Emerging themes in medicinal glycoscience

Kathryn M. Koeller and Chi-Huey Wong*

Department of Chemistry, The Scripps Research Institute and Skaggs Institute for Chemical Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037.
**Corresponding author (wong@Scripps.edu).*

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The recognition of complex carbohydrates and glycoconjugates as mediators of important biological processes has stimulated investigation into their therapeutic potential. New approaches for the simplification of glycoconjugate synthesis are overcoming the limitations of existing methods and providing a diverse array of these biomolecules. As the accessibility of glycoconjugates increases, carbohydrate-based constructs are becoming available for analysis as medicinal agents in a wide range of therapies.

Keywords: glycoconjugate, synthesis, therapeutic, antibiotic, vaccine

Numerous medicinally relevant physiological events rely on glycoconjugates for their viability[1–3]. The recognition of glycoprocessing enzymes as targets for therapeutic intervention has spurred the development of numerous drug candidates[4,5], including the well-known "flu" drugs that inhibit influenza virus neuraminidase. Likewise, the discovery of the selectins attracted researchers to the study of carbohydrate–protein interactions in the inflammatory response[6]. The observation that cell surface glycoforms are altered in certain cancers has served as both a diagnostic tool and the foundation of glycoconjugate-based vaccine development[7]. Glycans provide required recognition elements of many antipathogenic agents[8], and the glycopolymers heparin and hyaluronic acid are employed clinically as anticoagulants[9] or arthritis therapies, respectively. Furthermore, protein glycosyl phosphatidylinositol (GPI) anchors[10] and polylactosamine chains[11] play structural roles on the cell surface, and a nuclear β-*O*-GlcNAc modification may function in signal transduction pathways[12]. Moreover, the glycoprotein hormone erythropoeitin, used for clinical treatment of anemia, requires sialyl oligosaccharides for optimal activity in vivo[13].

Examination of the growing repertoire of glycoconjugate function provides assurance that this field will be at the forefront of research for years to come. Therapeutic agents based on carbohydrates or biological pathways in which they are involved have become important pharmaceutical targets. This review highlights areas in which glycoconstructs have been investigated for medicinal applications.

Problems in the development of glycotherapeutics

Many reliable options are available to protein and nucleic acid scientists for procuring a particular biomolecule. Automated synthesizers allow the manufacture of short peptide and nucleic acid sequences, and expression systems and polymerase chain reaction (PCR) make obtaining large quantities of macromolecular material a trivial consideration. Unfortunately, this is not the case for oligosaccharides and other glycoconjugates.

This discrepancy largely results from the intrinsic structure of carbohydrates themselves. To synthesize peptides or oligonucleotides, a method is available for iterative formation of a single type of bond (i.e., peptide or phosphodiester bond, respectively). However, synthesis of specific glycosidic linkages is a much more difficult task, as carbohydrates are densely functionalized with hydroxyl groups of similar reactivity. Synthetic carbohydrate chemists will attest to the many hurdles encountered in obtaining even relatively simple oligosaccharides, including laborious protecting-group manipulations and long overall synthetic routes. In addition, the degree of molecular diversity that can be generated from glycosidic linkage assembly is enormous: more than 10 million tetrasaccharides can be assembled from the 9 common monosaccharides, compared with 256 tetranucleotides and 16,000 tetrapeptides from the corresponding 4 nucleotides and 20 amino acid building blocks, respectively. Consequently, advancing glycoconjugate synthetic technology to the same level as proteins and nucleic acids remains a daunting challenge.

On a macromolecular scale, various cellular expression systems have proven useful for protein production. Yet, obtaining homogeneous *glyco*proteins is not a trivial matter. Glycosylation is a post-translational modification, rather than falling under direct transcriptional control, and glycan structure is therefore subject to several environmental factors. Enzyme competition for the same substrate, enzyme substrate specificity and availability, as well as variant glycosylation patterns with cellular host and culture conditions all play a role in the form of the resultant glycan[14]. Glycoproteins obtained from prokaryotic or eukaryotic expression systems therefore exhibit glycoform microheterogeneity[15]. This makes the contribution of specific glycan structure to underlying protein structure and function nearly impossible to assess.

Newly developed synthetic strategies

The ongoing investigation of carbohydrate-based therapeutic agents is dependent on the development of reliable synthetic methods to make glycoconjugates more readily available on a large scale. Toward this end, the incorporation of enzymes into synthetic protocols has the potential to drastically reduce the number of protecting-group manipulations and synthetic steps required[16–18].

