The bittersweet promise of glycobiology

Carbohydrates play a wide range of important roles in the body and, despite the challenges posed by glycobiology, are now tempting targets for drug developers.

Alan Dove

Although several genomes have been sequenced and many protein structures solved, there has been relatively little attention paid, to date, to the various ways in which proteins are "tweaked" through the attachment of sugars. However, the process of glycosylation is far from a decorative function. Carbohydrates help determine the three-dimensional structures of proteins, which are inherently linked to their function and their efficacy as therapeutics. Moreover, in contrast to some of the other chemical tags employed by cells (e.g., phosphates and lipids), carbohydrates exhibit a mind-boggling diversity of structures, can confer cell-type specificity, and are crucial components of cell-to-cell signaling. At the same time, carbohydrates make problematic drug targets; they are the most difficult biological molecules to analyze and synthesize, and are rapidly broken down in the bloodstream. Despite these challenges, recent technological advances have enabled several biotechnology companies to pursue new carbohydrate-based products, sweetening the outlook for glycobiology.

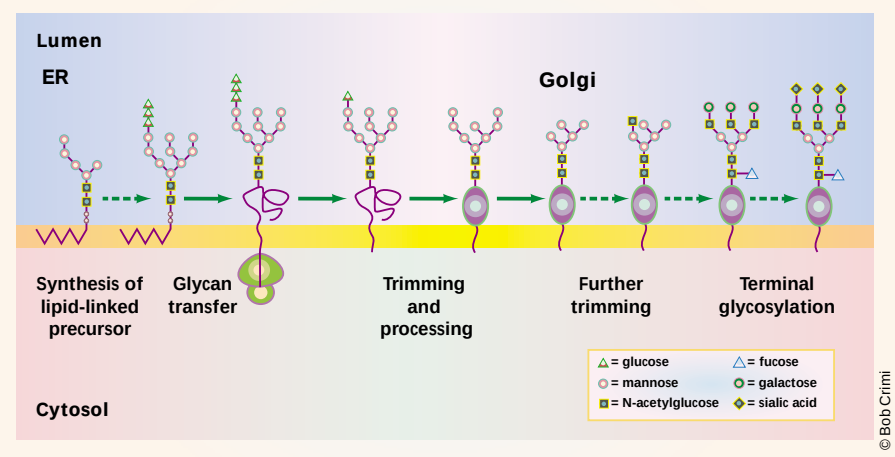


Figure 1. A simplified diagram showing the synthesis of an N-linked glycan. First, sugars are linked onto a lipid precursor (in the cytosol), which is then flipped over into the lumen of the endoplasmic reticulum (ER) and the core oligosaccharide finished. The glycan is then transferred to the nascent, growing polypeptide. Sugars are trimmed off, and the polypeptide is then folded (grey oval) before being moved to the Golgi complex. The glycoprotein goes through a series of further modifications, ending with the capping of the oligosaccharide branches with sialic acid and fucose. Source: Helenius, A. & Aebi, M. *Science*, **291**, 2364–2369, 2001.

Carbo loaded

In contrast to DNA, RNA, and proteins, carbohydrates can form branching structures, and for this reason a relatively simple set of sugars can form a huge number of complex structures. For example, in theory, the nine common monosaccharides found in humans could be assembled into more than 15 million possible tetrasaccharides, all of which would be considered relatively simple glycans. Different cell types express different complements of glycosylating enzymes, and have different sets of proteins that can be glycosylated. Glycosylation is therefore a flexible evolutionary tool serving a wide range of purposes in complex organisms.

The process of glycosylation begins when the protein is targeted to the glycosylation pathway during translation of messenger RNA (mRNA) into protein. The ribosome is attached to the endoplasmic reticulum (ER), and the nascent protein fed into the lumen of the ER as translation proceeds. In the ER, one set of glycosylation enzymes attaches sugars to specific portions of the protein. Other glycosylation enzymes then either add more sugars to these core structures, or partially trim the structures back so that new sugars can be added to a different part of the growing carbohydrate structure. The back-and-forth process of glycosylation continues into the Golgi apparatus, which sorts the new proteins, distributing them to their final destinations in the cell. In human cells, sialic acid is usually added to the tips of the carbohydrate branches, serving as a final cap important for a variety of glycoprotein functions (see Fig. 1).

