Carbohydrate vaccines: developing sweet solutions to sticky situations?

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Abstract | Recent technological advances in glycobiology and glycochemistry are paving the way for a new era in carbohydrate vaccine design. This is enabling greater efficiency in the identification, synthesis and evaluation of unique glycan epitopes found on a plethora of pathogens and malignant cells. Here, we review the progress being made in addressing challenges posed by targeting the surface carbohydrates of bacteria, protozoa, helminths, viruses, fungi and cancer cells for vaccine purposes.

Serotype
A group of closely related microorganisms distinguished by a characteristic set of antigens.

Conjugate vaccines
Carbohydrate-based vaccines in which the immunogens are glycan antigens covalently attached to a source of helper T-cell epitopes, often an immunogenic carrier protein, such as tetanus toxoid.

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Since Edward Jenner's seminal discovery in 1796 that inoculation with cowpox could protect against smallpox infection, vaccines have become a hugely important and successful countermeasure to the threat of infectious disease[1]. Vaccines provide protection by inducing humoral and/or cellular immunity to disease-causing pathogens. The dense surface distribution of often unique glycan structures on diverse pathogens and on malignant cells makes carbohydrates attractive vaccine targets (FIG. 1).

Using carbohydrates to induce immunity is a relatively new strategy, even though Heidelberger and Avery made the connection between pneumococcal serotype and capsular polysaccharide back in 1923 (REF. 2). Francis and Tillet had then noted that intradermal immunization with serotype-specific polysaccharide elicited antibodies against heterologous types of pneumococci species[3]. Heidelberger and co-workers subsequently established that vaccination with pneumococcal capsular polysaccharide could be used to elicit persistent antibody-mediated immunity[4]. Despite these key discoveries, the advent of chemotherapeutics and antibiotics during this same period dampened enthusiasm for developing carbohydrate vaccines. The steady increase in antibiotic resistance since then has catalysed a renewed interest. In 1983, the first polysaccharide vaccine, PneumoVax (Merck and Co.), was commercially launched. This vaccine was composed of unconjugated capsular polysaccharide isolated from 14 pneumonia serotypes; the current incarnation includes 23 out of approximately 90 known serotypes[5,6]. In healthy adults, this vaccine induces protection against approximately 90% of infections caused by these pathogens. However, in high-risk groups (that is, neonates and children under 2 years of age, the elderly and immunocompromised) polysaccharides generally elicit poor antibody responses and do not induce adequate protection[7].

The poor quality of antibody responses to carbohydrates is one of the many obstacles associated with developing carbohydrate-based vaccines (see below) and is largely attributed to the T-cell independent immune responses, which are typically triggered by repetitive carbohydrate antigens[8,9]. B-cell receptor crosslinking through binding repetitive motifs activates antigen-specific B cells independent of $CD4^+$ helper T cells. Such T-cell-independent responses are less robust, short-lived and primarily consist of immunoglobulin M (IgM) antibodies. By contrast, $CD4^+$ T cells, which are typically generated in response to proteins, enable the generation of high affinity, class-switched antibodies and subsequently, long-lived antibody-mediated protection. Zwitterionic capsular polysaccharides from some bacteria are an exception as these carbohydrates, like proteins, can be processed and presented on major histocompatibility complex class II molecules for activation of $CD4^+$ helper T cells and the generation of T-cell-dependent immune responses[10,11]. To recruit $CD4^+$ T cells for antibody responses against the vast majority of glycans, exogenous $CD4^+$ T-cell epitopes must be provided, usually in the form of a carrier protein. As early as 1931, Avery and Goedel reported that conjugation of glycans to a suitable protein scaffold enhanced the immunogenicity of carbohydrates[12]. It is now well known that immunization with neoglycoconjugates composed of capsular polysaccharide-derived glycans covalently coupled to an immunogenic protein carrier (conjugate vaccines) induces long-lasting protection against encapsulated bacteria, even among persons in high-risk groups[13,14]. The success of early conjugate vaccines was a key breakthrough that propelled the field forward[15]. Several conjugate versions of polysaccharide vaccines are now either commercially available (TABLE 1) or in development[16] (TABLE 2).

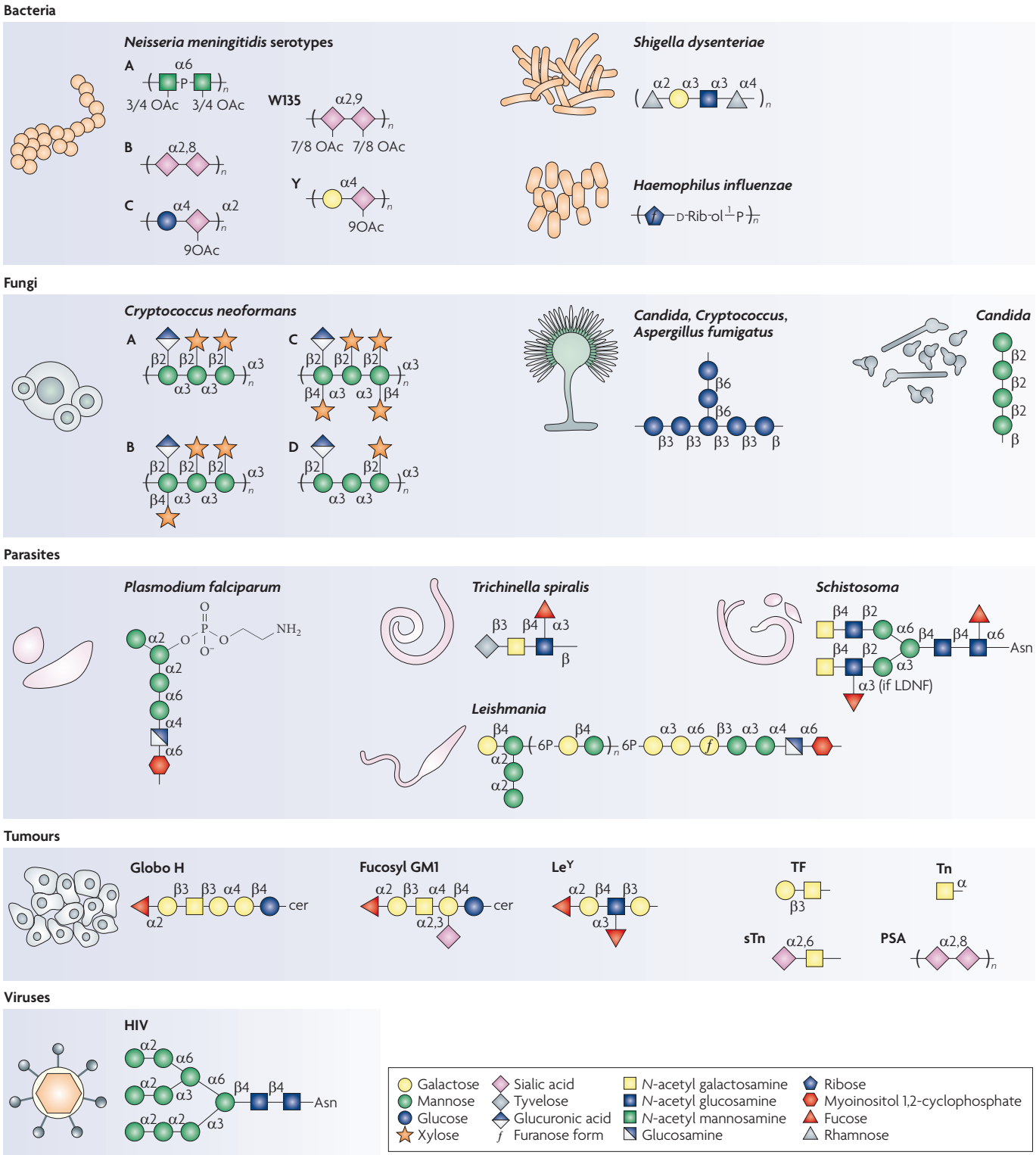

Figure 1 | **A diverse array of disease-causing agents and glycan antigens are targeted by existing and developmental carbohydrate vaccines.** Bacteria: capsular polysaccharide repeats associated with particular species (and serotypes). Fungi: common glucuronoxylomannan (GXM) motifs for serotypes A–D (*Cryptococcus*); β-glucan (*Candida*, *Cryptococcus and Aspergillus*); β-mannan (*Candida*). Parasites: synthetic glycosylphosphatidylinositol motif (*Plasmodium falciparum*); common tyvelose-containing antigen (*Trichinella*); LacdiNAc (LDN) and fucosylated LDN (LDNF) (*Schistosoma*); common lipophosphoglycan (*Leishmania*). Tumours: common glycan antigens associated with glycolipids (globohexaosylceramide (Globo H), fucosyl GM1, Lewis Y (Le^Y)) and glycoproteins (Thomsen–Friedenreich (TF), Le^Y, 2-6-α-*N*-acetylgalactosamine (Tn), sialyl Tn and polysialic acid (PSA)) found on various malignant tissues, see TABLE 3. Viruses: high mannose $GlcNAc_2Man_9$ (HIV). Note that mannose residues may be 6*O*-acetylated on GXM motifs.

