Carbohydrate Research, 254 (1994) 257–268
Elsevier Science B.V., Amsterdam

Binding of D-galactose-terminated ligands to rabbit asialoglycoprotein receptor *

Anita Krebs [a], William T. Depew [a], Walter A. Szarek [b,†], George W. Hay [b] and Lucjan J.J. Hronowski [b]

[a] *Department of Medicine. Queen's University, Kingston, Ontario K7L 3N6 (Canada)*
[b] *Department of Chemistry, Queen's University, Kingston, Ontario K7L 3N6 (Canada)*

(Received March 29th, 1993; accepted June 19th, 1993)

ABSTRACT

The binding affinities of a series of D-galactose-terminated glycerol glycosides and oligosaccharides for the asialoglycoprotein receptor isolated from rabbit liver were determined in vitro using a radioreceptor-inhibition assay with ^{125}I-asialoorosomucoid. The relative affinities of the synthetic ligands increased with the number of exposed D-galactose termini. Of the compounds examined, 1,2,3-tri-*O*-β-lactosylglycerol associated with the greatest affinity (estimated $K_d = 7.97 \times 10^{-5}$ M). Examination of the affinities of the synthetic series indicated that both the number and propinquity of the D-galactose termini influenced the strength of the binding interactions.

INTRODUCTION

Cell plasma membranes display arrays of receptors that recognize diverse ligands. Binding interactions between ligands and receptors may initiate transmembrane transport of the ligand, membrane protein phosphorylation reactions and a cascade of intracellular metabolic events. Characterization of these ligand–receptor interactions has permitted the elucidation of several important cellular physiological events. Moreover, membrane receptors provide portals for receptor-specific manipulations including the targeting of xenobiotics to particular receptor-bearing cell types[1–3].

The mammalian hepatocyte plasma membrane expresses the asialoglycoprotein receptor (ASGP-R), a unique integral membrane receptor exhibiting specificity for terminal, nonreducing β-D-galactopyranosyl or 2-acetamido-2-deoxy-β-D-galactopyranosyl residues[2,4]. ASGP-R binds molecules bearing such substituents and

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† Corresponding author.

SSDI 0008-6215(93)E0264-2

shuttles them primarily to lysosomes, where ligands are released prior to receptor recycling[5–8]. Complex ligands usually undergo lysosomal degradation providing low molecular weight fragments which escape into the cytoplasm by diffusion[9–12]. This sequence of events could be exploited to enable the routing of D-galactose-bearing synthetic or semisynthetic compounds to mammalian hepatocytes for the purpose of organ-specific drug delivery. The development of terminally D-galactose-substituted targeting vehicles, amenable to the chemical modifications necessary to incorporate a variety of drugs, is an essential step in achieving the goal of hepatocyte-specific drug delivery.

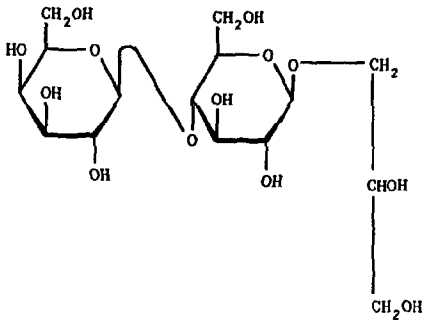
To design a targeting vehicle specific for ASGP-R, we have synthesized a series of D-galactose-terminated ligands including 1-*O*-β-lactosyl-(*R*,*S*)-glycerols (**1**), 1,2-di-*O*-β-lactosyl-(*R*,*S*)-glycerols (**2**), 1,3-di-*O*-β-lactosylglycerol (**3**), 1,2,3-tri-*O*-β-lactosylglycerol (**4**), 1-*O*-(6-*O*-β-lactosyl-β-lactosyl)-(*R*,*S*)-glycerols (**5**), 6-*O*-β-lactosyl-α,β-lactoses (**6**), 4-*O*-α-D-galactopyranosyl-D-galactopyranose (**7**), 4-*O*-β-D-galactopyranosyl-D-galactopyranose (**8**), 4,6-di-*O*-β-D-galactopyranosyl-α,β-D-glucoses (**9**), and 2,6-di-*O*-β-lactosyl-α,β-D-mannopyranoses (**10**). The binding affinities of these compounds have been determined in vitro according to the method of Connolly et al.[13] by a competitive radioreceptor-inhibition assay using isolated, affinity-purified rabbit ASGP-R, and ^{125}I-asialoorosomucoid as the competing ligand. The results indicate that synthetic oligomeric carbohydrate ligands bearing two or more D-galactose termini may be useful for hepatocyte-specific targeting.

