Drug delivery systems based on sugar-macromolecule conjugates

Benjamin G Davis* & Mark A Robinson

Address
Dyson Perrins Laboratory
Department of Chemistry
University of Oxford
South Parks Road
Oxford
OX1 3QY
UK
Email: Ben.Davis@chem.ox.ac.uk

*To whom correspondence should be addressed

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The specificity of carbohydrate-protein interactions can greatly outstrip that of many other ligand-binding systems; such is the enormous density of information that sugars can convey. In addition, macromolecules allow for the fine-tuning of active drug delivery through their great ability to undergo site-specific modification and their inherent physicochemical properties. Once combined, these two factors suggest that sugar-macromolecule conjugates, targeted using endogenous carbohydrate binding proteins, are a promising route to the 'magic bullet'.

Keywords Glycoconjugate, glycoprotein, glycotargeting, lectin, oligosaccharides, receptor-mediated endocytosis

Abbreviations

AF	Asialofetuin
ASGPR	Asialoglycoprotein receptor
ASOR	Asialoorosomucoid
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CRD	Carbohydrate-recognition domain
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ESMS	Electrospray mass spectrometry
Fuc	L-Fucose
Gal	D-Galactose
$GalNH_2$	D-Galactosamine
GCR	Glucocerebrosidase
Glc	D-Glucose
GlcNAc	*N*-Acetyl-D-glucosamine
Glu	Glutamate
GPT	Glutamic pyruvic transaminase
HDL	High density lipoprotein
HepG2	A human hepatoblastoma cell line
HMPA	Poly[*N*-(2-hydroxypropyl)methacrylamide]
Ig	Immunoglobulin
IME	Iminomethoxyethylthioglycoside
LDL	Low density lipoprotein
Lys	Lysine
MALDI	Matrix-assisted laser desorption ionization
Man	D-Mannose
MBP	Mannose binding protein
MW	Molecular weight
NMR	Nuclear magnetic resonance
ODN	Oligodeoxynucleotide
Orn	Ornithine
PEI	Polyethylenimine
PNA	Peptide nucleic acid
PGE1	Prostaglandin E1
PVLA	Poly(vinylbenzyl-lactonamide)
rhIL-2	Recombinant human interleukin-2
RME	Receptor-mediated endocytosis
SAR	Structure-activity relationship
SOD	Superoxide dismutase

Introduction

Selectively localizing a drug at or close to its proposed site of action has clear therapeutic advantages, including reduced toxicity and smaller dose levels. Broadly speaking, this can be achieved in one of two ways: (i) exploiting an existing (endogenous) interaction or activity at the desired site; or (ii) creating a new one. Typically, the former involves the exploitation of a binding interaction of either a ligand at that site for an introduced receptor (targeting receptor) or of a receptor at that site for an introduced ligand (targeting ligand). Since the feasibility of using carbohydrate ligands to target protein receptors at sites of localization, termed 'glycotargeting', was first demonstrated in 1971 [1], the potential of using carbohydrates to create a truly targeted (or actively-targeted) drug delivery system has been made clear. However, despite 30 years of research, to date, there is no general therapeutic system on the market, and many challenges still exist. In particular, small molecule drugs, no matter how heavily glycosylated, will always have the potential to pass into the kidneys, through glomerular filtration, and be rapidly cleared. Consequently, the use of macromolecular constructs that allow longer circulation times and give access to additional chemical functionality or more precise delivery, is an attractive alternative option.

Although much has been made of the potential of glycotargeting by many glycoconjugates, demonstrated successful outputs are relatively few and far between. Therefore, to provide a pragmatic focus, this review aims to cover developments over the past three years, but excludes articles for which no proven delivery or uptake effect has been demonstrated. In addition, many of the systems described here are currently investigated as part of ongoing, long-term projects, and the references in this review will, in many cases, refer only to the latest of a series of related papers.

Several reviews have examined glycotargeting [2,3], and the reader is referred to these for a comprehensive background view. A review by two of the pioneers of the field that focuses on the exploitation of receptor-mediated endocytosis (RME) by the asialoglycoprotein receptor (ASGPR) for gene and antisense delivery expertly picks out some of the most elegant examples [4•]. Hashida *et al* have more recently published another good review on this topic [5]. An excellent primer to the general area of receptor-mediated drug delivery [6•], and a comprehensive, although slightly unstructured and chemically-confused, general review of targeted non-immunogenic delivery [7] have also appeared. Of particular note, an excellently tempered review of the potential and current state-of-the-art of lectin-mediated drug delivery crucially highlights the vitally important interplay

of methods for the synthesis of oligosaccharides as targeting ligands, their assembly and methods for establishing the nature and level of their precise interactions [8••]. The article also illustrates the stunning level of information that sugars can convey, and hence the very high potential for selectivity, painting a justly rosy, yet circumspect picture of the future of the field.

The mechanisms of glycotargeting

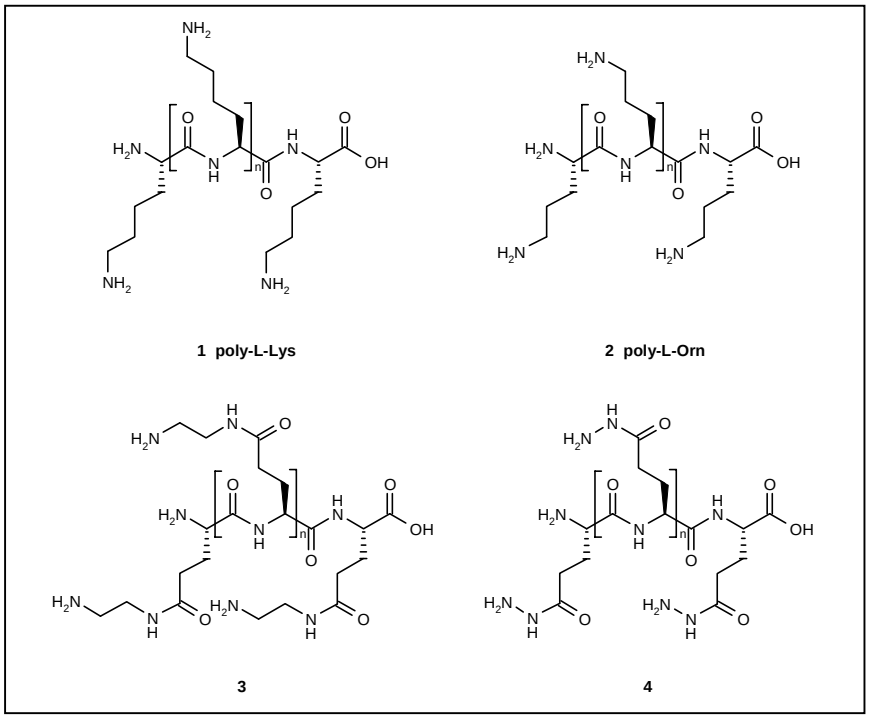
Glycotargeting exploits the highly specific interactions of endogenous lectins with carbohydrates (often with multiple carbohydrates). Primarily, RME is the biological mechanism that has been targeted, and consequently much interest has focused on the proteins involved in RME, such as the mannose binding protein (MBP). In particular, the ASGPR in the liver is a particularly attractive target, not least because of its very high density on hepatocyte surfaces (50,000 to 500,000 per cell) [9]. In addition to lectin receptors that are regularly involved in endocytosis, those that are not may also be targeted. For example, lectin-like 'homing' receptors on lymphocytes recognize so-called cell adhesion molecules (CAM) that contain carbohydrates, such as sialyl Lewis-x. Although any role in phagocytosis is unclear, these lymphocyte receptors provide another potential biological target for delivery by, at the very least, localizing higher concentrations at given cell surfaces.