Table 1 | **Licensed carbohydrate-based vaccines**

Indication	Vaccine	Manufacturer (Trade name)
Haemophilus influenzae type b (Hib)	Glycoconjugugate, polysaccharide with tetanus toxoid (TT)	Sanofi Pasteur (ActHIB); GlaxoSmithKline Biologicals (Hiberix)
	Diphtheria toxoid (DT), TT and acellular pertussis adsorbed, inactivated poliovirus and Hib–TT conjugate vaccine	Sanofi Pasteur (Pentacel)
	Hib conjugate (meningococcal protein conjugate)	Merck & Co (PedvaxHIB)
	Hib conjugate (meningococcal protein conjugate) and hepatitis B (recombinant) vaccine	Merck & Co (Comvax)
Neisseria meningitidis A, C, Y and W-135	Glycoconjugate, meningococcal polysaccharide with DT	Sanofi Pasteur (Menactra)
	Meningococcal polysaccharide	Sanofi Pasteur (Menomune-A/C/Y/W-135)
Salmonella typhi	Vi capsular polysaccharide	Sanofi Pasteur (TYPHIM Vi)
Streptococcus pneumoniae 4, 6B, 9V, 14, 18C, 19F and 23F	Pneumococcal polysaccharide 7-valent–CRM197 conjugate	Wyeth Pharmaceuticals (Prevnar)
Streptococcus pneumoniae 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F	Pneumococcal polysaccharide, 23-valent	Merck & Co (Pneumovax 23)

This table was adapted from the complete list of vaccines licensed for immunization and distribution in the United States, which is available from the US Food and Drug Administration website (see Further information). CRM197, non-toxic mutant of DT.

The field of carbohydrate vaccine design is now undergoing another quantum leap as a result of recent technological advances (BOX 1). The explosion in glycomics research is opening doors for carbohydrate vaccine researchers to better tackle the challenges inherent to carbohydrate vaccine development, and to expand the field to encompass a broader spectrum of diseases, in addition to bacterial infections. Although several reviews have described carbohydrate-based vaccines for specific indications[17–19], a broad survey is warranted to put in perspective the advances in the field as a whole (see also REFS 20,21). This Review will focus on the following questions: what are the shared and unique problems involved in creating carbohydrate vaccines for such diverse indications as bacterial, viral and parasitic infections and cancer? What solutions have been found and can they be applied across fields? What are the emerging challenges and future prospects for carbohydrate vaccine design and development?

Challenges of carbohydrate vaccine design

For most vaccines, the induction of protective antibodies is thought to be crucial for efficacy. The nature of glycans presents a number of challenges with respect to inducing protective antibodies. As already discussed, carbohydrates are often poorly immunogenic. Furthermore, carbohydrate-specific antibodies typically have low affinity (with dissociation constants in the micromolar range) compared with protein-specific antibodies (with dissociation constants in the nanomolar range). Protein–carbohydrate binding is mediated by a high degree of hydrogen bonding, and van der Waal's, hydrophobic and electrostatic interactions; that is, similar interactions to those involved in protein–protein binding[22–26]. Antibody binding to both carbohydrates and proteins involves a favourable enthalpy contribution to the free energy of interaction[22,24]. However, for carbohydrates, this is offset to a significant degree by an unfavourable entropy contribution. This has been attributed mainly to either the loss of conformational flexibility in the oligosaccharide upon complex formation[27] or to solvent re-arrangement upon binding. It has been suggested that water molecules hydrogen bonded to amphiphilic surfaces of unbound oligosaccharides are more mobile and less strongly hydrogen bonded than water molecules in bulk solution[28]. These unfavourable solvent re-arrangements may override favourable entropy contributions from hydrophobic effects when antibodies bind to carbohydrates as opposed to proteins. Owing to the inherent low affinity, glycan interactions rely on avidity effects that are enabled through multivalent interactions. Glycan microheterogeneity on glycoproteins and glycolipids is yet another obstacle to overcome in the identification and targeting of specific antigens (BOX 1). Moreover, the heterogeneous display of glycans on target organisms (or cells) can dilute the efficacy of any particular glycan-specific antibody response.

Four important considerations are generally applicable to the design of modern carbohydrate-based glycoconjugate vaccines: the antigen source, the carrier, the conjugation method and the adjuvant (FIG. 2). Glycan antigens are diverse and range from large elaborate capsular polysaccharides to small monosaccharide tumour antigens (FIG. 1). In general, polysaccharides exist as a family of closely related species that vary in their degree of polymerization. As the pertinent immunogenic

Microheterogeneity
Describes heterogeneity in glycan structures at a single glycosylation site owing to differential enzymatic processing by local glycosyl transferases and glycosidases.

Table 2 | **Examples of carbohydrate-based vaccines in development**

Indication	Vaccine	Phase of development
Enterohaemorrhagic *Escherichia coli*	O-specific polysaccharide–protein conjugate	Phase I[182]
Group A *Streptococcus* spp.	Glycoconjugate of Group A polysaccharide with TT	Preclinical[183]
Group B *Streptococcus* spp.	Glycoconjugates of type Ia, Ib, II, III and V polysaccharides linked to carrier proteins	Phase II[184]
Haemophilus influenzae (non-typeable)	Subunit-detoxified lipooligosaccharide conjugate	Preclinical[185]
Pseudomonas aeruginosa	Octavalent glycoconjugate of O-polysaccharide with toxin A	Phase III[186]
Salmonella typhi	rEPA–Vi conjugate vaccine	Phase III[33, 187]
Shigella dysenteriae	O-specific polysaccharide–protein conjugate	Preclinical[42]
Shigella flexneri	O-specific polysaccharide–protein conjugate	Phase II[188]
Shigella sonnei	O-specific polysaccharide–protein conjugate	Phase III[189]
Streptococcus pneumoniae	Glycoconjugates of synthetic 6B polysaccharide motifs	Preclinical[43]
Vibrio cholerae	Lipopolysaccharide–protein conjugate	Phase I[190]
Aspergillus fumigatus	β-Glucan–CRM197 conjugate	Preclinical [65, 66]
Candida albicans	Cell surface oligomannosyl epitope (various conjugates)	Preclinical[63,64]
	β-glucan–CRM197 conjugate	Preclinical[65,66]
Cryptococcus neoformans	Glycoconjugate of capsular polysaccharide with TT	Phase I[50]
	β-glucan–CRM197 conjugate	Preclinical[65,66]
Leishmania spp.	Lipophosphoglycan	Preclinical[97]
	Lipophosphoglycan conjugates	Preclinical[103]
Plasmodium falciparum	Glycosylphosphotidylinositol–KLH conjugate	Preclinical[93]
HIV-1	Man α(1→2)Man oligomannosyl epitope (various conjugates, engineered yeast strains and modified glycoproteins)	Preclinical[17, 113–118,165]
Breast cancer	Unimolecular hexavalent conjugates (Globo H–GM2–Lewis^y–sTn–TF–Tn–R)	Preclinical[149]
	sTn(c)–KLH plus QS-21 as adjuvant	Phase I[137]
Epithelial cancer	Globo H–GM2–Lewis^y–MUC1-32(aa)–sTn(c)–TF(c)–Tn(c)–KLH conjugate vaccine plus QS-21 as adjuvant	Phase I[147]
Melanoma	GM3NPhAc–KLH	Preclinical[152]
Prostate cancer	Unimolecular hexavalent conjugates (Globo H–GM2–Lewis^y–sTn–TF–Tn–R)	Preclinical[149]
	TF(c)–KLH plus QS-21 as adjuvant	Phase I[138]
	Tn(c)–KLH and Tn(c)–palmitic acid	Phase I[139]
	Globo H–GM2–Lewis^y–MUC1-32(aa)–TF(c)–Tn(c)–KLH conjugate vaccine plus QS-21 as adjuvant	Phase II[148]

This table was adapted from *The Jordan Report Accelerated Development of Vaccines 2007* (see Further information) and updated with additional information from publicly available resources. This table should not be considered inclusive of all ongoing carbohydrate vaccine research and development. (c), cluster; CRM197, a non-toxic mutant of diphtheria toxin; Globo H, globohexaosylceramide; KLH, keyhole limpet haemocyanin; MUC1-32(aa), mucin 1, 32 amino acids long; sTn, syalyl 2-6-α-*N*-acetylgalactosamine; rEPA, recombinant *Pseudomonas aeruginosa* exotoxin A; TF, Thomsen–Friedenreich; Tn, 2-6-α-*N*-acetylgalactosamine; TT, tetanus toxoid; Vi, capsular polysaccharide of *S. typhi*.

epitopes comprise only part of the glycan, oligosaccharides are often adequate for vaccine preparation. These molecules may be derived from digestion of naturally derived polysaccharides or produced as a chemically homogeneous species through synthetic methods. Carriers are most often proteins and could be toxoids, keyhole limpet haemocyanin (KLH) or virus capsids, although other materials are possible (FIG. 2). They should be immunogenic and express multiple loci for conjugation as polyvalent display is crucial for generating carbohydrate-specific antibody responses. Coupling of oligosaccharide antigens to the carrier necessitates activation of the sugars and/or the carrier. Several procedures have been developed to activate polysaccharides, but most result in the creation of reactive groups that are randomly distributed throughout the polymer. This random array of conjugation points is not conducive to creating homogeneous glycoconjugates. To generate well-defined conjugates, the linkage between sugar and carrier should be as specific as possible. One advantage

Box 1 | **Glycomics and carbohydrate vaccines**

Carbohydrate vaccine development is benefiting from the new glycomics technologies and the establishment of international glycomics consortia, such as the Consortium for Functional Glycomics and EuroCarbDB[160]. Advances in glycan analysis, synthesis, array fabrication and structure determination are especially valuable.