EXPERIMENTAL

Materials and methods. — The synthesis of the oligosaccharides has been published as follows: **1** and **3** (ref 14), **2** and **4** (ref 15), **5**, **6**, and **9** (ref 16) and **10** (ref 17). The disaccharides **7** and **8** were prepared according to the methods of Chacón-Fuertes and Martín-Lomas[18].

ASGP-R was isolated from frozen rabbit liver according to the method of Hudgin et al.[19]. Briefly, soluble receptor was extracted from liver homogenates by affinity chromatography using cyanogen bromide-activated Sepharose 4B (Pharmacia), coupled to asialoorosomucoid (ASOM) recovered following neuraminidase treatment (*Clostridium perfringens* Type X, Sigma) of human plasma orosomucoid (Cohn VI, Sigma). Neuraminidase was removed on a column of *N*-(*p*-aminophenyl)oxamic acid–agarose (Sigma). ASOM was labelled with ^{125}I (ICN Radiochemicals) using the lactoperoxidase–glucose oxidase method (Enzymobeads, BioRad Laboratories). Specific activities ranged from 3.0×10^8 to 8.2×10^8 cpm/μg. Labelled ASOM was diluted, when necessary, with unlabelled ASOM. Protein was determined using a dye-binding assay (BioRad).

The binding affinities of the synthetic compounds were determined according to the radioreceptor-inhibition assay described initially by Hudgin et al.[19] and subsequently modified by Connolly et al.[13]. Briefly, isolated ASGP-R (1 μg) was mixed



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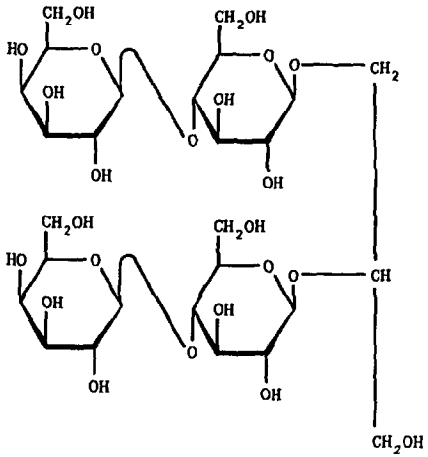

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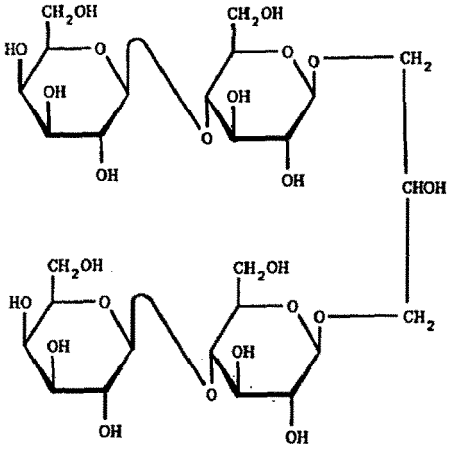
at 4°C with ^{125}I-ASOM (5 ng, specific activity, 1×10^5 cpm/ng) in buffer containing 2 mM calcium chloride in the presence of varying concentrations of synthetic D-galactose-terminated ligands. Mixtures were incubated at 4°C for 90 min. Receptor-bound ^{125}I-ASOM was precipitated with 40% saturated ammonium sulfate and collected on Whatman GF/C filters (2.5-cm diam). All binding assays were performed in triplicate. Nonspecific binding was measured in each experiment in the presence of a 1000-fold excess of unlabelled ASOM. The binding affinity of isolated ASGP-R for the standard ligand ^{125}I-ASOM was determined in each experiment, as a positive control, to confirm the affinities of all receptor isolates. Washed filters were counted in an LKB Compugamma counter. The high-affinity binding constant (K_d) was determined by Eadie-Scatchard analysis (Fig. 1).



