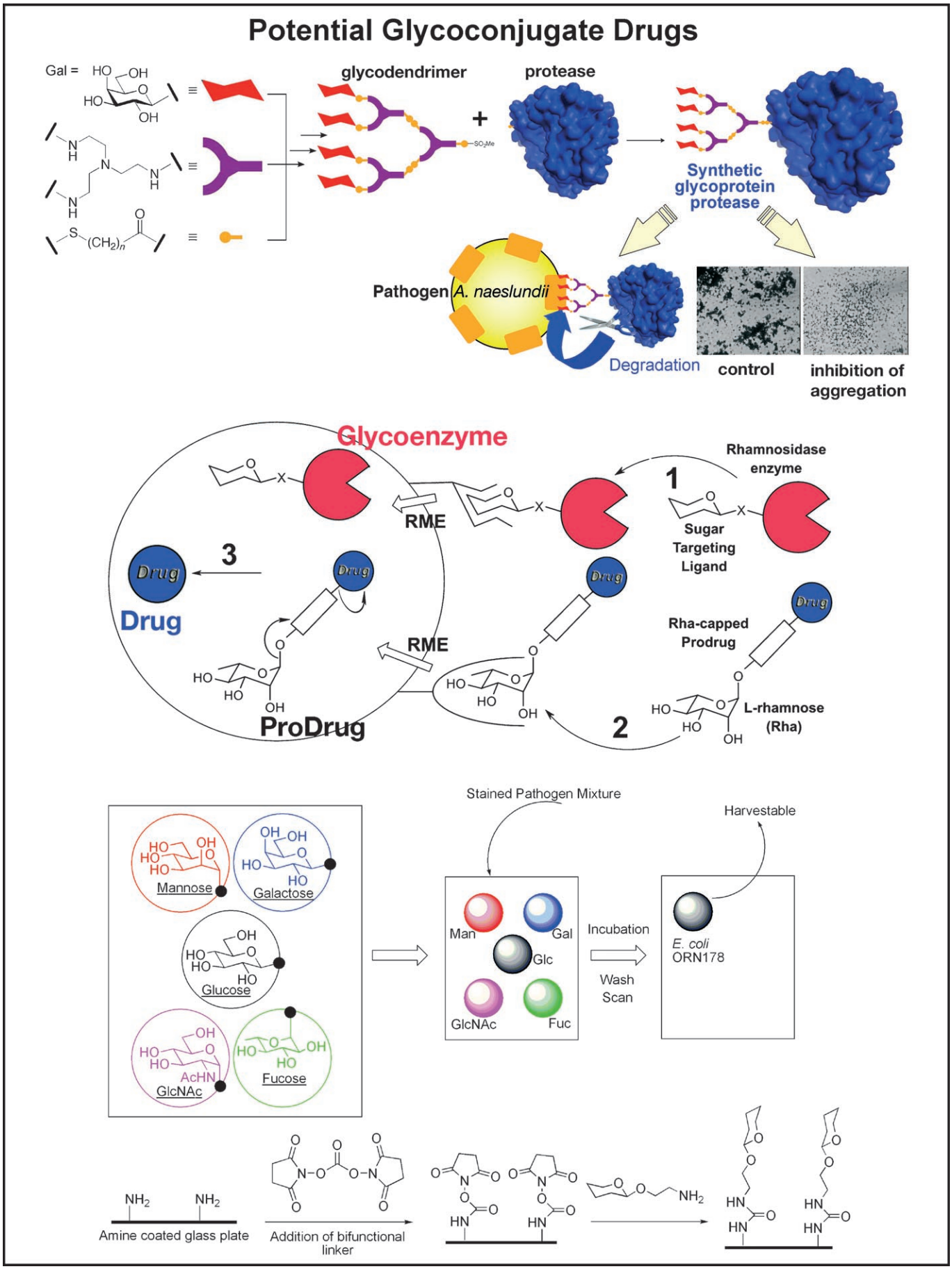

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Exploring and Exploiting the Therapeutic Potential of Glycoconjugates

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Abstract: Carbohydrates, either bound to proteins or in lipids, play essential roles as communication molecules in many intercellular and intracellular processes. In particular, carbohydrates are important mediators of cell–cell recognition events and have been implicated in related processes such as cell signaling regulation, cellular differentiation and immune response. This diverse utility has long suggested the power of carbohydrates in therapeutic approaches. This Concepts article highlights the recent potential uses of glycoconjugates as therapeutics, with particular reference to glycopeptides, glycoproteins, glycodendrimers, and glycoarrays.

Keywords: carbohydates · drug design · glycoconjugates · glycoproteins

Introduction

It is the aim of this article to focus on and highlight recent potential uses of glycoconjugates as therapeutics, with particular reference to glycopeptides, glycoproteins, glycodendrimers, and glycoarrays and is not intended as a comprehensive study of experimental methods. Until as recently as 30 years ago the primary interests in sugars in biology were probably as sources of energy for example, glucose and glycogen, or in cellular structure for example, chitin in crab shells. However, over the last decades it has become clearer that carbohydrates, either bound to proteins or in lipids, play essential roles as communication molecules in many intercellular and intracellular processes. In particular, carbohydrates are important mediators of cell–cell recognition events[1] and have been implicated in related processes such as cell signaling regulation,[2,3] cellular differentiation[4] and immune response.[5] This diverse utility has long suggested the power of carbohydrates in therapeutic approaches. For example, both the α-1 acid glycoprotein (during acute phase response)[6] and the IgG molecule (during rheumatoid arthritis)[7,8] display variations in glycan structure in disease when compared to healthy references. The tetrasaccharide sialyl Lewis X and related structures are key determinants in the recruitment of lymphocytes during inflammation[9] and furthermore, it has been known for more than 70 years that carbohydrates when attached to a protein carrier are able to induce an antibody response that might protect an organism from infection.[10] Despite these promising and suggestive observations, in many respects the full potential of glycobiology in a therapeutic context is yet to be realized.[11]

With the selected examples below we seek to show chemists can exploit these fundamental biological interactions in the potential development of future therapeutic agents.

