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Ligands of the asialoglycoprotein receptor for targeted gene delivery, part 1: Synthesis of and binding studies with biotinylated cluster glycosides containing N-acetylgalactosamine

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In order to develop the non-viral Bioplex vector system for targeted delivery of genes to hepatocytes, we have evaluated the structure-function relationship for a number of synthetic ligands designed for specific interaction with the hepatic lectin ASGPr. Biotinylated ligand derivatives containing two, three or six beta-linked N-acetylgalactosamine (GalNAc) residues were synthesized, bound to fluorescent-labeled streptavidin and tested for binding and uptake to HepG2 cells using flow cytometry analysis (FACS). Uptake efficiency increased with number of displayed GalNAc units per ligand, in a receptor dependent manner. Thus, a derivative displaying six GalNAc units showed the highest uptake efficacy both in terms of number of internalizing cells and increased amount of material taken up per each cell. However, this higher efficiency was shown to be due not so much to higher number of sugar units, but to higher accessibility of the sugar units for interaction with the receptor (longer spacer). Improving the flexibility and accessibility of a trimeric GalNAc ligand through use of a longer spacer markedly influenced the uptake efficiency, while increasing the number of GalNAc units per ligand above three only provided a minor contribution to the overall affinity. We hereby report the details of the chemical synthesis of the ligands and the structure-function studies *in vitro*.
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Introduction

Targeted delivery of corrective DNA into hepatocytes by receptor-mediated uptake is of considerable importance for the development of gene therapy for human diseases, including liver disorders. The galactose (Gal) or N-acetylgalactosamine (GalNAc) recognizing asialoglycoprotein receptor (ASGPr) is abundantly and selectivly expressed on hepatocytes. This hepatic lectin has been well characterized and studied as a promising candidate target for drug and gene delivery into hepatocytes [1,2]. The ASGPr interacts with terminal Gal or GalNAc residues of desialylated serum glycoproteins and mediates their clearance from the circulation by endosomal uptake [3,4]. The ASGPr also interacts with synthetic cluster glucoside ligands that mimic these natural ligands. A number of well-defined synthetic ligands displaying nanomolar affinity for the ASGPr has thus been designed over the years and evaluated for targeting [5–14].

Introduction of foreign DNA into mammalian cells *in vivo* requires delivery vectors that are able to direct import of the DNA into specific cells with high efficacy and promote stable and efficient expression. Although viral vectors are superior in efficacy here, they possess some serious disadvantages in safety and manufacturing that has turned the attention to development of non-viral techniques, such as the Bioplex system. This system for non-viral gene delivery takes advantage of the

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highly specific and stable interaction between peptide nucleic acids (PNA) and DNA for anchoring peptide functions to a plasmid vector [15–19]. This provides high flexibility in the composition of the vector and results in well-defined and stable carrier-complexes with the potential of becoming efficient delivery vectors.

In order to adapt the Bioplex vector for hepatocyte targeting, a synthetic high affinity ligand for ASGPr must be synthesized and covalently linked to a PNA [20]. Optimizing the targeting ligand is necessary for achieving specific uptake into a large portion of the target cells and a subsequent high level of gene expression. Studies have shown that the binding affinity of synthetic ligands for the ASGPr is highly dependent on the number, distance and three-dimensional arrangement of the sugars residues [11,21]. Affinity increases 100-1000 fold with each additional sugar from mono- to tri-antennary structure, beyond which affinity increases only modestly [22, 23]. Also, the length of the spacer separating the sugar moiety from the branching point of the ligand scaffold affects the affinity for the receptor. For Gal containing ligand constructs, long spacers of >20 Å has shown to result in high affinity binding to the ASGPr and specific targeting to hepatocytes [24], while short spacers of 4 Å lead to targeting of the related macrophage receptor expressed on Kupffer cells [25,26].

Given these multiple factors which affect the binding affinity of synthetic ligands for the ASGPr we have designed a number of synthetic ligands where the receptor-binding unit constitutes two, three, or six GalNAc units bound to a scaffold through linkers of defined lengths. The structures have distances of >20 Å between the GalNAc moiety and the branching point which should maximize the probability for specific and high affinity binding to ASGPr, and minimize the affinity for the related macrophage receptor [11]. To further increase ASGPr affinity and specificity, we have used GalNAc moieties throughout instead of Gal. The ASGPr binds oligosaccharides with terminal GalNAc residues with 10–50 fold higher affinity than ligands with terminal Gal residues [10,27]. In contrast, the macrophage receptor shows no such differential binding affinity and possesses considerably lower affinity for GalNAc than the ASGPr [28,29].