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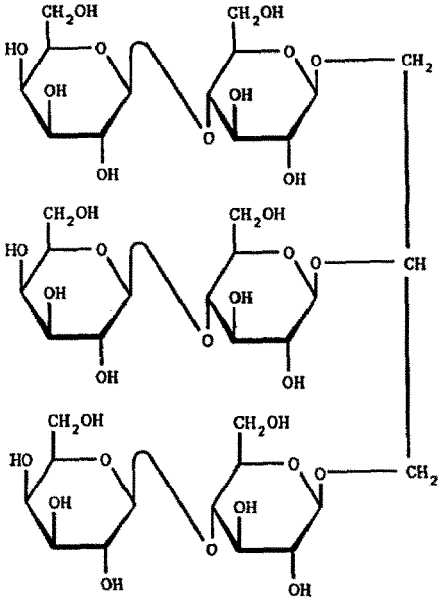
For some of the synthetic products, the binding affinities were also determined in cell culture according to the method of Schwartz et al.[20]. Briefly, the human hepatocellular carcinoma cell line HepG2 was seeded in tissue culture dishes



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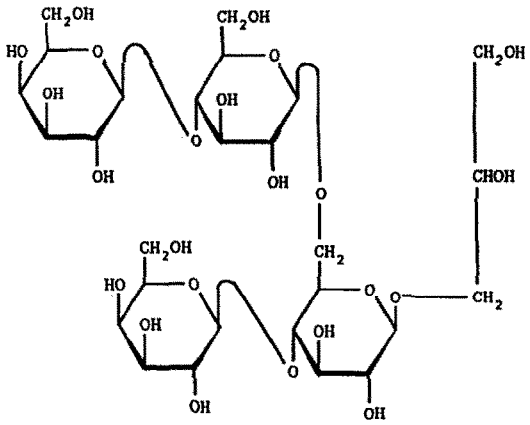

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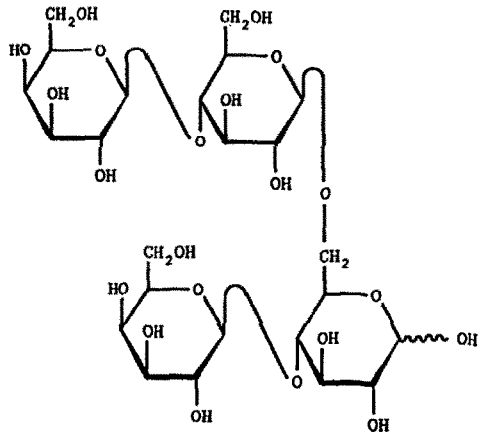

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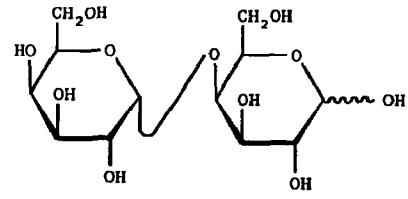
(35-mm, Falcon) and grown to confluence (usually 5–6 days) in Earle's MEM (Gibco) containing fetal calf serum (10%, heat-inactivated) and supplemental 2 mM L-glutamine. Prior to each experiment, the cultures were washed with calcium-free phosphate-buffered saline at 4°C. Cultures were then incubated for 15 min at 4°C in Earle's MEM containing 2 mM calcium chloride and 0.1% (w/v) bovine serum albumin in the presence of ^{125}I-ASOM (5 ng, 1×10^6 cpm) and increasing concentrations of synthetic ligand. Cultures were washed with phosphate-buffered saline containing 2 mM calcium chloride to remove unbound label.