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Glycoproteins and Glycopeptides

The co- and posttranslational modification of proteins with carbohydrates is of vital importance to protein stability,[12,13] structure and function,[7,8] and therefore can critically alter the potential therapeutic applications of glycoproteins. The fundamental problem associated with glycoproteins arises from the difficulty in generating homogenous sources since protein glycosylation is not under direct genetic control. Several approaches have attempted to overcome this limitation through both biological and chemical processes (or a combination of the two) and are now allowing the exploitation of synthetic glycoproteins in some potentially useful therapeutic strategies.[14–18]

A multivalent display of carbohydrate has long been known to enhance binding to cognate receptors.[19] Since many key biological processes involve binding of sugars to receptors it is likely that such multivalent displays of dendrimeric sugars can be exploited in therapeutic processes. Indeed, in one sense when one examines the branched dis-

play of glycans in natural glycoproteins these might be considered to be natural glycodendrimers. Rendle et al. have shown that a combination of site directed mutagenesis and chemoselective conjugation with glycodendrimeric methanethiosulphonate (MTS) reagents could be used to create a multivalent display of galactose on the surface of the protease subtilisin *Bacillus lentus* (SBL) with precision in high yields and purity. Such chemical glycosylation can overcome problems associated with the heterogeneous glycoprotein synthesis observed from, for example, the expression of glycoprotein in mammalian systems.[20] Different mono-, di-, tri- and tetragalactose tipped dendrimers were constructed on a variety of aromatic and aliphatic scaffolds and linked to SBL to create, so-called glycodendriproteins (Figure 1).

In this example, β-D-galactose was chosen for the tips of the dendrimers in order to target the pathogen *Actinomyces naeslundii* which has on its surface the fimbrial adhesin Fim A that binds galactosyl containing ligands. An ELISA-binding assay based on model Gal binding protein peanut agglutinin showed an increasing affinity with increasing Gal-antennae valency. Moreover, these Gal bearing glycodendriproteins successfully inhibited the binding of pathogen gram-positive *A. naeslundii* with its co-pathogen *Streptococcus oralis* at nanomolar levels ($IC_{50} = 20$ nM). The results were strongly dependant on the presence of a multi-antennary carbohydrate display, protease activity in the carrier protein SBL and correct sugar (Gal) presentation. Control experiments with alternative displays of sugars or inhibited, inactive enzyme were less effective. This co-aggregation inhibition is thought to be the most potent to date. In a parallel strategy, such protease targeting via homing carbohydrate ligands has also been further exemplified by equipping SBL with a mannose targeting ligand.[21] The resulting mannosylated protease showed increased degradation of a protein