In our continued work on developing of the Bioplex system for non-viral gene delivery, we needed to gain further insight in the structure-binding relationships between the different synthetic di- tri- and hexa-GalNAc-containing ligands. We have therefore synthesized biotin-labelled variants **7i**, **9i**, **10i**, and **11c** of the ASGPr ligands and measured their binding to target hepatocyte cells. The details of the chemical syntheses and binding studies are hereby reported.

Results

Synthesis of biotin-labelled GalNAc ligands

The central GalNAc synthon was the carboxylic acid **5**, carrying an O-acetylated GalNAc residue linked through a diethyleneglycol spacer. Compound **5** was assembled through the following sequences of synthetic steps (Figure 1).

Bromoacetic acid was treated with an excess of diethyleneglycol anion, and the product carboxylate anion was alkylated *in situ* with benzyl bromide to give the benzyl ester **1**, isolated after aqueous workup and silica gel chromatography in modest (19%) yield. Compound **1** was glycosylated with the oxazoline **3** (obtained from galactosamine pentaacetate **2**) using trimethylsilyl triflate as promotor. The desired glycoside **4** was obtained in 79% yield. Catalytic hydrogenation (Pd/C) of **4** in ethyl acetate produced **5** in 60% yield. With compound **5** in hand, construction of various biotinylated di- or tri-GalNAc ligands was possible. First, di-GalNAc compound **6e** (intended as a binding inhibitor) was synthesized from **5** (Figure 2).

Fmoc PAL solid-phase synthesis resin was treated with piperidine/DMF to expose amino groups, and then di-Fmoc-L-lysine was coupled. The product **6a** was subsequently treated with piperidine/DMF to produce the resin-linked diamine **6b**. Coupling with the GalNAc synthon **5** gave **6c**, which was

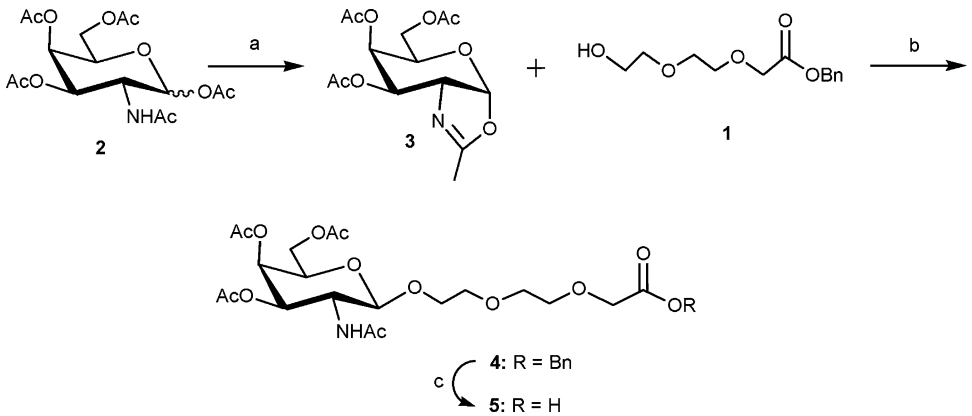

Figure 1. Reagents: (a) TMSOTf, (b) TMSOTf and (c) H_2/Pd/EtOAc.