Glycosyltransferases, especially combined with regeneration of sugar nucleotides (Fig. 1A), have great synthetic utility in the formation of glycosidic linkages[16–18]. Furthermore, genetic manipulation of the biosynthetic pathways in microorganisms has improved the production of sugar nucleotides[19]. The use of proteases as catalysts in peptide bond formation[20] and lipases for the mild removal of protecting groups[21] have been extremely benefi-

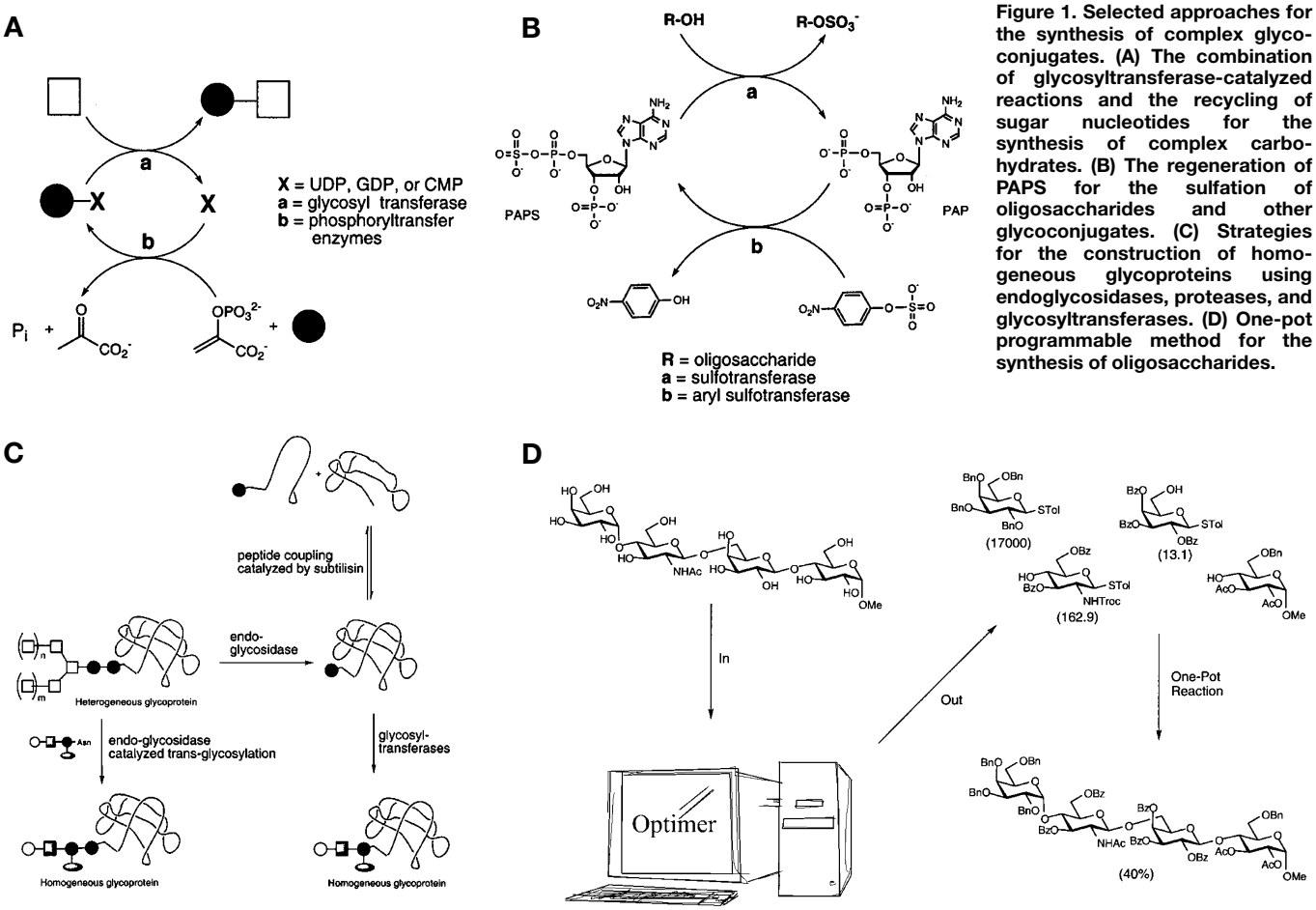

Figure 1. Selected approaches for the synthesis of complex glycoconjugates. (A) The combination of glycosyltransferase-catalyzed reactions and the recycling of sugar nucleotides for the synthesis of complex carbohydrates. (B) The regeneration of PAPS for the sulfation of oligosaccharides and other glycoconjugates. (C) Strategies for the construction of homogeneous glycoproteins using endoglycosidases, proteases, and glycosyltransferases. (D) One-pot programmable method for the synthesis of oligosaccharides.

