In Vivo Targeting Function of N-Linked Oligosaccharides with Terminating Galactose and N-Acetylgalactosamine Residues*

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N-Linked biantennary, triantennary, and core fucosylated biantennary oligosaccharides were isolated from animal glycoproteins and derivatized at their reducing end with Boc-tyrosine. The terminal Gal residues were enzymatically removed and replaced with GalNAc. Tyrosinamide-oligosaccharides were radioiodinated and administered intravenously to mice. Pharmacokinetic and biodistribution studies revealed structure-dependent differences in the steady-state volume of distribution, total body clearance rate, and targeting efficiency. Tyrosinamide-oligosaccharides were found to resist metabolism relative to a natural triantennary glycopeptide which was rapidly degraded in vivo. Triantennary oligosaccharides containing terminal Gal or GalNAc targeted the liver efficiently whereas biantennary oligosaccharides containing terminal Gal residues and differing only in their core fucosylation avoided recognition by the asialoglycoprotein receptor and were cleared unmetabolized by renal filtration. In contrast, biantennary oligosaccharides containing terminal GalNAc residues targeted the liver with much greater efficiency than Gal-terminated triantennary oligosaccharide. Core fucosylation reduced the metabolism rate of tyrosinamide-biantennary in the liver. The results establish the utility of tyrosinamide-oligosaccharides as probes to analyze the ligand specificity of mammalian lectins in vivo and demonstrate that a GalNAc-terminated biantennary is a potent ligand for the asialoglycoprotein receptor.

Glycoproteins contain oligosaccharides which often act as specific markers for targeting proteins either intra- or extracellularly (1, 2). The targeting activity is mediated through binding interactions with endogenous mammalian lectins (3, 4).

The first mammalian lectin was detected by Ashwell and co-workers, who correlated the rapid clearance of asialo glycoproteins from the serum with tissue localization into the liver. This led to the discovery of an asialoglycoprotein receptor (ASGP-R) on hepatocytes that binds Gal or GalNAc residues (5–7). Since its discovery, the ASGP-R has been used as a model system for studying the function of recycling receptors and to analyze the specificity of mammalian carbohydrate-protein interactions (8, 9).

Much of the framework for our current understanding of mammalian carbohydrate-protein interactions comes from in vitro binding studies that analyzed the ASGP-R affinity for synthetic or natural oligosaccharides (10–12). Complex oligosaccharides bind to the receptor with dissociation constants ranging from millimolar up to nanomolar as the number of terminal Gal residues on the ligand increases from 1 to 3 (11).

However, “clustering” of terminal Gal residues alone does not completely predict binding affinity. Triantennary oligosaccharides with isomeric branching patterns or those that differ by a single linkage have 20–100-fold reduced affinity for the receptor (13, 14). Of the natural ligands studied thus far, a triantennary oligosaccharide containing 3 N-acetyllactosamine residues in a 4,2,2 branching pattern from the core has the highest affinity per Gal residue (13). Earlier studies have also addressed the issue of the affinity of GalNAc-versus Gal-terminated glycoconjugates and measured subnanomolar binding constants for neoglycopeptides containing 3 terminal GalNAc residues, whereas nanomolar affinity was found for neoglycopeptides containing 2 GalNAc residues (15).

The present study has examined the in vivo targeting efficiency of triantennary and biantennary oligosaccharides containing either 2 or 3 terminal Gal or GalNAc residues. The results indicate that biantennary oligosaccharides containing terminal GalNAc residue are superior ligands for the ASGP-R compared with Gal-terminated triantennary. This may be a clue toward understanding the function of the receptor in mammals and has direct application toward the design of oligosaccharide-based targeted drug delivery carriers.