Carbohydrates can significantly affect protein structure and therefore influence protein function. Carbohydrates are also used as tags to sort proteins in the Golgi apparatus, targeting them to specific compartments within the cell or directing them to the cell surface. Once on the cell membrane, glycoproteins (and glycolipids) interact with receptors on other cells to communicate a wide range of messages. For example, cells of the immune system use the glycans on the cells that they encounter to identify everything from bacterial invaders to fellow leukocytes.

Sweet but sickly

Not surprisingly, given the importance of carbohydrates, defects in their metabolism can have disastrous consequences. For example, in Gaucher's and Fabry's diseases, inherited mutations in carbohydrate-processing enzymes cause glycolipids to accumulate, leading to damage to the kidneys, heart, and brain. The inability to form certain glycoproteins results in another set of inherited diseases called congenital disorders of glycosylation, which can result in symptoms ranging from chronic diarrhea to life-threatening neurological problems.

In addition to relatively rare genetic diseases, sugars play crucial roles in some leading health problems. Injured tissues stimulate endothelial cells to express selectins—glycoproteins that tell leukocytes in the bloodstream to infiltrate damaged tissue and mount an inflammatory response. However, this inflammation process can occasionally get out of control. In surviving stroke and heart attack patients, for example, blood flow is briefly cut off from a tissue, then restored. As the blood starts to flow again, selectins in the affected tissue aggressively recruit leukocytes from the bloodstream, leading to an intense inflammatory response. The resulting tissue damage, so-called reperfusion injury, is typically more severe than that

sustained by the initial interruption of blood flow. If the glycan-mediated signal from the selectins could be modulated, the damage might be mitigated.

Carbohydrates are also important for tumor development because cancer cells alter their surface glycoprotein expression to evade the immune system. By cloaking themselves with the right assortment of glycoproteins, the tumor cells can invade other tissues without being identified as aliens.

Sugar structures are also targets for pathogens seeking entry into the cells of their host. Because carbohydrates are both ubiquitous and diverse, viruses and other intracellular parasites can use glycoprotein receptors to target any host cell, or just one cell type in particular.

No simple solution

For many years, the potential of carbohydrate-based therapies has been yoked to seemingly intractable technical problems. Without high-throughput methods for analyzing and synthesizing carbohydrates, detailed knowledge of the structure and function of each carbohydrate moiety has been the hard-won products of labor-intensive experiments. Indeed, even the nomenclature of carbohydrates inhibits easy understanding (see "By any other name"). Given the extraordinary diversity of possible carbohydrate structures, and the cumbersome technology available, glycobiology has been a tough sell for biotechnology and pharmaceutical companies, made even tougher by a series of high-profile clinical trial failures during the mid-1990s (see below).

I would say that glycoproteomics is at least an order of magnitude more difficult than proteomics

Unlike DNA and proteins, no technology has been developed to allow carbohydrates to be "sequenced" using straightforward techniques. Instead, sugar structures must be solved by a combination of chromatography and mass spectrometry, procedures that have traditionally required considerable technical skill. "I would say that glycoproteomics is at least an order of magnitude more difficult than proteomics," says Anne Dell, a researcher in the department of biochemistry at the Imperial College of Science, Technology, and Medicine (London, UK). In a typical experiment, a glycoprotein is isolated from a chromatography gel, and the carbohydrate groups are then released from the protein by chemical or enzymatic treatment. The mixture of carbohydrates can then be subjected to high-performance liquid chromatography (HPLC) to separate out individual carbohydrates. Finally, individual carbohydrates can be studied in detail by chemical digestion and mass spectrometry, a technique that can give precise structural information about relatively small molecules such as monosaccharides.