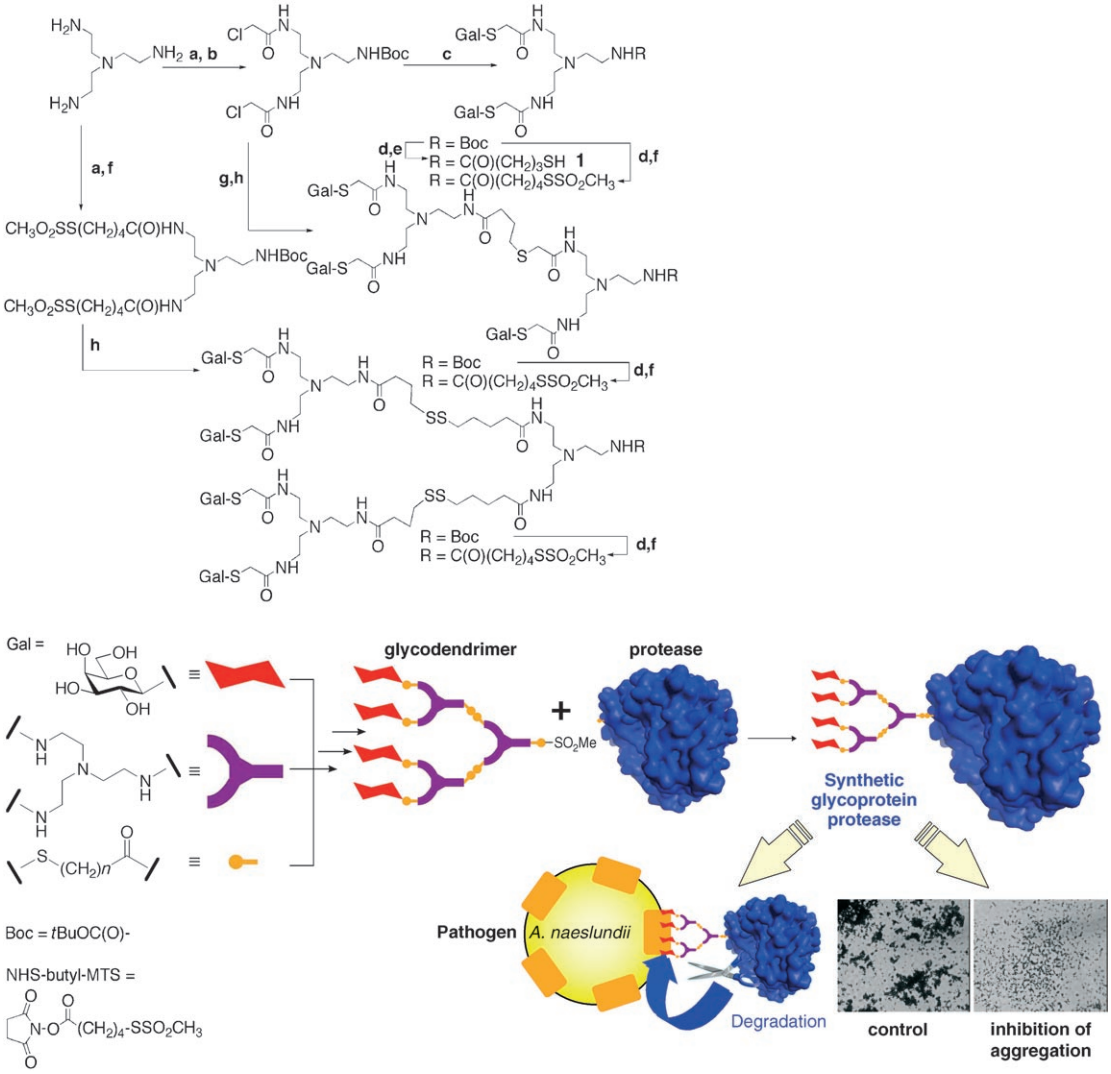


Figure 1. Synthetic route to glycodendriproteins and the mechanism of pathogenic inhibition. a) Boc_2O, CH_2Cl_2, −78 °C; b) $(ClCH_2CO)_2CO$, CH_2Cl_2; c) 2 equiv Gal-S^-Na^+, DMF; d) CF_3COOH, CH_2Cl_2; e) thiobutyrlactone, dithiothreitol, $NaHCO_3$, H_2O, EtOH; f) NHS-butyr-MTS, DMF; g) 1 equiv Gal-S^-Na^+, DMF; h) **1**, DMF.

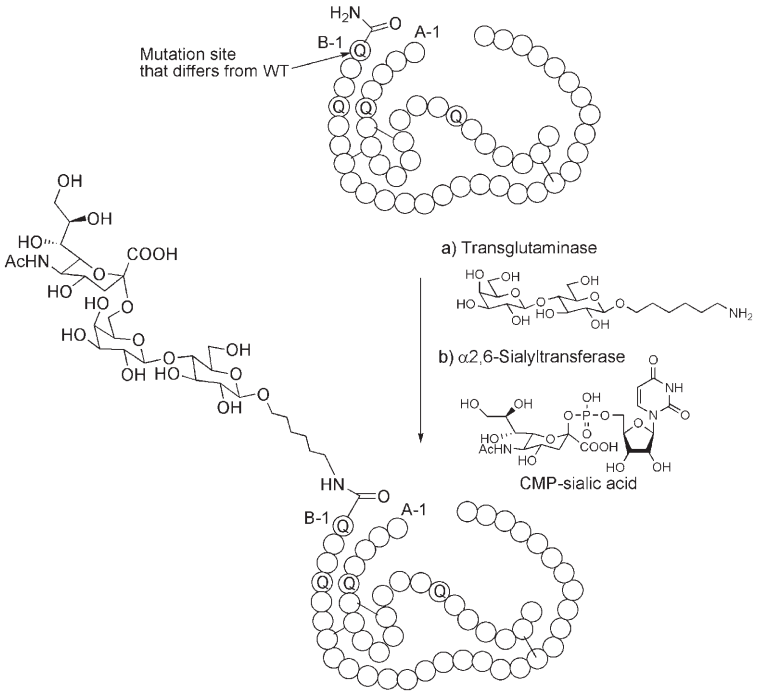
target, the mannose binding lectin concanavalin A (Con A), although the monovalent display used in this instance gave only a modest 1.5-fold increase in selectivity.

In an example of combined mutagenesis and enzymatic synthesis of glycoprotein, Nishimura and co-workers have glycosylated insulin,[22] a protein hormone that is the primary treatment of hyperglycaemia in diabetic patients. Patients can require significant amounts of insulin each year (0.5 g–1.0 g).[23] Wild type insulin is rapidly broken down by the liver within a few hours of administration thus requiring frequent injections. Current methods to increase in vivo activity have also been investigated,[24] however these are hindered by complicated administration regimes leading to a lack of control in blood circulating glucose levels caused by decreased water solubility.[25] It has been known for some time that higher levels of the sugar sialic acid on the terminii of glycoprotein glycans can increase circulation half life.[26,27] Nishimura et al. tackled the water solubility and degradation problems of insulin by introducing sialic acid moieties into a mutant peptide backbone, through a dual enzymatic extension procedure using initially transglutaminase (TGase) to introduce lactose (Lac) followed by use of Siaα2,6-transferase to make Sia2,6-Lac (Scheme 1). Glutamine residues naturally present in wild type (WT)-insulin were unfortunately inaccessible to TGase, thus more accessible Gln residues were added via site directed mutagenesis. Only modified variants of N-terminus mutants of the B chain of insulin were seen to have similar activity to WT-insulin and taken on for further study. Disappointingly after the TGase reaction, proteolytic digest revealed a lack of selectivity with both the terminal Q1 and Q4 being glycosylated. However, both isomers could be further extended with the use of Siaα2,6 transferase to create two different sialyllactose tipped artificial insulins. All mutants, with and without sugars, showed similar level of initial in vivo activity to WT, however, the Sia containing glycoproteins showed a more prolonged activity, consistent with the role of sialic acid in prolonging serum lifetimes.