MATERIALS AND METHODS

Sodium iodide was purchased from DuPont NEN. Sephadex G-10, chloramine T, sodium metabisulfite, 2-mercaptoethanol, uridine-diphosphate-N-acetylgalactosamine, and β-galactosidase transferase (EC 2.4.1.22) from bovine milk were purchased from Sigma. Thin layer chromatography plates (Silica Gel-60 F-254) were from Alltech, Deerfield, IL. Ketamine hydrochloride was purchased from Parke-Davis. Silastic catheters (0.305 mm inner diameter × 0.635 mm outer diameter) were from Baxter, β-Galactosidase (EC 3.2.1.23) from bovine testes was purchased from V-labs, Covington, LA. Hamilton polymer PRP-1 (10 μm) reverse-phase HPLC columns (0.47 × 25 cm) were purchased from Baxter Scientific, McGaw Park, IL. Silica-C8 (5 μm) reverse-phase HPLC columns (0.47 × 25 cm) were purchased from Rainin, Emeryville, CA.

Preparation of N-Linked Oligosaccharides with Terminal Gal Residues—N-Linked oligosaccharides were purified from animal glycoproteins as previously described (16). Briefly, biantennary I and triantennary III were prepared from bovine fetuin using N-glycosidase F to release the oligosaccharides from tryptic glycopeptides. The reducing end was converted into a glycosylamine, then derivatized with Boc-tyrosine-N-hydroxysuccinimide ester and purified by RP-HPLC. Each tyrosinamide-oligosaccharide was characterized by proton NMR and FAB-MS (16). A similar strategy was utilized to prepare biantennary II from porcine fibrinogen (17). Triantennary glycopeptide IV was prepared from bovine fetuin as described previously (18, 19).

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Preparation and Characterization of Oligosaccharides Containing Terminal GalNAc Residues—Oligosaccharides I, II, and III (1.5 µmol each) were dissolved in 50 µl of 0.5 M citrate phosphate buffer (pH 4.3) and digested with 100 milliunits (60 µl) of β-galactosidase at 37 °C for 24 h. Following heat denaturation of β-galactosidase at 50 °C for 1 h, 400 µl of 0.1 M acetic acid buffer (pH 7.5) containing 5 mM manganous chloride and 10 µl of EDTA-GalNAc was added and incubated at 37 °C for 24 h. An additional 10 µmol of UDP-GalNAc was added to drive the reaction to completion in 48 h. The oligosaccharide products (V, VI, and VII) were purified from successive 200 nmoles of injections on a polyammonium RP-HPLC column (0.47×25 cm) eluted isocratically at 1 ml/min with 0.1% acetic acid and 11% acetonitrile (50 °C) while detecting the oligosaccharide by λ280nm 0.2 absorbance units full scale.

Oligosaccharides (1 µmol each) were freeze dried twice in D2O (99.99%) then dissolved in 0.5 ml of D2O containing 0.01% acetic acid. Proton NMR was performed on a Bruker 500 MHz spectrometer operated at 23 °C, and spectra were processed using resolution enhancement parameters supplied by the Felix software package (Hare Research, Eugene, OR).

FAB-MS analysis of oligosaccharides V, VI, and VII was performed by preparing the sample (2 nmoles) in 20 µl of water containing 2 µl of octanedithiol. The water was removed by speed-vac evaporation, and the sample was applied to the probe of a Finnigan Matt 900 operating in the positive ion mode.

Radiolabeling N-Linked Oligosaccharides—iodinations were performed using a modification of the chloramine T method (20). The tyrosinamide-oligosaccharides (2 nmoles in 60 µl of 0.5 M sodium phosphate buffer (pH 7.0)) were added to 0.5 mCi of Na125I in 50 µl of 0.1 M sodium hydroxide. Chloramine T (20 µl of 10 mM in phosphate buffer) was added and allowed to react for 3 min followed by the addition of sodium metabisulfite (80 µl of 10 mM in phosphate buffer) in order to quench the reaction. Radiiodinated oligosaccharides were chromatographed on a Sephadex G-10 column (0.8×25 cm) eluted with 0.15 M sodium chloride (pH 7.0) while collecting 0.5-ml fractions. The oligosaccharides eluted between 3 and 4 ml and had a specific activity of 125 µCi/nmol, assuming quantitative recovery.