To take part in these precise interactions, access to controlled structures is essential, and such constructs have allowed the elucidation of some broad structure-activity relationships (SARs). For example, factors essential to the delivery of glycosylated proteins and polymers to the liver have been determined. Mannose-modified bovine serum albumin (BSA) was taken up by non-parenchymal cells, whereas galactose-modified BSA was taken up by parenchymal cells. In addition, it has been observed that linear macromolecules (ie, polymers) need higher levels of glycosylation than do more globular macromolecules (ie, proteins) [10]. Despite such useful studies, the level of information on glycosylated proteins and polymers at present is still too low to provide truly detailed SARs, and, thus, novel glycoconjugates are urgently required. Indeed, recent observations have highlighted the need for continued work on the elucidation of the mechanisms of uptake. For example, some glycosylated polylysines bearing sugar moieties that are poor ligands of the cell surface lectins of a given cell were found to be more efficient nucleic acid carriers than those bearing better sugar ligands [11].

Carrier synthesis

The use of sugar-macromolecule conjugates or glycoconjugates may be divided into two types: (i) those in which the macromolecule is itself the drug or therapeutic agent; or (ii) those in which the macromolecule plays a pivotal role in aiding the delivery of the drug or therapeutic agent. This review will primarily focus on the type of macromolecule that is glycosylated but, obviously, many of the techniques used for such glycosylations will be common to several conjugate types. These methods will be referred to in passing, but for more detail, the reader is referred to broader ranging reviews of the synthesis and use of glycoconjugates [12] and glycoproteins [13,14]. Certain articles have been written specifically with macromolecular glycotargeting in mind. For example, the utilization of unprotected reducing sugars to create glycosylamine derivatives suitable for conjugation has been reviewed in the context of glycotargeting [15].

Figure 1. Poly(amino acid) structures commonly used as macromolecule scaffolds for sugar-macromolecule drug delivery systems.



In the context of carrier construction, an avoidable problem continues to dog the field: characterization, particularly that of carbohydrate content, is often poor. For example, many researchers continue to use old-fashioned colorimetric assays with inherent errors of the order of up to 30%, eg, phenol with H_2SO_4, to characterize constructs from which conclusions are drawn based on differences of sometimes < 10%. This is despite the availability of more precise techniques such as mass spectrometry (electrospray mass spectrometry (ESMS), matrix-assisted laser desorption ionization (MALDI)) or nuclear magnetic resonance (NMR). Precision is also needed in analysis. For example, the need for pharmacokinetic analysis of glycotargeted gene delivery systems for rational design has been reviewed and emphasized [16].

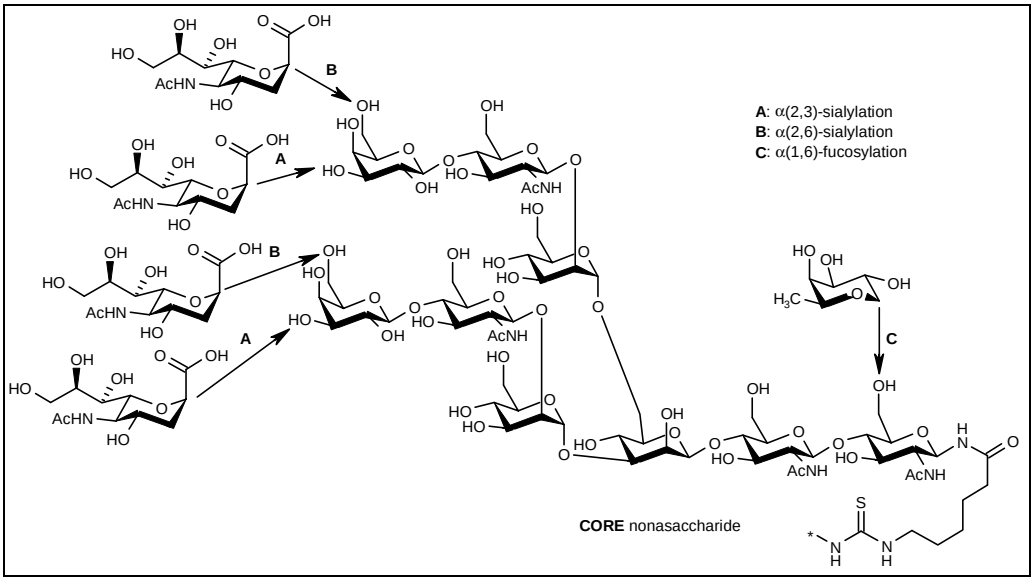
Proteins and polypeptides

The high density of chemical functionality of proteins and polypeptides allows them to be readily modified, making them attractive carriers. For example, serum albumins (human and bovine), asialofetuin (AF; by virtue of its existing glycans that have been stripped down to reveal D-galactosyl units), poly-L-Glu, poly-L-Lys (**1**; Figure 1) and poly-L-Orn (**2**; Figure 1) make suitable, inactive glycosylated carriers for a variety of purposes.

A study of the relationship between the structure and molecular recognition of the typical glycans found on N-linked glycoproteins has been made [17•]. In nature, N-linked glycans with a mannose core and α1,3- and α1,6-linkages provide the basis for a biantennary structure, which in this study was synthesized using a chemoenzymatic strategy. A reducing terminal isothiocyanate group allowed conjugation to the ε-amino Lys groups of BSA, with a glycan:BSA loading ratio of 3.9 for decasaccharides, 2.9 for α2,6-sialylated dodecasaccharides and 4.6 for α2,3-sialylated dodecasaccharides (Figure 2). Lectin and cell binding assays were used to show the effect of terminal sialylation and core fucosylation. For example, a decasaccharide showed a 5-fold higher affinity for galectin-1 compared with its α2,6-sialylated derivative. An organ distribution study in Ehrlich solid-tumor-bearing ddY mice showed little organ uptake other than to liver, kidney and spleen, and α2,6-sialylated conjugates showed more rapid serum clearance than α2,3-sialylated conjugates [17•].