Glycan analysis. The identification of potential carbohydrate epitope targets is typically the first step in the development of a carbohydrate vaccine. Analysis and purification of naturally occurring glycans is complicated by the microheterogeneity of carbohydrates arising from non-template-driven biosynthesis. In addition, there is a trade-off between the ability to perform high-throughput analysis of glycan mixtures and the ability to do fine structure characterization. Biochemical and analytical methods such as nuclear magnetic resonance (NMR), electrospray ionization–mass spectrometry (ESI–MS), matrix-assisted laser desorption ionization MS (MALDI–MS) and capillary electrophoresis have been developed to profile the repertoire of carbohydrate structures isolated from cells and tissues[160]. Each methodology is best suited for determining a certain set of attributes; for example, chain length and mass composition relationships from MALDI–MS or monosaccharide composition and linkage information using NMR. To improve the utility of these methods and to enable higher-throughput analysis, informatics-based sequencing methodologies are being used to integrate information from multiple complementary techniques[161,162].

Glycan synthesis. A key bottleneck in carbohydrate vaccine research is access to the carbohydrate antigens themselves. As natural sources are often limited and require complicated purification procedures, the onus has largely fallen on synthetic strategies to supply these ligands for vaccine research purposes. Advances in solution- and solid-phase glycan synthesis methods, as well as chemoenzymatic glycan assembly methods, have accelerated and simplified the production of complex carbohydrates such as β-glucan dodecasaccharide and globohexaosylceramide[163–165]. Automated approaches are based on two versatile strategies: reactivity-based one-pot synthesis and solid-support synthesis[163,164]. However, several issues remain to be addressed before these technologies are widely applicable, such as expansion of the repertoire of protected monosaccharide building blocks, glycosyltransferases and glycosidases. It should also be noted that each synthetic route must still be optimized and the required building blocks synthesized, which often requires several steps. Automated oligosaccharide synthesis often requires large, molar excesses of the building blocks, which also limits the appeal of this approach for many laboratories. Together, the ongoing improvements in isolation and purification methods are expected to allow carbohydrate vaccine researchers easier and faster access to defined carbohydrates in the near future.

Glycan arrays. Improved access to a large assortment of purified carbohydrates has enabled the development of high-throughput glycan-binding assays. The low affinity of protein–glycan interactions and high dependency on multivalent interactions is addressed by displaying glycans in an arrayed chip-based format. Several glycan arrays have been fabricated that utilize either covalent or noncovalent attachment protocols[166]. Fluorescent readouts are most common, although recently, surface plasmon resonance and MALDI–time of flight MS have been used as label-free alternatives[167–170]. From a vaccine perspective, these glycan arrays are invaluable tools for identifying relevant glycan targets and for evaluating candidate immunogens by revealing the fine specificity of relevant carbohydrate-specific antibodies elicited during natural infection, for example, HIV-specific antibody 2G12 (REFS 117,118,171).

Glycan structure determination. Structural data of protein–glycan interactions may provide valuable insights into the appropriate presentation of glycan antigens. Unfortunately, these data are difficult to obtain and consequently, relatively rare. A significant challenge stems from the flexibility of glycans, which assume multiple (defined) conformational states under physiological conditions. Currently, the combination of X-ray crystallography, NMR and molecular dynamics simulations, and other computational methods (such as docking simulations and absolute and relative free-energy calculations), are used in structural glycobiology to elucidate the conformations of free carbohydrates and protein–carbohydrate complexes[172,173]. Emerging technologies include oxidative footprinting of carbohydrate binding surfaces, which complements epitope mapping techniques, such as saturation transfer difference NMR[174]. Another promising approach for three-dimensional carbohydrate–protein complex determination combines partially oriented NMR spectroscopy with computational simulations[174].

Reactivity-based one-pot synthesis
In the one-pot strategy, several glycosyl donors are allowed to react sequentially in the same vessel in solution, resulting in a single main oligosaccharide product. These protocols generally make use of three main concepts, namely chemoselective principle, orthogonal glycosylation and pre-activation strategy.

Solid-support synthesis
In solid-support synthesis, an easily yet orthogonally cleavable linker tethers the first monosaccharide building block to a solid support. An activating agent then activates the donor for glycosylation with protected monosaccharide building blocks added in solution. Several cycles of activation, washing and deprotection ensue, followed by linker cleavage upon completion.

QS-21
A saponin derived from the tree bark of *Quillaja saponaria* that has been shown to augment carbohydrate-specific antibody responses.

of the synthetic route is that glycans can be produced with a readily activatable linker so that a single conjugation chemistry can be used for a wide range of products (BOX 2). Several synthetic linkers are available[29,30], but one must be cautious of the immunogenicity of these linkers relative to the glycan antigen[31,32]. The immune response may be predominantly directed against the linker and away from the carbohydrate antigen. Also, steric issues may be addressed with bifunctional spacers to enhance the efficiency of loading. Finally, adjuvants are often included to improve the immunogenicity of the target carbohydrate antigens. Alum is the only adjuvant approved for human use in the United States; however, several promising formulations are in clinical trials, for example, QS-21.

The remainder of this Review will focus on carbohydrate vaccine research pertaining to specific pathogens and cancer.

Bacterial pathogens

The surface of bacterial pathogens is covered with a dense array of polysaccharide that is a unique feature of not only the particular species, but also the strain of bacteria considered. Carbohydrate-specific antibodies are predominantly responsible for protection against bacteria with either a capsule or lipopolysaccharide on their surfaces. People lacking these antibodies, for example, the elderly and neonates, are at high risk of developing infections. With the increasing prevalence of antibiotic-resistant bacterial strains, the proven track

record of capsular polysaccharide-based vaccines has encouraged the development of these type of vaccines against a broader range of pathogens. For many bacterial infections, glycoconjugate vaccines have been made based on fragments of their capsular polysaccharides, for example, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*[16]. With the advent of conjugate vaccines for several strains of the above three bacteria, the incidence of bacteraemia, meningitis and otitis media has almost been eliminated in countries where these vaccines are routinely used. Vaccines against several other clinically important bacterial pathogens based on their capsular polysaccharide (for example, *Salmonella typhi* Vi)[33] or lipopolysaccharide O-chains (for example, on *Shigella dysenteriae*) are currently under development[16] (TABLE 2). The Vi conjugate vaccine candidate was shown to be safe, immunogenic and 90% efficacious in children aged 2–5 years old, whereas the licensed vaccines confer approximately 70% immunity and do not protect young children[33].

The development of effective carbohydrate-specific vaccines against bacterial pathogens has historically been complicated by the considerable heterogeneity and complexity of capsular polysaccharides. For example, more than 90 capsular types are described for *S. pneumoniae*[6,34], 23 of which are included in the current polysaccharide vaccine. Generally, heterogeneity issues have been successfully addressed by isolating polysaccharide from the most clinically relevant serotypes (geographically) followed by degradation into smaller products for activation and conjugation to immunogenic carrier proteins to create multivalent conjugate vaccines. For example, Prevnar (Wyeth/Pfizer) is a heptavalent pneumococcal conjugate.