cial in the area of glycopeptide synthesis[18], as well as for site-specific esterification[22]. Aldolases have provided access to numerous unnatural carbohydrate derivatives[16,23], and directed evolution is yielding novel catalysts with altered substrate specificity (S. Fong & C.-H. Wong, unpublished data). New enzymatic protocols for the recycling of 3′-phospho adenosine 5′-phosphosulfate (PAPS) as a sulfotransferase donor substrate have been developed (Fig. 1B) and will further allow elucidation of the role of glycoconjugate sulfation[24]. Endoglycosidases, proteases, and glycosyltransferases can be sequentially employed to produce glycoproteins with homogeneous glycoforms (Fig. 1C)[20]. As methods for the construction of homogeneous glycoproteins are greatly needed, other synthetic avenues, such as native peptide ligation[25], intein-catalyzed peptide bond formation[26], and endoglycosidase-catalyzed transglycosylation have also been explored[18,27] (Fig. 1C). At present, enzymes specific for every desired linkage are not currently available, and strategies combining chemical and enzymatic synthetic methods have been generally advantageous.

On the chemical forefront, many research groups have reported "one-pot" strategies for the synthesis of oligosaccharides[18]. These methods involve the selection of glycosyl donors that will react in a predefined order, thus potentially simplifying the overall synthetic procedure. The culmination of these efforts has resulted in the development of "one-pot programmable" methods (Fig. 1D)[28], which rely on the ability to control glycosylation reactions by careful selection of hydroxyl protecting groups, the ability to quantify glycosyl donor reactivity, and a universal donor activation strategy. Having already demonstrated its utility in the programmed synthesis of linear and branched oligosaccharides, this method continues to be developed, and represents the nearest precursor to automated carbohydrate synthesis that exists to date.

Although these unique one-pot and enzymatic synthetic methods have proven utility in the generation of glycoconjugates, traditional chemical synthesis continues to be a backbone staple. The following sections outline various approaches to carbohydrate-based therapeutics that were possible through chemical and chemoenzymatic synthetic approaches.

Antiviral agents

The influenza virus is one of the best-known pathogens dependent on carbohydrate-recognition for its infectivity. The viral hemagglutinin protein is responsible for initial recognition of host cell surface sialic acid (NeuAc), which is subsequently cleaved by the viral neuraminidase, allowing infection to proceed[29,30]. Anti-influenza drug design has focused on the inhibition of both hemagglutinin and neuraminidase, though in markedly different strategies (Fig. 2). Hemagglutinin exists as a homotrimer present in multiple copies on the viral cell surface and, in accordance, the most potent hemagglutinin inhibitors are polyvalent constructs[31,32]. Inhibition of neuraminidase, in contrast, has relied on mimicking the transition state of the hydrolytic reaction by rational design. Several of these transition-state analogs are potent neuraminidase inhibitors, and are now on the market[33,34].

Galactosyl-ceramide (Gal-Cer) on host cells is employed as an alternate receptor for HIV-1, and is a point of attachment for the V3 loop of gp120[35]. Soluble Gal-Cer analogs have been analyzed as inhibitors of viral infection through disruption of the naturally occurring gp120–Gal-Cer interaction[36,37]. Anti-HIV activity can also be accomplished by interfering with protein–RNA interactions[38]. The neomycin class of aminoglycoside antibiotics binds to the Rev responsive element (RRE) of HIV mRNA, and inhibits the interaction of the RRE with Rev protein[39]. This prevents the switch from viral latency to active viral replication. Synthetic agents that inhibit