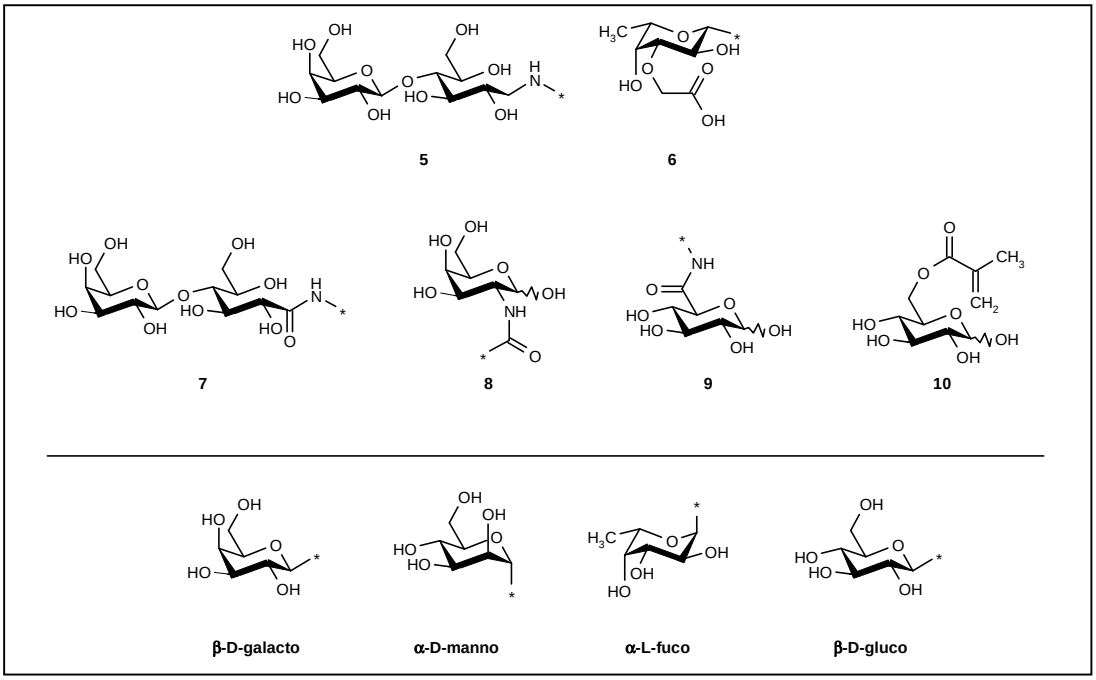
Three proteins (superoxide dismutase (SOD), BSA and immunoglobulin G (IgG)) with different molecular weights (MWs: 32, 67 and 150 kDa, respectively) have been mannosylated at different levels (17 and 21 × D-mannose (Man); 12, 16, 25, 35 and 46 × Man; and 32 and 42 × Man, respectively) using the iminomethoxyethylthioglycoside (IME) strategy. SOD conjugates showed good uptake to the liver at 0.05 and 0.1 mg/kg, as did the BSA conjugates, where a glycosylation level of 16 Man per BSA proved to give optimal uptake. Interestingly, IgG conjugates showed greatest uptake at 1 mg/kg, a far higher dose than that for SOD and BSA. This perhaps illustrates the benefit of a construct with a MW far greater than that required to bypass glomerular filtration. After incubation with serum (containing MBP) and gel filtration, the three proteins showed some interaction with MBP, particularly BSA with 25 and 46 Man residues [18]. The mannose receptor is a type I transmembrane protein with eight C-type carbohydrate-recognition domains (CRDs) in one polypeptide, while MBP has a single CRD. *In vivo* hepatic uptake and *in vitro* MBP binding assays using mannosylated BSAs and IgGs suggests that the recognition of MBP has a stronger cluster effect than that of hepatic mannose receptors. Different D-galactose (Gal) levels (four to 42 per protein) and different protein weights of galactosylated-BSA, galactosylated-SOD and lysozyme were used to show that liver clearance rates increased with Gal levels but varied little with mass. Furthermore, the small fraction of such proteins that were cleared intact to the bile after uptake decreased as Gal-density increased [19].

Figure 2. Complex-type glycans conjugated to BSA.



Six variations of complex-type glycans conjugated to BSA have been used to investigate the effect of structural variation of sialic acid and fucose substitution (A, B and C) upon organ distribution and uptake [17•]: basic core; core + A; core + B; core + C; core + A + C; core + B + C. Arrows indicate the structural variations and their corresponding points of attachment to the core carbohydrate motif used.

Figure 3. Commonly used carbohydrate targeting ligands.



Little specific targeting of the kidney is known apart from protein re-absorption and prodrug activation by kidney specific enzymes. Alkylthioglucosides have been used to assess the effect of MW and charge on kidney uptake [20]. These alkylthioglucoside derivatives, produced from 9-(1-thio-β-D-glucopyranosyl)nonanoic acid, were conjugated to L-Tyr through an ethylenediamine linker unit. The Tyr amino group was then attached to the C-terminus of acylated poly-L-Lys (4.5, 17 and 45 kDa). The uptake of polymers was above the level of glomerular filtration (the glomerular filtration rate (GFR) was determined using [^{14}C]inulin as a GFR marker), particularly for polymers of low MW. (The inhibition of uptake was determined by co-administration of *n*-octyl thioglucoside, which has a high affinity for the alkylglucoside binding site.) The effect of charge was studied using alkylthioglucosides with neutral, basic and acidic groups; acidity was shown to hinder binding to kidney membranes.

The use of albumin covalently modified with β-D-galacto units (using reductive amination with lactose to create structure **5** (Figure 3)) and methotrexate (using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated coupling) resulted in up to 16.5-fold higher drug levels in the liver than those obtained without the use of a delivery system. These uptake levels showed clear sugar-dependency [21]. In an elegant example of the use of BSA as a carrier, a range of novel L-fucose (Fuc)-derived carbohydrates were constructed and used to modify BSA to behave as a protein carrier that preferentially targeted tumor cells over hepatocyte or Kupfer cells in the liver [22••]. All except one of the BSA conjugates showed preferential parenchymal uptake. However, carbohydrate **6** (Figure 3) showed the desired tumor cell over parenchymal cell selectivity. Construction of this BSA conjugate was achieved by modification of tyrosines using diazo derivatives of **6**, which allowed the introduction of up to 22 carbohydrate motifs per BSA molecule. The cytotoxic agent batracylin was modified through its amine group with thiophosgene, and then used to introduce up to 30 copies of batracylin per BSA molecule through lysine modification. Analysis of the **6**-containing BSA demonstrated that it was taken up into cell lines, resulting in enhanced antitumor cytotoxicity. Other early indications suggest that fucosylated BSAs (constructed using IMEs) may be partially selective for Kupfer cell uptake, as compared with Gal or Man-derivatized BSAs [23].

A comparison of the hepatic uptake of glycosylated poly-L-Lys and poly-L-Glu carriers [24,25] showed that galactosylated poly-L-Glu accumulates within hepatocytes. By varying the spacer length between sugar and polymer backbone (through the use of ethylenediamine to create **3** (Figure 1), or hydrazine linkers to create **4** (Figure 1)) an increase in uptake can be achieved. Pharmacological effect was demonstrated by the conjugation of prostaglandin E1 (PGE1; Pharmacia & Upjohn Co), a drug effective against hepatitis, to these polymer constructs and by measuring glutamic pyruvic transaminase (GPT) activity in the plasma of mice with CCl_4-induced hepatitis. Animals that were administered with the hydrazine-linked polymer showed a reduction in GPT, relative to the dosing of free PGE1 [24,25]. In contrast, the ethylenediamine-spacer conjugate failed to release PGE1, despite good targeting [26].

