation of the polysaccharide backbone of bacterial peptidoglycan (Chapter 21). In addition, 5-S-GlcNAc is another inhibitor of OGT (Figure 55.3). Its peracetylated form (Ac-5SGlcNAc), as well as a similar chemical inhibitor, peracetyl

4-thio-GlcNAc (Ac-4SGlcNAc), crosses cell membranes and undergoes deacetylation by non-specific esterases to generate active inhibitors.

Several O-GlcNAcase inhibitors are based on *N*-acetylglucosamine. The first compound in this class, PUGNAc (*O*-[2-acetamido-2-deoxy-D-glucopyranosylidene]amino-*N*-phenylcarbamate; Figure 55.3) inhibits O-GlcNAcase at nanomolar concentrations, but also inhibits lysosomal β-hexosaminidases (HexA and HexB; Chapter 44). Thiamet-G and the related *N*-acetylglucosamine-thiazoline (NAG-thiazoline) are more specific and inhibit at lower concentrations than PUGNAc. A rationally designed glucoimidazole, GlcNAcstatin, inhibits O-GlcNAcase with a K_i of 4.6 pM and exhibits 10^5-fold selectivity over HexA and HexB. These compounds inhibit the enzyme in cells and tissues, providing new tools to study the function of *O*-GlcNAc, and are potential candidates for drug therapy.

SUBSTRATE ANALOGS: DIRECTED SYNTHESIS OF INHIBITORS

A number of inhibitors of specific transferases have been developed based on the concept that substrate analogs might act as tight-binding inhibitors. The general strategy is to modify the hydroxyl group that acts as the nucleophile during formation of the glycosidic bond or groups in its immediate vicinity (Table 55.3). Many designer compounds lack inhibitory activity, since modification of the targeted hydroxyl group prevents binding of the analog to the enzyme by interfering with hydrogen bonding networks that position the substrate. In other cases, the analogs exhibit K_i values in the approximate range of the K_m values for the unmodified substrate. As one might expect, the analogs usually act competitively with respect to the unmodified substrate, but in a few cases the inhibition pattern is more complex, suggesting possible binding outside the active site.

Nucleotide sugar analogs provide opportunities for blocking classes of enzymes that utilize a common donor (e.g., all fucosyltransferases utilize GDP-fucose). A large number of nucleotide sugar derivatives have been made (e.g., *N*- and *O*-substituted analogs of UDP-GalNAc) and several inhibit the enzymes in vitro, but have proven less useful in living cells due to poor uptake. “Bisubstrate” analogs consist of the nucleoside sugar donor or PAPS covalently linked to the acceptor substrate by way of a neutral bridging group. This arrangement may generate inhibitors whose binding characteristics reflect the product of the affinity constants for donor and acceptor (approximated by the product of the individual K_m values). Bisubstrates that have been made have K_i values in the range of the K_m values for the nucleotide donors, suggesting that the correct geometry for the bridging group may have not been attained or that the analog binds in ways that differ from the natural substrates.

The search for active compounds often benefits from serendipidy and the synthesis of di-, tri-, and tetrasaccharides with the desired modifications is rather labor

intensive. Nevertheless, the approach has yielded insights into the binding and reactivity of the enzymes, and substrate analogs with selectivity for particular enzymes have been developed in this way. Because many of the transferases have now been purified and cloned, we can look forward to more detailed kinetic and crystallographic studies, which will provide clues for deriving mechanism-based inhibitors in the future (Chapter 6).

GLYCOSIDE PRIMERS: MIMICKING WHAT ALREADY WORKS

The utility of any glycosyltransferase inhibitor ultimately depends on its ability to cross the plasma membrane and enter the Golgi where the glycosyltransferases reside. Unfortunately, many of the compounds described above lack activity in live cells, presumably because their polarity and charge prevents their uptake. More than 40 years ago, Okayama and colleagues found that D-xylose in β-linkage to a hydrophobic aglycone (the noncarbohydrate portion of a glycoside; e.g., *p*-nitrophenol) was taken up rather efficiently and inhibited the assembly of glycosaminoglycans on proteoglycans. Xylosides mimic the natural substrate, xylosylated serine residues in proteoglycan core proteins, and thus act as a substrate. "Priming" of chains occurs on the added xyloside, which diverts the assembly process from the endogenous core proteins and causes inhibition of proteoglycan formation. In general, cells incubated with xylosides secrete large amounts of individual glycosaminoglycan chains and accumulate proteoglycans containing truncated chains. The enormous success of β-D-xylosides in altering proteoglycan biosynthesis suggested that other glycosides might act as "primers" as well (Table 55.4). Subsequent studies have shown that β-*N*-acetylgalactosaminides prime oligosaccharides found on mucins and inhibit O-glycosylation of glycoproteins. Other active glycosides include β-glucosides, β-galactosides, β-*N*-acetylglucosaminides, and even disaccharides and trisaccharides. These compounds require conjugation to appropriate aglycones and acetylation to neutralize the polar hydroxyl groups on the sugars. Cells contain several carboxyesterases that remove the acetyl groups and render the compounds available to the transferases in the Golgi.

Priming by glycosides occurs in a concentration-dependent manner, but the efficiency varies widely among different compounds and cell types. These variations may relate to the relative abundance of endogenous substrates, enzyme concentration and composition, the solubility of different glycosides, their susceptibility to hydrolysis, their uptake across the plasma membrane and into the Golgi, and their relative affinity for the glycosyltransferases. The type of chain made on a given primer also depends on concentration and aglycone structure, which may reflect selective partitioning of primers into different intracellular compartments or into different branches of biosynthetic pathways. Like priming, inhibition of glycoprotein, glycolipid, or proteoglycan formation occurs in a dose-dependent

fashion, but the blockade is rarely complete, probably because of the inability of glycosides to mimic the entire endogenous substrates.

Primers represent starting points for making analogs with tight-binding inhibitor properties described above. Many of the compounds described in Table 55.3 could be converted to permeable acylated glycosides and tested in live cells for inhibitory activity. Active compounds could potentially become carbohydrate-based drugs to treat glycosylation-dependent diseases. Oligosaccharide priming may have beneficial effects as well. Xylosides, for example, can be absorbed through the gut, and when consumed at sufficient concentration, they exhibit antithrombotic activity. Many glycosides occur naturally, since various organisms (especially plants) produce hydrophobic compounds as part of chemical defense and conjugate them to sugars in order to render them soluble. Thus, the human diet may contain various types of glycosides with interesting (and unknown) biological activities.

Care must be taken in interpreting the results of experiments utilizing glycoside primers. For example, β-D-xylosides also prime glycans related in structure to glycosphingolipids and HNK-1. In some cases priming per se is not the mechanism responsible for inhibition of glycosylation, but rather inhibition occurs due to competitive binding of the primer to a target enzyme. Finally, primers could deplete cells of nucleotide sugars and have multiple effects on glycosylation. For example, 4-methyl-umbelliferone is often used to block hyaluronan biosynthesis. The precise mechanism of action is unknown, but is thought to involve depletion of UDP-GlcA due to glucuronidation of the compound. Reduction of UDP-GlcA in turn could affect formation of sulfated glycosaminoglycans and other glucuronic acid containing glycans and alter the pools of other nucleotide sugars, such as UDP-GlcNAc.

INHIBITORS OF GLYCOLIPIDS AND GPI ANCHORS

Reagents that can alter the assembly of glycolipids in cells have been described. Xylosides have a mild effect on glycolipid formation, possibly because of the similarity between xylose and glucose and the assembly of a GM3-like compound (Neu5Acα2–3Galβ1–4Xylβ-O-R) on the primer. Because cells will take up intermediates in glycolipid biosynthesis, they behave like synthetic glycoside primers. For example, glucosylceramide will give rise to complex glycolipids when fed to cells. On the basis of this observation, an analog containing a reactive exocyclic epoxide group was prepared. The compound inhibits glycolipid formation ($IC_{50} \sim 8$ μM), presumably by reaction of the epoxide with a nucleophile in the active site of lactosylceramide synthase. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an analog of glucosylceramide, was synthesized originally to inhibit glucosylceramide synthesis in patients with Gaucher disease. However, this compound directly inhibits the activity of purified lactosylceramide synthase.

As mentioned above, the α-glucosidase inhibitor, *N*-butyldeoxynojirimycin, has been used to inhibit glycosphingolipid formation because it blocks glucosylceramide synthesis. This compound is now in clinical use for treating type I Gaucher's disease, a lysosomal storage disorder in which glucocerebrosidase is missing (Chapter 44). Its beneficial activity occurs through "substrate deprivation" by blocking synthesis of glycosphingolipids, thereby "depriving" the lysosome of substrate. Sphingolipid analogs (Figure 55.4) are more potent inhibitors. Lengthening the hydrocarbon chain from 10 to 16 carbons further enhances efficacy. These compounds can almost completely deplete glycolipids in cells, and require only micromolar concentrations for activity.