Another problem that hinders the development of some vaccines is the structural similarity between certain glycan antigens and host glycans, and therefore may be tolerated by the host's immune system, resulting in vaccine formulations with poor immunogenicity. For example, similarities between the meningococcus Group B (MenB) capsular polysaccharide and self sialic-acid-containing glycans found during normal growth and development and in the central nervous system probably make these bacterial glycans particularly poor immunogens[35,36]. One general strategy to overcome this immunotolerance is to immunize with a chemically modified version of the glycan, essentially rendering it more foreign to the host. If the modification is sufficiently structurally conservative, the elicited antibodies may cross-react with the natural glycan on the pathogen. Efforts to increase their immunogenicity via *N*-propionylation of the polysialic acids (PSAs) resulted in moderately higher antibody titres that cross-react with unmodified MenB capsular polysaccharide, but not with self sugars[37]. One general point to consider when immunizing with glycans similar to those found on host tissue is that the benefit of the vaccine should outweigh the risk of inducing autoimmunity. This risk may be less pronounced if the target structures are poorly expressed on host cells. For instance, the risk of inducing autoimmune antibody responses combined with the identification of alternative MenB target antigens shifted the focus away from carbohydrate-based vaccine development for MenB[38]. Interestingly, a small increased risk of developing Guillain–Barré syndrome may be associated with vaccination with Menactra (Sanofi Pasteur), the new multivalent meningococcal conjugate[39]. The ability to break tolerance in a reliable, safe and effective manner against pathogen-associated carbohydrates that resemble self is a hurdle that vaccinologists in other fields, especially in cancer and HIV (see below), must also overcome. Vaccine strategies that incorporate chemical modification of the target antigen show potential for addressing this issue.

Several conjugate vaccines composed of naturally derived polysaccharides have excellent efficacy (usually approximately 90%) and safety profiles in the clinic[7]. However, using naturally derived polysaccharide to produce conjugate vaccines that meet quality control and safety standards as required by the US Food and Drug Administration in a cost-effective manner is challenging, as discussed above. Thus, a movement towards synthetic carbohydrate vaccines is underway[18]. Synthetic glycans with uniform linkers can be used to manufacture well-characterized conjugates in a more economical and reproducible manner, as well as free from bacterial contaminants[40] (BOX 2).

Synthetic methods may also aid in addressing one of the main scientific challenges in this field: understanding the relationship between chain length (and/or saccharide density) and the potency of the protective antibody response. Synthetic methods enable the pursuit of empirical approaches to map the structural parameters that influence carbohydrate-specific immunogenicity such as chain length, saccharide composition and secondary structure. For instance, an investigation into a vaccine against *S. dysenteriae* included conjugates that displayed various densities of tetra-, octa-, dodeca- and hexadecasaccharides based on the tetrasaccharide repeat unit of the O-specific oligosaccharide[41]. It was found that the octa-, dodeca- and hexadeca-, but not tetrasaccharide, conjugates were immunogenic and elicited protective antibodies. Moreover, optimal loading densities were identified that were dependent on the chain length. The importance of the non-reducing terminal residue of oligosaccharides has also been demonstrated[42]. The findings from these studies, however, cannot necessarily be extended to other systems. For example, an immunogenicity and protection study on the *S. pneumoniae* 6B capsular polysaccharide showed that single repeats of either a di- or tetrasaccharide were sufficient to elicit protective antibody responses in rabbits and in mice[43].

Fungal pathogens

Pathogenic fungi are significant infectious threats. Of particular concern are the opportunistic fungal agents such as *Candida*, *Cryptococcus* and *Aspergillus*, which target immunocompromised individuals worldwide. The disease burden has not declined despite the availability of effective chemotherapeutics. Furthermore, current therapeutics are limited by toxicity concerns and the emergence of resistance. Interest in vaccine

Multivalent conjugate vaccines
Vaccines described as multivalent are composed of multiple, different glycan antigens. For example, capsular polysaccharide fragments from different serotypes or different tumour-associated antigens.

Guillain–Barré syndrome
Guillain–Barré syndrome is an uncommon, serious autoimmune disorder that attacks the peripheral nervous system, particularly myelin sheaths. It causes progressive muscle weakness and numbness that can eventually lead to full body paralysis. Some forms of this disease are mediated by ganglioside-specific antibodies that recognize antigens on peripheral nerves.

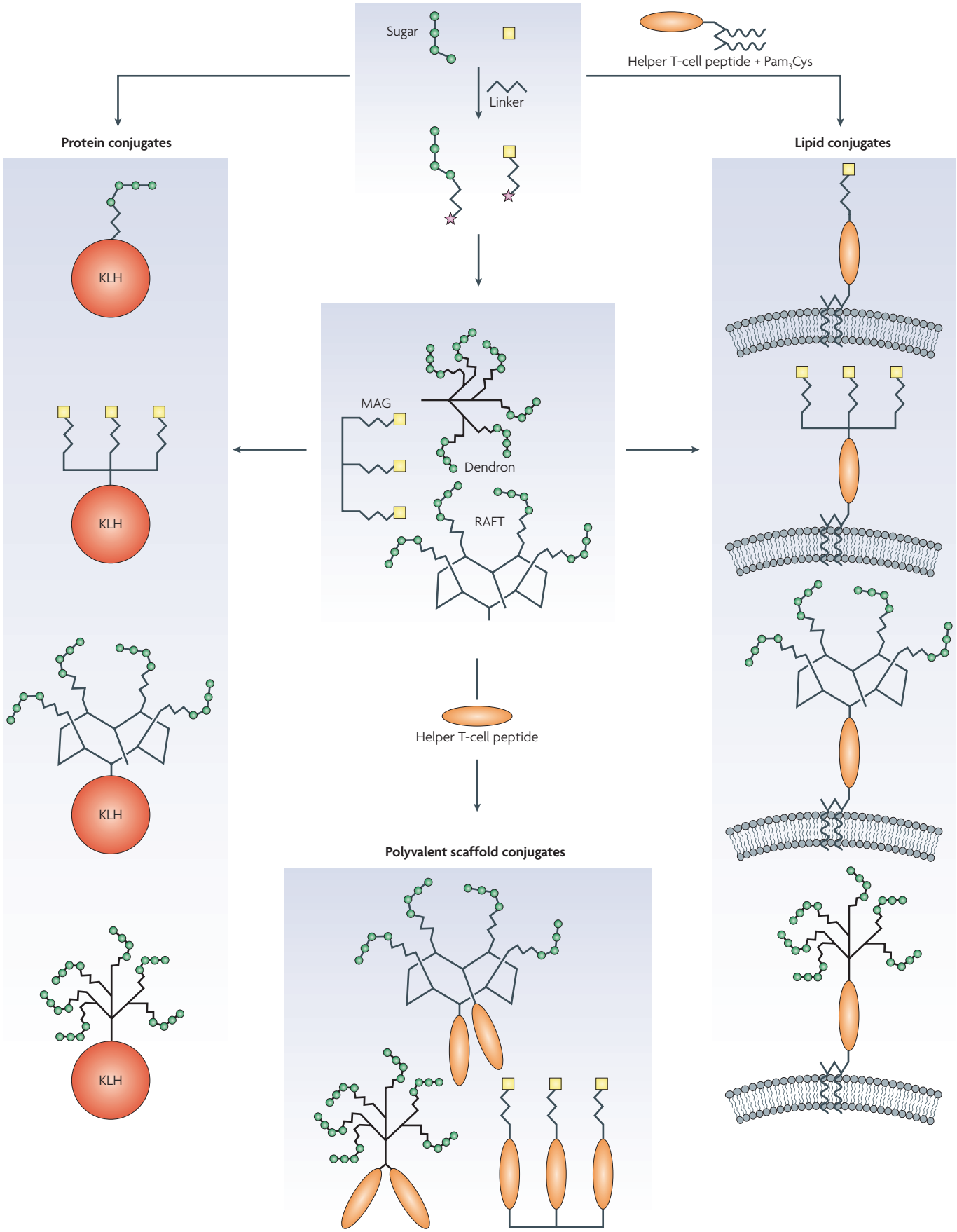

Figure 2 | **Schematic representation of glycoconjugate immunogen design.** Starting from activated glycans (star denotes activated group) from natural or synthetic sources, the production of three categories of glycoconjugate immunogens is shown: protein conjugates, lipid conjugates and polyvalent scaffold conjugates. The requirement for both polyvalent display and helper T-cell epitopes, crucial for achieving strong, long-lasting and class-switched antibody responses, are satisfied in each category. For protein conjugates, activated glycans are covalently attached to immunogenic protein carriers — for example, keyhole limpet haemocyanin (KLH) — which provide helper T-cell epitopes and enable polyvalent display. Lipid conjugates, made by covalent linkage of activated glycans to helper T-cell peptides attached to lipid moieties, allow polyvalency through formulation into lipid membranes. In addition, activated glycans may first be conjugated to synthetic polyvalent scaffolds — for example, dendron, multiple antigen glycopeptide (MAG) and regioselectively addressable functionalized template (RAFT) — which may then be used to make protein and lipid conjugates. Alternatively, polyvalent scaffold conjugates may be made through addition of helper T-cell peptides alone. Adjuvants are usually included in the final glycoconjugate vaccine formulations (for example, alum or QS-21). Note that tripalmitoyl-*S*-glyceryl-cysteinylserine (Pam_3Cys also) has adjuvant properties.

development was lacking until the 1990s as antibodies were previously considered unimportant to host defence against mycoses[44]. A major shift was catalysed by the discovery that a monoclonal antibody against *Cryptococcus neoformans* polysaccharide protects against experimental cryptococcal infection in mice[45]. Mounting evidence for fungal-specific antibodies against surface polysaccharides and other antigens, which mediate protection has since encouraged the development of vaccines to elicit such antibodies[46]. As the major component of the cell walls and capsules of fungi are polysaccharides, these have been the focus of considerable research.