The purity of each iodinated oligosaccharide was analyzed by spotting 1 µl (2 nCi) at the origin of a TLC plate developed with ethyl acetate/acetic acid/pyridine/water at a ratio optimized for each oligosaccharide (I, 4:3:2:2; II, 4:3:2:2.5; III, 4:3:3:5:5, and IV 3:2:1:5:2; V 4:3:2:2:5; VI 4:3:2:2:5; VII 3.5:3.5:2:2.5). Quantitative densitometry was performed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) following 12-h autoradiographic exposure at room temperature. ImageQuant software (Molecular Dynamics, Sunnyvale, CA) was used to integrate the densitometry trace and established >95% purity for each iodinated oligosaccharide.

PharmacoKinetic Analysis Of N-Linked Oligosaccharides—In vitro stability was determined by (incubating 1 µl (50 nCi) of each radiiodinated oligosaccharide with 100 µl of humanized whole mouse blood at 37 °C). At time points ranging from 1 to 6 h, aliquots (10 µl) were extracted from blood and analyzed using TLC and quantitative autoradiography as described below.

ICR albino mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 28–32 g were housed in cages located in a limited access area with a 12-h light-dark cycle and controlled temperature (23–28 °C). Mice were fed standard laboratory mouse food (Charles River Inc.) ad libitum for at least 3 days before use.

Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (10 mg/100 g body weight) and then underwent dual cannulation of their left and right jugular veins. A bolus intravenous dose (15 µl) of oligosaccharide (1.8 µCi) was administered via one of the catheters, and blood samples (10 µl) were withdrawn with a microsyringe from the other catheter at 1, 3, 6, 10, 15, 20, 30, 40, and 60 min after dosing. Each blood sample was replaced with 10 µl of normal saline into the sampling catheter.

The in vivo stability of each oligosaccharide was ascertained by TLC. Blood time points were added to 60 µl of water, and the iodinated oligosaccharide was extracted from serum proteins by adding 200 µl of acetonitrile to the micropre centrifuge tube. The samples were vortexed and centrifuged for 10 min (10,000 g) to remove protein, and the precipitate was discarded with 60 µl of 0.1 M acetic acid. The supernatants were dried by speed-vac evaporation, dissolved in 10 µl of water, and then spotted (1.5 µl) onto adjacent lanes of a TLC plate. The plate was developed, autoradiographed, and analyzed by quantitative densitometry against a calibration strip prepared by spotting serial dilutions of an iodinated oligosaccharide.

PharmacoKinetic parameters were derived from direct blood counts versus time for three data sets for each oligosaccharide. Iterative non-linear least-squares fits for individual data sets were obtained with PCNONLIN (SCI Software, Lexington, KY) using a two-compartment open model described by the integrated equation

\[
C_i = A e^{-a t} + B e^{-\beta t} \quad \text{(Eq. 1)}
\]

where \(C_i\) is the concentration of oligosaccharide in blood. \(A\) and \(B\) are constants and \(a\) and \(\beta\) are hybrid first-order rate constants that characterize the slopes of the fast and slow phases of decline in a plasma concentration versus time profile (21). The mean residence time (MRT) was calculated according to Equation 2, and it is the average time that the oligosaccharide was in the body of the mouse (22).

\[
MRT = \frac{A}{\alpha + \beta} \quad \text{(Eq. 2)}
\]

The total body clearance (\(Cl_{TB}\)) was calculated using

\[
Cl_{TB} = \frac{\text{dose}}{A e^{-\alpha} + B e^{-\beta}} \quad \text{(Eq. 3)}
\]

and the volume of distribution at steady-state (\(V_{dz}\), defined as the sum of volumes of the two compartments) was calculated according to Equation 4 (23).