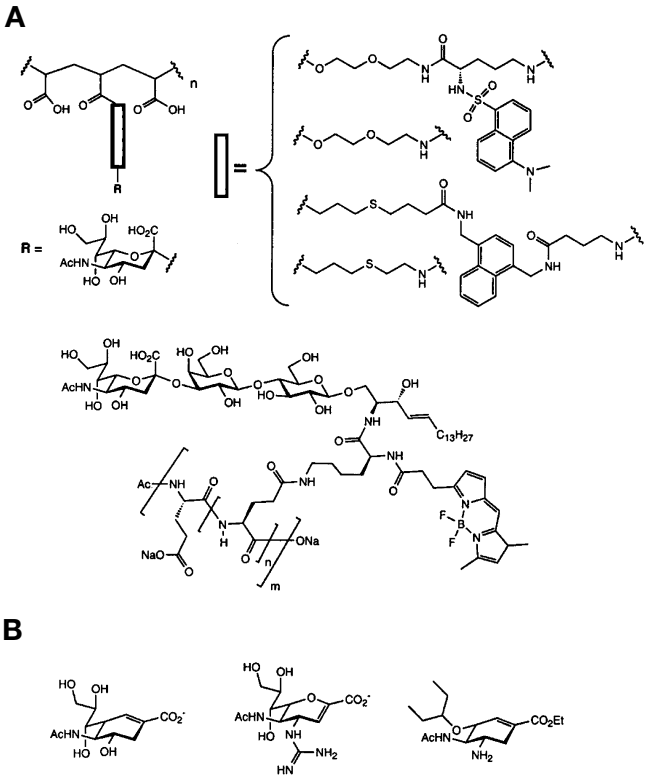

Figure 2. Strategies for the inhibition of influenza virus. (A) Polyvalent hemagglutinin inhibitors. (B) Transition-state analog neuraminidase inhibitors.

RRE–Rev protein interactions with high affinity and specificity are currently being developed as potential antiretroviral agents[39,40].

Antibacterial and antimicrobial agents

Many microorganisms rely on the recognition of glycoconjugates for the ability to infect a given host cell[41]. Not surprisingly, antibiotics that target invasive organisms also often contain glycostructure. In this area, a recent intensified focus on the glycopeptide antibiotic vancomycin stems from its consideration as the "antibiotic of last resort" with respect to resistant bacterial strains[42]. Vancomycin functions by adhering to D-Ala-D-Ala sequences within the bacterial cell wall of Gram-positive bacteria and inhibits the transpeptidase activity required for the assembly of peptidoglycan (Fig. 3). Several synthetic studies have focused on the mechanism of vancomycin action, including the role of membrane anchoring[43], electrostatic interactions[44], multimerization[45–48], and the pathway by which resistance develops (i.e., bacterial cell wall mutation of D-Ala-D-Ala to D-Ala-D-lactate)[49,50]. A recent investigation suggests that mimics of the vancomycin glycan may exert antibacterial activity by inhibition of the transglycosylase, rather than the transpeptidase[51].

Erythromycin is a macrolide antibiotic that is also employed in the treatment of infections caused by Gram-positive bacteria and serves as a clinical alternative for patients allergic to penicillins[52]. Characterization of the polyketide synthase responsible for erythromycin biosynthesis has resulted in the generation of novel macrolide structures. Through continuing investigation of the modular synthase, potent new antibiotics may be discovered for the fight against resistant bacteria[53].

Lipopolysaccharide is the main outer-membrane component of Gram-negative bacteria. Antibacterial agents often cause disruption of bacterial membranes and the release of lipopolysaccharide, which can trigger an acute inflammatory response that leads to Gram-negative sepsis[54]. The reducing terminal glycophospholipid of lipopolysaccharide (Lipid A) contains all of the structural elements necessary to cause septic shock, and Lipid A antagonists are therefore considered therapeutic targets.

Various strategies toward this perplexing therapeutic dilemma (bacterial infection vs. endotoxic shock) have been undertaken to date. Lipid A from *Rhodobacter sphaeroides* is nontoxic, and synthesis of a structural mimic was one avenue explored[55]. This construct served as a lipopolysaccharide antagonist and protected mice from lipopolysaccharide-induced lethality. A second strategy involves disruption of lipopolysaccharide biosynthesis using the cytosine monophosphate (CMP)-3-deoxy-D-manno-octulosonate (KDO) synthetase inhibitor β-KDO[56]. Since β-KDO itself is membrane-impermeable, a "pro-drug" β-KDO–Ala-Ala conjugate capable of membrane permeation was synthesized. Treatment with this construct resulted in accumulation of a Lipid A precursor and bacterial cell death. Another protocol employed Lipid A substrate analogs as inhibitors of the deacetylase in the biosynthetic pathway[57].

Helicobacter pylori is the main cause of gastritis, ulcers, and stomach cancer in humans. This bacterium recognizes both NeuAc-containing oligosaccharides[58] and the Le^b blood[59] group on the gastric epithelium. As such, soluble Le^b and 3′-sialyllactose may serve as therapeutic agents, by competitively inhibiting bacterial attachment to the gastric cell layer[60].