In mice, mannosylated-poly-L-Lys/gene complexes have been evaluated, and intrahepatic levels of up to 80% of total dose have been reported. The system was used to generate high chloramphenicol acetyltransferase levels in the liver [27]. Poly-L-Orn was modified with galactose and then with the fusigenic peptide mHA2, which allows endosomal escape, to obtain a carrier that led to a 280-fold increase in luciferase gene expression compared to that obtained with

DNA/DOTMA:Chol liposomes (a system often used as an unglycosylated liposome standard). The resulting luciferase activity in hepatocytes contributed more than 95% of the total activity in all of the tissues examined [28•].

Man-polyethylenimine (PEI) conjugates have been used for RME-mediated gene delivery via the surface-bound mannose receptor that is highly expressed on antigen presenting dendritic cells [29••]. It has been suggested that PEI may have a higher transfection ability than poly-L-Lys, although no direct comparison in sugar-targeting systems has ever been made. Conjugates were synthesized by reductive amination with mannobiose or by coupling with mannosylphenylisothiocyanate to introduce α-Man units (shown incorrectly in the paper as β-Man). These conjugates enhanced transfection by 500- to 1000-fold, which was blocked by Man-BSA, indicating a Man-specific mechanism. Little variation in transfection ability with carrier MW or conjugation method was observed. The absolute expression levels, however, were low, but were enhanced by the addition of adenovirus to the complexes, perhaps through greater endosomolytic release due to the presence of viral coat peptide. Of exceptional note, by using this enhanced system, the achieved gene expression levels of ovalbumin were high enough to activate T-cells [29••].

Glycosylation often allows site-selective delivery of a protein that is itself the drug. A triantennary galactose-terminated glycoside with a terminal straight chain alkylamine was conjugated to recombinant human interleukin-2 (rhIL-2) using a microbial transglutaminase (M-TGase) [30••]. M-TGase catalyzes the acyl transfer reaction between the γ-carboxyamide group of glutamine residues and various primary amines via a Michaelis-type, acyl-enzyme intermediate. In this instance, Gln^{74} of rhIL-2 was site-selectively modified in ~ 20% yield without any loss in activity. Uptake to the liver, kidneys and spleen was assessed, with and without asialoorosomucoid (ASOR), a ligand with high affinity for the ASGPR. Moderately enhanced selectivity by the $(Gal)_3$-rhIL-2 was observed for the liver. This technique is limited to proteins with available Gln residues, but the same research group had previously reported the production of chimeric proteins with artificially introduced Gln-containing sequences that may also be substrates for transglutaminases, thereby suggesting a wider applicability of this delivery system.

Administration of mannosylated-SOD to mice undergoing hepatic ischemia/reperfusion significantly prevented neutrophil infiltration in the liver. Histological evaluation of liver tissues confirmed the ability of the system to suppress neutrophil-induced hepatic injury [32]. Mannosylated and galactosylated catalase have also been investigated in the same injury model [33].

Alternatively, the level of desired glycosylation may be increased by removing other carbohydrate caps from the oligosaccharide structures already present in naturally-occurring glycoforms of proteins. For example, neuraminidase was used to remove the sialic acid carbohydrate tips of the oligosaccharide structures in human interferon-β, thereby revealing additional galactose units as ligands for the ASGPR [34•]. This led to increased uptake of the protein in the HepG2 liver cell line and enhanced antiviral effect in mice infected with hepatitis B. The first example of a glycosylated protein drug was β-glucocerebrosidase (β-GCR; used to treat Gaucher's syndrome), which bears the hexasaccharide $Man_3GlcNAc_2Fuc$ at Asn^{19} and Asn^{146}. Although a commercial drug for several years, strong evidence (MBP binding K_d and cell-type distributions) has only recently emerged that suggests that the success of β-GCR is, in part, due to the selective delivery to macrophages, which is mediated by the exposed Man termini of these glycan structures in GCR [35•].

Polysaccharides

Pullulan is a polysaccharide composed of three α1,4-linked glucose molecules that are polymerized via α1,6-linkages to form a polymer with an average MW of 58.2 kDa. The hepatic uptake of fluorescein-labeled pullulan was assessed both *in vivo* and *in vitro*. Uptake from serum into liver parenchymal cells was influenced by dose size, and co-administration of both AF and arabinogalactan reduced the level of serum clearance. Furthermore, analysis of liver slices by fluorescence microscopy showed endocytosis of pullulan, leading to the conclusion that pullulan binds to the ASGPR and is internalized via receptor-mediated endocytosis [36].

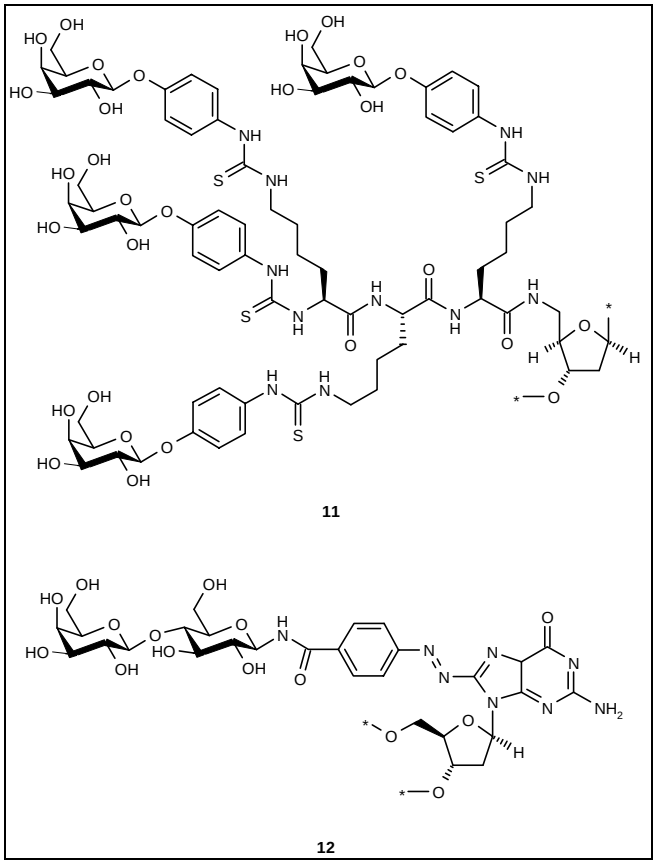
The organ distribution of arabinogalactan has been determined, and although it appears to be processed by ASGP RME, its low average MW (12.5 kDa) resulted in substantial clearance to the kidneys in rats [37]. It has been suggested that partial oxidation to aldehyde, which does not affect uptake, followed by loading with amine-derivatized compounds could be a viable delivery strategy.

Reductive amination has been used to glycosylate succinylated chitosan (poly-β(1,4)-D-glucosamine) [38]. The product of reductive amination with lactose introduces a galactosyl unit such as **5**, whereas with galactose, only an acyclic modification, which is poorly recognized by lectins, results. This is in accordance with the greatly enhanced liver retention that was observed for the former; indeed this retention is greater than that seen with polypeptides or proteins, and is probably due to reduced biodegradability. Similarly, chitosan has been reacted with lactobionic acid to introduce unit **7** (Figure 3) to chitosan before being further derivatized through reductive amination with dextran [39]. This combination led to enhanced stability of the resulting complexes with DNA (decreased aggregation) and, as a gene carrier, allowed successful transfection into HepG2 liver cell lines.