\[
V_{dz} = Cl_{TB} \times MRT \quad \text{(Eq. 4)}
\]

The "tissue to plasma" ratio (T/P) for a two-compartment model is defined as ratio of oligosaccharide in the peripheral compartment versus the central compartment at time approaching infinity and was calculated according to

\[
T/P = \frac{k_{12}}{k_{21} - \beta} \quad \text{(Eq. 5)}
\]

where \(k_{12}\) and \(k_{21}\) are the micro rate constants that characterize the rate of oligosaccharide transfer between the two compartments (21).

Biodistribution of N-Linked Oligosaccharides—Mice were anesthetized with ketamine hydrochloride, and a single silastic catheter was inserted into the right jugular vein. An intravenous bolus dose (50 µl) of oligosaccharide (7 µCi in normal saline) was administered after which the catheter was removed and the vein was ligated. After 30 min, mice were euthanized and subjected to whole body autoradiography (24). The deeply anesthetized mice were immersed in a hexane dry ice bath (~70 °C) for 5 min and mounted in a 4% (w/v) carboxymethylcellulose block which was then cooled to ~20 °C. Eight longitudinal sections (25 µm thick) were cut on a cryo-microtome (LKB 2250, Sweden) from multiple depths in order to reveal all organs. The sections were collected on adhesive tape (Scotch 810, 3M Co., Minneapolis, MN), dried at ~1°C for 24 h, then autoradiographed for 48 h using a PhosphorImage.

Quantitative biodistribution analysis was performed at 30 min or longer after dosing mice intravenously with oligosaccharides (1.8 µCi) as described above. The mice were sacrificed by cervical dislocation, and the major organs (liver, heart, lungs, spleen, kidney, stomach, small intestine, and large intestine) were removed, washed with saline, and weighed. Radioactivity in each intact organ was measured by direct γ counting.

RESULTS

Galactose-terminated N-linked oligosaccharides were prepared from bovine fetuin (I and III) (16) and porcine fibronogen (II) (17) as tyrosinamide-oligosaccharides possessing a Boc-tyrosine linked to the reducing end GlcNAc through a β-glycosylamide linkage. A triantennary glycopeptide (IV) was also prepared for study which contained the same oligosaccharide as III linked to a pentapeptide through the side chain of asparagine (1). In addition, three GalNAc-terminated oligosaccharides were prepared using oligosaccharides I, II, and III as substrates for β-galactosidase and galatosyltransferase (25). The transformed oligosaccharides (Fig. 1, structures V, VI, and VII) each eluted later on RP-HPLC relative to the parent Gal terminated...
oligosaccharides (Fig. 2).

The structure of oligosaccharides V, VI, and VII were established by proton NMR and FAB-MS analysis. Each oligosaccharide possessed anomeric resonances for GlcNAc 1, 2, and Man 4 and 4′ (Fig. 3) with identical chemical shifts as the parent oligosaccharides (I, II, III) (16, 17). In addition, oligosaccharide VI contained a fucose anomeric resonance and a methyl resonance coincident with those in II. The presence of two GalNAc residues on V and VI was established from the downfield shift of the GalNAc anomeric protons relative to Gal anomeric resonances in I and II. Oligosaccharides V and VI each displayed two new N-acetyl signals attributed to GalNAc (Fig. 3). The assignment of these signals was based on chemical shift comparison to similar biantennary oligosaccharides isolated from human kallidinogenase (26) (Table 1).

Oligosaccharide VII possessed 3 terminal GalNAc residues as evidenced by three additional N-acetyl resonances compared with III. However, the GlcNAc and GalNAc anomeric protons were highly overlapped, and on this basis the proton NMR spectrum was tentatively assigned as shown in Fig. 3. FAB-MS analysis of oligosaccharides V, VI, and VII provided pseudomolecular ions of 2007.77, 2153.70, and 2413.97 corresponding to (within 0.2 mass units) the calculated exact mass (M + Na) for the proposed GalNAc-terminated oligosaccharides, respectively.