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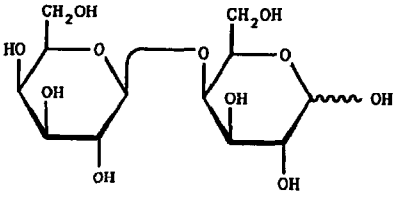

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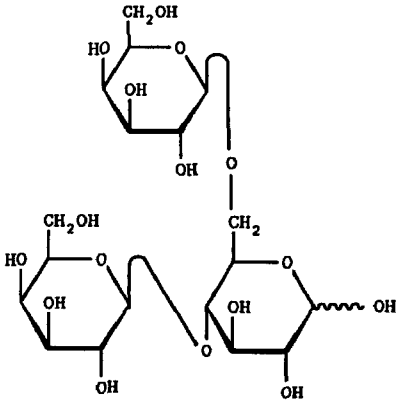
The cells were solubilized with 1 M sodium hydroxide at room temperature and the radioactivity in the resulting solutions was measured.

Expression of Results. — In all binding studies, conditions were employed which enabled the estimation of the K_d of the high-affinity binding site of ASGP-R[21]. The binding affinities determined using isolated, solubilized ASGP-R and HepG2 cells in culture were expressed as I_{50}. Each I_{50} was determined according to the



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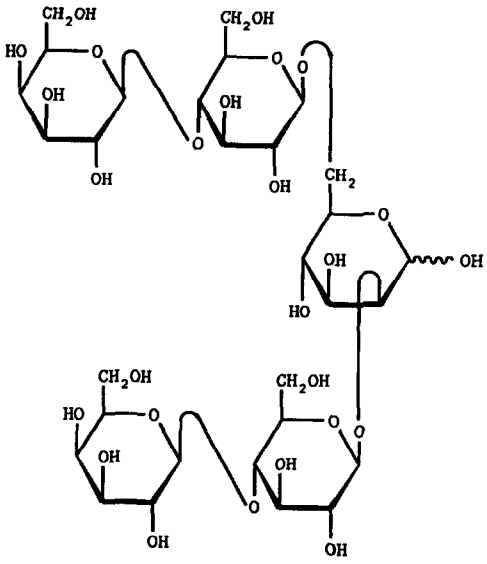

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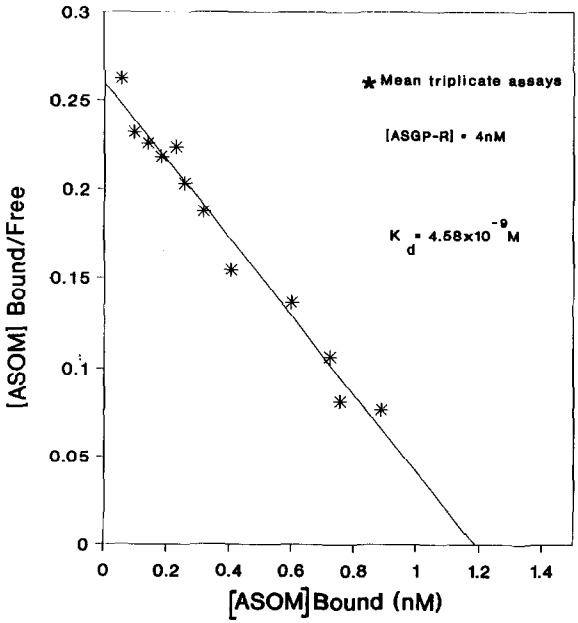

Fig. 1. Eadie–Scatchard plot for the high-affinity binding site of rabbit asialoglycoprotein receptor (ASGP-R) using asialoorosomucoid (ASOM) as the ligand. All assays were performed in triplicate using [ASGP-R] = 4 nM.