Polynucleic acids or mimetics

Branched L-Lys clusters were reductively aminated with eight lactose units [40] (note that the structure of lactose is drawn incorrectly in this paper) and used to tag the *N*-terminus of peptide nucleic acid (PNA) 13-mers as potential antisense molecules for telomerase activity. The resulting galactosylated PNAs showed enhanced telomerase inhibition in HepG2 cell lines compared to that of untagged PNA, but less inhibition when compared with a cationic lipid-based system [40]. An 18-mer antisense oligodeoxynucleotide (ODN) labeled with **11** (Figure 4) at its 5'-end showed enhanced liver uptake (from 19 to 77%) as a result of ASGPR targeting [41].

Figure 4. Two examples of carbohydrate systems that have been suggested for nucleic acid targeting [41,42•].



11

12

In a highly novel approach, the coupling of a β-lactosyl-diazonium reagent to two plasmids highlighted the potential of directly glycotargeting polynucleotides [42•]. It was presumed, on the basis of literature on diazotizations, that modification occurred at position 8 of the guanine base of G (**12**; Figure 4) [43]. Substitution levels of 2.5 to 3.1 mol% resulted in greater resistance of the glycosylated plasmids to restriction enzymes SmaI, which cleaves G-containing sequences, and SspI, which interestingly does not. However, although the modified plasmids showed enhanced binding to a galactose-specific lectin, transfection in the HepG2 cell line was not enhanced.

Non-natural polymers

The first example of the targeting of sugar-macromolecule conjugate carriers in humans was reported in 1999 [44•,45•]. A phase I clinical study of a poly[*N*-(2-hydroxypropyl)methacrylamide] (HMPA) copolymer bearing doxorubicin and galactosamine was monitored using ^{123}I-based imaging, and showed 30% delivery of the polymer prodrug to the liver. Single photon emission computed tomography (SPECT) showed a ratio of tumor uptake:surrounding liver uptake of ~ 1:3 at 24 h. However, it should be noted that in this particular case, complications may arise from the attachment of $GalNH_2$ via its *N*(2) group and not via its *O*(1) anomeric center (**8**; Figure 3). This generated a 4-fold increase in sugar microheterogeneity, of which only one of the four forms (β-pyranose-$GalNH_2$) could target the ASGPR [44•,45•]. A 5-fold reduction in cardiotoxicity of this copolymer prodrug over free doxorubicin was also observed [46]. Enhanced HepG2 binding (determined by flow cytometry) and internalization (determined by confocal microscopy) was observed for other HPMA co-polymers containing 10 to 30 mol% of mono-, di- and tri-Gal or -$GalNH_2$ bearing acrylamide units. However, the maximum enhancement level was 5.6-fold for triantennary over monoantennary, which amounts to only 1.9-fold enhancement when corrected to a per-sugar basis; this is much less remarkable than other instances of the cluster effect [47].

Trans-retinoic acid has been delivered to hepatocyte cell lines using poly-L-lactic acid nanoparticles coated with a lactonamide-polystyrene. This system was prepared simply by mixing the three components in solution, followed by diafiltration [48]. Flow cytometry and cell adhesion assays using mouse hepatocytes to test a polystyrene incorporating structure **9** (Figure 3) were inhibited by AF, which is consistent with the involvement of the ASGPR. Unusually, glucuronamide **9** appears to target the ASGPR despite having the wrong stereochemistry and functionality, as compared with the normal β-galactoside ligand. Moreover, a polymer of the methacrylate ester of the *O*(6) of glucose (Glc) **10** (Figure 3) was able to selectively bind ASGPR from cell lysates. Only vague reasons for this binding of ASGPR by these C(6)-modified glucoses were suggested, but it should be noted that, as for **8**, both **9** and **10** display a 4-fold increase in sugar microheterogeneity as a result of their free anomeric *O*(1) groups [49].

Recent work using positively-charged galactodendrimeric systems based on commercially-available dendrimers has reconfirmed previous studies in other systems that carbohydrate multivalency in the DNA-carrier is beneficial in transfections of HepG2 cell line [50].

Liposomes and nanoparticles

The following liposomal preparations are featured by virtue of their properties, since they give some insight into the behavior of macromolecular constructs, although it should be noted that liposomes are typically the products of non-covalent assembly of small MW glycoconjugates. In addition, liposomal constructs, by their very nature, may restrict the type of drugs that they can efficiently encapsulate. A brief review of the synthesis and use of glycoprotein-liposome conjugates has appeared [51]. For example, periodate oxidation of the sialic acid side chain of ganglioside lipid liposomes, followed by reductive amination of BSAs that had been previously glycosylated with *N*-acetyl-D-glucosamine (GlcNAc) created constructs that were further elaborated with galactosyltransferase enzymes (to introduce Gal) and sialyltransferases (to introduce sialic acids). The properties of these constructs were compared [51].

Liposomes are commonly cleared by the reticular endothelial system (RES), often into non-parenchymal cells. However, many therapies require delivery to parenchymal cells. Liposomes composed of dipalmitoylphosphatidyl choline (DPPC), cholesterol (Ch) and soybean-derived sterylglucoside (SG) have been produced in DPPC:Ch:SG

Table 1. Survey of targets.

Organ	Cell-type	Receptor	References
Liver	Parenchymal	ASGPR	[1,19,21,22••,24-26,30••,32,33,34•,36-41,42•,44•,45•,46-49,52-56,60-62]
	Non-parenchymal	MBP	[18,27,33]
	Tumor		[22••,28•,44•,45•,46,47,57,59]
Kidney			[20]
Blood	Macrophages	MBP	[35•]
	Dendritic cells		[29••]
Others	Numerous		[17•]

ratios of 6:3:1 and 6:4:0. 1,1'-Dioctadecyl-3,3,3',3'-tetraethylindocarbocyanine perchlorate (DiI) was used as a fluorescent probe to monitor the association of the lipid layer components of liposomes with HepG2 cells, which showed a marked reduction on co-incubation with AF. However, when doxorubicin was incorporated into these liposomes, no change in uptake was seen upon co-incubation with AF, even though previous results have suggested that uptake of doxorubicin into hepatocytes, when incorporated in liposomes as opposed to the free drug, is higher. It has been suggested that doxorubicin leaks from the liposome near the cell, accumulating within the cell in a non-specific manner [52].

Triantennary β-galactoside clusters mounted on cholesteryl esters have been incorporated into 8- to 10-nm liposomes. The clusters used can be regarded as more chemically stable then previous variants but, more importantly, they give rise to enhanced ASGPR-mediated uptake, with less undesired exchange of the glycolipid from the liposomes to other lipid compartments. Critically, a clear pitfall of overloading sugar density was observed: at a loading ratio of 5% (w/w) the glycolipid/liposome particles are efficiently processed by the ASGPR, whereas 50% loading resulted in an uptake that was not blocked by AF, indicating a less specific uptake, perhaps by the Kupfer cell Gal/Fuc receptor [53].