Recently, it has been shown that the AB_5 Shiga toxin pentamer from *Shigella dysenteriae* could be neutralized by a multivalent carbohydrate construct[61]. The potent five-armed "STARFISH" ligand was designed based on the X-ray co-crystal structure of the toxin bound to a monomeric ligand. Notably, in vitro inhibition of Shiga toxin by the polyvalent molecule was increased six orders of magnitude compared with the monovalent ligand.

The GPI biosynthetic pathway in trypanosomes has also been investigated as a point of therapeutic intervention in African sleeping sickness[62]. Using synthetic substrates as probes, Smith and colleagues[63] found that 2-*O*-methylation of D-*myo*-inositol was tolerated by trypanosomal α1,4-mannosyltransferase, but not by the human enzyme. This and other differences distinguish the biosynthetic pathways in parasites and mammals, providing potential leads for the design of antiparasitic agents.

The neomycin class of aminoglycoside antibiotics interacts with the *Escherichia coli* 16S rRNA A-site, thereby disrupting the fidelity of protein translation, and causing eventual bacterial cell death[64,65]. However, like glycopeptide antibiotics, prolonged treatment with aminoglycosides results in bacterial resistance. In this case, rather than mutate the RNA target, bacteria have evolved enzymes to modify the aminoglycosides. Following acetylation, phosphorylation, or adenylylation by these resistance-causing enzymes, the aminoglycosides have drastically reduced affinity for their RNA targets, and can no longer exert their antibacterial effects[66].

To overcome resistance, as well as to combat such side effects of aminoglycoside administration as oto- and nephrotoxicity, synthetic antibiotic analogs have been developed[67–70]. Aminoglycoside derivatives have been analyzed with respect to A-site rRNA affinity, antibiotic activity, disruption of protein translation, and inhibition of aminoglycoside-modifying enzymes. These studies have resulted in the identification of an aminoglycoside dimer with nanomolar A-site affinity that was also a competitive inhibitor of resistance gene-encoding aminoglycoside 6′-acetyltransferase [AAC(6′)]-aminoglycoside 2″-phosphorylase (APH(2″); Fig. 4)[68]. In the same vein, Mobashery[69] has accomplished the synthesis of an original kanamycin A derivative that intrinsically "resists" phosphorylation. Design of dual-function constructs of this nature may be the strategy required to combat resistant pathogens in the future. Aminoglycosides may also prove useful for the disruption of oncogenic mRNA translation[71].