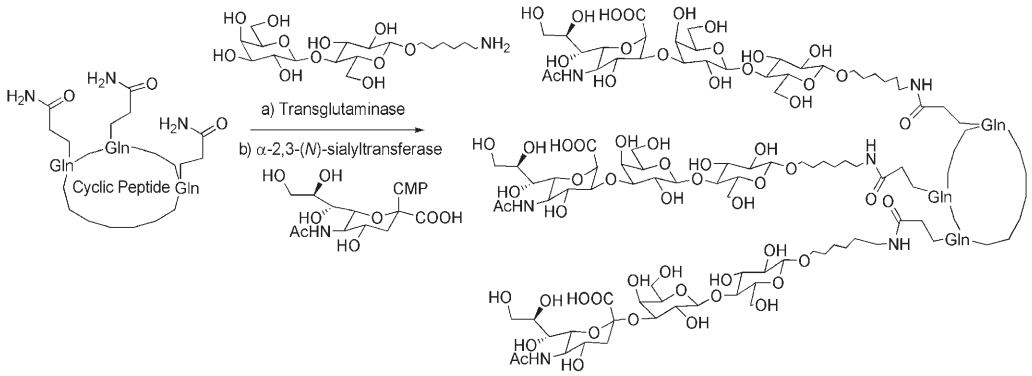
Interestingly, this technique was further enhanced by using a dendrimeric display of sialic acid to create glycodendriproteins. The same synthetic methods were also used to attach di- and tri-antennary dendrimers of lactose to the protein, which were subsequently extended with sialyltransferase. Although it was shown the binding affinity of the modified insulin to its receptor decreases as dendrimer size increases, overall in vivo activity was increased due to the enhanced half life caused by the higher degree of sialic acid incorporation.

Essentially similar methods were also exploited in the development of a potential influenza inhibitor.[28] Influenza is initiated by the docking of the virus' heamagglutinin (HA) to sialic acid containing oligosaccharides on host cell surfaces.[29] Molecules with a high affinity for HA have the potential to be potent candidates for the treatment of influenza through competitive inhibition, with a dendrimeric display increasing this potential yet further. To this end, glutamine containing cyclic peptides were designed to act as a backbone to support this carbohydrate sialic acid scaffold due to their synthetic flexibility and potential biological compatibility. These cyclic peptides were then treated with TGase and amine-modified lactose unit to afford a mixture of mono, di- and tri-antennary carbohydrate structures, which were separated by HPLC and subsequently treated with 2,3αSiaT to afford sialyl-tipped glycopeptides (Scheme 2). As expected the trivalent ligand showed most significant binding due to the multivalent effect. It was suggested that the cyclic peptide backbone was key to potent binding due to orientation with respect to HA; cyclic peptide [Gly-Ser-Ser-Gln-Ser-Ser-Gly]$_3$ was most potent.

Vancomycin, a natural product of *Amycolatopsis orientalis*, is used as a last defence against methicillin-resistant bacteria.[30] It is a glycopeptide antibiotic, that works primarily by inhibiting the terminal enzymatic step (transpeptidase) in peptidoglycan synthesis.[31] Increasing importance has been placed upon this "drug of last resort" due to the disturbing increase in appearance of even vancomycin-

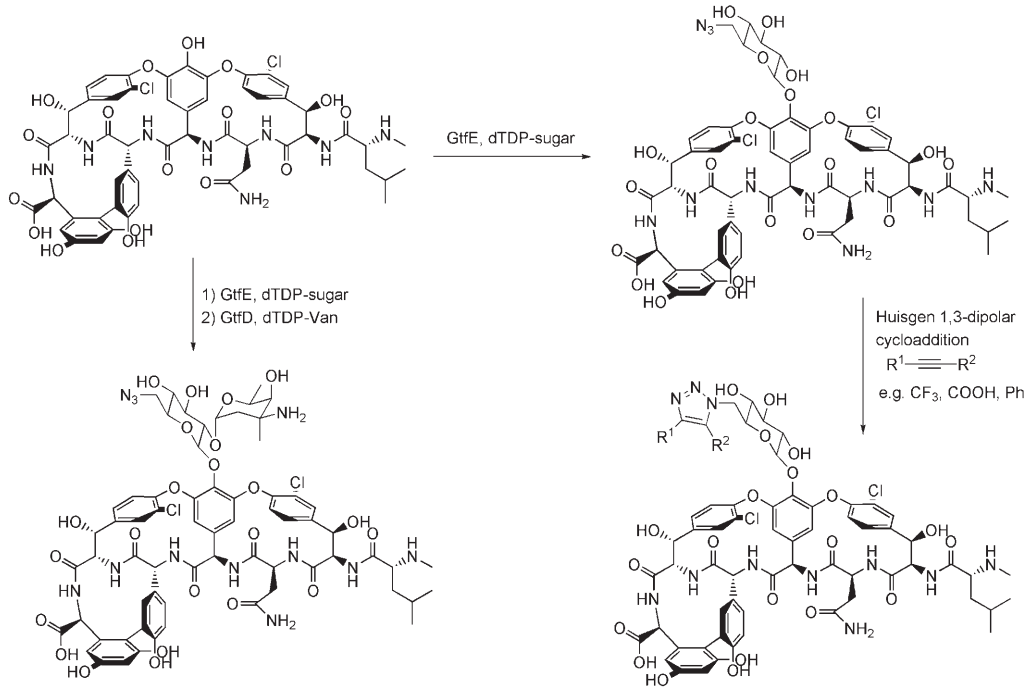


Scheme 1. Dual enzymatic extension procedure to synthesise modified insulin.