The liver uptake of antisense ODNs coupled to the oleoyl ester of lithocholic acid can be enhanced by association with lactosylated low density lipoprotein (LDL), as compared with unglycosylated LDL [54]. Oleoyl esters of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) complexed with galactosylated-high density lipoprotein (HDL) resulted in delivery of 69% of the total dose to the liver, compared with < 5% for free PMEA [55], and galactosylated cholesteryl substances allowed liver specific uptake of PGE1 at parenchymal:non-parenchymal selectivity ratios of up to 15:1 [56]. Interestingly, a sialyl Lewis-x-lipid-targeted, merphalan-lipid-loaded, phospholipid-based liposome significantly prolonged the survival times of mice with grafted adenocarcinomas [57].

Such RME-based strategies are not always successful. For example, a novel β-galactosylated (incorrectly described in the paper as an α-galactoside) spermine bolaamphiphile prepared from lactose showed lower transfection levels of cell lines than unglycosylated spermine lipid control systems. The observed decreases *and* increases in transfection levels upon AF treatment questions the participation of the ASGPR [58]. In another example, the addition of a β-gluco-sitosterol steroid lipid to liposomes also resulted in lower gene expression levels in HepG2 cell lines than those without [59]. A novel glycolipid containing a triantennary GalNAc-terminated cluster glycoside has been used in uptake studies, which showed liposomal particles > 70 nm could not be taken up by the ASGPR in mice [60]. A study of the ability of galactosylated liposomes to deliver lipophilic prodrug probucol to mouse liver showed a marked variation in success with the nature of the phosphatidylcholine used to prepare the liposome [61].

Nanoparticles coated with poly(vinylbenzyl-lactonamide) (PVLA) and loaded with cell modulators (eg, colchicines and Taxol) have been investigated to determine their potential in receptor-mediated delivery to hepatocytes. The nanoparticle matrix was formed from either poly(lactic acid) or poly(γ-benzyl-L-glutamate), and the nanoparticles were formed using known methods to give, for example, standard PVLA nanoparticles of 55.6-nm average diameter and Taxol/PVLA-coated PVLA nanoparticles of 306.0-nm average diameter. Fluorescence and confocal laser microscopic studies suggest that these nanoparticles are internalized by hepatocytes via receptor-mediated endocytosis [62].

Conclusions

Sugar-macromolecule conjugates as selective drug delivery systems are another exciting avenue among an ever-expanding array of medicinal applications for carbohydrates [12,63,64,65]. Yet, to date, despite their clear promise, there have been precious few examples of their exploitation. Given the wealth of highly-specific interactions that are available, each a precise delivery route waiting to be used (consider also reverse approaches, ie, the use of lectin-macromolecular conjugates to target endogenous carbohydrate ligands [66]), this is a field rich in opportunities (Table 1). Central to its fruition is the need for precision, both in construction and analysis.

References

- •• of outstanding interest
- • of special interest

1. Rogers JC, Kornfeld S: **Hepatic uptake of proteins coupled to fetuin glycopeptide.** *Biochem Biophys Res Commun* (1971) **45**:622-629.

2. Monsigny M, Roche A-C, Midoux P, Mayer R: **Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes.** *Adv Drug Deliv Rev* (1994) **14**:1-24.

3. Wadhwa MS, Rice KG: **Receptor-mediated glycotargeting.** *J Drug Targeting* (1995) **3**:111-127.

4. Wu GY, Wu CH: **Receptor-mediated delivery of foreign genes to hepatocytes.** *Adv Drug Deliv Rev* (1998) **29**:243-248.
• *A review that concisely picks out the very best examples of sugar-targeted gene therapy.*

5. Hashida M, Nishikawa M, Yamashita F, Takakura Y: **Cell-specific delivery of genes with glycosylated carriers.** *Adv Drug Deliv Rev* (2001) **52**:187-196.

6. Rihova B: **Receptor-mediated targeted drug or toxin delivery.** *Adv Drug Deliv Rev* (1998) **29**:273-289.
• *This review contains excellent references and provides a very balanced yet concise introduction to the broader field.*

7. Vyas SP, Sihorkar V: **Endogenous carriers and ligands in non-immunogenic site-specific drug delivery.** *Adv Drug Deliv Rev* (2000) **43**:101-164.

8. Yamazaki N, Kojima S, Bovin NV, André S, Gabius S, Gabius H-J: **Endogenous lectins as targets for drug delivery.** *Adv Drug Deliv Rev* (2000) **43**:225-244.
•• *An excellent review that strikes a great balance between excited optimism and the current limitations; it powerfully notes the real need for precision in future elucidations and more diverse constructs, which will only be achieved through a combination of excellence in all disciplines.*

9. Eisenburg C, Seta N, Appel M, Feldmann G, Durand G, Feger J: **Asialoglycoprotein receptor in human isolated hepatocytes from normal liver and its apparent increase in liver with histological alterations.** *J Hepatol* (1991) **13**:305-309.

10. Gablus S, Kayser K, Bovin NV, Yamazaki N, Kojima S, Kaltner H, Gabius H-J: **Endogenous lectins and neoglycoconjugates: A sweet approach to tumour diagnosis and targeted drug delivery.** *Eur J Pharm Biopharm* (1996) **42**:250-261.

11. Monsigny M, Midoux P, Mayer R, Roche AC: **Glycotargeting: Influence of the sugar moiety on both the uptake and the intracellular trafficking of nucleic acid carried by glycosylated polymers.** *Biosci Rep* (1999) **19**:125-132.

12. Davis BG: **Recent developments in glycoconjugates.** *J Chem Soc Perkin Trans 1* (1999):3215-3237.

13. Seitz O: **Glycopeptide synthesis and the effects of glycosylation on protein structure and activity.** *ChemBioChem* (2000) **1**:214-216.

14. Davis BG: **Synthesis of glycoproteins.** *Chem Rev* (2002) **102**:579-602.

15. Monsigny M, Quetard C, Bourgerie S, Delay D, Pichon C, Midoux P, Mayer R, Roche AC: **Glycotargeting: The preparation of glyco-amino acids and derivatives from unprotected reducing sugars.** *Biochimie* (1998) **80**:99-108.

16. Takakura Y, Nishikawa M, Yamashita F, Hashida M: **Development of gene drug delivery systems based on pharmacokinetic studies.** *Eur J Pharm Sci* (2001) **13**:71-76.

17. Unverzagt C, André S, Seifert J, Kojima S, Fink C, Srikrishna G, Freeze H, Kayser K, Gabius H-J: **Structure-activity profiles of complex biantennary glycans with core fucosylation and with/without additional α2,3/α2,6 sialylation: Synthesis of neoglycoproteins and their properties in lectin assays, cell binding and organ uptake.** *J Med Chem* (2002) **45**:478-491.
• *A rare example of the incorporation of complex oligosaccharide structures into sugar-targeted macromolecules.*

18. Opanasopit P, Shirashi K, Nishikawa M, Yamashita F, Takakura Y, Hashida M: ***In vivo*** **recognition of mannosylated proteins by hepatic mannose receptors and mannan-binding protein.** *Am J Physiol Gastrointest Liver Physiol* (2001) **280**:G879-G889.