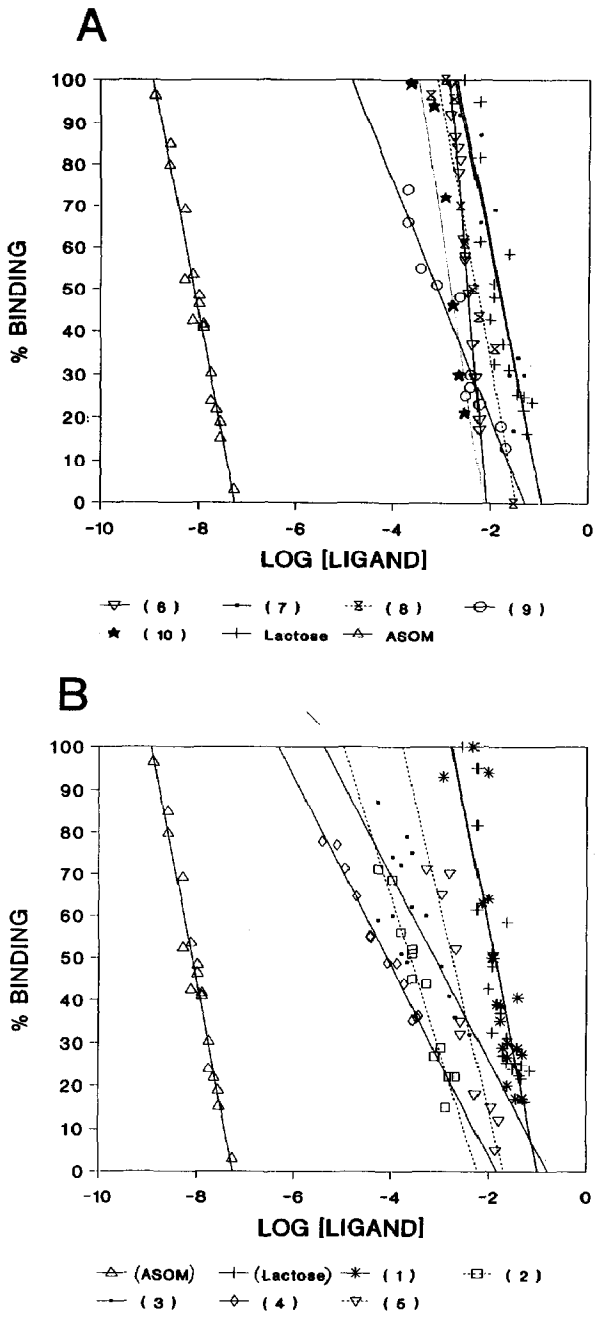

Fig. 2. (A) Inhibition of ^{125}I-asialoorosomucoid binding to rabbit ASGP-R by synthetic oligosaccharide ligands. All assays were performed in triplicate using [ASGP-R] = 4 nM. Binding inhibition by ASOM and lactose presented for comparison. Lines show results of linear regression analyses; △———△ (ASOM), +–+ (Lactose), ▽———▽ (6), ■———■ (7), ⧖———⧖ (8), ○———○ (9), ★———★ (10). (B) Inhibition of ^{125}I-asialoorosomucoid binding to rabbit ASGP-R by synthetic glycerol-containing ligands. All assays were performed in triplicate using [ASGP-R] = 4 nM. Binding inhibition by ASOM and lactose presented for comparison. Lines represent results of linear regression analyses; △———△ (ASOM), +–+ (Lactose), ★———★ (1), □———□ (2), ■———■ (3), ◇–◇ (4), ▽———▽ (5).

method of Lee et al.[22] as the molar concentration of synthetic ligand required to inhibit 50% of the binding of the standard ligand, ^{125}I-ASOM. For each test ligand, a binding isotherm was constructed from the results of the radioreceptor-inhibition assays. The binding curves were replotted (Figs. 2A and 2B) following logarithmic transformation of the concentrations of competing ligands. The I_{50} values for the synthetic ligands were derived from linear regression analyses of these plots.

RESULTS AND DISCUSSION

The identification of ligand-specific cell-membrane receptor-mediated transport processes has provided explanations for many cell-specific metabolic activities[1–3]. In the mammalian liver, ASGP-R mediates calcium-dependent membrane binding and internalization of D-galactose-terminated ligands by the mechanism of receptor-mediated endocytosis[1,2,4]. Internalized, membrane-associated, receptor-ligand complexes are routed intracellularly by way of a series of vesicular compartments to lysosomes. During this vesicular journey, intravesicular pH falls and ligand is released. Membrane-associated receptor is recycled to the plasma membrane domain while unbound ligand is metabolized in the lysosomal compartment prior to cytoplasmic release of the degradation products[1,2,5–12].