Scheme 2. Potential influenza vaccines based upon carbohydrate modified cyclic peptides.

resistant strains. Recently, it has been shown that novel carbohydrate derivatives of vancomycin may overcome this resistance.[32] Natural vancomycin binds to the terminal D-Ala-D-Ala unit of the bacterial cell wall precursor impeding further processing of the intermediate into peptidoglycan.[33] Resistant strains exchange this dipeptide to a glycopeptide component D-Ala-D-Lac, causing a change in the hydrogen-bonding pattern, resulting in a three-fold decrease in affinity.[34,35] Kahne et al. have shown that modifying the carbohydrate portion of vancomycin with hydrophobic substituents, results in increased activity.[36] This action is believed to occur by enhancing association of glycopeptide to the bacteria, and hence in closer proximity to the cell wall precursors. The modification causes a change in the mode of action of the drug by blocking instead a key transglycosylation step leading to the formation of immature peptidoglycan, and thereby potentially overcoming resistance to standard vancomycin. The carbohydrate motif is fundamental to this activity, which is in fact independent of peptide portion. Indeed, analogues without peptide still retained some activity. Thorsen et al. have developed a procedure to rapidly access libraries of vancomycin derivatives based upon an efficient chemoenzymatic route.[37] The vancomycin biosynthetic enzyme, GtfE, a glycosyltransferase known to have a broad specificity,[37] was used with a variety of natural and un-natural NDP sugar donors, to create a modified vancomycin library that was screened (Scheme 3). Although all members of a first library showed decreased activity with respect to vancomycin, a secondary library was synthesised by the use of un-natural donors containing reactive groups that could be further modified later. For example, secondary chemoselective ligation of azido sugars gave a stage II library that showed increased activity with respect to unmodified-vancomycin and pleasingly some derivatives showed in-



Scheme 3. Synthetic scheme for the carbohydrate modification of the antibiotic vancomycin.

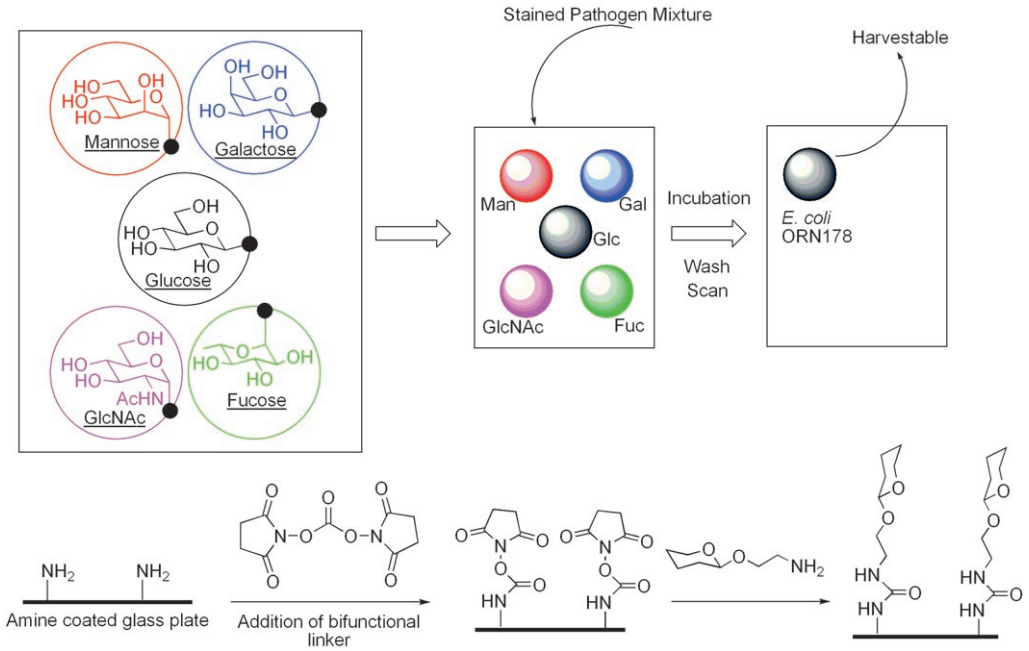
creased organism specificity. In contradiction to previous work it was found that hydrophilic substituents were crucial to the therapeutic efficacy thus illustrating the need for further potential exploration in this area.

Nicolaou et al. have carried out studies involving dimeric derivatives of vancomycin synthesised by disulphide formation and olefin metathesis.[38] It has been shown that a tendency of glycopeptide antibiotics to dimerise correlates with their increased activity.[39] Taking advantage of this multivalent effect, dimeric vancomycin-derived antibiotics were synthesised and shown to be potent antibiotics effective against vancomycin-resistant bacteria.

Microarrays

Cell-surface carbohydrates are exploited by pathogens for adherence and entry into cells. Carbohydrate microarrays have been used to study such interactions of bacteria with carbohydrates. Seeberger and co-workers reacted sugars bearing an ethanolamine linker with amine-reactive homobifunctional disuccinimidyl carbonate linkers coated on glass slides to create arrays of sugars.[40] *E. coli* was shown to selectively bind to mannose from an array of monosaccharides. Furthermore, differences in carbohydrate binding affinities were observed for *E. coli* mutants (Scheme 4). The resulting "fingerprints" of these bacteria can be used to characterize bacterial type. They were also able to "capture" these bacteria and then culture them for study of antibacterial susceptibility. This technique nicely allows for rapid screening and testing of pathogens. Feizi and co-workers have exploited neoglycolipid (NGL) technology to generate arrays of immobilized oligosaccharide probes derived from biological sources and chemical syntheses showing similar applications and results.[41]