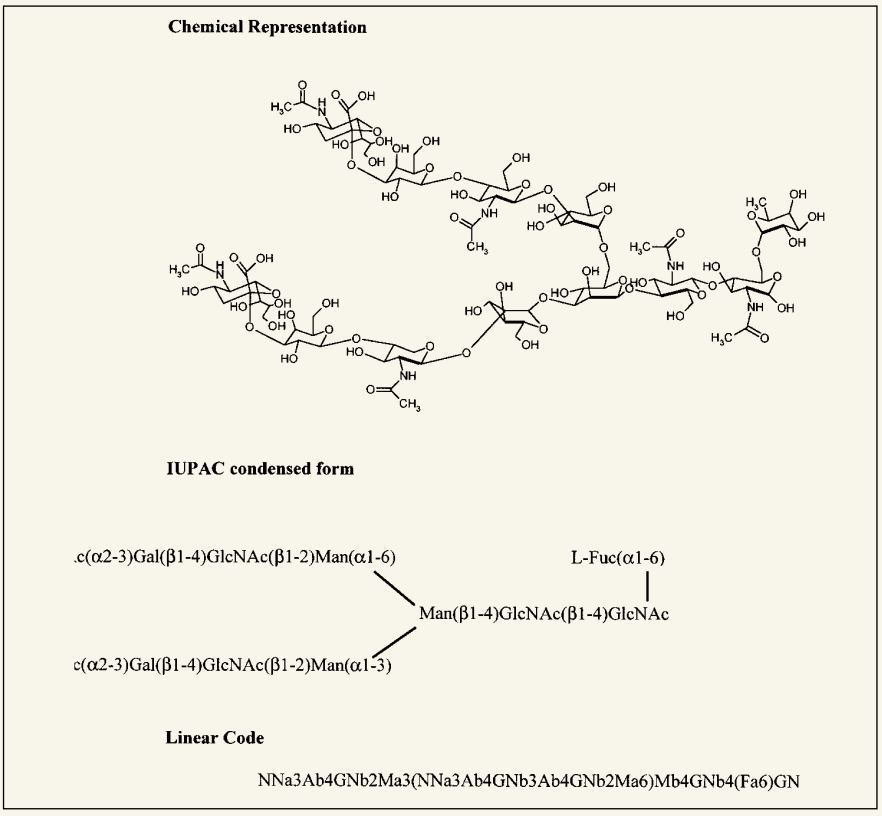
Besides the complex multistep analysis required for each sugar, glycobiologists must also contend with the heterogeneity of sugar structures within a sample. For example, Pauline Rudd, a scientist at the Glycobiology Institute at the University of Oxford (UK), points out that the human prion protein can carry one of 52 different sugar structures at one site on the protein: "That, of course, makes it very difficult to analyze, because if you've got 'x' amount [of sample], you've got 'x' divided by 52 of any one carbohydrate." Obtaining sufficient quantities of a given glycan for analysis is often a major problem, especially in studying glycoproteins present in low concentrations in cells. "We can look at the things that are there in very high abundance, but not at the more interesting things are generally there in low abundance," says Rudd.

Once a carbohydrate structure is solved, duplicating the structure artificially can also be a daunting task. Cells that are capable of

By any other name

Among the many difficulties that have faced glycobiologists, one of the most obvious may also be the easiest to fix. Molecular biologists use simple alphabetic codes to describe the nucleic acid or protein sequences they study, but glycobiologists have been stuck using the complete, clumsy chemical names of their complex molecules.

In an effort to improve clarity, Glycominds (Maccabim, Israel) has developed the Glycominds Linear Code, a nomenclature that allows any sugar to be described with a code of letters and punctuation that accurately represents its building blocks and branching structure. "The Glycominds Linear Code builds a unique syntax that can describe any glycan in just one way," says Avinoam Dukler, CEO of Glycominds. The Glycominds Linear Code is a proprietary system, but Dukler says that it is licensed at no charge to any researcher requesting it. Another unique advantage is that code is computer-readable, which no other glycan-naming system can claim. "Now the world of computation and bioinformatics can begin [for glycobiology]," says Dukler. *AD*

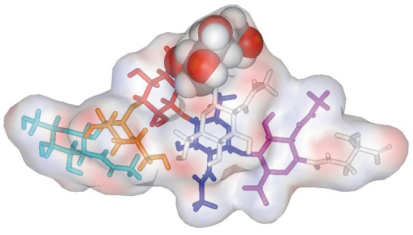


glycosylating proteins generate a diverse array of carbohydrate structures, and it is difficult to control the production of a specific glycoprotein *in vivo*. Until recently, organic chemistry was the most common approach for carbohydrate synthesis in the lab, but the complexity of biologically relevant glycans pushes synthetic chemistry to its limits. In addition, the synthesis of each structure had to be designed from scratch, making this approach inappropriate for high-throughput drug design.