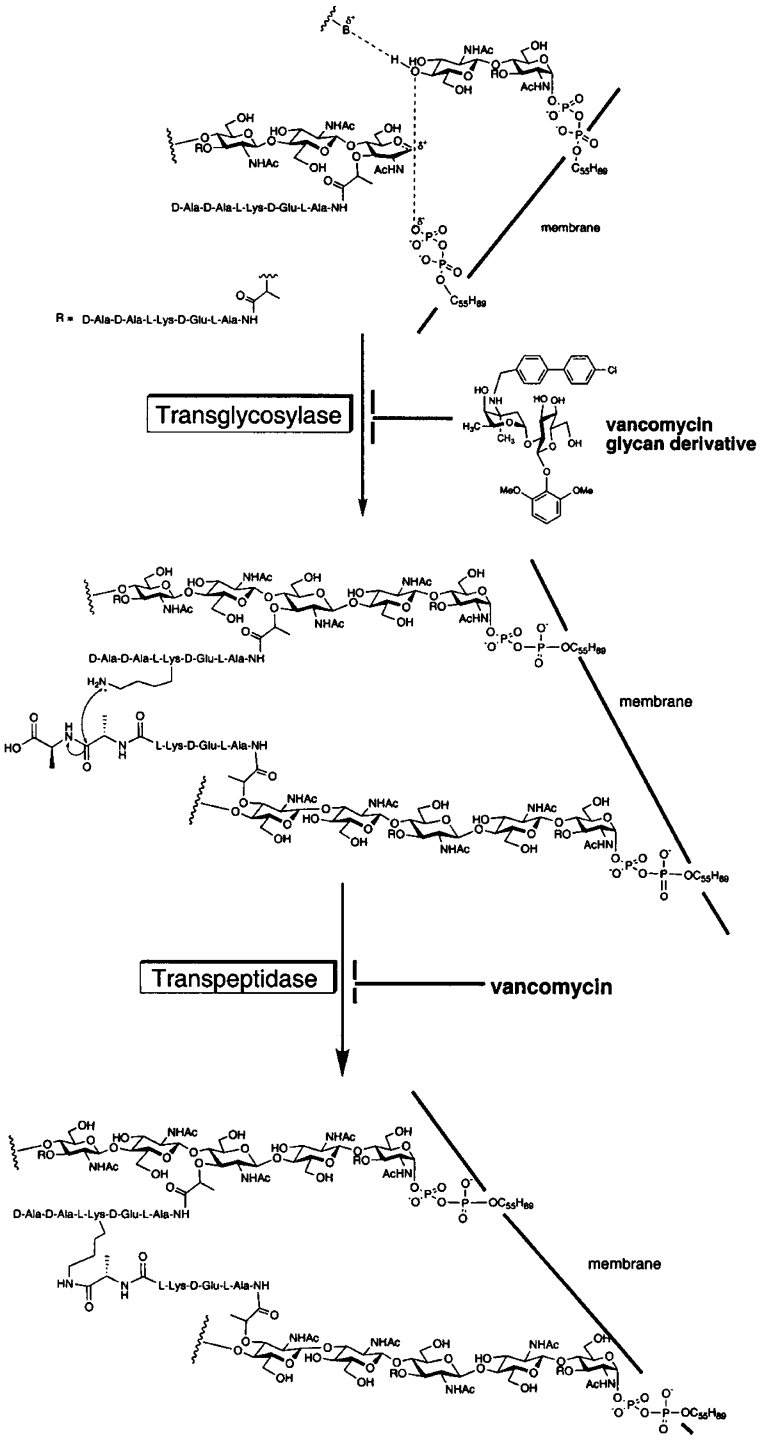

Figure 3. The biosynthesis of bacterial cell wall peptidoglycan involves both transglycosylase and transpeptidase activity. The antibiotic vancomycin inhibits the transpeptidase, and unique mimics of the vancomycin glycan inhibit the transglycosylase.

The immune system as a therapeutic target

As human organ donors are generally in short supply, animal tissues (especially those of porcine origin) have been suggested as an alternative solution; however, hyperacute rejection of foreign tissues from the human body currently precludes this practice. The discovery that the immune response against α-galactosyl (α-Gal) epitopes present on xenotransplants is the primary cause of rejection has led to research into evasion of the immune system using synthetic constructs[72]. Two general therapies have been undertaken. The first involves depleting human blood of anti-α-Gal antibodies by passage through an α-Gal affinity matrix. The other is to construct soluble α-Gal containing oligosaccharides that bind to the anti-α-Gal antibody with higher affinity than the natural α-Gal epitope. In this strategy, polymeric α-Gal constructs effectively inhibit anti-α-Gal binding to pig kidney cells, and may have clinical potential[72].

In contrast to evasion of the immune system, vaccines generated to glycoconjugates rely on the ability of the body to mount an immune response against a desired structure. The capsular polysaccharides present on invasive bacteria are prime targets for vaccine generation and have been used clinically in the treatment of bacterial meningitis and other diseases such as pneumonia and shigellosis[73,74]. For example, routine vaccination against *Haemophilus influenzae* type B with polysaccharide–protein conjugates is a highly effective treatment employed worldwide[74]. In a new report, a vaccine against *Staphylococcus aureus* has been developed to a polysaccharide antigen that is present on the bacteria during human infection, but not expressed during normal bacterial cell growth[75]. This strategy has proved effective in laboratory animals and is a candidate for clinical vaccination.

In general, because oligosaccharides themselves are T-cell-independent antigens and therefore serve as poor immunogens, several practical methods for conjugation to protein carriers (haptens) have been investigated. To date, most vaccines are generated to a panel of oligosaccharide structures present within a given capsular polysaccharide, a situation that raises the issue of whether the most relevant structure is adequately represented in the mixture of resulting polyclonal antibodies. In this respect, synthetic methods are valuable for the construction of a homogeneous oligosaccharide population before immunization.

In CD_1-glycolipid antigen presentation to T cells, the immune response appears to be dependent on the presence of the glycan portion of the glycolipid, and this represents a possible alternative therapeutic avenue for vaccine development[76,77]. In this context, various polysialyltransferases from neuroinvasive bacteria can be employed to synthesize polysialylated glycolipids[78,79]. If coupled to CD_1-mediated T-cell activation, new vaccines may be developed to eradicate specific cancers or bacteria by the induction of killer T cells[80].

Glycoconjugate vaccines are also emerging as a therapy in the battle against specific cancers[7]. Cell surface glycans are often altered or truncated in cancerous states as a result of a change in the regulation of specific glycosyltransferase genes. Interestingly, although glycosphingolipids are present on both normal tissue and tumors, they act as immunogens only when present on the surface of tumor cells. Consequently, immunization to these antigenic determinants can provide vaccines useful in the eradication of cancerous cells from the body[81]. Notably, the fully synthetic Globo H vaccine has been shown to be immunogenic in humans for the treatment of prostate cancer (Fig. 5)[82]. Furthermore, the synthetic sialyl-T_n vaccine for breast and ovarian cancers[83] is highly target-specific, and its use correlates with prolonged survival in human clinical trials.