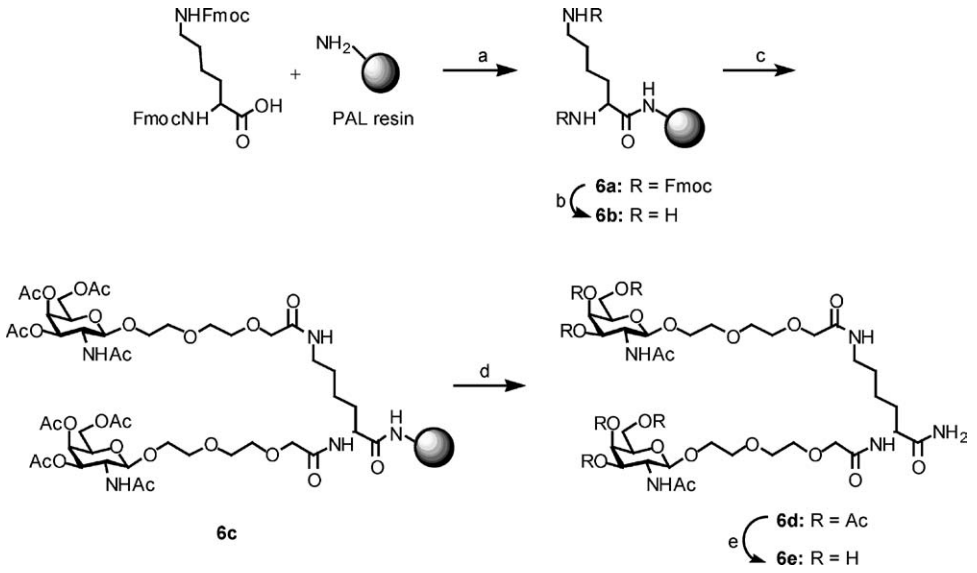

Figure 2. Reagents: (a) PyBOP/DIPEA, (b) piperidine, (c) compound **5**/PyBOP/DIPEA, (d) TFA and (e) MeONa/MeOH.

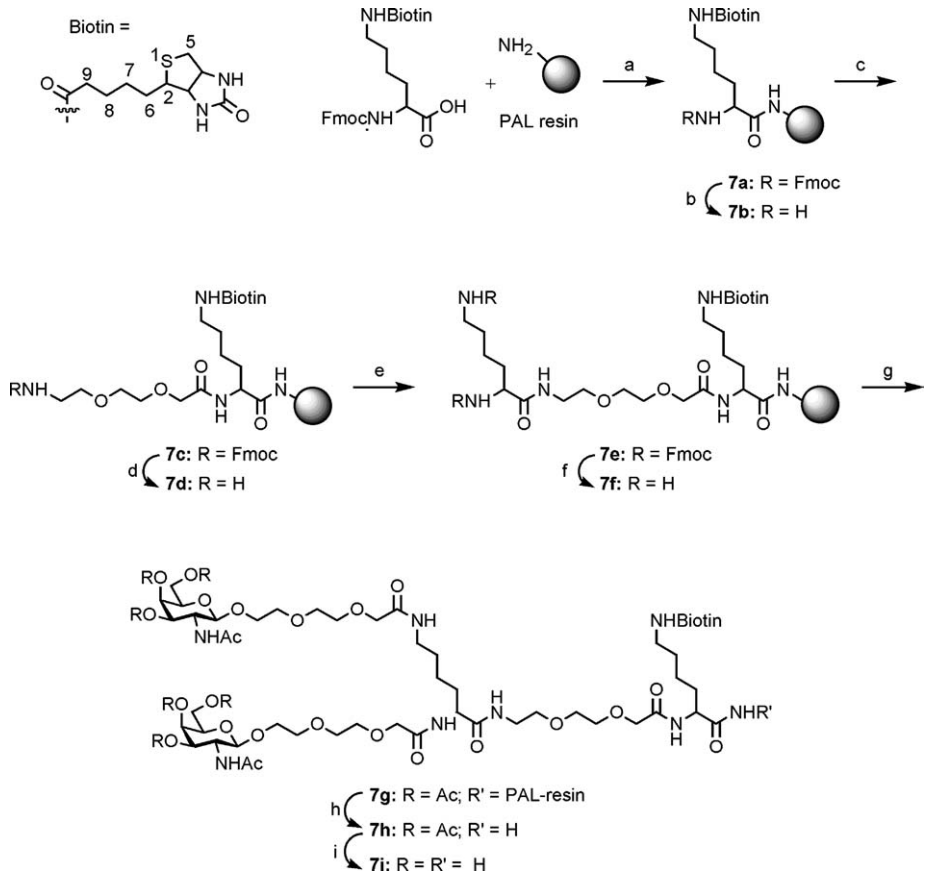

Figure 3. Reagents: (a) PyBOP/DIPEA, (b) piperidine, (c) 8-(fluorenylmethoxycarbonylamino)-3,6-dioxaoctanoic acid/ PyBOP/DIPEA, (d) piperidine, (e) N(α),N(ε)-di-Fmoc-L-lysine/PyBOP/DIPEA, (f) piperidine, (g) compound **5**/PyBOP/DIPEA, (h) TFA and (i) MeONa/MeOH.

detached from the resin using 95% aqueous TFA to give **6d**. *In situ* de-O-acetylation (using sodium methoxide) of **6d** produced **6e** (15- 29% yield, calculated from the initial amount of resin used) after purification on Bio-Gel P2.