The struggle does not abate once a potentially useful carbohydrate is synthesized. "Using carbohydrates themselves as therapeutics … there are some fairly unique issues," says Dale Cumming, vice president for discovery research at GlycoDesign (Toronto, ON, Canada). The digestive system excels at breaking down most naturally occurring carbohydrates, and such drugs will likely have to be injected directly into the bloodstream. Glycosidases in the blood can reduce a carbohydrate-based drug's half-life to just a few minutes, depending on its structure. Carbohydrate-based drugs such as heparin—the world's best-selling prescription drug—and the *Haemophilus influenzae* type B (Hib) vaccine have avoided this pharmacokinetic pitfall because their structures are not readily recognized by the body's normal complement of glycosidases. However, evidence is mounting that other carbohydrate-based therapeutics will not fare as well.

Indeed, carbohydrates also present a major drug delivery problem for ordinary protein therapeutics, most of which must be correctly glycosylated to work. Glycosylation also affects protein breakdown; for example, proteins lacking terminal sialic acid residues on their sugar groups are often targeted by the immune system and rapidly degraded (see "Sugar in bulk").

Glycobiology is "a bit like the proverbial onion. You get through one layer and you discover that there are whole new layers of complexity to be addressed," says GlycoDesign's Cumming. One layer of the onion that left a bitter taste in the mouths of pharmaceutical companies and investors were early failures. In the early 1990s, biotechnology startup Cytel (San Diego, CA) set out to develop carbohydrate inhibitors of selectins, the glycoproteins that promote inflammation. The company's lead drug—Sialexin—failed in clinical trials. A double blow came when a second glycobiology startup, Glycomed, failed in its efforts to develop a modified form of heparin. The two companies were subsequently bought out by competitors, but their story cast a long shadow over the field of glycobiology. "They spent a lot of money and a lot of years in clinical trials that simply demonstrated no efficacy whatsoever," says Stephen Roth, chairman and chief executive officer (CEO) of Neose (Horsham, PA), which purchased Cytel. As a result, says Roth, glycobiology will probably have to prove itself with a significant clinical success before large companies and investors will be willing to return to the field.



A model of a carbohydrate that is a substrate for a glycosylating enzyme, GnT-V. Bubbles depict binding site.

Glycan do it

Glycobiologists seem to have risen to the challenge, making several crucial technological advances in the past few years and overcoming at least some of the obstacles facing early pioneers. The major technology for analyzing sugar structures—mass spectrometry—has improved in both sensitivity and accuracy, and has become technically easier, making carbohydrate analysis accessible to more researchers outside of the field of glycobiology. In Oxford, Rudd and her colleagues regularly teach their analytical glycan extraction and mass spectrometry techniques to visiting scientists, who can then incorporate glycan analysis into their regular work. While conceding that it is still far from a turnkey technology such as DNA sequencing, Rudd argues that this type of analysis will ultimately become part of the molecular biologist's toolkit.

Another analytical technique for carbohydrates may be widely available sooner. Glycominds (Maccabim, Israel) has developed a chip-based assay for glycan binding, an important first step in determining the function of glycans. The chip allows the researcher to screen the carbohydrate-binding pattern of a protein against a few hundred unique carbohydrate structures simultaneously. The pattern of colored spots on the chip indicates which sugar structures bind to the protein. "We're using these carbohydrates as a tool to identify novel proteins that have carbohydrate-binding domains. For drug discovery, we're screening low-molecular-weight compounds that might inhibit these interactions," says Avinoam Dukler, CEO of Glycominds. The chips might also form the basis of a diagnostic tool to screen for antibodies against a particular glycan.