Inhibitors of GPI anchor formation have also been described. Mannose analogs (2-deoxy-2-fluoro-D-glucose and 2-deoxy-D-glucose) inhibit the formation of dolichol-P-mannose in vivo and thus inhibit GPI biosynthesis. However, they lack specificity because these agents also can affect other pathways dependent on dolichol-P-mannose. Mannosamine inhibits GPI anchor formation both in *Trypanosoma brucei* and in mammalian cells by the formation of $ManNH_2$-Man-GlcN-PI. Apparently, mannosamine in its activated form (GDP-$ManNH_2$) is used as a substrate in the second mannosyltransferase reaction, but the $ManNH_2$-Man-GlcN-PI intermediate will not act as a substrate for the next α2-mannosyl-transferase (Chapter 12). GlcNR-phosphatidylinositols with different substituents (R) act as substrate analogs and some act as suicide inhibitors *in vitro*. Another class of inhibitors is based on fatty acid analogs that only trypanosomes incorporate into GPI anchors. Trypanosomes, unlike their mammalian hosts, incorporate myristic acid into GPI anchors by exchanging myristic acid for other fatty acids in the phosphatidylinositol moiety. By making a series of analogs, an inhibitor was found that is highly toxic to trypanosomes in culture and nontoxic to mammalian cells (10-[propoxy]decanoic acid). Such reagents are drug candidates for treating trypanosomiasis, which is endemic in sub-Saharan regions of Africa.

RATIONAL DESIGN USING CRYSTAL STRUCTURES

Neuraminidase (Sialidase) Inhibitors

Studies of influenza neuraminidase exemplify the power of rationally designed drugs. The crystal structure for influenza neuraminidase was obtained in 1983, and since then many other enzymes have been characterized from other sources. Even before the crystal structure had been obtained, a neuraminidase inhibitor was deduced by assuming that the hydrolysis reaction probably involved a transition state with a carbocation intermediate at C-2. This would result in C-2 and C-3 adopting a trigonal planar (sp^2) configuration, and therefore compounds that mimicked this geometry were hoped to have inhibitory activity. Indeed, Neu5Ac-2-

ene (DANA; Figure 55.5) has a micromolar K_i value. Interestingly, this compound works on most sialidases, but not on the trypanosome *trans*-sialidase and only weakly on bacterial sialidases.

Visual inspection of influenza neuraminidase with the inhibitor bound showed that two glutamate residues lined a pocket near carbon 4 of the sialic acid analog (Figure 55.6). The pocket was fairly open, suggesting that a bulkier substituent at this position might be tolerated, at least sterically. A substrate analog was produced containing a positively charged guanidinium group instead of the hydroxyl at carbon 4 (4-guanidino-DANA; Figure 55.5), creating an analog with remarkable influenza neuraminidase inhibitor potency (K_i value of 10^{-11} M). The higher affinity was presumably due to an additional salt bridge formed between the charged guanidinium group and the carboxylates lining the pocket. The analog is nearly a million times less potent on human sialidases, leading to its use as the anti-influenza drug Relenza® (Chapter 57). It does not work on bacterial sialidases, however, because the equivalent pocket is filled with an arginine group.

Subsequent studies substituted a cyclohexene to mimic the planar ring of the proposed intermediate in hydrolysis. Good inhibitors were found and an orally active analog is widely used, the oral anti-influenza drug Tamiflu® (Figure 55.5, Chapter 57). As the crystal structures for other sialidases are solved, the design of species-specific analogs may be possible. This rational approach to inhibitor design holds great potential, not only for neuraminidase inhibitors, but also for the design of compounds that might block the activity of various glycosyltransferases.

Sulfotransferase Inhibitors

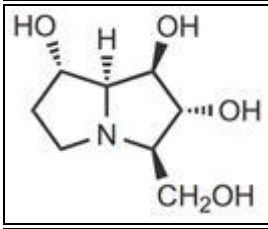
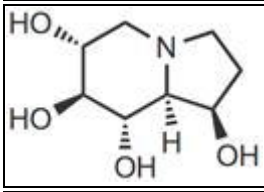
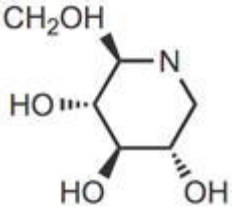
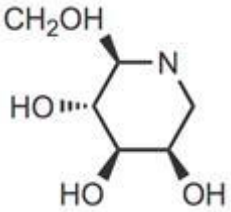
A large family of Golgi sulfotransferases installs sulfate esters on a variety of glycans using PAPS as the active sulfate donor. High-resolution crystal structures are now available for both glycan-modifying sulfotransferases as well as soluble drug detoxifying sulfotransferases. These enzymes have in common a conserved fold that binds PAPS (see Chapter 6). A series of small aromatic compounds (such as 2,6-dichloro-4-nitrophenol and pentachlorophenol) and several disaccharide analogs of GlcNAc-6-sulfotransferase substrates have inhibitory activity. Screening targeted libraries of purine derivatives yielded compounds with high selectivity towards individual sulfotransferases, suggesting that subtle differences in the PAPS-binding sites can be exploited.

The ability to screen large libraries against sulfotransferases has been facilitated by the development of high-throughput assays. Most low-throughput assays measure the transfer of [^{35}S] from [^{35}S]PAPS or measure the radiolabel transfer to a carbohydrate substrate bearing a hydrophobic tail that can easily be isolated using a reverse-phase cartridge. Some sulfotransferases will catalyze the reverse reaction in the presence of high concentrations of a sulfated donor. For example, β-

TABLE 55.1 Classes of inhibitors

Class of inhibitor/modulator	Target
Metabolic inhibitors	Steps involved in formation of common intermediates such as PAPS or nucleotide sugars
Tunicamycin	N-linked glycosylation through inhibition of dolichol-PP-GlcNAc formation; peptidoglycan biosynthesis through inhibition of undecaprenyl-PP-GlcNAc assembly
Plant alkaloids	N-linked glycosylation through inhibition of processing glycosidases
Substrate analogs	Specific glycosyltransferases or glycosidases
Glycoside primers	Glycosylation pathways by diverting the assembly of glycans from endogenous acceptors to exogenous primers

Table 55.2 Examples of alkaloids that inhibit glycosidases involved in N-linked glycan biosynthesis

Alkaloid	Source	Target
Australine		α-glucosidase I
Castanospermine		α-glucosidase I and II
Deoxynojirimycin		α-glucosidase II (and I)
Deoxymannojirimycin		α-mannosidase I

arylsulfotransferase IV (β-AST-IV) will catalyze the reverse transfer of the sulfuryl group from *p*-nitrophenol sulfate to PAP, generating PAPS and *p*–nitrophenolate ion. When coupled to another sulfotransferase of interest, β-AST-IV regenerates PAPS and stoichiometric amounts of the ion, which can be monitored by UV absorbance. The enzyme also will transfer sulfate to water. A library of 35,000 compounds with purine and pyrimidine scaffolds was screened using β-AST-IV and a fluorescence assay that measured desulfation of 4-methylumbelliferone sulfate. Multiple hits were obtained with moderate inhibition, and subsequent structure elaboration of the library resulted in the generation of a very tight binding small molecule inhibitor with a K_m value five orders of magnitude lower then the natural substrate. In theory, this approach can be exploited for other enzymes for which high-throughput assays can be developed.