An alternative strategy involves vaccination with antibodies that, though generated by immunization to peptide antigens, cross-react with cell surface carbohydrate epitopes[84]. Numerous naturally occurring glycoconjugates, such as Reishi polysaccharides, also act as

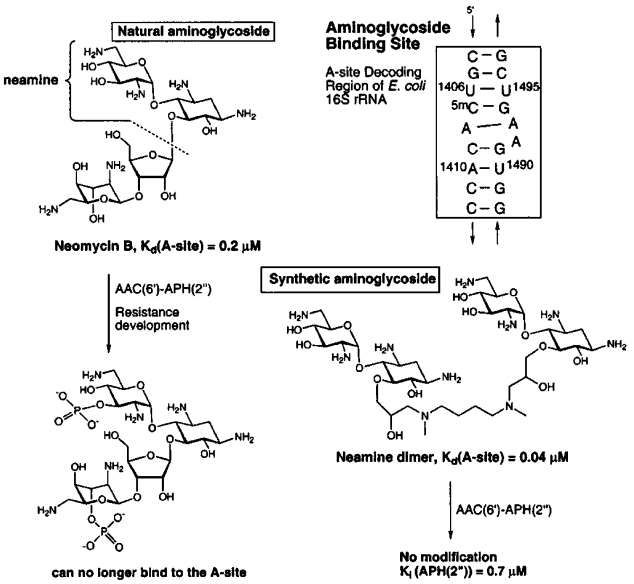

Figure 4. Aminoglycosides exert their antibiotic effects through binding of the bacterial 16S rRNA A-site. The neamine dimer pictured binds to the A-site with high affinity, and also inhibits the APH(2″) activity of the resistance-causing bacterial enzyme AAC(6′)-APH(2″), thereby serving as a dual-function ligand in the fight against emerging resistant bacterial strains.

immunomodulators, although their mechanism of action is unknown[85]. Understanding the molecular mechanism of their function may lead to development of new carbohydrate-based therapeutics.

Other targets

Inhibitors of glycosyltransferases and glycosidases also have proven use as therapeutic agents. For example, treatment with *N*-butyldeoxynojirimycin (NBJ) prevents the accumulation of ganglioside G_{M2} in the brain, by serving as a glucosyltransferase inhibitor (Fig. 6)[86,87]. As such, it has proven useful in the treatment of Tay–Sachs disease and may provide a general strategy for treating other sphingolipidoses[88]. The potent inhibition by NBJ of α-glucosidases I and II probably accounts for its effectiveness in inhibiting HIV replication in vitro.

The inflammatory response employs the selectin family of carbohydrate-binding proteins in its early stages of leukocyte recruitment. Selectin–carbohydrate interactions have been extensively reviewed[89], and the development of inhibitors of selectin–ligand interactions generally relies on mimicking the sialyl-Lewis x (sLe^x)–selectin interaction in either mono- or polyvalent form. One class of cell adhesion inhibitors induces the proteolysis of L-selectin from the surface of leukocytes[90]. Alternative strategies toward anti-inflammatory agents have taken the form of sLe^x–RGD (Arg-Gly-Asp) conjugates that recognize both selectins and integrins[91], or the inhibition of mannose phosphate receptors[92]. Furthermore, di-Le^x has entered clinical trials in rheumatoid arthritis patients[93]. In the same vein, it has been shown that a sLe^x glycosylated complement inhibitory protein (sCR1) inhibits both complement activation and selectin-mediated platelet–leukocyte interactions, thus reducing the level of neuronal injury due to reperfusion in stroke[94].