Studies with glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans* (FIG. 1), illustrate some of the difficulties related to designing and developing carbohydrate vaccines against fungal pathogens. Immunization with a GXM–tetanus toxoid conjugate has been shown to elicit protective GXM-specific antibody responses, and GXM-specific monoclonal antibodies have been shown to protect mice against cryptococcosis[47]. However, non-protective and even deleterious antibodies that bind to different epitopes on GXM[48] have also been elicited by GXM conjugates[49,50]. In addition, antibody-mediated protection has been shown to be contingent not only on the antibody specificity and titre, but also on the antibody isotype[51–53]. Elegant studies using GXM-specific monoclonal antibodies of the same specificity, but different isotype, showed that those that did not activate complement or opsonophagocytosis (human IgG2 and IgG4 and mouse IgG1) protected best against *C. neoformans* infection[51,53]. The potential use of GXM conjugate vaccines is further complicated by unwanted immunomodulatory effects exhibited by this polysaccharide, such as interference with leukocyte migration[54,55]. To circumvent some of these issues, alternative vaccine constructs containing defined antigens, including synthetic oligosaccharides[50] and peptide mimitopes[56] that are designed to represent the protective epitope(s) of GXM, are being pursued.

A systematic approach that integrates immunogenicity studies with oligosaccharide synthesis and structure determination was used to identify a protective motif on the cell wall of a *Candida* spp. and to develop several carbohydrate vaccine candidates, which are in various stages of testing. Early vaccine formulations based on mannan extracts elicited antibodies that were capable of protecting mice against vaginal and disseminated *Candida albicans* infection[57,58]. Extensive research on protective responses against *Candida* mannan components in experimental animals indicated that antibodies against the unique minor β-mannan component — specifically, short β1,2 linked oligomannosides — but not the major α-mannan component, are protective against candidiasis[59]. Antibodies against β1,2 mannotriose or mannobiose protect against systemic candidiasis caused by *Candida tropicalis* and *C. albicans*, and are also expected to show efficacy against other strains that synthesize these motifs (for example, *Candida glabrata* and *Candida lusitaniae*)[57]. Using the synthetic route, this motif has been methodically pursued as the basis of a defined glycoconjugate vaccine. In a synthetic panel of di- to hexamannosides, the small di- and trimannosides were the most effective inhibitors of protective monoclonal antibodies that bind the β1,2 mannan motif; this was attributed to their well-ordered helical conformation[60,61]. Efficient synthetic strategies were devised to enable the preparation of gram quantities of β1,2 oligomannosides and of several prototype oligomannoside conjugate vaccines[62–64]. Immunization of experimental animals with clustered and unclustered di- and trimannoside–tetanus toxoid conjugates elicited antibody titres that cross-react strongly with *C. albicans* β-mannan cell wall extract[63]. In addition, synthetic glycopeptide vaccines that combine β1,2 trimannoside and peptide epitopes have recently been shown to induce protection against candidiasis[64].

β-glucan, a conserved structural component of many pathogenic fungi, has been described as a promising target for an effective vaccine against candidiasis — and potentially a wide range of other mycoses. An algal β1,3 glucan conjugated to a non-toxic mutant of diphtheria toxin, CRM197, was found to be immunogenic in mice and to provide antibody-mediated protection against infection by *C. albicans* and *Aspergillus fumigatus*[65], two phylogenetically distinct fungal species. Furthermore, the elicited antibodies inhibited the growth of *C. albicans, A. fumigatus* and *C. neoformans*, *in vitro* in the absence of immune effector cells, which suggests that vaccine efficacy may not require cellular or other components of an intact immune system[65,66]. These findings have sparked considerable speculation about the possibility of developing a broad-spectrum anti-fungal vaccine based on β-glucan that may be efficacious even in immunocompromised individuals[67].

Parasitic pathogens (protozoans and helminths)

It is disheartening to think that no vaccine is available for any of the major global parasitic infections such as malaria, leishmaniasis, African trypanosomiasis, amoebiasis, schistosomiasis and lymphatic filariasis. Several vaccine strategies are being pursued but, in general, progress in this field has been impeded by the complex biology of parasites, the immune evasion mechanisms used by many parasites, and a poor understanding of the

Glucuronoxylomannan (GXM). A linear polymer composed of repeating mannose trisaccharide motifs bearing a single β1,2 glucuronic acid with variable xylose and *O*-acetyl substitutions to form six triads.

Opsonophagocytosis A specific mechanism by which the host protects itself against infection. It involves the coating of a target (for example, bacterium) with serum opsonins (antibodies and complement), which mediates engulfment and destruction of the target by phagocytes (for example, macrophages).

Box 2 | **First synthetic carbohydrate vaccine**

Haemophilus influenzae type b (Hib) is a pathogen that, before the introduction of Hib conjugate vaccines in 1988, was the leading cause of bacterial meningitis in children in the United States of America. These vaccines, which contain capsular polysaccharide isolated from the pathogen, have reduced the incidence of bacterial meningitis and pneumonia in the developing world by more than 95%[175]. However, introduction of the vaccine in developing countries has been slow owing to its high cost and limited availability[176]. The World Health Organization estimates that, in the developing world, Hib is currently responsible for approximately three million serious illnesses and an estimated 386,000 deaths per year, almost all of which are children under the age of 5 years old[176].

The first commercial vaccine containing a synthetic carbohydrate antigen was developed in Cuba against Hib. This vaccine, Quimi-Hib (Heber Biotech) (FIG. 3A), exhibits several advantages associated with the synthetic route:

- potentially lower production costs compared with conventional vaccines using carbohydrates from natural sources
- controlled production of a homogeneous single compound including the linker
- minimal batch-to-batch variability during manufacturing process
- higher quality control standards are permitted, compared with the use of naturally derived agents

In addition, synthetic carbohydrates can be modified to increase their immunogenicity.

In 1989, a team from Cuba embarked on a project to produce a new, more economical conjugate anti-Hib vaccine from a fully synthetic capsular polysaccharide antigen (FIG. 1). In collaboration with a Canadian chemist they spent 2 years developing a streamlined synthetic scheme that is amenable to large-scale production[40]. The resulting synthetic pathway involves a one-step polycondensation reaction with the use of H-phosphonate chemistry, and produces oligomers with, on average, eight repeating units of polyribosylribitol with an 80% yield after purification by size-exclusion chromatography[40]. The antigen was first conjugated to human serum albumin for antigenic evaluation and then to tetanus toxoid for immunogenicity studies in animals, and finally clinical studies in adults, children and infants[40,177,178].

Fourteen years and seventeen clinical trials later, the result is a 99.7% success rate in children[40]. In 2004, Quimi-Hib became part of Cuba's national vaccination programme.

correlates of immunity[68]. In fact, disease is often the result of the interplay between the host and the invading parasite, which involves complex immune responses[69–73].

Glycans make attractive vaccine targets on parasitic protozoans and helminths because unique glycan antigens are highly abundant and accessible on the surface of multiple developmental stages. These antigens also tend to be immunodominant, at least in helminth infections[74]. The ability of antibodies to protect against natural infection is not yet established for many parasites; however, there are some infections, such as malaria, filariasis and trypanosomiasis, in which antibodies have been shown to be important for host defence[71,75–77]. In addition, immunization with glycoprotein-rich materials has been shown to induce protective responses[74]. For example, immunization studies with schistosome soluble egg antigens and radiation-attenuated cercariae have shown that protective immune responses correlate with strong glycan-specific antibody responses[78–80]. Passive immunization with tyvelose-reactive antibodies against a group of antigenic glycoproteins (termed *Trichinella spiralis* larvae group 1, TSL-1) provides protective immunity in rats by expelling invading larvae from the intestine[81,82], and such antibodies block epithelial invasion by the parasites *in vitro*[83,84].

Identification of protective glycan epitopes in mixtures of glycan-rich material, such as soluble egg antigens, radiation-attenuated cercariae or TSL-1, is challenging. Immunization with a simple tyvelose–bovine serum albumin conjugate failed to induce protective immunity against intestinal forms of *T. spiralis* despite the presence of tyvelose-specific antibodies[85]. Carbohydrate vaccine research has been limited owing to difficulties in obtaining enough material to study, as parasites are often notoriously difficult to culture. However, recent technological advances (BOX 1) are allowing the investigation of potential vaccine antigens[86]. For instance, numerous surface glycans associated with *Schistosoma* spp. (for example, LacdiNAc, fucosylated LacdiNAc and Lewis X) and *Leishmania* spp. (for example, lipophosphoglycan (LPG)) have been identified and are being evaluated as immunogens[74,87] (FIG. 1).