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Figure Legends

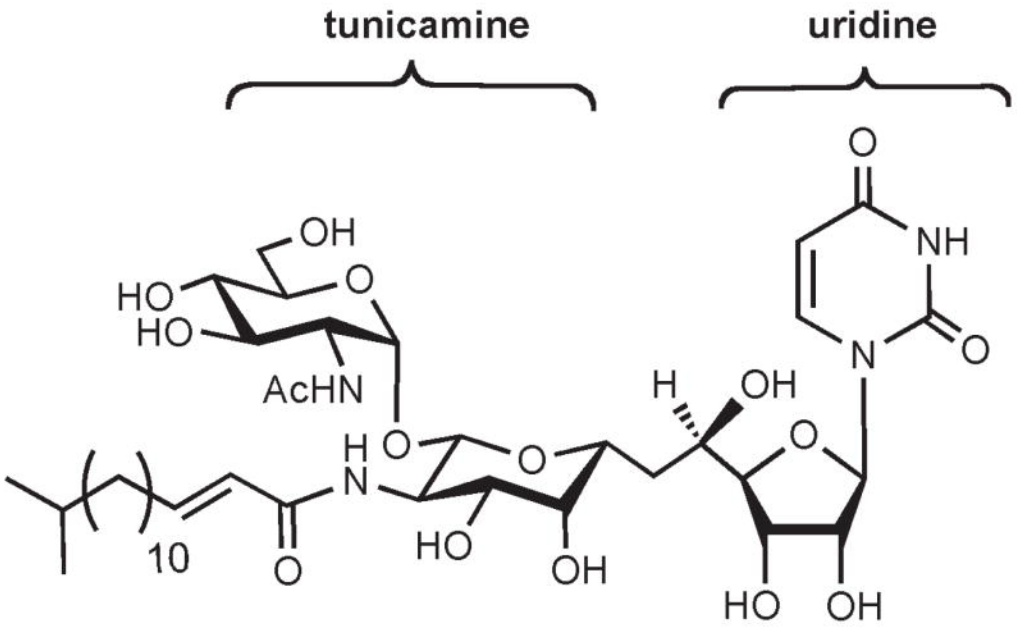


FIGURE 55.1. Structure of tunicamycin, which consists of uridine conjugated to the disaccharide, tunicamine.

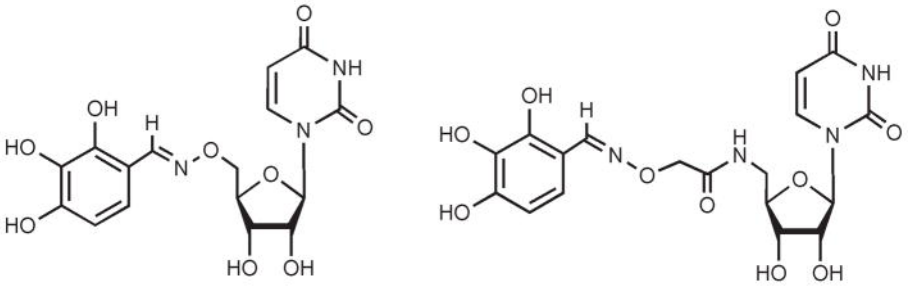


FIGURE 55.2. Broad-spectrum inhibitors of the ppGalNAcTs identified from screening a uridine-based library.

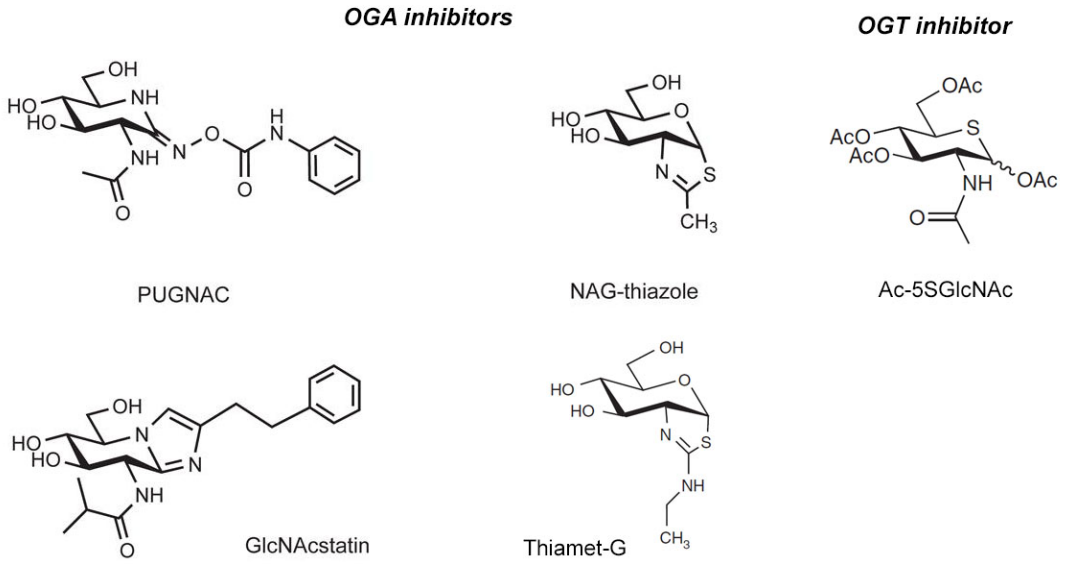


FIGURE 55.3. Inhibitors of O-GlcNAc-specific β-hexosaminidase (OGA) and O-GlcNAc transferase (OGT).

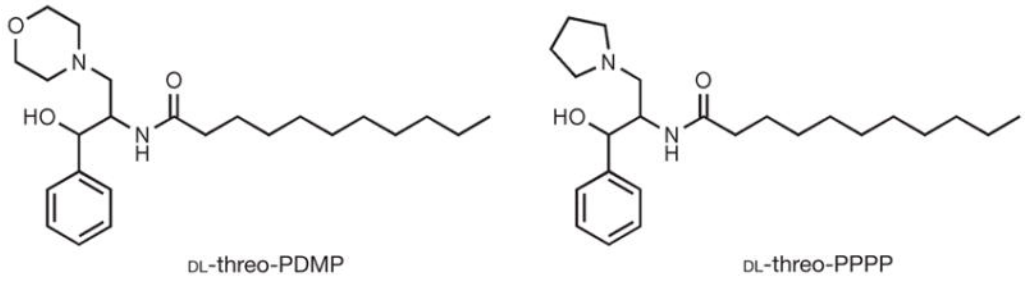


FIGURE 55.4. Inhibitors of glycosphingolipid formation. These analogs are potent inhibitors of the glucosyltransferase that initiates glycosphingolipid formation.

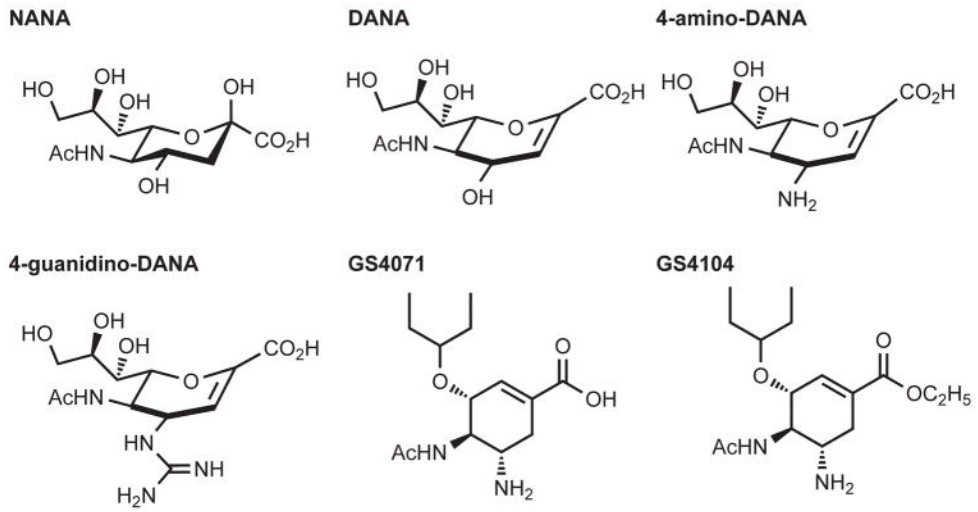


FIGURE 55.5. Structure of influenza neuraminidase inhibitors. Chemical structure of Neu5Ac, NANA; 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid, DANA; 4-amino-DANA; 4-guanidino-DANA (Relenza®, zanamivir); (3R, 4R, 5S)-4-acetamido-5-amino-3-(1-ethylpropoxyl)-1-cyclohexane-1-carboxylic acid ethyl ester (GS4104, Tamiflu®, oseltamivir). DANA is thought to resemble the transition state in hydrolysis, and addition of the guanidinium group in Relenza provides higher affinity binding to the active site. The ethyl ester in Tamiflu enhances oral availability, then is quickly removed in the body by non-specific esterases.