Radiiodination of the tyrosine residue resulted in oligosaccharides with a high specific activity and purity. Control experiments confirmed that the iodinated oligosaccharides, including glycopeptide IV, were not metabolized on prolonged incubation in blood.

Preliminary pharmacokinetic studies established that the oligosaccharides were rapidly cleared when administered intravenously. Therefore, serial sampling from the jugular vein was used to improve precision and increase the number of early time points. A complete oligosaccharide concentration versus time profile (9 or 10 time points) was obtained from a single mouse (Fig. 4).

The concentration of oligosaccharides in blood was determined by direct gamma counting of blood time points which is valid if the oligosaccharides are metabolically stable. To confirm this, oligosaccharides were extracted from blood time points with >80% efficiency and analyzed on TLC with autoradiographic detection (Fig. 4). The blood concentrations of oligosaccharides I, II, III, V, VI, and VII decreased over 60 min without appreciable formation of metabolites. Quantitative densitometric analysis of the TLC plates produced a concentration versus time profile that closely mirrored that obtained by direct γ counting of blood samples (Fig. 4, B and F).

In contrast, radioactivity in the blood increased 10 min after administration of glycopeptide IV (Fig. 4C). TLC analysis revealed the presence of faster migrating iodinated products indicating that the glycopeptide was rapidly metabolized (Fig. 4D).

Pharmacokinetic parameters were determined from triplicated data sets for each tyrosinamide-oligosaccharide, then averaged to obtain the mean and standard deviation (Table II). In contrast, the metabolic instability of glycopeptide IV precluded detailed analysis of its pharmacokinetics.

Each tyrosinamide-oligosaccharide showed a biexponential decline in blood after intravenous dosing which was fit by a two-compartment open model with elimination from the central compartment (Fig. 4, panels A, C, and E). All of the oligosaccharides were rapidly cleared from the blood with α half-lives that were typically less than 1 min and β half-lives which ranged from 15 to 40 min, resulting in an overall mean residence time of less than 1 h (Table II).

An elevated steady-state volume of distribution and accelerated total body clearance were observed for oligosaccharides that target liver (III, V, VI, and VII) (Table II). In contrast, the total body clearance for oligosaccharides I and II approximates the renal filtration rate in mice (0.4 ml/min), indicating this to be the major elimination route for oligosaccharides that interact weakly with endogenous lectins.

The biodistribution of each oligosaccharide was analyzed 30 min after dosing because at this time less than 5% of the dose remained in the blood as determined by pharmacokinetic analysis (Fig. 4). Whole body autoradiography was used to screen all tissues to identify the major sites of distribution (Fig. 5). Quantitative biodistribution analysis of dissected tissues con-
Targeting Function of N-linked Oligosaccharides

Fig. 2. RP-HPLC analysis of tyrosine-derivatized oligosaccharides. The RP-HPLC separation of each oligosaccharide and glycopeptide presented in Fig. 1 is shown.

The percentage of dose recovered in dissected organs at 30 min post-administration is expressed as the targeting efficiency. Triantennary oligosaccharide III and glycopeptide IV were both targeted to the liver with only minor distribution to other reference tissues (Fig. 5B). Quantitative analysis established a liver targeting efficiency of 59 and 52%, respectively, whereas the kidney and small intestine were the only other reference organs that contained radioactivity in excess of 1% of the dose (Fig. 5).

Biantennary oligosaccharides I and II failed to biodistribute appreciably to the liver and instead underwent renal filtration as determined by whole body autoradiography (Fig. 5A). Quantitative tissue counting confirmed a liver targeting efficiency of 5% for I or II, whereas the radioactivity in the kidney was 3.5%, the intestine was 2%, and all other tissues contained background counts. The radioactive oligosaccharide recovered from urine comigrated with oligosaccharides I or II on TLC, establishing that the excreted oligosaccharides were essentially unmetabolized.