Variation in size, antigenicity and accessibility to the immune system of the various developmental stages of parasitic pathogens complicates the selection of appropriate carbohydrate antigens for immune evaluation. For example, *Leishmania* avoid direct contact with immune effectors by invading host cells, whereas African trypanosomes keep genetically switching their highly immunogenic glycosylphosphatidylinositol (GPI)-anchored surface glycoprotein called variant surface glycoprotein[88]. Furthermore, complex carbohydrates have been shown to have key roles in the interaction of protozoan parasites with their hosts[69,89–92].

Some of the considerations discussed above have led to the pursuit of non-traditional vaccines, including anti-pathogenesis and transmission-blocking vaccines, in addition to traditional prophylactics. The pathology of malaria is largely considered to have a toxic basis[69]. The toxin was identified as the GPI anchor of the *Plasmodium* spp., which is invariant, abundant and essential for anchoring several essential proteins involved in erythrocyte invasion[69]. Preclinical studies with a synthetic version of GPI conjugated to KLH (FIG. 3) elicits high titres of IgG and shows promise in reducing the pathology of malaria. Survival rates in a mouse challenge model were also increased, but without preventing infection[93]. The LPG of *Leishmania*, which is not normally immunogenic during natural infection[94], is the predominant glycoconjugate on the surface of promastigotes[89,95]. It is also an important virulence factor and is essential for survival

Soluble egg antigens
A variety of soluble products secreted by schistosome eggs in host tissues that promote endothelial cell attachment and induce T-cell-mediated granuloma formation.

Radiation-attenuated cercariae
These are irradiated schistosome larvae capable of infecting mammals but unable to develop into adult forms.

Tyvelose
A monosaccharide found on some pathogenic bacteria and the parasitic nematode *Trichinella spiralis*.

Promastigote
One of the morphological stages in the development of certain protozoa, characterized by a free anterior flagellum.

and infectivity[89,90]. Development of parasites into non-infectious nectonomad forms was observed in the gut of sandflies that had previously ingested LPG-specific antibodies from mice immunized with LPG, suggesting the potential for an LPG-based transmission-blocking vaccine[96]. Because of epitope heterogeneity and the observation of both protective and disease-promoting effects associated with LPG (depending on the immunization route)[97–100], several LPG-based constructs are being synthesized and evaluated as immunogens[101,102]. Early preclinical studies show that a synthetic glycoconjugate based on the LPG cap of *Leishmania donovani* elicits primarily IgG and IgM responses that cross-react with parasites from infected hamsters[103]; further protection experiments are anticipated.

Viral pathogens

Several clinically important viruses express glycoproteins on their surfaces, and the associated glycans have crucial roles in infectivity and immune evasion. In contrast to other pathogens, these viruses are decorated by self glycans, which are expected to be tolerated, because they co-opt the glycosylation machinery of their host. However, a broadly neutralizing antibody against HIV-1, 2G12, isolated from a pool of B cells from infected individuals, neutralizes a wide range of HIV-1 strains, and provides protection in animal models, by binding specifically to a conserved cluster of oligomannose glycans on the envelope glycoprotein, gp120 (REFS 104–107). These observations provide the foundation for targeting conserved high mannose clusters on HIV.

A dense array of N-linked glycans, referred to as the glycan shield, covers much of the surface of gp120 in envelope spikes. The close spacing between carbohydrates on gp120, which is unusual for mammalian glycoproteins, is thought to impose conformational constraints on these glycans. It has been postulated that this dense and relatively rigid presentation of oligomannose on gp120, stabilized by a network of intermolecular hydrogen bonds, provides the basis for immunological discrimination by 2G12 (REF. 108). Biochemical, glycan array, structural and modelling studies indicate that 2G12 binds with nanomolar affinity to terminal Manα(1→2)Man residues of 3–4 high mannose glycans (for example, $GlcNAc_2Man_9$) within a cluster on gp120 via a novel V_H domain-exchanged structure that creates a multivalent binding surface[105,106,109]. Two novel glycan binding sites within the V_H–V_H' interface may also interact with gp120 (REF. 25). Additional glycans within the cluster are also important, presumably for maintaining the conformation of this epitope[110,111]. Using 2G12 as a guide, it may be feasible to design an immunogen that elicits similar antibodies provided the same immunological constraints that drove the development of 2G12 are replicated. Meeting these criteria is challenging owing to the involvement of self glycans, the cluster dependence for non-self discrimination and the unique recognition mode of 2G12. Because the glycan shield is considered immunologically silent in the context of gp120, apart from 2G12, alternative presentations of clustered oligomannose (that is, mimetics) have been sought.

Several oligomannoside ligands containing Manα(1→2)Man for 2G12 have been identified, in addition to $GlcNAc_2Man_9$, all of which bind 2G12 with similar affinities (FIG. 3). Of these, the D1 arm (Manα(1→2)Manα(1→2)Manα(1→3)Man; Man_4) represents the minimum recognition motif[105,109]. Several strategies to create glycoconjugate immunogens based on $GlcNAc_2Man_9$, Man_8, Man_9 and Man_4 are being explored[17,112–115]. Although high mannose glycans are the natural ligands for 2G12, the use of synthetic derivatives (especially Man_4) may be advantageous for focusing the immune response and overcoming tolerance issues (for example, short Manα(1→2)Man motifs are immunogenic in the context of *Candida* α-mannan). In addition, synthesis of Man_4 is much easier than full-length $GlcNAc_2Man_9$. Examples of synthetic strategies yielding high-affinity multivalent mimetics include the display of oligomannose on regioselectively addressable functionalized templates (RAFTs), oligodendrons and Qβ bacteriophage, and the generation of cyclic glycopeptides[17,112–115] (FIG. 3). In addition, selective inhibition of glycosylation in mammalian and yeast cells using kifunensine[116], or deletion of glycosylation enzymes, have produced near-homogeneous $GlcNAc_2Man_9$ or $GlcNAc_2Man_8$ glycoproteins[117], respectively, that bind 2G12. Some *Candida* spp. are also recognized by 2G12 and these are being investigated as immunogens[116].

Limited progress has been made in eliciting gp120 cross-reactive antibodies and none of these constructs has elicited neutralizing antibodies. A synthetic Man_4 conjugate, bovine serum albumin–$(Man_4)_{14}$, and a $GlcNAc_2Man_9$ cyclic glycopeptide conjugate both elicited reasonable IgG titres in laboratory animals against oligomannose, but these IgGs do not bind gp120 (REFS 115,118). In one rabbit, gp120 cross-reactive mannose-specific antibodies were elicited by a yeast mutant (exclusive $GlcNAc_2Man_8$ glycosylation); however, these antibodies did not neutralize HIV[117]. The current difficulties in generating the proper specificity of antibodies may reflect inadequate mimicry of the glycan shield (for example, flexible glycans), tolerance mechanisms and/or the inability to induce domain exchange. Testing of these hypotheses is particularly challenging as the only model antibody currently available, 2G12, has a unique architecture.

Cancer

The vaccines described so far target exogenous causative agents of disease, which is not the case for cancer. The targets in this case are host cells that have undergone mutations leading to uncontrolled cell division and the ability to invade other tissues. Another defining feature of cancer is altered glycosylation, including increased expression of certain glycans, called tumour-associated carbohydrate antigens (TACAs)[119] (TABLE 3), relative to normal tissues (FIG. 1). Commonly, changes in glycosyltransferase expression levels can lead to an increase in the size and branching of N-linked glycans, which creates additional sites for terminal sialic acid residues[120]. A corresponding increase in sialyltransferase expression ultimately leads to an overall increase in sialylation[121]. Overexpression of glycosyltransferases involved in linking terminal residues

Broadly neutralizing antibody
An antibody that binds to the native envelope of a broad range of isolates of a highly variable virus such as HIV, hepatitis C virus or influenza virus and prevents infection of target cells.

gp120
The highly glycosylated envelope surface glycoprotein responsible for receptor and co-receptor binding that together with gp41 comprise the heterodimeric envelope trimer spikes of HIV.

Glycan shield
A dense array of N-linked glycans (high mannose and complex types) that covers much of the surface-accessible face of gp120 in the context of the HIV envelope spike.

Kifunensine
A mannose analogue of bacterial origin that competitively inhibits type-1 endoplasmic reticulum and, to a lesser extent, Golgi α-mannosidases, thus preventing the normal trimming of immature $Man_9GlcNAc_2$ glycans to $Man_5GlcNAc_2$ and subsequent glycan modifications that create hybrid and complex-type glycans.