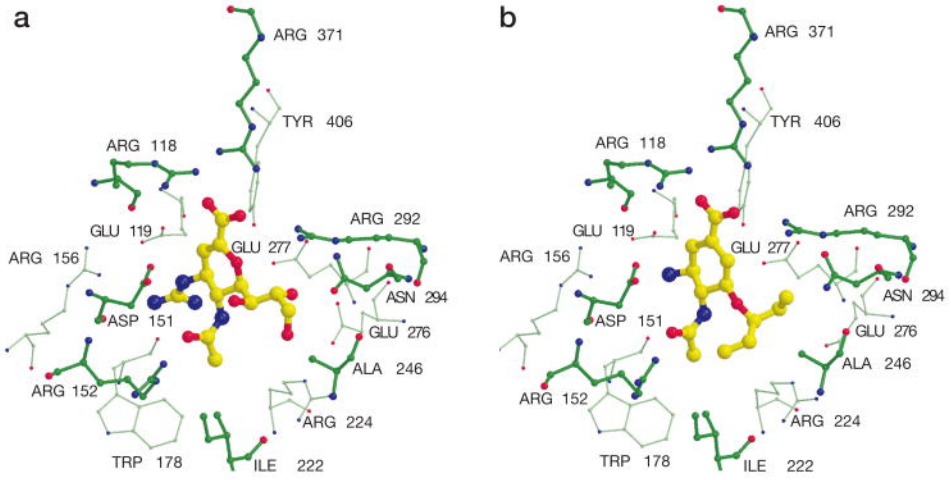


FIGURE 55.6. Crystallographic structures of influenza virus neuraminidase (N9 subtype) with two different rationally designed inhibitors bound in the active site. The inhibitors are shown as *colored ball and stick* models, where *yellow* is carbon, *blue* is nitrogen, and *red* is oxygen. The catalytic site of the enzyme is shown with the closer carbon atoms in *dark green* and those further away in *light green*. (*a*) Relenza (4-guanidino-Neu5Ac2en). (*b*) De-esterified Tamiflu. (Redrawn, with permission, from Garman E. and Laver G. 2004. *Curr. Drug Targets* **5:** 119–136.)

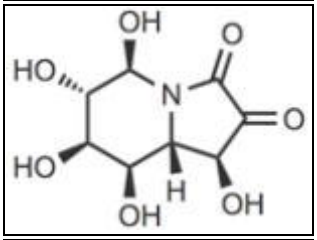
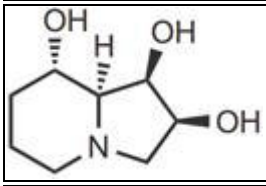
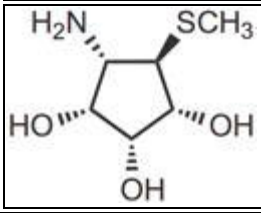
Alkaloid	Source	Target
Kifunensin		α-mannosidase I
Swainsonine		α-mannosidase II
Mannostatin A		α-mannosidase II

TABLE 55.3 Synthetic substrate-based inhibitors of glycosyltransferases

Enzyme	Substrate	Inhibitor	Substrate K_m (μM)	Inhibitor K_i (μM)
α2FucT	β3GlcNAcβ-*O*-R	2-deoxyGalβ3GlcNAcβ-*O*-R	200	800
β4GalT	GlcNAcβ3Galβ-*O*-R	6-thioGlcNAcβ3Galβ-*O*-Me	1000	1000
α3GalT	Galβ4GlcNAcβ-*O*-R	3-aminoGalβ4GlcNAcβ-*O*-R	190	104[a]
β6GlcNAcT	Galβ3GalNAcα-*O*-R	Galβ3(6-deoxy)GalNAcα-*O*-R	80	560
β6GlcNAcT-V	GlcNAcβ2Manα6Glcβ-*O*-R	GlcNAcβ2(6-deoxy)Manα6Glc β-*O*-R	23	30
β6GlcNAcT-V	GlcNAcβ2Manα6Glcβ-*O*-R	GlcNAcβ2(4-*O*-methyl)Manα6 Glcβ-*O*-R	23	14
β6GlcNAcT-V	GlcNAcβ2Manα6Glcβ-*O*-R	GlcNAcβ2(6-deoxy,4-*O*-Me)Manα6Glcβ-O-R	23	3
α6SialylT	Galβ4GlcNAcβ-O-R	6-deoxyGalβ2GlcNAcβ-*O*-R	900	760[a]

α3GalNAcT-A	Fucα2Galβ-*O*-R	Fucα2(3-deoxy)Galβ-*O*-R	2	68
α3GalNAcT-A	Fucα2Galβ-*O*-R	Fucα2(3-amino)Galβ-*O*-R	2	0.2[a]

The aglycone (R) varies in the different compounds.

[a] Inhibition mixed or noncompetitive.

TABLE 55.4 Examples of glycoside primers

Glycoside	**Pathway affected**
Xylβ-*O*-R	Glycosaminoglycans, glycolipids
Galβ-*O*-R	Glycosaminoglycans
GalNAcα-*O*-R	O-linked chains found on glycoproteins and mucins
GlcNAcβ-*O*-R	Polylactosaminoglycans
Peracetylated Galβ4GlcNAcβ-*O*-R	Sialyl Lewis^x
Peracetylated GlcNAcβ3Galβ-*O*-R	Sialyl Lewis^x

Chapter 57, Glycans in Biotechnology and the Pharmaceutical Industry

Essentials in Glycobiology, 3rd edition

Chapter 57

Glycans in Biotechnology and the Pharmaceutical Industry

Authors: Peter H. Seeberger, Richard D. Cummings

Several classes of successful commercial products are based on isolated or synthetic glycans. This chapter summarizes the use of glycans as vaccines and therapeutics. Applications of glycan mimics as drugs is also discussed.

GLYCANS AS COMPONENTS OF SMALL-MOLECULE DRUGS

Many well-known small-molecule drugs, such as antibiotics and anticancer therapeutic agents, are natural products that contain glycans as part of their core structure and/or as a sugar side chain (i.e., a glycoside). Some examples of natural products that bear glycan side chains are shown in Figure 57.1. The well-established area of natural product chemistry will not be reviewed in detail here. Modified glycans gave rise to synthetic drugs such as small-molecule inhibitors of influenza virus neuraminidase (see also Chapter 55). Recent advances in the functional understanding of carbohydrate–protein interactions have enabled the development of glycomimetics, a new class of small-molecule drugs that are briefly described.

Small-Molecule Inhibitors of Influenza Virus Neuraminidase

Influenza virus has two major surface proteins, hemagglutinin and neuraminidase (see Chapter 34). The hemagglutinin initiates infection by binding to cell-surface sialic acids. The neuraminidase assists virus release by cleaving sialic acids to prevent unwanted retention of newly synthesized virus on the cell surface. Neuraminidase may also function during the invasion phase by removing sialic acids on soluble mucins that would otherwise inhibit cell-surface binding. Since neuraminidase is essential to the viral life cycle, based on the crystal structure of the enzyme a rational drug design program yielded Zanamivir (Relenza™). The addition of a bulky guanidino side chain at C-4 of a previously known neuraminidase inhibitor, 2-deoxy-2,3-dehydro-*N*-acetyl-neuraminic acid, markedly increased the affinity for influenza neuraminidase, without affecting host-cell neuraminidases (Figure 57.2; see also Chapter 55). Relenza blocks the influenza virus life cycle by preventing infection and by interrupting the spread of the virus during the early phase of an infection. Due to poor oral availability, Relenza has to be inhaled to work at the mucosal sites of infection in the upper airway. The orally available drug Oseltamivir (Tamiflu™) achieves the same effects and has taken over most of the market due to the ease of use. The fear of avian influenza virus (“bird flu”) spreading into human populations has prompted stockpiling of Tamiflu. Fortunately, widespread use of the drug has not become necessary to date. The development of Tamiflu is a textbook example for rational drug design resulting in a powerful drug against a devastating disease.

THERAPEUTIC GLYCOPROTEINS

Most biotherapeutic products are glycoproteins and include erythropoietin as well as various other cytokines, antibodies, glycosyltransferases, and glycosidases. This class of molecules sells in the tens of billions USD per year world wide. Therapeutic glycoproteins are typically produced as recombinantly in cell culture systems or, less commonly, in the milk of transgenic animals. Control of glycosylation is of major importance during the development of these drugs, because their glycan chains have marked effects on stability, activity, antigenicity, and pharmacodynamics in intact organisms. In most cases, glycosylation must be optimized to ensure prolonged circulatory half-life in the blood. Manipulation of glycans to promote targeting to specific tissues and cell types has also been a useful element of drug design.