GalNAc-substituted oligosaccharides (V, VI, and VII) displayed different biodistribution characteristics than Gal-termi-
Targeting Function of N-linked Oligosaccharides

FIG. 3. Partial 500 MHz proton NMR spectra of GalNAc-terminated oligosaccharides. The structural reporter groups in the Man anomeric region (4.85–5.15 parts/million), GalNAc and GlcNAc anomeric region (4.48–4.64 parts/million), and N-acetyl region (1.96–2.10 parts/million) is shown for biantennary oligosaccharides V and VI and triantennary oligosaccharide VII. The numbering used to assign the resonances is shown in Fig. 1 (structure VII). See Table I for chemical shift values corresponding to the resonance assignments.

nated oligosaccharides. Biantennary oligosaccharides V and VI targeted to liver as evidenced by whole body autoradiography. Direct tissue counting established a liver targeting efficiency of 80%, whereas <5% of the dose was recovered in kidney and small intestine and all other tissues were targeted at <1% (Fig. 5). Similar results were obtained for the GalNAc-terminated triantennary oligosaccharide (VII) of which 85% distributed to the liver 30 min after administration (Fig. 5C).

The time course of liver targeting was analyzed for oligosaccharides V and VI at 30, 60, 120, and 180 min after dosing. Biantennary V was eliminated from the liver over 3 h, and the parent compound or its metabolites were delivered to the small intestine through biliary excretion (Fig. 6). In contrast, core fucosylation of biantennary VI slowed its elimination rate from the liver, which was also reflected in its low level of accumulation in the small intestine (Fig. 6).

DISCUSSION

One approach to studying mammalian lectins in vivo is to utilize radiolabeled glycoconjugates as probes. Receptor activity may be identified from the accumulation of the ligand in or at the target tissue(s) (27).

We have initiated studies aimed at identifying the tissue distribution and specificity of mammalian lectins in vivo using an experimental design analogous to that performed by

<table>
<thead>
<tr>
<th>Proton* of V°</th>
<th>VI</th>
<th>VII</th>
</tr>
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<tbody>
<tr>
<td>H-1 of</td>
<td></td>
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</tr>
<tr>
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<td>5.015</td>
</tr>
<tr>
<td>2</td>
<td>4.617</td>
<td>4.628</td>
</tr>
<tr>
<td>4</td>
<td>5.110</td>
<td>5.111</td>
</tr>
<tr>
<td>4'</td>
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<td>4.912</td>
</tr>
<tr>
<td>5</td>
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<table>
<thead>
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<tbody>
<tr>
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<td>8</td>
<td>2.067</td>
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° See Fig. 1 for labeling nomenclature.
Chemical shifts in parts/million reported relative to an internal standard of acetone (2.225 parts/million) at 23 °C.
Fucose residue in structure VI.
Ashwell and co-workers (5-7) which led to the discovery of the ASGP-R nearly three decades ago. Since their pioneering experiments, technological advances have led to several improvements in experimental design such as the utilization of purified N-linked oligosaccharides as probes in place of glycoproteins or neoglycoproteins. An immediate advantage of this is the ease with which oligosaccharides can be manipulated with glycosidases and glycosyltransferases (25). Consequently, experiments are possible that compare the biodistribution characteristics of oligosaccharides containing single or multiple sugar substitutions or deletions.

Oligosaccharide biodistribution studies necessitate radiolabeling at a location that does not alter their affinity for receptors. The oligosaccharides used in this study all contained a tyrosine residue attached to GlcNAc 1 through a \(\beta\)-glycosylamide linkage that allows incorporation of \(^{125}\)I. Tyrosinamide-oligosaccharides appear to be ideal ligands for pharmacokinetic and biodistribution studies due to their relative metabolic stability compared with a natural glycopeptide (Fig. 4). Ligands that resist metabolism are desirable for investigations aimed at elucidating in vivo biodistribution because radioactive metabolites may redistribute and complicate pharmacokinetic analysis and the interpretation of target site and targeting efficiency.