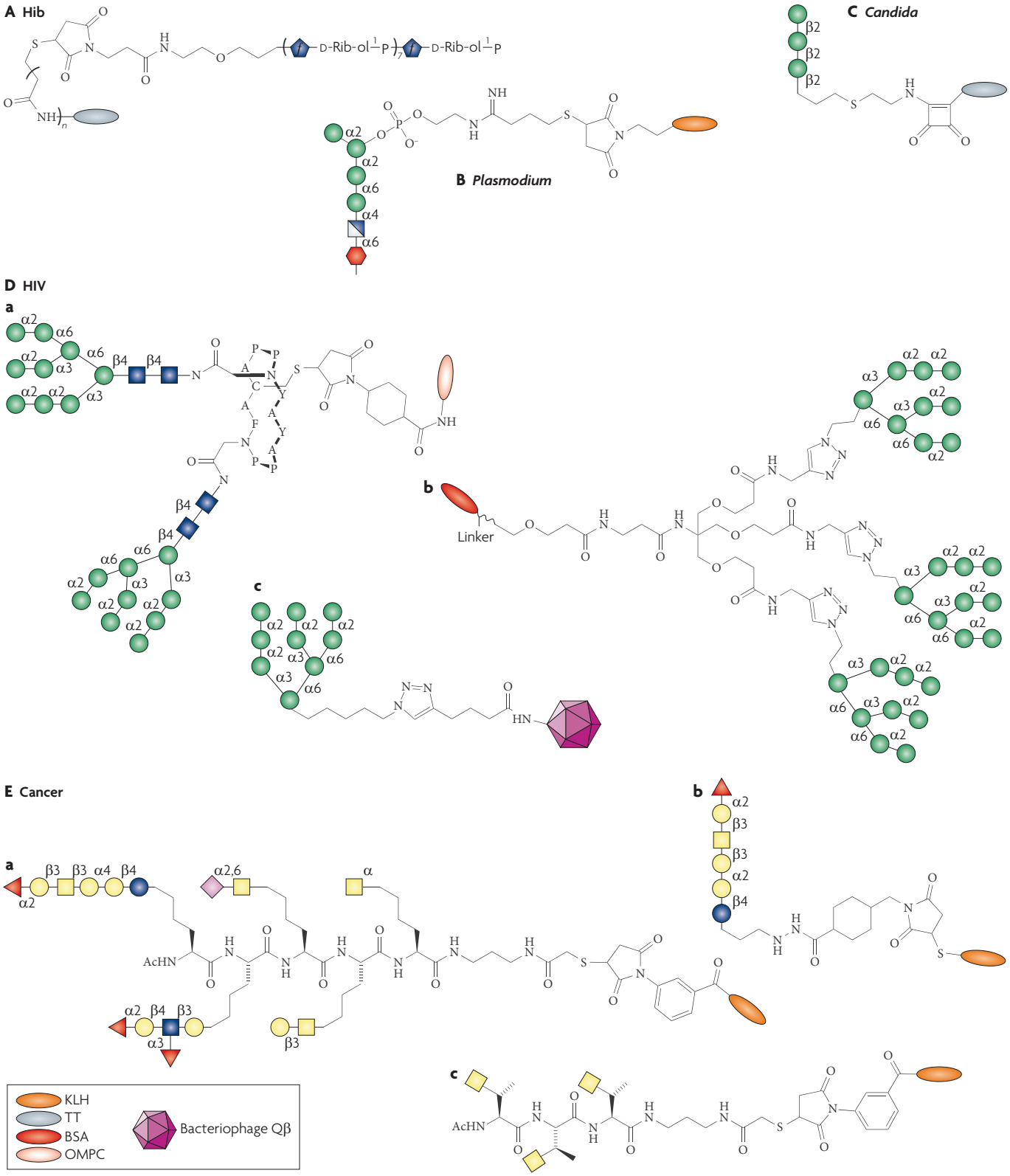

Figure 3 | **Synthetic glycoconjugate immunogens.** This figure shows examples of synthetic carbohydrate immunogens used in vaccine development. **A** | *Haemophilus influenzae* type b (Hib): polyribosylribitol–tetanus toxoid (TT) conjugate (Quimi-Hib; Herber Biotech)[40]. **B** | *Plasmodium*: glycosylphosphatidylinositol– keyhole limpet haemocyanin (GPI–KLH)[179]. **C** | *Candida*: Man_3–TT[60]. **D** | HIV: $GlcNAc_2Man_9$ divalent glycopeptide OMPC (outer membrane protein complex, derived from *Neisseria meningitidis)* conjugate[180] (**a**); Man_9 glycodendron bovine serum albumin (BSA) conjugate[113] (**b**); and Qβ–Man_9 (overall representations also depicted)[114] (**c**). **E** | Cancer: KLH– globohexaosylceramide (Globo H)[181] (**a**); KLH-clustered 2-6-α-*N*-acetylgalactosaminyl (Tn)[127] (**b**); unimolecular pentavalent KLH conjugate[149] (**c**).

to glycans leads to the overexpression of certain terminal glycan epitopes on tumours, such as sialyl Lewis X, sialyl Lewis A, sialyl 2-6-α-*N*-acetylgalactosamine (sTn), sialyl Lewis Y, globohexaosylceramide and PSA[122–124] (TABLE 3). Certain glycoproteins, such as mucins, which can serve as a scaffold for several of the aforementioned TACAs, and glycolipids, such as gangliosides, a glycosphingolipid-containing sialic acid (for example, GD2, GD3, GM2 and fucosyl GM1), are also overproduced[125,126]. An expanding body of preclinical and clinical research show that antibodies against TACAs can eliminate circulating tumour cells and micrometastases[127]. Although TACAs are self glycans, they may serve as potential vaccine antigens as they are generally poorly expressed or inaccessible on normal, healthy tissues. These observations form the rationale for carbohydrate-based therapeutic vaccines that are primarily for use in the adjuvant setting, that is, after completion of primary therapy (for example, chemotherapy)[19].

The ability to elicit antibodies against TACAs to effectively and selectively eliminate malignant cells leading to an improved clinical outcome is an ambitious goal. TACAs are poorly immunogenic and the heterogeneity of TACA expression (TABLE 3) and glycan microheterogeneity make it difficult to isolate unique glycoforms from natural sources (for example, globohexaosylceramide). Thus, synthetic methods have a large role in vaccine development. The general criteria for breaking tolerance to TACAs were delineated in the pursuit of the first-generation monomeric vaccines: polyvalent display on an immune carrier (high sugar/carrier ratio), such as KLH, of TACAs closely resembling the natural presentation on target cells in the presence of a strong adjuvant such as QS-21 (REF. 128). Key studies on GD3-based conjugates established KLH and QS-21 as the most potent carrier and adjuvant pair for breaking tolerance to TACAs[129,130]. Chemical modification of the glycans is sometimes also necessary to increase immunogenicity, for example, GD2- and GD3-lactone and *N*-propylated PSA, which is reminiscent of the strategy used for developing MenB capsular polysaccharide vaccines[131–133]. Several KLH monovalent vaccines, including those displaying synthetic carbohydrates (for example, globohexaosylceramide and Lewis Y, FIG. 3) are in various stages of clinical trials and they have generally been found to be safe and immunogenic[134]. Nonetheless, alternative carrier and adjuvant strategies are in development. For instance, a fully synthetic three component vaccine comprising a Toll-like receptor 2 agonist, a promiscuous peptide helper T-cell epitope and a tumour-associated glycopeptide was recently shown to elicit robust antibody responses in mice that recognize tumour cells expressing TACAs[135].

Second-generation vaccines rely heavily on synthetic methods to mimic the natural presentation of TACAs and the strategies developed have influenced other fields, especially HIV[136]. For example, TACAs typically associated with mucins (such as, Thomsen–Friedenreich (TF), sTn and Tn) are found in clusters and mimicking this presentation is important for generating strong antibody responses that cross-react with these TACAs on mucins and tumour cells. Clinical studies with synthetic glycopeptide cluster KLH conjugates of Tn, sTn and TF have demonstrated the safety and the improved immunogenicity of these glycans[137–139]. In addition, smaller fully synthetic cluster vaccines, based on presentation on the lipopeptide tripalmitoyl-S-glyceryl-cysteinylserine (Pam_3Cys)[140], multiple antigen glycopeptides[141,142] and RAFTs[143] have shown potential in preclinical studies (FIGS. 2,3). Synthetic glycopeptide vaccines based on mucin 1 (MUC1), the membrane-bound glycoprotein extensively overexpressed on epithelial tumour cells, are also being pursued. Interestingly, antibodies against a sTn–MUC1 tandem-repeat glycopeptide conjugate specifically recognize not only the glycan but also the peptide backbone[144].

The heterogeneity of TACA expression on malignant cells (TABLE 3) has led to the development of multivalent vaccines as either polyvalent monomeric formulations or unimolecular multivalent formulations that can be tailored to a particular cancer (FIG. 3; TABLE 3). In preclinical studies, tetravalent and heptavalent monomeric vaccines were shown to induce similar antibody titres against individual TACAs compared with those achieved with the individual monovalent vaccines[145,146]; however, recent clinical studies show lower IgM and especially IgG titres against individual antigens in patients that have been immunized with multivalent vaccines[147,148]. Unimolecular pentavalent and hexavalent KLH and Pam_3Cys conjugate vaccines have recently been synthesized, using unnatural amino acids to link the carbohydrate antigens, and early immunogenicity studies are producing encouraging results. In mice, the individual TACAs were more immunogenic when delivered as a pentavalent unimolecular (on KLH or Pam_3Cys) vaccine compared with the corresponding pool of monomeric KLH conjugates[149].