Optimizing Glycans of Therapeutic Glycoproteins for Prolonged Serum Half-life

Erythropoietin (EPO) is the most successful biotechnology product to date. It is a circulating cytokine that binds to the erythropoietin receptor, inducing proliferation and differentiation of erythroid progenitors in the bone marrow. EPO was developed to treat anemias caused by bone marrow suppression after chemotherapy or lack of erythropoietin (e.g., renal failure). Natural and recombinant forms of erythropoietin carry three sialylated complex N-glycans and one sialylated O-glycan. Although in vitro the activity of deglycosylated erythropoietin is comparable to that of the fully glycosylated molecule, its activity in vivo is reduced by about 90%, because poorly glycosylated erythropoietin is rapidly cleared by filtration in the kidney. Undersialylated erythropoietin is also rapidly cleared by galactose receptors in hepatocytes and macrophages (see Chapter 31). Fully sialylated chains and increased tetra-antennary branching reduces these problems and increases EPO activity in vivo nearly tenfold. Addition of an N-glycosylation site also increases half-life and activity in vivo. Covalently linking polyethylene glycol to the protein also reduces clearance by the kidney. Erythropoietin is unusual because it is small enough to be cleared by the kidney if it is underglycosylated. For most glycoprotein therapeutics, a more important consideration is minimizing clearance by galactose-binding hepatic receptors by ensuring full sialylation of glycans. Because glycans greatly influence the efficacy of these drugs, control over glycosylation during production is vry important in light of regulatory requirements for batch-to-batch product consistency. Changes in culture pH, the availability of precursors and nutrients, and the presence or absence of various growth factors and hormones can each affect the extent of glycosylation, the degree of branching, and the completeness of sialylation. Sialidases and other glycosidases that are either secreted or released by dead cells can also cause degradation of the previously intact product in the culture medium. These issues were hotly debated in recent years with the advent of “biosimilars” or generic versions of glycoproteins. The need to proof composition has fueled efforts devoted to glycan analysis and sequencing.

Impact of Glycosylation on Licensing and Patentability of Biotherapeutic Agents Therapeutic Glycoproteins for Prolonged Serum Half-life

Patenting of new therapeutics is typically based on the composition of matter in the claimed molecule. Small molecules of defined structure and nonglycosylated proteins are easily captured in this manner. However, glycoproteins, especially those with multiple glycosylation sites, render it virtually impossible to obtain preparations that contain only a single glycoform. Thus, most biotherapeutic glycoproteins consist of a mixture of glycoforms. Licensing bodies allow for a certain range of variation in glycoforms and the complexity of the mixture. However, the manufacturer and the agency must agree on the extent such variation is acceptable for a given drug formulation. Biopharmaceutical companies therefore spend considerable effort in assuring that their products fall within these defined ranges, once these are approved by licensing bodies. The inherent difficulty in reproducing complex glycoform mixtures also complicates efforts to make generic forms of recombinant glycoprotein drugs. Given the complexities of producing glycotherapeutic agents in mammalian cells, even the smallest changes in growth conditions can have significant effects on the range of glycoforms found in any given product batch. The licensing agencies use consistency in glycoform composition as an indirect measure of the quality of process control in production. Differences in glycosylation can have implications for the patentability of agents where the polypeptide remains constant. Marked differences in glycosylation have been used to define agents as being uniquely different. However, it is usually necessary to show that the differences in glycosylation being claimed also have a significant effect in changing the functionality of the drug in question. The associated pharmaceutical licensing and legal issues are rapidly evolving to keep pace with scientific advances in this area.

GLYCOSYLATION ENGINEERING

T There are limits as to how much of a biotherapeutic glycoprotein an animal cell line can produce. Production becomes an issue in cases where very large amounts of a particular glycoprotein is needed. Glycoprotein production in plants or yeast is attractive but makes it necessary to eliminate risks arising from the nonhuman glycans of plant and fungal cells that could cause excessively rapid clearance and/or antigenic reactions. Many plant and yeast glycans are immunogenic and elicit glycan-specific IgE and IgG antibodies in humans when delivered parenterally. A variety of mammalian genes have been added back into yeast and/or genes that are producing nonhuman glycosylation have ben eliminated. Extensively engineered yeast strains are capable of producing biantennary N-linked glycans with the human sialic acid *N*-acetylneuraminic acid (Neu5Ac) but the productivity of such yeast strains are often low. Efforts to engineer yeast to make human-like O-glycans are under way, but glycosaminoglycans have not yet been addressed.

Plants and algae have also been used to engineer recombinant glycoproteins, but, as in yeast, the glycans produced by plants differ from those found in vertebrates. The antigenic differences that arise in recombinant glycoproteins produced in plants become less problematic if used for topical or oral administration, since humans are normally exposed to plant glycans in the diet. The cost of production is much lower than in animal cell culture systems and animal sera are not needed. As in yeast, "humanizing plants" with respect to glycosylation may allow the production of nonimmunogenic glycoproteins. Chemical methods for synthesizing entire glycoproteins from scratch have been developed and single glycoforms of EPO have been prepared by total synthesis. Given the complexity of glycoprotein synthesis, scale up of these processes is challenging and an area of intense research activity. (see Chapter 49).

GLYCAN THERAPEUTIC APPROACHES TO METABOLIC DISEASES

Salvage versus De Novo Synthesis

All monosaccharides needed for cellular glycan synthesis can be obtained from glucose through metabolic interconversions (see Chapter 4). Alternatively, monosaccharides can be derived from the diet or salvaged from degraded glycans. The relative contributions of different sources can vary with the cell type. For instance, even though all mammalian cells use sialic acid, only some contain high amounts of UDP-GlcNAc epimerase/*N*-acetylmannosamine kinase (GNE), which is required for the de novo synthesis of CMP-sialic acid. But sialic acid salvage from degraded glycans is quite efficient, decreasing the demand on the de novo pathway. Similarly, galactose, fucose, mannose, *N*-acetylglucosamine, and *N*-acetylgalactosamine can come from the diet or be salvaged for glycan synthesis, whereas glucuronic acid, iduronic acid, and xylose cannot. All monosaccharides derived from the diet or degraded glycans can be catabolized for energy, and again, cells vary in their reliance on the different pathways.

The variable contributions of these pathways are important for therapy of some diseases. For instance, patients with congenital disorder of glycosylation type Ib (CDG-Ib), who are deficient in phosphomannose isomerase, benefit greatly from oral mannose supplementation to bypass the insufficient supply of glucose-derived mannose-6-phosphate. A few CDG-IIc patients have been treated with fucose to restore synthesis of sialyl Lewis^x on leukocytes (see Chapter 42). Some patients with Crohn's disease show clinical improvement with oral *N*-acetylglucosamine supplementation, but the mechanism is unknown. Mice deficient in GNE activity have kidney failure, but providing *N*-acetylmannosamine in the diet prevents this outcome. Clinical trials using *N*-acetylmannosamine to treat GNE-deficient patients with hereditary inclusion body myopathy type II (HIBM-II) have been conducted but have yielded inconclusive results.

Special Diets

Some monosaccharides and disaccharides can be toxic to humans who lack specific enzymes. For example, people who lack fructoaldolase (aldolase B) accumulate fructose-1-phosphate, which ultimately causes ATP depletion and disrupts glycogen metabolism. Prolonged fructose exposure in these people can be fatal, and fructose-limited diets are critical. Deficiencies in the ability to metabolize galactose (see Chapter 4) are mostly due to a severe reduction in galactose-1-phosphate uridyl transferase activity and cause galactosemia. Although these patients are asymptomatic at birth, ingesting milk leads to vomiting and diarrhea, cataracts, hepatomegaly, and even neonatal death. Low-galactose or galactose-free diets can prevent these life-threatening symptoms. However, even these diets do not prevent unexplained long-term complications, which include speech and learning disabilities and ovarian failure in females with galactosemia.

Infants hydrolyze lactose (Galβ1-4Glc) quite well, but the level of intestinal lactase can be much lower or absent in adults because of down-regulation of lactase gene expression. About two thirds of the human population has lactase nonpersistence, making milk products a dietary annoyance. Unabsorbed lactose provides an osmotic load and is metabolized by colonic bacteria, causing diarrhea, abdominal bloating, flatulence, and nausea. Lactase persistence has evolved in certain pastoral populations from northwestern Europe, India, and Africa, allowing milk consumption in adult life. However, many adults either avoid lactose-containing foods or use lactase tablets to improve lactose digestion.

Substrate Reduction Therapy

The failure to turn over glycans by lysosomal degradation causes serious problems for patients with lysosomal storage disorders. Deficiencies in individual lysosomal enzymes lead to pathological accumulation of their substrates in inclusion bodies inside the cells (see Chapter 41). One approach to treating these disorders is to inhibit initial glycan synthesis, a strategy termed substrate reduction therapy (SRT). Reduced synthesis of the initial compound decreases the load on the impaired enzyme, and some patients show significant clinical improvement. A small molecule drug used for SRT is *N*-butyldeoxynojirimycin (or *N*-butyl-DNJ) (Miglustat, Zavesca®), that was approved in 2002 for treatment of Gaucher's disease (glucocerebrosidase deficiency).