In addition to commercial applications for its chip, Glycominds is hoping to aid academic and government scientists profil-

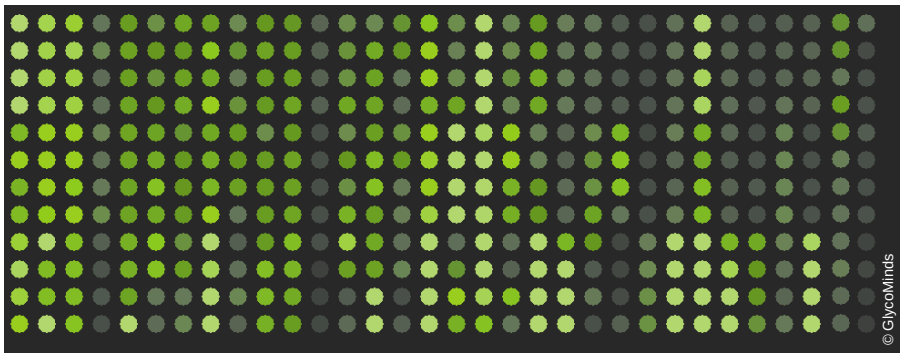
Sugar in bulk

Biotechnology companies working on glycans are primarily interested in developing new drugs (see main text), but at least one company is pursuing broader applications. Neose (Horsham, PA) has established partnerships with at least two dozen pharmaceutical and biotechnology companies to improve the glycosylation of medically useful glycoproteins. The company is also developing strategies to drastically slash the cost of protein production, for both pharmaceutical and industrial uses.

Neose's major technology, Glycoadvance, uses enzymes to add sugars to proteins *in vitro*, significantly improving the yield of usable protein from fermentations. There has been no shortage of interest in the service: "Most large pharmaceutical companies and big biotech companies have products that are sort of stalled somewhere in advanced clinical trials because of protein production problems. Those production problems always come back to less-than-optimal glycosylation of their product by the Chinese hamster ovary (CHO) cells," says Stephen Roth, chairman and CEO of Neose.

Biotechnology companies have long used fungal systems like yeast and *Aspergillus* to manufacture huge quantities of proteins cheaply. However, fungi glycosylate proteins with a lot of mannose, which makes the protein therapeutically useless: The human immune system identifies this glycosylation pattern as that of a fungal or bacterial invader, rapidly removing the proteins from the circulation. By adding humanlike sugar groups, such as sialic acid, to proteins produced in yeast, Roth hopes to eliminate the troublesome and expensive CHO cells from the production process.

If that strategy works, the impact on the cost of protein drug production could be substantial. "The cost of producing a typical pharmaceutical glycoprotein [in CHO cells] will be between $1 million and $3 million a kilogram. Your typical industrial glycoprotein made in *Aspergillus* will be around $200–$300 a kilogram," says Roth. *AD*



The GlycoChip is the first microarray for complex carbohydrates. Synthesis of the glycans on the chip is done *in situ* .

ing carbohydrate expression and binding patterns. One such effort, the Consortium for Functional Glycomics (http://functionalglycomics.mit.edu), recently applied for a National Institutes for Health grant to finance its multi-institution glycobiology project. Stressing that the project has not yet been funded, James Paulson, professor of molecular biology at The Scripps Research Institute (La Jolla, CA) and one of the initiators of the consortium, says that it is "interested in defining the paradigms through which carbohydrate binding modulates [protein] function and in particular modulates communication between cells."

Sugars by design

While new techniques are beginning to yield more information about glycan structure and function, improved means of synthesizing carbohydrates for further study and drug development are also emerging. The new synthetic approaches fall into four major categories: One-pot synthesis is a direct descendant of traditional organic chemistry techniques, in which successive reactions are carried out in a single test tube to produce the final carbohydrate product; in solid-phase synthesis, the initial sugar groups are anchored to beads or surfaces, allowing them to be isolated and moved easily at each step of the process; enzymatic processes use glycosylating enzymes, derived from cells, to carry out the reactions; finally, several groups have developed novel biological systems, in which carbohydrates are produced in genetically modified animal, yeast, or bacterial cells. Each method has advantages and disadvantages, but all of them have been improved in recent years.

A major advance in solid-phase synthesis was the development of an automated system that may eventually become as widely used as oligopeptide and oligonucleotide synthesizers[1]. "What we have developed is a very general procedure in the lab...based on an application which builds on monosaccharide molecules," says Peter Seeberger, professor in the department of chemistry at the Massachusetts Institute of Technology (Cambridge, MA). Seeberger and one of his students, Obadiah Plante, are cofounders of Advanced Carbohydrate Technologies (Cambridge, MA), which is commercializing the technology for drug development and research.