To tackle the problem of immunotolerance to TACAs, a novel immunotherapeutic strategy that combines cell glycoengineering with vaccines made of unnatural TACA analogues has been developed[150]. Using GM3 as a target, Guo and colleagues[151] have shown that tumour cells incubated with *N*-phenylacetyl-D-mannosamine efficiently expressed the unnatural GM3 analogue, GM3NPhAc, in place of the natural TACA[151]. They also showed that GM3NPhAc–KLH elicits strong T-cell-dependent antibody responses. In addition, a GM3NPhAc-specific monoclonal antibody mediated selective killing of melanoma cells that were glycoengineered to express the corresponding GM3 analogue[152].

Future perspectives and conclusions

In this article we have presented an overview of the current state of carbohydrate vaccine research for a diverse set of diseases, discussing the challenges involved and progress made towards addressing them. Much has been done to broaden the scope of carbohydrate vaccinology to diseases outside bacterial infections and bespoke vaccines are currently in the clinic. However, significant issues remain to be addressed.

Of general importance, the mechanism(s) that control the relative immunotolerance of carbohydrate antigens is not fully understood, although, low-level expression of the

Therapeutic vaccine
In cancer, a therapeutic vaccine is intended to elicit antibodies against tumour-associated carbohydrate antigens for the purpose of eliminating tumour cells that remain after primary therapy (for example, chemotherapy), when the disease burden is minimal. Ultimate clinical goals are increased disease-free survival and overall survival.

Monomeric vaccine
A vaccine formulation containing a single carbohydrate antigen displayed in multiple copies on an immunogenic carrier (for example, globo-hexaosylceramide–keyhole limpet haemocyanin).

Polyvalent monomeric formulation
A mixture of two or more monomeric immunogens (for example, sialyl 2-6-α-*N*-acetylgalactosaminyl–keyhole limpet haemocyanin), each displaying the tumour antigen in multiple copies. Here, polyvalent refers to the inclusion of more than one type of antigen in the formulation rather than the display of multiple copies of an antigen.

Unimolecular multivalent formulation
A construct containing different tumour antigens displayed on a single molecular backbone (often a peptide) that can then be conjugated to a carrier protein or other platform for immunization. Note that multivalent and polyvalent are usually used synonymously to denote the display of multiple copies of a single antigen.

Table 3 | **Expression profiles of tumour-associated carbohydrate antigens on malignant tissues**

Tumour	Tumour-associated carbohydrate antigens*													
	sLe^x	Le^x	sLe^a	Le^a	sTn	Tn	TF	Le^y	Globo H	PSA	GD2	GD3	Fucosyl GM1	GM2
B-cell lymphoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	✓	ND	ND	✓
Breast	ND	ND	✓	ND	✓	✓	✓	✓	✓	ND	ND	ND	ND	✓
Colon	ND	ND	✓	ND	✓	ND	✓	✓	ND	ND	ND	ND	ND	✓
Lung	✓	ND	ND	ND	✓	ND	ND	✓	✓	ND	ND	ND	ND	✓
Melanoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	✓	✓	ND	✓
Neuroblastoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	✓	✓	✓	ND	✓
Ovary	ND	ND	ND	ND	✓	ND	✓	✓	✓	ND	ND	ND	ND	✓
Prostate	ND	ND	ND	ND	✓	✓	✓	✓	ND	ND	ND	ND	ND	✓
Sarcoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	✓	✓	ND	✓
Small cell lung	ND	ND	✓	ND	ND	ND	ND	ND	✓	✓	ND	ND	✓	✓
Stomach	ND	✓	✓	✓	✓	✓	✓	✓	✓	ND	ND	ND	ND	✓

Globo H, globohexaosylceramide; Le, Lewis; ND, not detected at given threshold; PSA, polysialic acid; s, sialyl; TF, Thomsen–Friedenreich; Tn, 2-6-α-*N*-acetyl-galactosaminyl. *Antigens present on at least 50% of cancer cells in at least 60% of biopsy specimens based on REFS 191,192.

same antigens on self tissues, or during a developmental stage, and their structural similarity to self antigens is at least partially involved. The balance between exposure to an antigen on foreign organisms and the expression of the same, or very similar, antigen by the host probably influences the level of immune tolerance to that structure. For example, the Galα(1→3)Galβ(1→4)GlcNAc-R (α-Gal) epitope is abundantly expressed on glycoconjugates of non-primate mammals, prosimians and New World monkeys but is not expressed on glycoconjugates of humans, apes and Old World monkeys owing to the inactivation of a specific Gal transferase required for adding the α1,3 Gal cap on the epitope[153]. It has been postulated that the complete lack of α-Gal epitope expression and the continual exposure to α-Gal epitopes found on intestinal microbial flora is largely responsible for the abundance of Gal-specific antibodies in humans, apes and Old World Monkeys[153]. A novel strategy takes advantage of these antibodies to increase the immunogenicity of vaccine immunogens. By engineering in α-Gal epitopes into glycoprotein immunogens, such as influenza haemagglutinin, immune complexes can be made that help target the immunogen to antigen-presenting cells[153].

There are also more specific challenges to be addressed within each area of carbohydrate vaccine design. The minimal protective epitopes for many bacterial pathogens have yet to be determined. Carbohydrate vaccine development for fungal and parasitic infections is comparatively new and, as such, the main issues involve the identification and validation of epitopes that elicit protective, rather than neutral or disease-enhancing, antibodies and the elucidation of antibody-mediated mechanisms of protection. For HIV vaccinologists, the key difficulty lies in mimicking the presentation of the protective epitope, a dense cluster of glycans, in order to elicit neutralizing antibodies against HIV that discriminate against self glycoproteins. Cancer vaccinologists have addressed many issues similar to those currently facing researchers in the aforementioned fields; for example, definition and presentation of several synthetic antigen candidates. These potential targets must still be validated in a clinical setting, which involves defining the populations that may benefit from these vaccines and how they should be used in combination with other available therapies or cytotoxic T-cell immunogens[154,155]. Cancer-specific cytotoxic T cells may also be required to optimize immune-mediated tumour clearance.

As vaccine candidates approach clinical evaluation, more precise criteria for efficacy and clinical impact will need to be defined. As implied above, immune mechanisms associated with cancer and pathogen infections (especially viral and parasitic infections) are complex and often only partially defined. However, discussion of mechanisms outside antibody-mediated protection is beyond the scope of this Review. In general, low titres of antibodies that function in complement-mediated lysis and opsonization correlate with protection against bacterial infections and against the development of disease[1]. For viruses, higher titres of neutralizing antibodies and perhaps antibody-dependent cell-mediated cytotoxicity are important[1,156]. In cancer, antibody-mediated protection is thought to work mostly by antibody-dependent cell-mediated cytotoxicity, complement-dependent cell lysis and opsonization[157]. The situation is more complex for fungal vaccines because protective mechanisms seem to be specific for both the mycosis and the antigen. For example, the ability to bind complement and Fc receptors by GXM-specific IgG negatively correlates with protection against *C. neoformans*. By contrast, complement-mediated lysis is thought to be an important function of β1,2 mannoside-specific antibodies against *Candida*[46]. Antibody-mediated mechanisms of protection are poorly understood for parasitic infections. Suggested mechanisms depend on the pathogen, life stage and antigen; for example, antibody-dependent cellular inhibition involving monocytes (*Plasmodium* blood stages)[158], toxin neutralization (*Plasmodium*)[93] and complement-mediated lysis (*Trypanosoma brucei*)[159].

The evaluation of new glycoconjugate vaccines may be hindered by poorly defined clinical endpoint criteria, such as those based solely on ELISA assay titres rather than on functional assays. Despite the immense diversity of carbohydrate vaccine indications, several common obstacles are apparent: poor immunogenicity, heterogeneity, antigenic mimicry of self glycans, and identification and access to protective epitopes. Common solutions to these problems may be realized. For example, conjugation of carbohydrates to immunogenic protein carriers has become a universal method for increasing the immunogenicity of glycans and the quality of the resulting antibody response. Furthermore, the synthetic approaches developed for clustering TACAs are finding application in HIV vaccinology. Although a number of significant challenges remain, the future looks bright as researchers continue to learn from the experiences of carbohydrate vaccinologists in other fields. In addition, advances in glycomics will continue to accelerate research and the development of new carbohydrate vaccines. However, it is too early to tell whether carbohydrate vaccines will provide sweet solutions to a variety of sticky situations beyond bacterial infections.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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Complete List of Vaccines Licensed for Immunization and Distribution in the US:
http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093833.htm
The Jordan Report Accelerated Development of Vaccines 2007:
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