19. Staud F, Nishikawa M, Takakura Y, Hashida M: **Liver uptake and hepato-biliary transfer of galactosylated proteins in rats are determined by the extent of galactosylation.** *Biochim Biophys Acta* (1999) **1427**:183-192.

20. Shirota K, Kato Y, Suzuki K, Sugiyama Y: **Characerization of a novel kidney-specific delivery system using an alkylglucoside vector.** *J Pharmacol Exp Ther* (2001) **299**:459-467.

21. Han J, Lim S-J, Lee M-K, Kim C-K: **Altered pharmacokinetics and liver targetability of methotrexate by conjugation with lactosylated albumins.** *Drug Delivery* (2001) **8**:125-134.

22. Lerchen H-G, Baumgarten J, Piel N, Kolb-Bachofen V: **Lectin-mediated drug targeting: Discrimination of carbohydrate-mediated cellular uptake between tumor and liver cells with neoglycoconjugates carrying fucose epitopes regioselectively modified in the 3-position.** *Angew Chem Int Ed* (1999) **38**:3680-3683.
•• *Excellent carbohydrate chemistry and good in vivo work are strikingly combined in the creation and evaluation of novel targeting ligands. From several L-fuco structures, one tumor selective ligand was found.*

23. Opanasopit P, Nishikawa M, Yamashita F, Takakura Y, Hashida M: **Pharmacokinetic analysis of lectin-dependent bio-distribution of fucosylated bovine serum albumin: A possible carrier for Kupffer cells.** *J Drug Targeting* (2001) **9**:341-351.

24. Hashida M, Akamatsu K, Nishikawa M, Yamashita F, Takakura Y: **Design of polymeric prodrugs of prostaglandin E-1 having galactose residue for hepatocyte targeting.** *J Control Release* (1999) **62**:253-262.

25. Akamatsu K, Yamasaki Y, Nishikawa M, Takakura Y, Hashida M: **Synthesis and pharmacological activity of a novel water-soluble hepatocyte-specific polymeric prodrug of prostaglandin E-1 using lactosylated poly(L-glutamic hydrazide) as a carrier.** *Biochem Pharmacol* (2001) **62**:1531-1536.

26. Hashida M, Akamatsu K, Nishikawa M, Yamashita F, Yoshikawa H, Takakura Y: **Design of polymeric prodrugs of PGE1 for cell-specific hepatic targeting.** *Pharmazie* (2000) **55**:202-205.

27. Nishikawa M, Takemura S, Yamashita F, Takakura Y, Meijer DKF, Hashida M, Swart PJ: **Pharmacokinetics and *in vivo* gene transfer of plasmid DNA complexed with mannosylated poly(L-lysine) in mice.** *J Drug Targeting* (2000) **8**:29-38.

28. Nishikawa M, Yamauchi M, Morimoto K, Ishida E, Takakura Y, Hashida M: **Hepatocyte-targeted *in vivo* gene expression by intravenous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system.** *Gene Ther* (2000) **7**:548-555.
• *A good demonstration of the potentially very high levels of in vivo expression that can be achieved with sugar-targeted gene carriers.*

29. Diebold SS, Kursa M, Wagner E, Cotten M, Zenke M: **Mannose polyethylenimine conjugates for targeted DNA delivery into dendritic cells.** *J Biol Chem* (1999) **274**:19087-19094.
•• *A very exciting paper that describes the targeting of a new cell class, and demonstrates the potential of sugar-based gene delivery to elicit antigen-specific T-cell response.*

30. Sato H, Hayashi E, Yamada N, Yatagai M, Takahara Y: **Further studies on the site-specific protein modification by microbial transglutaminase.** *Bioconjug Chem* (2001) **12**:701-710.
•• *An excellent example of the preparation of a precisely glycosylated protein. Although the low yields necessitated purification, this paper gives a clear indication of how the precise constructs that will be needed for elucidating SARs may be constructed and characterized. Slightly unclear arguments based on data, which were not shown, claim involvement of the ASGPR but without RME. It should also be noted that the use of transglutaminases to glycosylate Gln-containing peptides, but not their in vivo activity, has been suggested previously, see reference [31].*

31. Ramos D, Rollin P, Klaffke W: **Enzymic synthesis of neoglycopeptide building blocks.** *Angew Chem Int Ed* (2000) **39**:396-398.

32. Yabe Y, Kobayashi N, Nishihashi T, Takahashi R, Nishikawa M, Takakura Y, Hashida M: **Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives.** *J Pharmacol Exp Ther* (2001) **298**:894-899.

33. Yabe Y, Nishikawa M, Tamada A, Takakura Y, Hashida M: **Targeted delivery and improved therapeutic potential of catalase by chemical modification: Combination with superoxide dismutase derivatives.** *J Pharmacol Exp Ther* (1999) **289**:1176-1184.

34. Eto T, Takahashi H: **Enhanced inhibition of hepatitis B virus production by asialoglycoprotein receptor-directed interferon.** *Nature Med* (1999) **5**:577-581.

• *This paper describes the key step of removing rather than the addition of carbohydrates to allow potent targeting of this important protein treatment, with little loss in activity.*

35. Friedman B, Vaddi K, Preston C, Mahon E, Cataldo JR, McPherson JM: **A comparison of the pharmacological properties of carbohydrate remodeled recombinant and placental-derived β-glucocerebrosidase: Implications for clinical efficacy in treatment of Gaucher disease.** *Blood* (1999) **93**:2807-2816.

• *MBP binding data and cellular distribution show that glucocerebrosidase is targeted to the macrophage site of therapy by the presence of just two triantennary Man-tipped $Man_3GlcNAc_2Fuc$ structures at natural glycosylation Asn sites 19 and 146.*

36. Kaneo Y, Tanaka T, Nakano T, Yamaguchi Y: **Evidence for receptor-mediated hepatic uptake of pullulan in rats.** *J Control Release* (2001) **70**:365-373.

37. Kaneo Y, Ueno T, Tanaka T, Iwase H, Yamaguchi Y, Uemura T: **Pharmacokinetics and biodisposition of fluorescein-labeled arabinogalactan in rats.** *Int J Pharm* (2000) **201**:59-69.

38. Kato Y, Onishi H, Machida Y: **Biological characteristics of lactosaminated N-succinyl-chitosan as a liver-specific drug carrier in mice.** *J Control Release* (2001) **70**:295-307.

39. Park YK, Park IH, Shin BA, Choi ES, Kim YR, Akaike T, Cho CS, Park YK, Park YR: **Galactosylated chitosan-graft-dextran as hepatocyte-targeting DNA carrier.** *J Control Release* (2000) **69**:97-108.

40. Zhang X, Simmons CG, Corey DR: **Liver cell specific targeting of peptide nucleic acid oligomers.** *Bioorg Med Chem Lett* (2001) **11**:1269-1272.

41. Biessen EAL, Vietsch H, Rump ET, Fluiter K, Kuiper J, Bijsterbosch MK, Van Berkel TJC: **Targeted delivery of oligodeoxynucleotides to parenchymal liver cells *in vivo*.** *Biochem J* (1999) **340**:783-792.