In enzymatic synthesis, Neose has enjoyed considerable success (see "Sugar in bulk"), but Roth concedes that "the place where we are relatively helpless is the synthesis of complex carbohydrates that do not exist in nature, and for which there are no [glycosylating] enzymes." That limitation may soon be lifted, at least partially. By rationally modifying one of the key glycosylation enzymes in *Salmonella* bacteria, researchers at the Memorial Sloan–Kettering Cancer Center (New York, NY) and Brookhaven National Laboratory (Brookhaven, NY) created an enzyme that uses sugars that are not natural substrates for a wild-type glycosylating enzyme[2]. Non-natural sugar structures are of particular interest to drug developers because they may resist degradation in the bloodstream.

Biological carbohydrate synthesis strategies have focused primarily on producing better glycoproteins, rather than generating pure carbohydrates. Genentech (S. San Francisco, CA), for example, has developed genetically modified Chinese hamster ovary (CHO) cells that attach more sialic acid to their glycoproteins than ordinary CHO cells, improving the pharmacokinetic profiles of the manufactured proteins[3].

Advances such as these will undoubtedly accelerate the pace of glycobiology research, but most experts agree that successful labs will eventually employ a mixture of techniques to synthesize glycans and glycoproteins.

Developing sugar pills

Even though most large pharmaceutical companies are still wary of glycobiology, it seems clear that carbohydrates will play an

Table 1. Companies focusing on carbohydrates

Company (location)	Focus
Abaron Sciences (La Jolla, CA)	Targeting glycosylation enzymes for a variety of novel therapies.
Biomira (Edmonton, AB, Canada)	Vaccines like Theratope, to halt metastatic cancers.
GlycoDesign (Toronto, ON, Canada)	Carbohydrate-processing inhibitors (CPIs) for the treatment of cancer, infection, inflammation, and cardiovascular disease: GD39 in phase 2 for various metastatic cancers.
Glycominds (Maccabim, Israel)	High-throughput study of complex carbohydrates. Products include GlycoChip and GlycoSd with Glycominds database.
GlycoTech (Rockville, MD)	Using non-natural molecules to mimic carbohydrates.
Neose Technologies (Horsham, PA)	Enzyme-based protein glycosylation and bulk sugar synthesis. In conjunction with Bristol-Myers Squibb, have a melanoma cancer vaccine in phase 3.
Neuronyx Biotech (Malvern, PA)	Glycolipid research.
Novazyme (Oklahoma City, OK)	Glycosylation enzymes and lysosomal storage diseases.
Progenics (Tarrytown, NY)	Ganglioside-based vaccine for the treatment of melanoma in phase 3.
SafeScience (Boston, MA)	Carbohydrate-based cancer treatments. GBC-590 in phase 2 for treatment of pancreatic and colorectal cancers.
Synsorb Biotech (Calgary, AB, Canada)	Oligosaccharides to bind toxins in the gut; Synsorb Cd in phase 3 for treatment of *Clostridium difficile* infection.
Synthon Chiragenics (Monmouth Junction, NJ)	Carbohydrate-based chiral chemistry technology.

increasingly important role in future drug development, and a number of biotechnology companies have begun to dabble in it (see Table 1). In addition to heparin and the Hib vaccine, other successful carbohydrate-based drugs include the influenza antivirals Tamiflu and Relenza and the antibiotics erythromycin and vancomycin.

Biotechnology companies are also starting to pay more attention to glycosylation, which is now being recognized as a major problem in protein production. Amgen (Thousand Oaks, CA), for example, discards about 80% of the erythropoietin it produces because the protein is not correctly glycosylated. Like many other therapeutically important proteins, erythropoietin will not function correctly if it lacks the right sugar groups. Although the importance of glycosylation has been understood for decades, companies have tolerated low yields of recombinant proteins in the past. However, with more proteins in their pipelines and fermentation facilities strained to their limits, companies are looking for ways to improve efficiency.

Carbohydrates are also increasingly being targeted as potential new medicines. Carbohydrate-based antibiotics are well established in medicine, but recently researchers have taken a new strategy. Pathogenic bacteria often use glycoprotein receptors on the surfaces of host cells to colonize a tissue. Eric Vimr, a researcher at the University of Illinois (Urbana-Champagne, IL), says that new antibiotics might act to prevent this attachment: "[One could] design some oligosaccharide that is identical or mimics that docking molecule on the host cell ... to thwart the ability of the microorganism to colonize." Carbohydrates are well tolerated by the human digestive system, and so carbohydrate-based medicines could have fewer side effects than traditional antibiotics for treating gastrointestinal infections.