Several characteristics of ASGP-R make it attractive as a target for an organ-specific drug delivery system. This receptor is hepatocyte-specific[1,2]. Studies examining multiantennary D-galactose-terminated glycoproteins and neoglycoproteins in several animal models have demonstrated avid hepatic uptake and rapid hepatocyte degradation of such ligands[23,24]. Indeed, greater than 90% of labelled D-galactose-bearing glycoprotein is sequestered in the hepatic parenchyma within 20 min following intravenous administration[25]. As a result of the many functional enzymes available in metabolically active hepatocytes, ligand internalized by this route may be rapidly degraded making subunits readily available within the cell.

Ligand specificity for ASGP-R is determined by the availability of exposed, terminal nonreducing D-galactose units. Other structural characteristics have been determined which enhance ligand–receptor binding and cellular uptake[4]. Binding increases as the number of the exposed D-galactose units per molecule of ligand increases[26,27]. The intramolecular arrangement of exposed D-galactose units also influences binding. Clustering of substituents, enabling close approximation of three or more D-galactose residues strengthens the binding interactions considerably[27,28], independent of the total number of terminal D-galactose residues. Structural features facilitating such close approximations are associated experimentally with greater binding affinities. Binding affinity is also enhanced by substitution of particular sites in the terminal D-galactose unit[4,22,29]. *N*-Acetyl-D-galactosamine readily substitutes for D-galactose in this binding process.

The present study was designed to explore the use of relatively low molecular weight D-galactose-terminated oligosaccharides as ligands for specific binding to

TABLE I

Inhibitory potencies of D-galactose-terminated disaccharides on ^{125}I-ASOM binding to rabbit ASGP-R

Compounds	I_{50} (M)
Lactose	1.15×10^{-2}
1-*O*-β-Lactosyl-(*R*,*S*)-glycerols (**1**)	1.33×10^{-2}
4-*O*-α-D-Galactopyranosyl-D-galactopyranose (**7**)	1.55×10^{-2}
4-*O*-β-D-Galactopyranosyl-D-galactopyranose (**8**)	4.95×10^{-3}

ASGP-R. The availability of such molecules would facilitate the design of practical ligand–drug complexes targetable to human hepatocytes.

The binding characteristics of the multiantennary D-galactose-terminated glycoprotein, ASOM, are depicted in Fig. **1**. Eadie–Scatchard analysis gave a K_d for the high affinity binding site of 4.58×10^{-9} M, a value similar to those reported by others[13,22]. A second multiantennary D-galactose-terminated glycoprotein, asialofetuin, showed similar characteristics ($K_d = 1.4 \times 10^{-8}$ M).

All of the D-galactose-terminated oligosaccharides in this series demonstrated binding to isolated rabbit ASGP-R, and, for selected compounds, binding was also demonstrable with HepG2 cells.

Table I shows the relative binding affinities of the ligands, **1**, **7**, and **8**, which possess a single exposed D-galactose terminus. The range of values for the corresponding I_{50} (4.95×10^{-3} to 1.33×10^{-2} M) agrees with results obtained by other investigators[22,28,30] for similar molecules. As expected, these values are of the same order of magnitude as those recorded for lactose.