Lysosomal Enzyme Replacement Therapy

Another approach for treating lysosomal storage disorders is enzyme replacement therapy. Unlike most therapeutic glycoproteins that interact with target receptors on the surface of cells, lysosomal enzymes developed for replacement therapy must be delivered intracellularly to lysosomes, their site of action. During the normal biosynthesis of lysosomal enzymes, their N-glycans become modified with mannose-6-phosphate (Man-6-P) residues, which target them to lysosomes using Man-6-P receptors (see Chapter 30). The challenge for enzyme replacement therapy is to get the enzymes targeted properly to lysosomes, where they can degrade accumulated substrate. Enzyme replacement therapy for Gaucher's disease targets the lysosomes

of macrophages via the cell-surface mannose receptor (see Chapter 31). The four recombinant enzyme products Imiglucerase (approved in 1995), Velaglucerase (approved in 2010), Taliglucerase alfa (Elelyso, approved in 2012), and Eliglustat (Cerdelga, approved in 2014) are marketed.
The success of glucocerebrosidase treatment stimulated the development of lysosomal enzymes for treatment of other lysosomal storage diseasessuch as Fabry's disease, mucopolysaccharidoses type I, II, and VI, and Pompe's disease. The replacement therapies clearly have beneficial effects and prolong life but are extremely expensive.

Chaperone Therapy

A third approach for treating lysosomal storage disorders takes advantage of the fact that some genetic defects lead to misfolding of the encoded enzyme in the endoplasmic reticulum (ER). Low-molecular-weight competitive inhibitors of some of these enzymes can act as "chaperones," that stabilize the folded enzyme in the ER and effectively rescue the mutation and increase the steady-state concentration of active enzyme in the lysosome. The dose of the inhibitor must be carefully adjusted to ensure that the inhibitory effects on enzyme function do not overshadow beneficial effects on folding. Only a low level of enzyme restoration is needed to significantly reduce the accumulation of undigested glycan substrates, indicating that lysosomal hydrolases are normally present in large catalytic excess.

THERAPEUTIC APPLICATIONS OF GLYCOSAMINOGLYCANS

The use of purified glycans as therapeutics has received less attention than the development of glycoprotein-based treatments. Difficulties in establishing structure–activity relationships due to the large number of chiral centers and functional groups, undesirable pharmacokinetics of available formulations, poor oral absorption of the compounds, and low-affinity interactions with drug targets have limited their development. Some successful glycan drugs, such as the anticoagulant heparin, are given by injection, although efforts are under way to convert heparin into an orally absorbable form by complexing it with positively charged molecules. It may be possible to deliver other hydrophilic and/or negatively charged glycan drugs in this way to allow penetration of the intestinal barrier. Glycans are also sometimes attached to hydrophobic drugs to improve their solubility and alter their pharmacokinetics.

The anticoagulant heparin is as discussed in Chapters 16 and 43, one of the most widely prescribed drugs today. Heparin binds and activates antithrombin, a protease inhibitor of the coagulation cascade. Antithrombin activation leads to rapid inhibition of thrombin and factor Xa, shutting down the production of fibrin clots. Billions of doses of heparin (several metric tons) is produced by autodigestion of pig intestines, followed by graded fractionation of the products. Unfractionated heparin produces a variable anticoagulant response as it also binds to several plasma,

platelet, and endothelial proteins. Low-molecular-weight (LMW) heparins are derived by chemical or enzymatic cleavage of heparin to form smaller fragments. The pharmacological properties and the relative efficacy of the various LMW heparins are superior to those of unfractionated heparin and fewer secondary complications are reported. LMW heparins have replaced unfractionated heparins as therapeutic of choice in virtually all developed countries. In price sensitive markets the unfractionated products are still heavily used. The preparation of recombinant heparin based on heparin biosynthesis enzymes is still under development. Arixtra, a synthetic heparin pentasaccharide that binds antithrombin exactly as isolated heparin is used to prevent deep-vein thrombosis and pulmonary embolism and has gained market share in recent years. Still, the higher cost of the synthetic drug Arixtra has prevented an even larger success therapeutic.
To prevent excessive bleeding, rapid neutralization of heparin is desirable. Administration of the basic protein protamine, which binds to heparin, neutralizes its activity, and results in clearance of the complex by the kidney and liver. Heparin is also used to treat protein-losing enteropathy (PLE), likely working by competing for proinflammatory heparin-binding cytokines that trigger PLE in susceptible patients (see Chapter 43).

Hyaluronan (see Chapter 15) is a naturally occurring glycosaminoglycan that is extensively used in surgical applications. Because of its viscoelastic properties, hyaluronan has lubricating and cushioning properties that have made it useful for protecting the corneal endothelium during ocular surgery. Hyaluronan has antiadhesive properties and is useful in postsurgical wound healing. The mechanism of action is not well understood, but it may involve hyaluronan-binding proteins that mediate cell adhesion (see Chapter 15). Intra-articular injections of hyaluronan are used to treat knee and hip osteoarthritis. Modest improvement in patients treated with hyaluronan, may be the result of a mechanical (as a viscosupplement) and/or a biological (via signaling pathways) effect. Hyaluronan is used in very large quantities as a tissue filler in cosmetic medicine.

"GLYCONUTRIENTS"

"Glyconutrient" is a term used by the nutritional supplement industry to describe some of their products with wide-ranging claims concerning potential benefits. In most cases, these claims have not been substantiated through placebo-controlled, double-blind trials with defined, quantifiable outcomes. Much work is needed in this area to obtain insight into the potential role of dietary glycans on human health and to help consumers make wise decisions regarding their use. Mixtures of plant polysaccharides such as larchbark arabinogalactan, and glucomannan are often termed "glyconutrients" that are claimed to contain "essential monosaccharides" needed for "cell communication." Because all monosaccharides can be made from glucose (except in patients with rare genetic deficiencies; see Chapter 42), none of the other monosaccharides are actually known to be "essential." Moreover, these polysaccharides are not degraded to available monosaccharides in the stomach or

small intestine. Instead, anaerobic bacteria in the colon metabolize them and produce short-chain fatty acids. No peer-reviewed clinical studies support the efficacy of such "glyconutrients" for any disease or condition. Nevertheless, the following examples demonstrate how dietary glycans might have beneficial effects.

Glucosamine and Chondroitin Sulfate

Glucosamine (often mixed with chondroitin sulfate) has been promoted to relieve symptoms of osteoarthritis, which involves the age-dependent erosion of articular cartilage. Cartilage provides a cushion between the bones to minimize mechanical damage, and a net loss of cartilage occurs when the degradation rate exceeds the synthetic rate. A number of clinical trials report that glucosamine improves osteoarthritis symptoms, and some claim to restore partially the structure of the eroded cushion, in particular in the knees. Superficially, this would seem to make sense, because primary glycans of cartilage include hyaluronan (see Chapter 15) and chondroitin sulfate, both of which contain hexosamines within their structure (see Chapter 16). Conflicting reports suggest that the outcome may depend on study design and the type and source of material. Nevertheless, veterinarians report positive results after treating animals with glucosamine for over two decades. Double-blind, placebo-controlled studies in humans have shown a decreased rate of joint space narrowing. Glucosamine might also alter UDP-GlcNAc and potentially UDP-GalNAc levels, thus affecting cellular responses involving major classes of glycans.

Positive effects of chondroitin sulfate on osteoarthritis are less well-documented. It remains unclear how the acidic chondroitin sulfate polymer can be absorbed and delivered to its proposed site of action. Further studies are needed to determine whether chondroitin sulfates are absorbed by the target tissue, and if they actually lead to changes in cartilage metabolism.

Xylitol and Sorbitol in Chewing Gum

Many studies suggest that chewing gum containing sugar alditols such as xylitol and sorbitol, can help control the development of dental caries. Mothers who chew xylitol-sweetened gum may even block transmission of caries-causing bacteria to their children. The benefit of these reduced sugars seems to be based on stimulation of salivary flow, but an antimicrobial effect is also possible. Xylitol also inhibits the expression and secretion of proinflammatory cytokines from macrophages and inhibits the growth of *Porphyromonas gingivalis,* one of the suspected causes of periodontal disease. Children who drank xylitol solutions also had a lower occurrence of otitis media.