Next, the biotin-labelled di-GalNAc compound **7i** was synthesized from **5** (Figure 3).

After treatment of Fmoc PAL solid-phase synthesis resin with piperidine/DMF to expose amino groups, N-α-Fmoc-N-ε-biotinyl-L-lysine was coupled to give **7a**. Treatment with piperidine/DMF gave amine **7b**, which was coupled with the "spacer" unit 8-fluorenylmethoxycarbonylamino-3,6-dioxaoctanoic acid to produce **7c**. The Fmoc group was removed and the product was coupled with di-Fmoc-L-lysine to give **7e**. Treatment with piperidine/DMF gave the diamine **7f**, which was then coupled with the GalNAc synthon **5** to give **7g**, which was then detached from the resin using 95% aqueous TFA to give **7h**. *In situ* de-O-acetylation (sodium methoxide) and Biogel P2 purification gave **7i** (25–46% yield, calculated from the initial amount of resin used).

The intermediate tri-GalNAc synthon **8f** was synthesized from **5** (Figure 4), either by solid-phase or solution-phase chemistry.

For the preparation of **8f** by solid-phase chemistry, N-(α)-Fmoc-N-(ε-Mtt)-Lysine-Wang resin (**8a**) was treated with piperidine/DMF to give the derivative with free α-amino groups (**8b**), and then di-Fmoc-lysine was coupled to give **8c**. The N-protection groups were removed (TFA/triisopropylsilane and piperidine/DMF, respectively) to give the triamine **8d**. Coupling with the GalNAc synthon **5** gave **8e**, which was detached from the resin using 95% aqueous TFA to give, after C-18 solid-phase extraction purification, the tri-GalNAc synthon **8f** (34% from **8a**).

For the preparation of **8f** by solution-phase chemistry, di-L-lysine dihydrochloride was treated with allyl alcohol to produce the allyl ester **8g** (98%). After coupling (DCC/HOBt) with the GalNAc synthon **5**, compound **8h** (80%) was produced. Removal of the allyl group by treatment with tetrakis(triphenylphosphine)palladium gave **8f** (69%).