Binding affinities were greater in molecules containing two D-galactose termini (**2**, **3**, **5**, **6**, **9**, and **10**; Table II). This group exhibited binding affinities between 1.4 and 45 times greater than the linear molecules shown in Table I. Compound **2**

TABLE II

Inhibitory potencies of D-galactose-terminated bi- and tri-antennary oligosaccharides and multiantennary glycoproteins on ^{125}I-ASOM binding of rabbit ASGP-R

Compounds	I_{50} (M)
Biantennary oligosaccharides	
1,2-Di-*O*-β-lactosyl-(*R*,*S*)-glycerols (**2**)	2.90×10^{-4}
1,3-Di-*O*-β-lactosylglycerol (**3**)	9.13×10^{-4}
1-*O*-(6-*O*-β-Lactosyl-β-lactosyl)-(*R*,*S*)-glycerols (**5**)	2.09×10^{-3}
6-*O*-β-Lactosyl-α,β-lactoses (**6**)	3.45×10^{-3}
4,6-Di-*O*-β-D-galactopyranosyl-α,β-D-glucoses (**9**)	1.50×10^{-3}
2,6-Di-*O*-β-lactosyl-α,β-D-mannopyranoses (**10**)	1.53×10^{-3}
Triantennary oligosaccharide	
1,2,3-Tri-*O*-β-lactosylglycerol (**4**)	7.97×10^{-5}
Multiantennary glycoproteins	
Asialoorosomucoid	8.19×10^{-9}
Asialofetuin	1.40×10^{-8}

TABLE III

Inhibitory potencies of selected D-galactose-terminated ligands on ^{125}I-ASOM binding by HepG2 cells

Ligand	I_{50} (M)	I_{50} (M) [a]
Lactose	1.0×10^{-3}	1.15×10^{-2}
Asialoorosomucoid	6.1×10^{-10}	8.19×10^{-9}
1-*O*-β-Lactosyl-(*R*,*S*)-glycerols (**1**)	1.65×10^{-3}	1.33×10^{-2}
1,3-Di-*O*-β-lactosylglycerol (**3**)	1.81×10^{-4}	9.13×10^{-4}
1,2,3-Tri-*O*-β-lactosylglycerol (**4**)	9.83×10^{-6}	7.97×10^{-5}

[a] I_{50} values obtained from competitive binding assays using isolated rabbit ASGP-R for comparison.

demonstrated the most avid binding of these biantennary molecules, and **6** the least. The 12-fold difference in binding affinity seems likely to be related to differences in the conformations of these molecules. The 3-fold increase in binding between the two regioisomers **3** and **2**, and the 2.3-fold enhancement between the structurally-related **6** and **9**, are consistent with greater proximity of the D-galactose termini in **2** and **9**, respectively. The greater difference in binding affinities between the pair of regioisomers **2** and **3**, and the structurally-related pair **6** and **9**, may be a reflection of greater separation of the D-galactose termini in the latter pair.

Binding affinity measured in cell culture was consistently higher when compared with the results obtained using isolated receptor (Table III). This enhancement of binding affinity has been recognized by others[4] and probably is related to the different microenvironments supporting receptor activity in the membrane. The results from cell-culture experiments provide measures of affinity which more closely reflect the binding interactions in vivo.

In contrast with the biantennary molecules, the triantennary compound **4** displayed a 140-fold enhancement of binding affinity compared with lactose. The apparent binding affinity of the tri-lactosylated glycerol **4** is at least 2.3-fold greater than values obtained[28] for tri-β-D-galactosylated and tri-β-lactosylated derivatives of (6-aminohexanamido)tris(hydroxymethyl)methane ($K_d = 3.0 \times 10^{-4}$ M and 1.8×10^{-4} M, respectively). This result demonstrates the requirement for a multiantennary D-galactose-terminated oligosaccharide in the quest for a low molecular weight ligand to be used as a targeting vehicle with biologically important receptor interactions. Clearly, the addition of a third D-galactose residue markedly enhances ligand binding, and this finding, which was predicted on the basis of the known characteristics of ASGP-R, suggests that this or similar molecules could be useful as targeting vehicles for directed drug delivery. Although the binding affinities of the multiantennary glycoproteins are $\sim 10^4$-fold greater than that of **4**, they are not practical candidates as potential targeting vehicles because of their large masses. Drugs targeted with these vehicles would require very large (kg-range) doses to achieve therapeutic effects in humans. The choice of a targeting vehicle bearing three D-galactose termini may afford sufficient vehicle affinity and low molecular weight in a synthetically efficient fashion.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council Canada for support of the research in the form of a grant (to W.A.S.).

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