In addition to using refined N-linked oligosaccharide ligands, whole body autoradiography was used to analyze biodistribution in mice. This technique simultaneously surveys the distribution of radioactivity in all tissues and thus avoids missing targeting activity to minor organs that are not routinely dissected. Combining this technique with computer-assisted autoradiography increases the speed and sensitivity of the analysis and can potentially lead to the discovery of otherwise undetected oligosaccharide biodistribution sites, implicating
The targeting efficiency, as defined as the percent of dose distributed to the major organs of mice at animals ASGP-R. Predictably, the low molecular weight and hydrophilic containing terminal Gal and GalNAc residues to test their relativity of N-linked oligosaccharides makes them susceptible to rapid clearance by renal filtration, resulting in short serum half-lives compared with glycoproteins (28). Their hydrophilicity however, is also potentially an advantage which improves their selectivity as probes for identifying lectin activity in vivo, and instead renal filtration dominates as the major clearance mechanism. Furthermore, we failed to detect the biodistribution of I or II to other tissues, suggesting that mice do not possess high affinity receptors involved in the uptake of these common N-linked oligosaccharides.

In contrast, triantennary oligosaccharide III targets liver as efficiently as glycopeptide IV, indicating that tyrosine derivatization does not compromise ASGP-R binding (Fig. 5). A targeting efficiency of 59% for III is a reflection of the reported nanomolar dissociation constant of I and II (11) is insufficient to compete for receptor recognition and uptake in vivo, and instead renal filtration dominates as the major clearance mechanism. Furthermore, the affinity of the oligosaccharide 1000-fold resulted in an enhancement in targeting efficiency of nearby 55%.

Even more remarkable are the results of biodistribution of oligosaccharides V, VI, and VII which illustrate a dramatic influence of terminal sugar residue on targeting efficiency to the liver (Fig. 4C). GalNAc-terminated biantennary oligosaccharides V and VI targeted liver with 20% higher efficiency than triantennary III, and the addition of a third GalNAc residue further enhanced the targeting efficiency 5% for triantennary VII.

These results indicate that ligands possessing two properly positioned GalNAc residues on a biantennary oligosaccharide have much greater affinity for the mouse ASGP-R than triantennary III. Although a direct correlation between ASGP-R binding affinity and targeting efficiency is not possible from the

**Table II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Residence Time</th>
<th>Steady-state Volume of Distribution</th>
<th>Total Body Clearance</th>
<th>Tissue/Plasma Ratio</th>
<th>Targeting Efficiency</th>
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<tbody>
<tr>
<td>I</td>
<td>20.7 ± 4.7</td>
<td>7.8 ± 2.3</td>
<td>0.4 ± 0.2</td>
<td>2.4</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>20.8 ± 4.0</td>
<td>8.3 ± 2.1</td>
<td>0.4 ± 0.2</td>
<td>2.7</td>
<td>5</td>
</tr>
<tr>
<td>III</td>
<td>46.1 ± 27.3</td>
<td>25.6 ± 11.1</td>
<td>0.7 ± 0.3</td>
<td>6.1</td>
<td>59</td>
</tr>
<tr>
<td>V</td>
<td>19.5 ± 5.9</td>
<td>28.5 ± 10.8</td>
<td>1.5 ± 0.3</td>
<td>9.6</td>
<td>79</td>
</tr>
<tr>
<td>VI</td>
<td>31.7 ± 10.6</td>
<td>72.5 ± 29.1</td>
<td>2.3 ± 0.2</td>
<td>12.4</td>
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<tr>
<td>VII</td>
<td>24.6 ± 8.6</td>
<td>43.5 ± 15.8</td>
<td>1.8 ± 0.1</td>
<td>16.6</td>
<td>85</td>
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*The experimentally determined targeting efficiency at 30 min.*