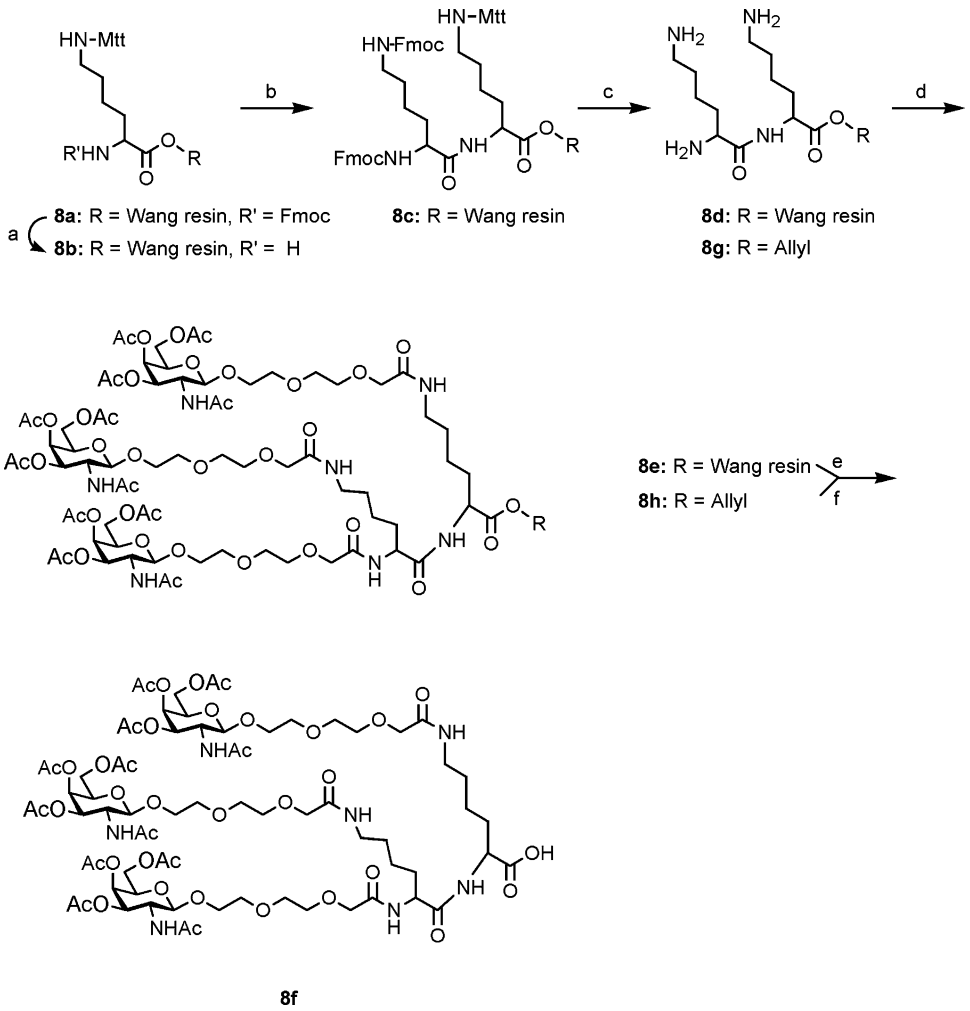

Figure 4. Reagents: (a) piperidine, (b) N(α), N(ε)-di-Fmoc-L-lysine/PyBOP/DIPEA, (c) 1. TFA/triisopropylsilane, 2. piperidine, (d) **5**+ coupling agent, (e) TFA and (f) tetrakis(triphenylphosphine)palladium.