42. Akasaka T, Matsuura K, Emi N, Kobayashi K: **Conjugation of plasmid DNAs with lactose via diazocoupling enhances resistance to restriction enzymes and acquires binding affinity to galactose-specific lectin.** *Biochem Biophys Res Commun* (1999) **260**:323-328.

• *A wonderful idea for the direct glycosylation of plasmids that could circumvent the need for gene carriers. Unfortunately, in this case, no enhanced delivery was observed.*

43. Matsuura K, Akasaka T, Hibino M, Kobayashi K: **Facile synthesis of stable and lectin-recognizable DNA-carbohydrate conjugates via diazo coupling.** *Bioconjug Chem* (2000) **11**:202-211.

44. Julyan PJ, Seymour LW, Ferry DR, Daryani S, Boivin CM, Doran J, David M, Anderson D, Christodoulou C, Young AM, Hesslewood S, Kerr DJ: **Preliminary clinical study of the distribution of HPMA copolymers bearing doxorubicin and galactosamine.** *J Control Release* (1999) **57**:281-290.

• *This paper, along with reference [45•], evaluates the first example of a sugar-macromolecule carrier in humans.*

45. Ferry DR, Seymour LW, Anderson D, Hesselwood S, Julyan P, Boivin C, Poyner R, Guest P, Doran J, Kerr DJ: **Phase I trial of liver targeted HPMA copolymer of doxorubicin PK2, pharmacokinetics, SPECT imaging of I-123-PK2 and activity in hepatoma.** *Br J Cancer* (1999) **80**:413.

• *This paper, along with reference [44•], evaluates the first example of a sugar-macromolecule carrier in humans.*

46. Hopewell JW, Duncan R, Wilding D, Chakrabarti K: **Preclinical evaluation of the cardiotoxicity of PK2: A novel HPMA copolymer-doxorubicin-galactosamine conjugate antitumour agent.** *Hum Exp Toxicol* (2001) **20**:461-470.

47. David A, Kopeckova P, Rubinstein A, Kopecek J: **Enhanced-biorecognition and internalization of HPMA copolymers containing multiple or multivalent carbohydrate side-chains by human hepatocarcinoma cells.** *Bioconjug Chem* (2001) **12**:890-899.

48. Cho CS, Cho KY, Park IK, Kim SH, Sasagawa T, Uchiyama M, Akaike T: **Receptor-mediated delivery of all trans-retinoic acid to hepatocyte using poly(L-lactic acid) nanoparticles coated with galactose-carrying polystyrene.** *J Control Release* (2001) **77**:7-15.

49. Kim SH, Goto M, Akaike T: **Specific binding of glucose-derivatized polymers to the asialoglycoprotein receptor of mouse primary hepatocytes.** *J Biol Chem* (2001) **276**:35312-35319.

50. Ren T, Zhang GS, Liu DX: **Synthesis of galactosyl compounds for targeted gene delivery.** *Bioorg Med Chem* (2001) **9**:2969-2978.

51. Yamazaki N, Jigami Y, Gabius HJ, Kojima S: **Preparation and characterization of neoglycoprotein-liposome conjugates: A promising approach to developing drug delivery materials applying sugar chain ligands.** *Trends Glycoscience Glycotechnol* (2001) **13**:319-329.

52. Maitani Y, Kawano K, Yamada K, Nagai T, Takayama K: **Efficiency of liposomes surface-modified with soybean-derived sterylglucoside as a liver targeting carrier in HepG2 cells.** *J Control Release* (2001) **75**:381-389.

53. Sliedregt L, Rensen PCN, Rump ET, van Santbrink PJ, Bijsterbosch MK, Valentijn AR, van der Marel GA, van Boom JH, van Berkel TJC, Biessen EAL: **Design and synthesis of novel amphiphilic dendritic galactosides for selective targeting of liposomes to the hepatic asialoglycoprotein receptor.** *J Med Chem* (1999) **42**:609-618.

54. Rump ET, de Vrueh RLA, Manoharan M, Waarlo IHE, van Veghel R, Biessen EAL, van Berkel TJC, Bijsterbosch MK: **Modification of the plasma clearance and liver uptake of steroid ester-conjugated oligodeoxynucleotides by association with (lactosylated) low-density lipoprotein.** *Biochem Pharmacol* (2000) **59**:1407-1416.

55. de Vrueh RLA, Rump ET, van de Bilt E, van Veghel R, Balzarini J, Biessen EAL, van Berkel TJC, Bijsterbosch MK: **Carrier-mediated delivery of 9-(2-phosphonylmethoxy-ethyl)adenine to parenchymal liver cells: A novel therapeutic approach for hepatitis B.** *Antimicrob Agents Chemother* (2000) **44**:477-483.

56. Kawakami S, Munakata C, Fumoto S, Yamashita F, Hashida M: **Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs.** *J Pharm Sci* (2001) **90**:105-113.

57. Vodovozova EL, Moiseeva EV, Grechko GK, Gayenko GP, Nifant'ev NE, Bovin NV, Molotkovsky JG: **Antitumour activity of cytotoxic liposomes equipped with selectin ligand SiaLeX, in a mouse mammary adenocarcinoma model.** *Eur J Cancer* (2000) **36**:942-949.

58. Gaucheron J, Santaella C, Vierling P: ***In vitro* gene transfer with a novel galactosylated spermine bolaamphiphile.** *Bioconjug Chem* (2001) **12**:569-575.

59. Hwang SH, Hayashi K, Takayama K, Maitani Y: **Liver-targeted gene transfer into a human hepatoblastoma cell line and *in vivo* by sterylglucoside-containing cationic liposomes.** *Gene Ther* (2001) **8**:1276-1280.

60. Rensen PCN, Sliedregt LA, Ferns M, Kieviet E, van Rossenberg SMW, van Leeuwen SH, van Berkel TJC, Biessen EAL: **Determination of the upper size limit for uptake and processing of ligands by the asialoglycoprotein receptor on hepatocytes *in vitro* and *in vivo*.** *J Biol Chem* (2001) **276**:37577-37584.

61. Hattori Y, Kawakami S, Yamashita F, Hashida M: **Controlled biodistribution of galactosylated liposomes and incorporated probucol in hepatocyte-selective drug targeting.** *J Control Release* (2000) **69**:369-377.

62. Cho C-S, Kobayashi A, Takei R, Ishihara T, Maruyama A, Akaike T: **Receptor-mediated cell modulator delivery to hepatocyte using nanoparticles coated with carbohydrate-carrying polymers.** *Biomaterials* (2001) **22**:45-51.

63. McAuliffe JC, Hindsgaul O: **Carbohydrates in medicine.** In: *Molecular and Cellular Glycobiology*. Fukudam M, Hindsgual O (Eds), Oxford University Press, UK (2000):249-285.

64. Davis BG: **Hand in Glove.** *Chem Ind* (2000):134-138.

65. Koeller KM, Wong C-H: **Emerging themes in medicinal glycoscience.** *Nature Biotechnol* (2000) **18**:835-841.

66. Clark MA, Hirst BH, Jepson MA: **Lectin-mediated mucosal delivery of drugs and microparticles.** *Adv Drug Deliv Rev* (2000) **43**:207-223.