Wong and co-workers have used robotic technology to print diverse glycan arrays to probe glycan binding protein (GBP)–ligand interactions.[42] Amine functionalised sugars were delivered in a controlled fashion using standard microarray printing onto *N*-hydroxysuccinimide functionalised plates, leading to the formation of amide bound carbohydrate ligands. Arrays comprising of 200 synthetic and natural glycan sequences representing the major glycans from glycoproteins and glycolipids were tested with various plant and human lectins, glycan-specific antibodies and bacterial and viral GBPs. The results not only confirmed specificities from previous experiments using other techniques but also showed finer specificities not previously noted. For example cyanovirin (CVN), a cyanobacterial protein which binds to high mannose groups on gp120 thus blocking the initial step of HIV-1 infection, specifically recognized several fragments containing terminal Manα-1→2- as well as the high mannose glycans containing Manα-1→2- termini, that are the reported specificity. These arrays were shown to be 100 times more sensitive than traditional ELISA-based arrays, and as expected, enhancement was observed when multivalent displays of ligands were used. Multivalent displays amplify the differences in intrinsic binding compared to specific binding and reveal biologically important motifs. This pioneering technique reproducibly detected antiglycan antibody specificities in crude human serum, giving the array the potential to act as a fast diagnostic tool in the identification of a host of diseases.



Scheme 4. General technique of carbohydrate microarrays for high throughput screening of *E. coli* carbohydrate binding affinities.

Approaches to Anti-HIV Vaccines

2G12 is a broadly neutralizing human monoclonal antibody against HIV-type 1, that binds to the viral coat protein gp120, a key viral receptor for CD4 and chemokine receptors CCR5 and CXCR4. 2G12 has a unique "domain-exchanged" structure that allows it to target the high mannose carbohydrate antigen that is found on the so-called "silent face" of HIV-1 gp120. Carbohydrate regions of glycoproteins are not normally immunogenic due to the heterogeneity of glycoproteins, which will dilute immune response, and typically, large, flexible glycans have the potential to inhibit other potential protein epitopes. Antibodies to the virus such as 2G12 to be effective need to be tolerated in the presence of host carbohydrates. 2G12 binding is strongly dependant on the glycan content of the high mannose motifs; mutational and biochemical studies on the protein also show the need for glycan occupancy at positions N332, N392 and N339.[43–45] Wang et al. have designed and synthesised a template-assembled oligomannose cluster to mimic the proposed 2G12 epitope.[46] Cholic acid was selected due to mimic dimensions of the gp120 epitope, its rigidity and multiple functionality allowing trivalent glycosylation with oligomannose structures and a fourth functionality to allow attachment to a carrier protein so that it can be used as a vaccine (Scheme 5). Analysis of the resulting synthetic epitope mimic by competitive inhibition of 2G12 binding to immobilised gp120 showed enhanced affinity compared to the high-mannose modified amino acid $Man_9GlcNAc_2Asn$ unit alone (approx. 46 times) although this is several orders of magnitude below that of gp120. This approach is suitable for further designs of mimics for the epitope of antibody 2G12.

Crystal structures have revealed that it is the D1 arm of the $Man_9GlcNAc_2$ that is significant in binding and, in particular, the terminal Manα1→2Man residues (Figure 2). Lee et al. have attempted to use these data to design their own novel immunogens using a programmable reactivity based one-pot oligosaccharide synthesis approach.[47] Evaluation of the various oligosaccharides synthesised for their ability to inhibit the interaction between 2G12 and gp120 showed tetramannose, with an additional α1→2-linked mannose, had the highest inhibition. This is consistent with the crystallographic data and work is in progress for the development for a HIV vaccine.

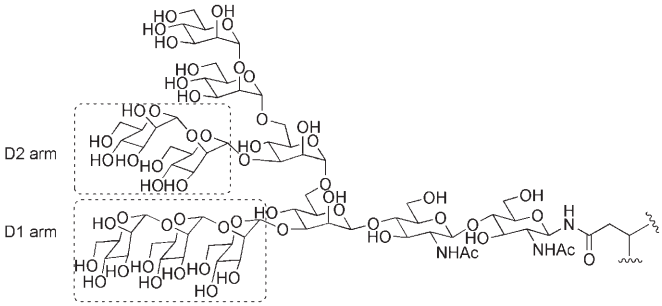
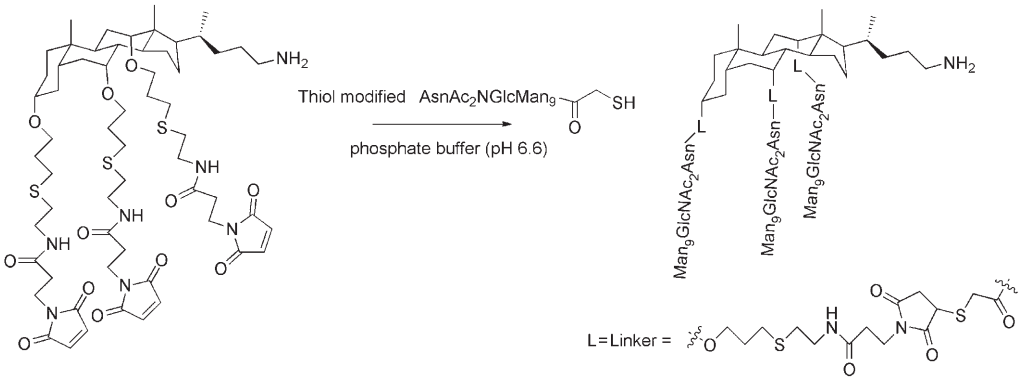


Figure 2. High-oligomannose structure key to gp120 recognition.