Unfortunately, these so-called glycomimetic antibiotics have had little success so far, probably because the individual sugar molecules can only bind individual receptors on the bacteria, leaving adjacent, unoccupied receptors free to attach to host cells. Vimr hopes to address this problem by genetically modifying benign bacteria to express glycomimetics on their cell surfaces, then using the antibiotic-coated bacteria as the drug.

Many intracellular pathogens also require sugars, and glycosylation is itself a tempting target for novel antivirals. In one promising approach being pursued by Raymond Dwek and colleagues at Oxford University (UK), a small-molecule inhibitor of cellular glycosylation enzymes leads to the mis-glycosylation and subsequent misfolding of the matrix protein of hepatitis B virus. Host cell proteins are also mis-glycosylated when the drug is given, but the cell can identify and eliminate the defective proteins, whereas the virus cannot. The resulting defective viral particles are unable to initiate another round of replication, and glycosylation inhibitors appear to be promising antivirals[4]. The same group is also pursuing a similar approach for HIV treatments, although neither therapy has reached clinical trials yet. One advantage of targeting the glycosylation enzymes, rather than the carbohydrates themselves, is that small-molecule enzyme inhibitors can be developed and delivered in the same manner as traditional pharmaceuticals, avoiding the difficulties of delivering carbohydrate-based drugs.

For biotechnology companies glycobiology is an area really ripe for potential therapeutic intervention

Targeting cancers

Another effort to target glycosylation enzymes is taking place at GlycoDesign, which is carrying out clinical trials on its lead anticancer compound, GD0039. GD0039 blocks a critical carbohydrate-processing enzyme, and inhibits the ability of cancer cells to evade the immune system. The result seems to be a cellular stalemate, in which a tumor is not able to grow but is also not destroyed entirely. Cumming explains that "we don't know the precise mode of action or even if there is a singular mode of action [for the drug]." Although the importance of glycosylation for normal biology would suggest that inhibitors of glycosylation might have significant and undesirable side effects, GlycoDesign has found that, so far, the drugs are well tolerated in experimental animals and in humans.

Another class of carbohydrate-based cancer treatments aims to alert the immune system to the tumor's glycan disguise. Several companies and academic research teams are now developing anticancer vaccines that stimulate an immune response against the carbohydrate antigens found on tumor cells. Although these antigens are normally tolerated by the immune system, evidence is emerging that chemically modified glycans and adjuvants can boost immunity, causing tumors to be held in check or possibly even eliminated.

In addition to helping cancer evade the immune system, glycans help orchestrate normal and pathological immune responses, a fact that is also drawing the attention of researchers. Many autoimmune diseases are driven by a process called the Th1 response, in which the immune system initiates a chain reaction of inflammatory signals. Donald Harn, a researcher at Harvard University (Cambridge, MA), is hoping to use glycoconjugates to initiate compensatory anti-inflammatory Th2 responses: "We've now done a large number of studies in three different animal models in which ... the glycoconjugates appeared to be therapeutic." Harn, whose laboratory is also collaborating with Neose to develop adjuvants for improved vaccines, hopes to use similar carbohydrate-based drugs to modulate a wide range of immune responses.

Even selectins, which led to the downfall of Cytel, are getting a second chance. After purchasing Cytel, Neose retained some of the company's research staff and continued working on the selectin problem. Neose's Roth explains that when Cytel entered the field the structure of the carbohydrate that interacts with P-selectin had not been completely solved: "Cytel chose a structure that looked like the right structure at that time, but ended up not being the correct structure." In the ensuing years, says Roth, Neose has discovered that "the real structure is at least a thousand times more active," and the company is now developing it as an anti-inflammatory drug.

While more companies are beginning to target glycosylation and carbohydrates for drug development, experts in the field agree that sugars will continue to present a bigger challenge than DNA or proteins. "Glycobiology started in a less mature state in the early 1990s than did proteins or nucleic acids," says Cumming, adding that "it's clear we understand a lot more now than we did 15 years ago, but it's clear there's a lot more to understand. For biotechnology companies, glycobiology is an area really ripe for potential therapeutic intervention."

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