Milk Oligosaccharides

Human milk contains about 70 g/liter of lactose and 5–10 g/liter of free oligosaccharides. More than 130 different glycan species have been identified with lactose at the reducing end, including poly-*N*-acetyllactosamine units. Some glycans

are α2-3- and/or α2-6-sialylated and/or fucosylated in α1-2, α1-3, and/or α1-4 linkages. In contrast, bovine milk, the typical mainstay in human infant formulas, contains much smaller amounts of these glycans. These differences may account for some of the physiological advantages seen for breast-fed versus formula-fed infants. The glycans may also favor growth of a nonpathogenic bifidogenic microflora and/or block pathogen adhesion that causes infections and diarrhea. Surprisingly, a substantial number of human milk oligosaccharides remain almost undigested in the infant's intestine and are excreted intact into the urine. Whether supplementing infant formula with specific, biologically active free glycans enhances infant health is unknown.

GLYCANS AS VACCINE COMPONENTS

Microbial Vaccines

Polysaccharide vaccines consisting solely of glycan components typically elicit poor immunity, especially in infants. Since glycans are T-cell-independent antigens they do not effectively stimulate T-helper-dependent activation and class switching of B-cell-mediated immunity. Conjugate vaccines consisting of glycans coupled to carrier proteins have proven to be highly effective. Three major conjugate vaccines are marketed today: *Haemophilus influenzae* type b (Hib) causes an acute lower respiratory infection among young children. Children up to this age constitute a high risk group for Haemophilus influenzae type B infections. Consequently, the Hib PSV introduced in 1985 was withdrawn from the market in 1988 and replaced by CPS-protein conjugate vaccine formulations. A conjugated form of an Hib-derived oligosaccharide coupled to a protein carrier is part of routine vaccination schedules and has been so successful that infectious diseases caused by this bacterium are nearly eradicated in vaccinated populations. A potent semi-synthetic Hib glycoconjugate vaccine marketed in Cuba contains glycan chains with an average length of 16 monosaccharides. Pneumococcal conjugate vaccines have been developed to cover an increasing number of serotypes, and current formulations are 10- (Synflorix®, GSK) and 13-valent (Prevnar13®, Pfizer). Prevnar 13 provides protection against serotypes that account for more than 70% of cases of invasive pneumococcal disease worldwide and is the best selling vaccine axceeding revenues of five billion US$ in 2015. Conjugate vaccines to protect from *Neisseria meningitides* are also very successful on the market. Due to the fast onset and rapid progression of meningococcal infections, vaccination is required to protect against this disease. Several conjugated CPS vaccines are licensed in different parts of the world: The tetravalent serogroup A, C, W, and Y (Menactra, Menveo, and Nimenrix) and a few monovalent vaccines based on serogroup C CPS (Meningitec, Menjugate, NeisVac-C). Two combination vaccines for *N. meningitidis* and *Haemophilus influenzae* type b (Hib) are available against meningococcal serogroups C/Y (MenHibrix) (Hale et al., 2014) and against meningococcal serogroup C (Menitorix). A monovalent serogroup A vaccine (MenAfriVac) is widely used in the sub-Saharan meningitis belt of Africa.

Currently, several new vaccines based on synthetic oligosaccharide antigens are being developed to protect children and the elderly from a variety of bacterial infections. Vaccines to protect from hospital acquired infections that are increasingly antibiotic resistant are in preclinical evaluation.

Cancer Vaccines

Several carbohydrate-based cancer vaccines are at different stages of development to treat cancer. Ganglioside immunogens present on certain types of cancer cells such as gangliosides GM2 and GD2 in melanomas and globo H in breast cancer are being explored. The shorter glycan sequences such as sialyl-Tn (sialylα2-6GalNAcα-) found on cancer mucins (see Chapter 44) has seen little progress in twenty years. The synthetic GloboH hexasaccharide (see Figure 57.1) resembling the breast and prostate cancer antigen has reached Phase3 clinical trials and is expected to gain approval for marketing in late 2016 following two decades of development. This first successful example is expected to boost to a very challenging area of vaccine development.

BLOCKING GLYCAN RECOGNITION IN DISEASES

Blocking Infection

As discussed in Chapter 34, many microbes and toxins bind to mammalian tissues by recognizing specific glycan ligands. Thus, small soluble glycans or glycan mimetics can be used to block the initial attachment of microbes and toxins to cell surfaces (or block their release), and thus prevent or suppress infection. Because many of these organisms naturally gain access through the airways or gut, the glycan-based drugs can be delivered directly without being distributed systemically. Milk oligosaccharides are believed to be natural antagonists of intestinal infection in infants (see above) and polymers that will block the binding of viruses such as influenza. Although backed by a strong scientific rationale and robust in vitro studies, such “antiadhesive” therapies have not yet found much practical application.

Inhibition of Selectin-mediated Leukocyte Trafficking

When specific glycan-protein interactions are responsible for selective cell–cell interactions and a resulting pathology, then administration of small-molecule glycomimetics of the natural ligand is useful means of intervention. Selectin-mediated recruitment of neutrophils and other leukocytes into sites of inflammation or ischemia/reperfusion injury involves specific selectin–glycan interactions in the vascular system (see Chapter 31). The use of sialyl Lewis^x tetrasaccharide derivatives failed due to poor oral availability and a short serum half-life. Glycomimetics that preserve the essential functionality of the parent tetrasaccharide but eliminate unwanted polar functional groups and synthetically

cumbersome glycan components have been successful. The design of a monosaccharide glycomimetic starting from sialyl Lewis^x is shown in Figure 51.3. First, the sialic acid residue was replaced with a charged glycolic acid group, the *N*-acetylglucosamine residue was then replaced with an ethylene glycol linker, and finally the galactose residue was replaced with a linker moiety. The resulting glycomimetic had E-selectin binding affinity comparable to sialyl Lewis^x.
Simple monovalent sialyl Lewis^x glycomimetics have proven effective in various animal inflammatory models as well as clinical trials. Bimosiamose (TBC-1269) an E-, P- and L-selectin inhibitor is in phase 2 trials as treatment against asthma and psoriasis. GMI-1070 is targeting the same lectins and is being explored as a treatment for sickle cell crisis in phase 2 clinical trials.

TRANSFUSION AND TRANSPLANTATION REJECTION BY ANTIGLYCAN ANTIBODIES

As discussed in Chapter 13, a variety of glycans, including the classical A and B blood group determinants, can act as barriers to blood transfusion and transplantation of organs. Rejection of mismatched blood or organs occurs because hosts have a high titer of preexisting antibodies against the glycan epitopes, presumably as a prior reaction to related structures found on bacteria or other microbes. In the case of the ABO blood groups, incompatibility is routinely managed by blood and tissue typing and finding an appropriate donor for the recipient. Bacterial enzymes can be used in vitro to remove the A and B blood group determinants from A and B red cells, converting them into “universal donor” O red cells.

A related problem is found in xenotransplantation (i.e., the transplantation of organs between species) which is actively being pursued as a solution for the shortage of human organs for patients. The animal donors of preference are pigs, because many porcine organs resemble those of humans in size, physiology, and structure. However, unlike humans and certain other primates, pigs and most other mammals produce the terminal “α-Gal” epitope on glycoproteins and glycolipids. Because humans have naturally occurring high-titer antibodies in blood directed toward this epitope, this results in hyperacute rejection of porcine organ transplants, via reaction of the antibodies with endothelial cells of blood vessels. Attempts to prevent this reaction, include blood filtration over glycan affinity columns to remove xenoreactive antibodies and blockade of the interaction by infusing soluble competing oligosaccharides. Transgenic pigs lacking the reactive epitope have also been produced, as have animals with an excess of complement-controlling proteins on their cell surfaces. Pig organs also have high levels of the nonhuman sialic acid (Neu5Gc), against which most humans have antibodies. Even if this problem is solved, there are other glycan and protein structural differences between humans and pigs that cause later stages of graft rejection, thus necessitating immunosuppression.