The lack of appreciable liver targeting for I and II is consistent with previous studies that analyzed the biodistribution of asialo-transferrin which contains similar galactose terminated biantennary oligosaccharides (30). Assuming that the ASGP-R is responsible for the observed uptake of oligosaccharides into the liver, it is apparent that the micromolar dissociation constant of I and II (11) is insufficient to compete for receptor recognition and uptake in vivo, and instead renal filtration dominates as the major clearance mechanism. Furthermore, the affinity of the oligosaccharide 1000-fold resulted in an enhancement in targeting efficiency of nearby 55%.

Fig. 5. Biodistribution analysis of N-linked oligosaccharides. The targeting efficiency, as defined as the percent of dose distributed to the major organs of mice at 30 min post-administration, is presented in bar graphs for each oligosaccharide. Each bar represents mean of three animals ± standard deviation. See Fig. 1 for structures. The insets show representative whole body autoradiographic biodistribution analysis at 30 min for biantennary oligosaccharide II (panel A), triantennary III (panel B), and triantennary VII (panel C).

Fig. 6. Elimination rate of oligosaccharide V and VI. The percent of radioactive dose recovered in liver (solid lines) and small intestine (dashed lines) is shown for oligosaccharides V (○, ○) and VI (●, ■) at times ranging from 30 to 180 min. Each data point represents the mean of three animals ± standard deviation. As discussed in the text, core fucosylation slows the elimination of VI from the liver into the bile duct and small intestine relative to V.
present results, the experimental design does reveal the relative binding preference of the ASGP-R under physiological conditions. Given the great range of targeting efficiencies that were determined for closely related ligands, one might consider the possibility that the receptor functions by modulating the serum concentration of glycoproteins by virtue of its broad range of affinities for N-linked oligosaccharides possessing different branching patterns and terminal sugar structures. The ability of this receptor to accommodate ligands with greatly different affinities is probably related to the organization of its multiple subunits (9).

A further result of this study is the finding that tyrosinamide-oligosaccharides V and VI are eliminated from the liver at different rates. Although it is unclear why core fucosylation of VI decreases its relative liver elimination rate, it is also evident that this results in a decreased accumulation of the oligosaccharide in the small intestine. This indicates for the first time a physiological function for core fucosylation on N-linked oligosaccharides.

A portion of this study was devoted to comparing the pharmacokinetic behavior of N-linked oligosaccharides having greatly different affinities for the ASGP-R. The results established that each N-linked oligosaccharide exhibits a biexponential decay from the blood with a half-life less than 1 h. A larger volume of distribution and faster total body clearance rate were associated with targeting activity for tyrosinamide-oligosaccharides III, V, VI, and VII. However, oligosaccharides which target fewer receptors in organs that are perfused by less blood, may not produce meaningful differences in these parameters. The pharmacokinetic parameters determined for oligosaccharides I and II reflect general clearance properties for branched oligosaccharides in the range of 9 to 11 sugar residues. These data may be useful when designing carbohydrate drugs intended as antagonists for mammalian lectins (31).

Future studies will focus on characterizing the biodistribution of N-linked oligosaccharides containing different and unique terminal sugar residues using the approach outlined. These studies may yield biodistribution patterns which implicate the presence of tissue-specific mammalian lectins. This may be one approach to investigate and assign function to individual N-linked oligosaccharides on animal glycoproteins and may lead to further applications of oligosaccharides in targeted drug delivery.

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REFERENCES

26. Taniyama, N., Awaysa, J., Kurotsu, M., Hantawa, N., Shinoda, I., Arata, Y., Ye-
Biochem. Biophys. 205, 70–84