Approaches to Anti-Malarial Vaccines

Fatalities from malaria are caused by an inflammatory cascade initiated by a malarial toxin released from the parasite *Plasmodium falciparum*. Glycosylphosphatidylinositols (GPI) are thought to be the primary toxin that underlies malarial pathology. Seeberger and co-workers have chemically synthesised the GPI oligosaccharide and conjugated it to carrier proteins.[48] Using this vaccine model anti-GPI antibodies from immunized mice were obtained and shown to neutralise the pro-inflammatory activity of *P. falciparum* in vitro; deaths from malarial parasites were greatly decreased in animal models and the data support the idea that GPI-conjugates could be used for anti-malarial vaccine design. It should be stressed that as part of this vaccine strategy, a rapid and high yielding method was used for the synthesis of the GPI carbohydrate motif using automated solid-phase techniques.[49] This may be adapted with alternative units to generate vaccine precursors for structure/activity-relationship studies. Such syntheses will clearly aid in testing these type of vaccine hypotheses and in epitope mapping of human anti-glycan antibodies.



Scheme 5. Template-assembled oligomannose cluster to mimic the 2G12 epitope.

Carbohydrate-Mediated Drug Delivery

Targeted delivery is important approach to enhancing drug therapy. Synthetic glycopolymer and glycoproteins have in the past been used as carriers of covalently conjugated drugs bearing carbohydrate ligands.[50] These typically rely on endogenous enzymatic mechanisms for the release of active drug and so release may also occur at unwanted sites. In addition, there is a limit to the loading of the drug on such scaffolds that creates problems of dose control and cost. A new bipartite drug delivery system, lectin-directed enzyme-activated prodrug therapy, LEAPT, has been designed to exploit endogenous carbohydrate lectin binding by combining it with biocatalysis using novel glycosylated enzymes and prodrugs.[51] First a glycosylated enzyme is delivered to specific cell types within the body that are predetermined by the selected carbohydrate ligand. Second a prodrug capped with a non-mammalian sugar is added. The use of linkages in the prodrug that can only be cleaved by the activity of the glycosylated enzyme ensures that it is only released at the target site (Figure 3). This first example system has used a rhamnosidase enzyme which was first stripped of its natural sugars using enzyme endo H and then chemically glycosylated using IME methodology.[52,53] When co-adminstered with model rhamnose-capped prodrugs, in vivo analysis showed a high level of drug in the target organ, the liver. Moreover, use of a prodrug of anticancer drug doxorubicin in the system allowed promising treatment of an animal tumour model. These results further highlight the possibility of exploiting carbohydrate interactions in developing tools for targeted drug delivery. The methodology shows possible adaptation to other disease targets by varying the sugar or to target other suitable receptors of medical relevance.

Glycoviruses

Gene therapy of diseases relies on vectors, typically viruses, to deliver nucleic acids that modulate the function of malfunctioning or missing genes.[54] For certain applications of gene therapy, virus vectors will need to be delivered preferentially to diseased cells, thus requiring cell specific targeting, whilst avoiding neutralizing antibodies, in order to have an effective in vivo activity. These ideal properties are difficult to achieve, and are some of the biggest challenges faced by gene therapy today.[55] Adenovirus (AV) is a commonly used vector in gene therapy normally binding through a lysine-dependant interaction to the coxsackie adenovirus receptor (CAR) of certain host cell.[56–58] The virus has a broad tropism of infection and so generally lacks targetability.[59]

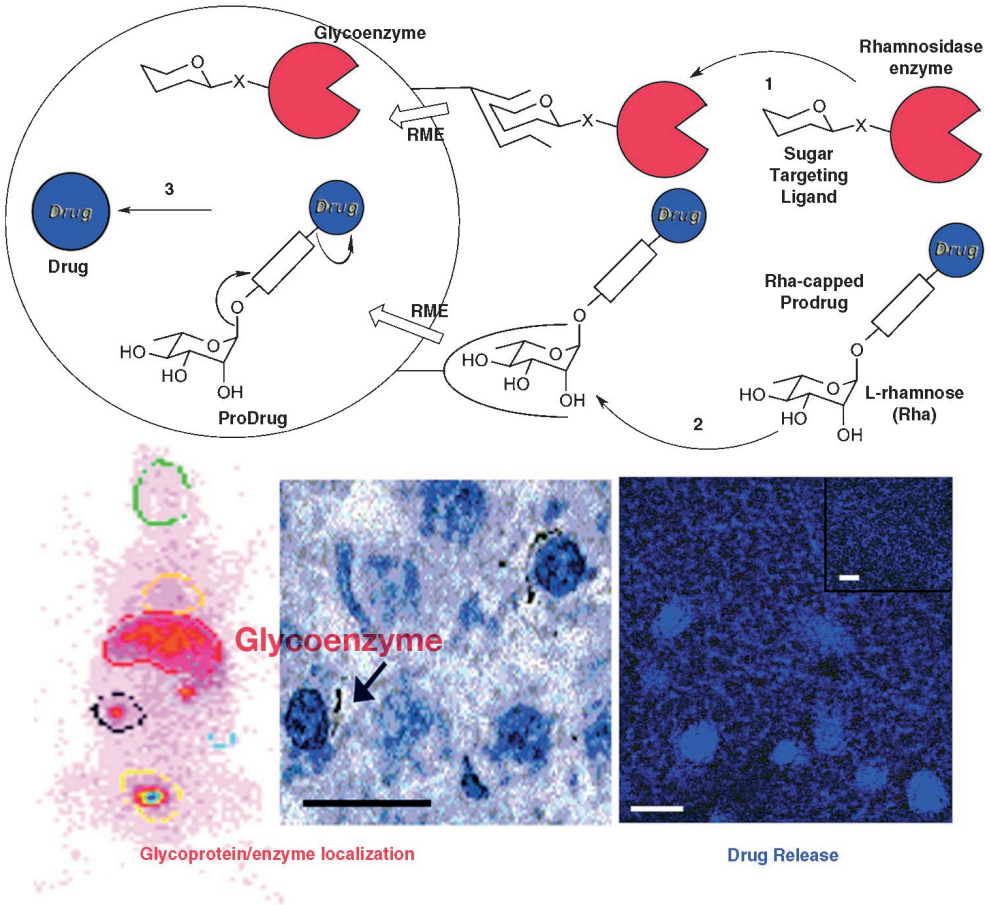


Figure 3. LEAPT overview: highlighting mode of action and in vivo results.