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FIGURE LEGENDS

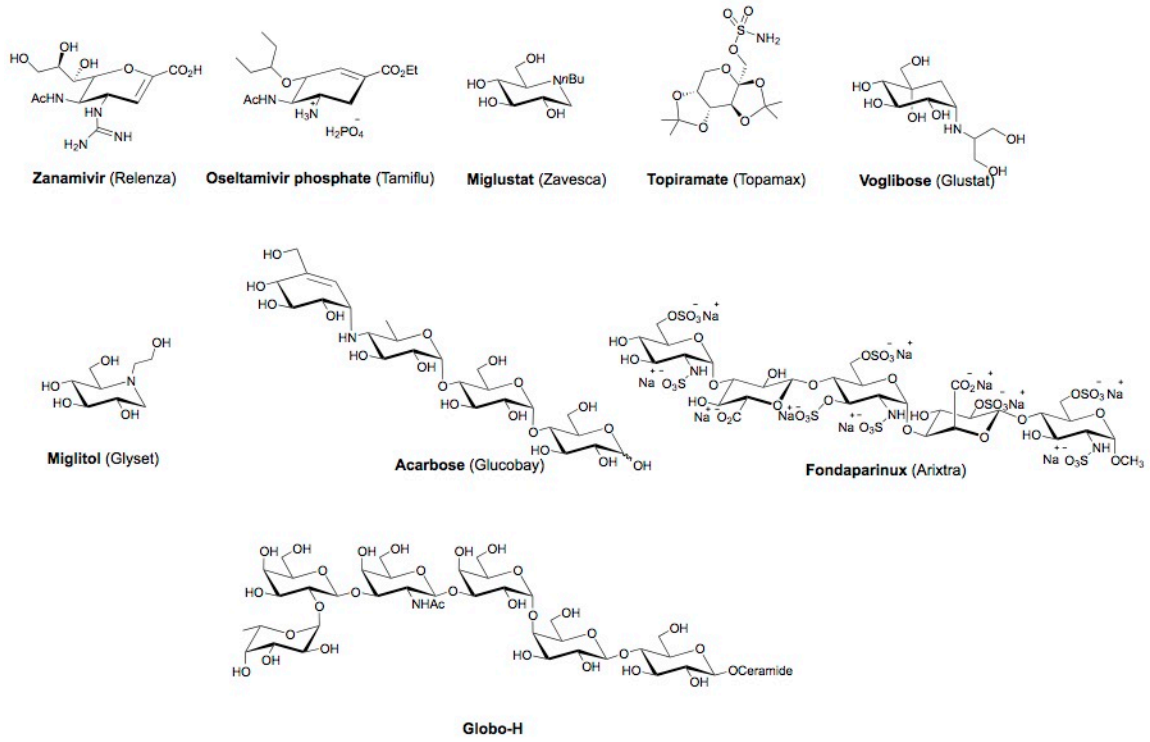

FIGURE 57.1. Examples of natural products that contain glycan components. Streptomycin and erythromycin A are antibiotics, doxorubicin is chemotherapeutic drug, and digoxin is used to treat cardiovascular disease.

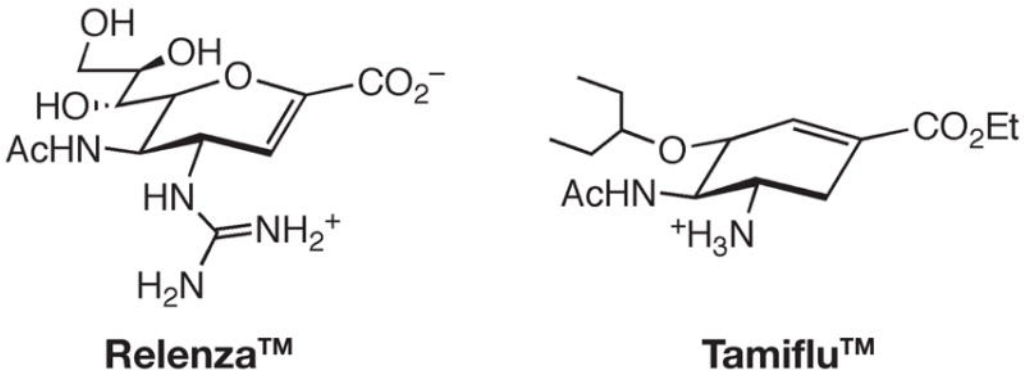

FIGURE 57.2. The synthetic influenza neuraminidase inhibitors Relenza™ and Tamiflu™.

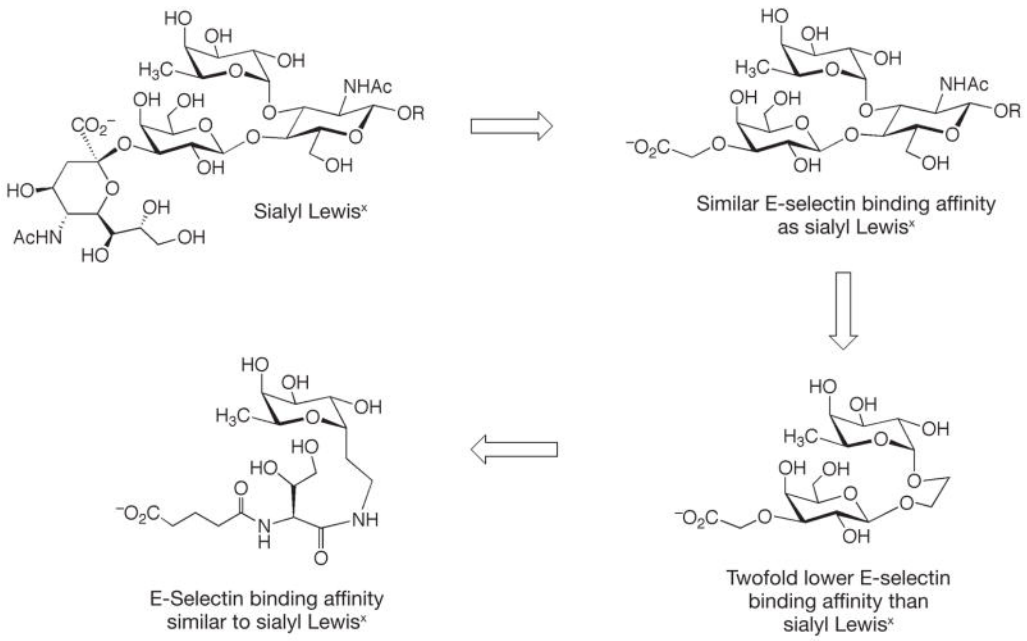

FIGURE 57.3. Glycomimetic E-selectin inhibitors based on sialyl Lewis^x.

TABLE 57.1 Examples of glycan-based drugs, their target diseases, and modes of action

Drug			
Targeting sialic acids			
Zanamivir (Relenza®)	Biota/GlaxoSmithKline	influenza type A and B	inhibits neuraminidase
Oseltamivir (GS 4104, Tamiflu®)	Gilead/Roche	chemoprophylaxis	inhibits neuraminidase
Targeting glycosaminoglycans			
Heparin	multiple brands	anticoagulant; possible value in cancer metastasis prevention	activates antithrombin; inhibits heparanase and selectins and blocks interactions between growth factors and heparan sulfate
Hyaluronan (HA)	multiple brands	ocular surgery; osteoarthritis; plastic surgery	tissue space filler; anti-inflammatory agent
Laronidase (Aldurazyme®)	Genzyme	mucopolysaccharidosis type I (MPSI); α-idu-ronidase deficiency	enzyme replacement therapy (ERT)
Galsulfase (Naglazyme®)	Biomarin	mucopolysaccharidosis type VI; arylsulfatase B deficiency	ERT
Hyaluronidase (Cumulase®)	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
Tramiprosate (Alzhemed)	phase III trials (Neurochem)	amyloid diseases, Alzheimer's disease, and possibly other amyloidoses	binds to amyloid plaque, blocks its formation
Eprodisate (Kiacta®)	phase II/III trials (Neurochem)	amyloid A amyloidosis	interferes with glycosamino-glycan–amyloid interactions
Targeting glycosphingolipids			
N-butyl-deoxynojirimycin (DNJ) (Miglustat, Zavesca®)	phase III trials (Acetelion)	type 1 Gaucher's disease; Niemann–Pick's disease type C; late-onset Tay–Sach's disease; type 3 Gaucher's disease	substrate reduction therapy; inhibits glucosylceramide synthase
Imiglucerase (Cerezyme®)	Genzyme	type 1 Gaucher's disease	ERT
β-Agalsidase (Fabrazyme®)	Genzyme	Fabry disease; α-galactosidase A deficiency	ERT
Others			
Acarbose (Glucobay®)	Bayer	type 2 diabetes	blocks intestinal α-glucosidases involved in digestion of dietary glycans
Alglucosidase alfa (Myozyme®)	Genzyme	Pompe's disease (glycogen storage disease); α-	ERT

		glucosidase A deficiency	
Allosamidin	Industrial Research	insecticide	chitinase inhibitor

Modified from Brown J.R., Crawford B.E., and Esko J.D. 2007. *Crit. Rev. Biochem. Mol. Biol.* **24:** 481–515.
Compounds targeted at microbial glycans, such as the aminoglycoside antibiotics or other inhibitors of cell wall assembly, have not been included.