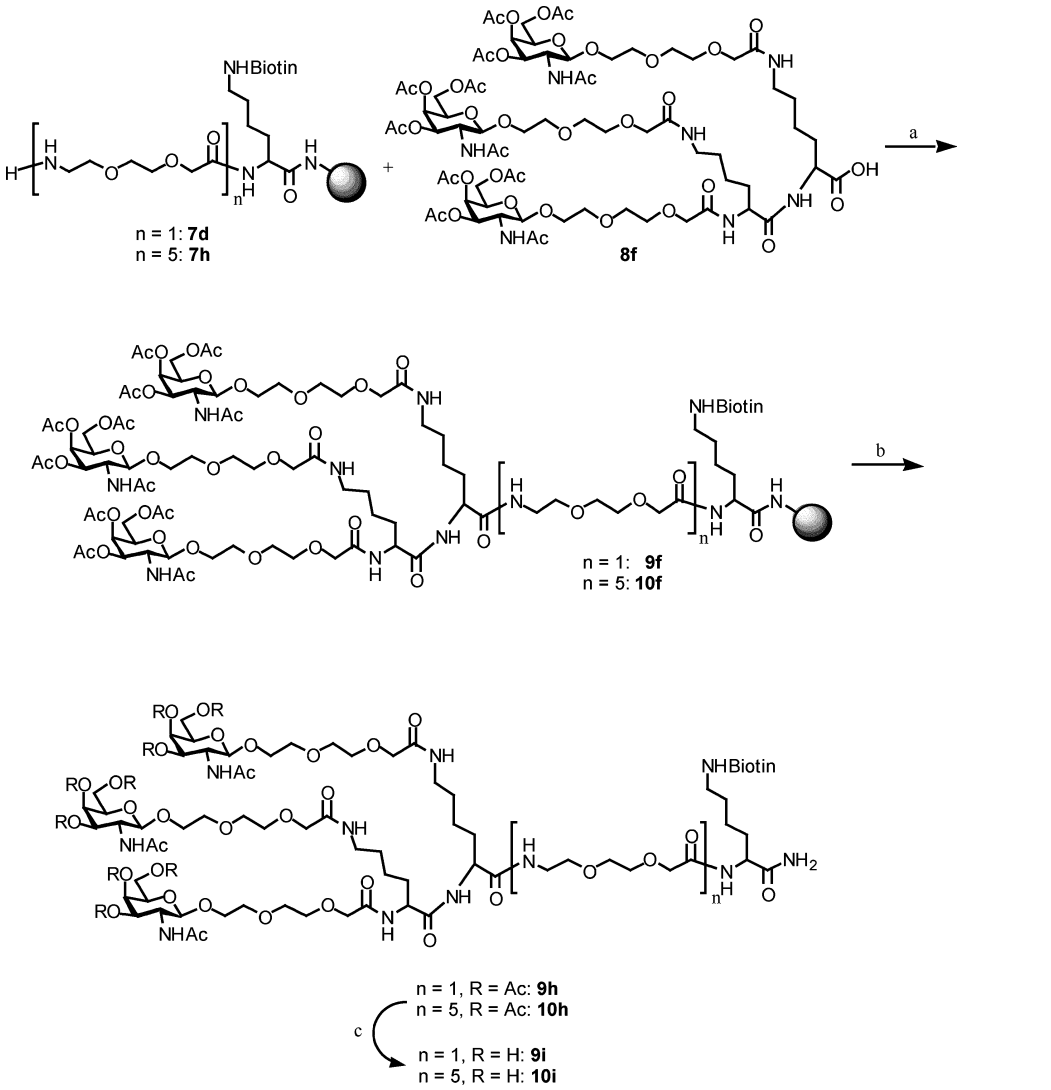

Figure 5. Reagents: (a) PyBOP/DIPEA, (b) TFA and (c) NaOMe/MeOH.

Then, the tri-GalNAc synthon **8f** was used to synthesize the biotin-labelled tri-GalNAc compound **9i** and **10i** (Figures 3 and 5).

Compound **7d** (Figure 3) was coupled with the tri-GalNAc synthon **8f** to give **9f** (Figure 5), which was then detached from the resin using 95% aqueous TFA to give **9h**. *In situ* de-O-acetylation (sodium methoxide) and Biogel P2 purification gave **9i** (24% yield, calculated from the initial amount of resin used). Compound **10i** was synthesized analogously in low (3-5%) yield, with the difference that the 8-fluorenylmethoxycarbonylamino-3,6-dioxaoctanoic acid spacer coupling cycle was repeated five times (to give **7h**) before coupling with **8f**.

Finally, the biotinylated hexa-amine **11a** (obtained by custom synthesis from a commercial source) was coupled, using TSDU activation, with the GalNAc synthon **5** to give **11b** in 63% yield, O-deacetylation of which gave the hexa-GalNAc derivative **11c** in 50% yield (Figure 6).

Biological studies

To evaluate the ability of each synthesized GalNAc-construct to mediate ASGPr specific binding and uptake into the human hepatocyte, the ligand constructs were designed to contain a biotin label on their C-terminal lysine. Fluorescent-labeled streptavidin was functionalized with the biotin-labeled GalNAc constructs, incubated with cells of the ASGPr expressing human liver cell line HepG2, and uptake monitored by FACS analysis of the cells [30]. Binding of functionalized streptavidin to cells was performed on ice to saturate binding and inhibit uptake by endocytosis. Unbound excess was removed by washing before incubation at 37°C to initiate uptake. FACS analysis of cells taking up functionalized streptavidin showed a distinct population of cells with higher intensity of green fluorescence compared to untreated cells. The fluorescence intensity of the cell population was proportional to the amount of internalized material. For the most efficient ligand, containing six GalNAc units,