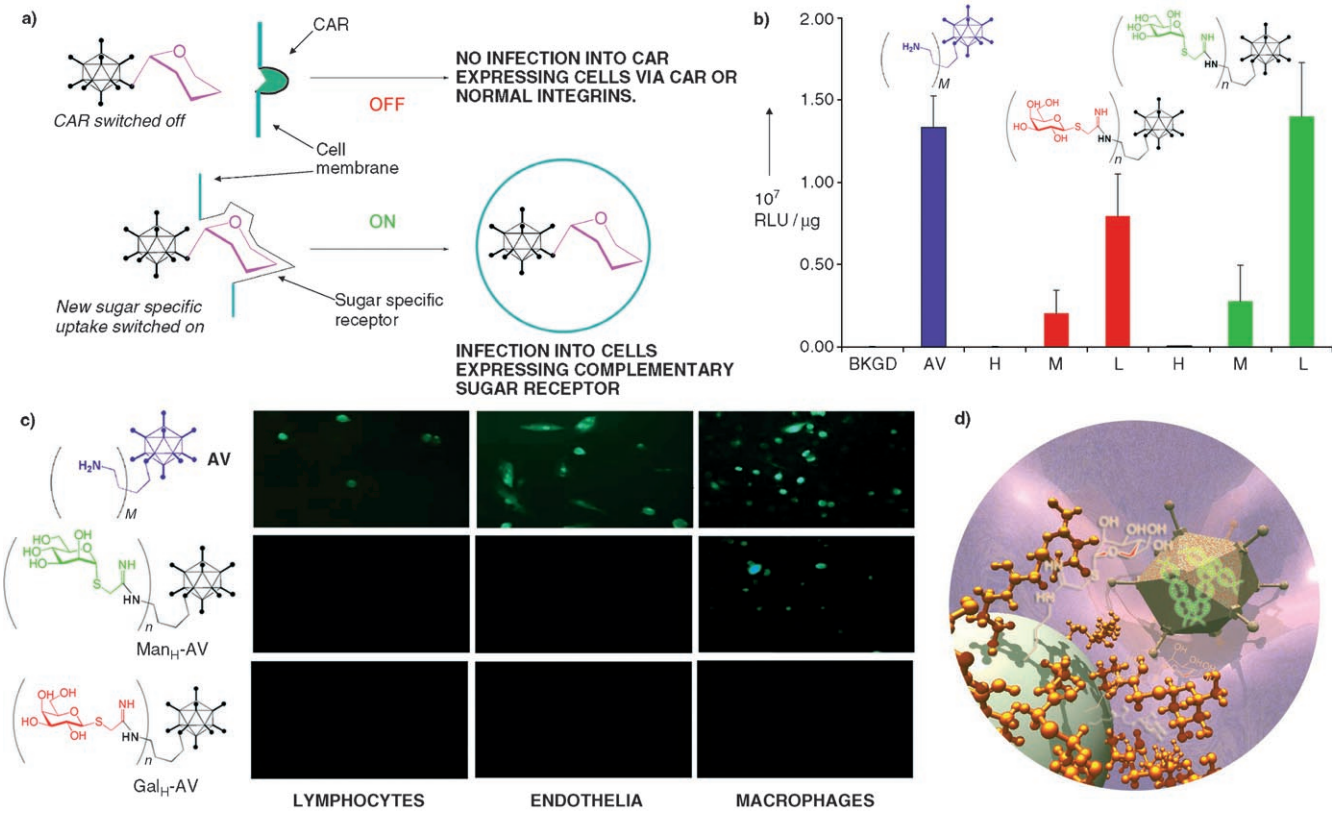


Figure 4. a) Sugar controlled transfection; b) the effects of variable glycosylation levels on CAR; c) re-targeting of mannosylated virus; d) artist impression of a mannosylated adenovirus targeted to a macrophage.

Pearce et al. have overcome this lack of specificity by reducing the CAR uptake mechanism and switching it to a sugar specific mediated uptake mechanism through the use of glycosylating reagents that chemically mask the lysine residues involved in CAR and some non-specific integrin mediated uptake interactions with carbohydrates (Figure 4a).[60] Increasing the level of glycosylation decreased the virus' ability to transfect model systems via CAR (Figure 4b). Pleasingly, the results showed that while non-glycosylated AV virus broadly transfects green fluorescent protein activity into a variety of human blood-related cell types, with the use of Gal-modified viruses resulted in no transfection into these cell types. Excitingly, when Man-modified virus was used only transfection into macrophages was seen, probably via mannose-binding protein interaction (Figure 4c).

In conclusion, although for many years the vast potential of carbohydrate science to create therapeutics has been touted[7,11] there have been disappointingly few examples of translation into genuine clinical application. The preponderance of glycoproteins as biopharmaceuticals and pioneering (but rare) examples of small molecule approaches (e.g. Relenza,[61] Tamiflu,[62] and Vivesca[63]) have demonstrated the powerful existing role that sugars can play in medicine. This review has attempted to generate a snapshot of some potential future approaches. With courage and vision the real potential of carbohydrates to treat disease might start to be tapped.

Acknowledgements

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