Heparin is a heavily sulfated glycopolymer, employed for decades as an anticoagulant, that functions by inhibiting thrombin and Factor Xa through recognition of antithrombin III. However, administering large and heterogeneous heparin preparations can lead to detrimental side effects because of unfavorable interactions with blood vessel components. Recently, structurally defined heparin mimics with antithrombin activity similar to natural

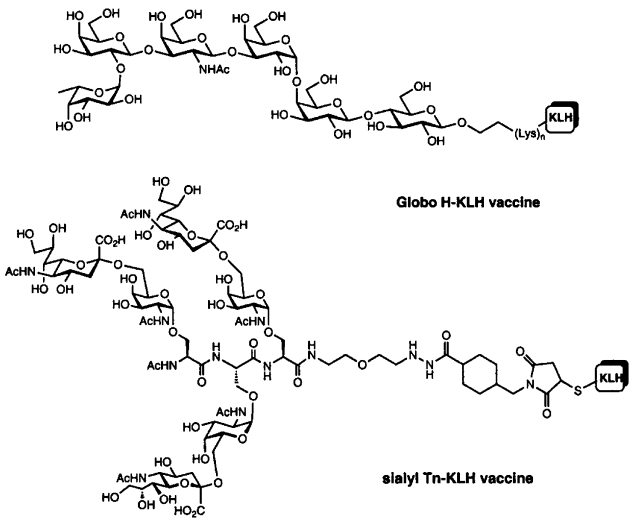

Figure 5. Keyhole limpet hemocyanin (KLH) conjugates of the Globo H and sialyl-T_n cancer antigens serve as effective vaccines against prostate and breast cancers, respectively.

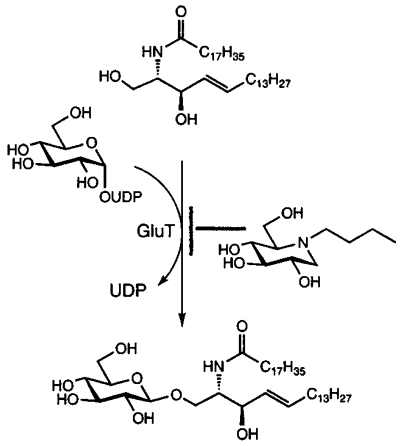

Figure 6. *N*-butyldeoxynojirimicin inhibits the glucosyltransferase involved in sphingolipid biosynthesis, and serves as a therapeutic agent in the treatment of Tay–Sachs disease.

heparin, but with reduced side effects, have been obtained through chemical synthesis[95,96].

Glycoproteins also have clinical utility, the prime example being erythropoietin (EPO)[97], which is widely used as a treatment for anemia associated with renal failure, chemotherapy, and 3′-azidothymidine (AZT) administration. Interaction of EPO with its receptor (EpoR) stimulates erythropoeisis, resulting in the production of red blood cells. Studies of EPO have revealed that glycosylation, and especially the presence of sialic acid, is required for its normal function in vivo[98,99]. Currently, EPO must be administered intravenously or by subcutaneous injection, and agents that act similarly but are more conveniently delivered are under investigation. Toward this end, phage display techniques have identified short peptides that mimic the action of EPO by binding with high affinity to EpoR[100,101].

Future directions

In the past decade, glycoconjugate research has yielded an abundance of medicinally relevant information. New methods have eased the synthesis of complex oligosaccharides, and techniques for the facile synthesis of other glycoconjugates are rapidly developing. From a synthetic point of view, availability of an enzyme for each glycosidic

linkage or an automated method to obtain any desired glycoconjugate would be advantageous. Furthermore, control of glycoprotein glycoform heterogeneity remains a problem and results in a poor understanding of the contribution of carbohydrates to protein structure and function. The development of methods for the construction of homogeneous glycoproteins is relevant not only from an academic standpoint, but also for large-scale production processes and marketing as pharmaceutical agents. Current synthetic efforts in this area have significantly advanced the field toward this end. Moreover, remodeling of cell surfaces employing glycosyltransferase strategies[102] or by exploiting biosynthetic pathways[103] may provide new insights into the function of cell surface glycoconjugates.

Therapeutically, it will be necessary to identify antibacterial agents that can evade resistant bacteria, because the clock on existing antibiotics is ticking. New strategies toward antibiotic agents are in high demand, especially those in which multiple mechanisms of action contribute to efficacy. In this area, the most significant results have been derived from the aminoglycoside antibiotics, which show promise as both RNA-binding molecules as well as therapeutics that avoid modification by resistance-causing enzymes. New antibiotics with novel function may also be discovered through manipulation of biosynthesis involving modular polyketide synthases.

Further evaluation of glycoconjugate vaccines will also be necessary, in light of the fact that great success has already been achieved in eradication of specific cancers. It is remarkable that the synthetic carbohydrate vaccines induce little undesirable response, even though the carbohydrate structures employed for immunization are present ubiquitously.

As our understanding of carbohydrate-mediated biological recognition processes advances to the molecular level, new carbohydrate-based therapies employing existing glycoconjugates and carbohydrate mimetics (noncarbohydrate organic molecules that resemble carbohydrates) will become readily available and widely utilized medicinal agents.

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