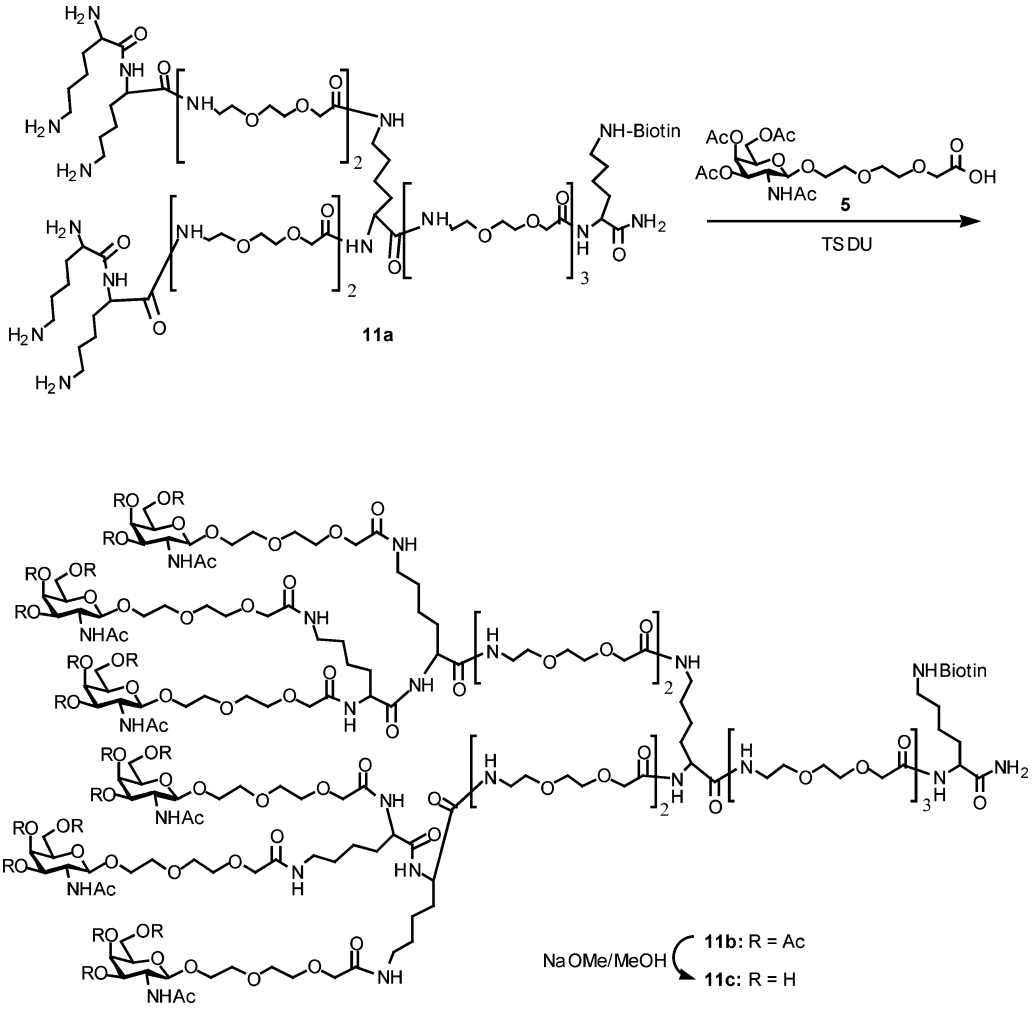

Figure 6.

80–90% of all cells performed efficient uptake of functionalized streptavidin, judged from the large shift of positive cells to high fluorescence intensities (Figure 7a–c). To confirm whether the observed fluorescence signal originated from complex internalized into cells, or from complexes bound to the outside cell surface only, cells were incubated with the targeting complex for 1.5 h on ice, washed and immediately trypsinized without further incubation at 37°C. These cells showed no increase in fluorescence compared to untreated cells, confirming that the trypsin treatment removed all receptor bound complexes on the cell surface and that the observed signal in the experimental set up originated from internalized complex (results not shown).

To establish whether the uptake was truly dependent on ASGPr mediated uptake, the dependence of calcium ion concentration for uptake was investigated. The ASGPr is a lectin receptor and consequently dependent on Ca^{2+} for binding ligands [30]. Incubation with 5mM EDTA for 10 minutes on ice, after the incubation with functionalized streptavidin, completely abolished uptake, in accordance with an ASGPr dependent uptake (Figure 7d). Furthermore, competitive binding assays were performed where functionalized streptavidin were allowed to compete for receptor binding with asialofetuin, a naturally occurring serum protein and known ASGPr ligand. Uptake of functionalized streptavidin was abolished only when preincubating the cells on ice in 1000-fold excess of asialofetuin to saturate the receptors previous to addition of the functionalized streptavidin to the cells. In contrast, when incubating functionalized streptavidin in presence of asialofetuin, without any previous incubation, 1000-fold excess of asialofetuin caused only approximately 50% reduction in uptake of functionalized streptvidin, demonstrated in (Figure 8). Finally, binding assays performed on cells that do not express the ASGPr (Neura 2A cells) resulted in no detectable uptake (data not shown).

To gain further insight in the structure-binding relationship and the importance of the number of displayed GalNAc units for efficient uptake, a set of biotin-labelled ligands (**7i**, **9i** and **11c**) were tested containing two to six GalNAc units. Fluorescent streptavidin was functionalized with each of the ligands

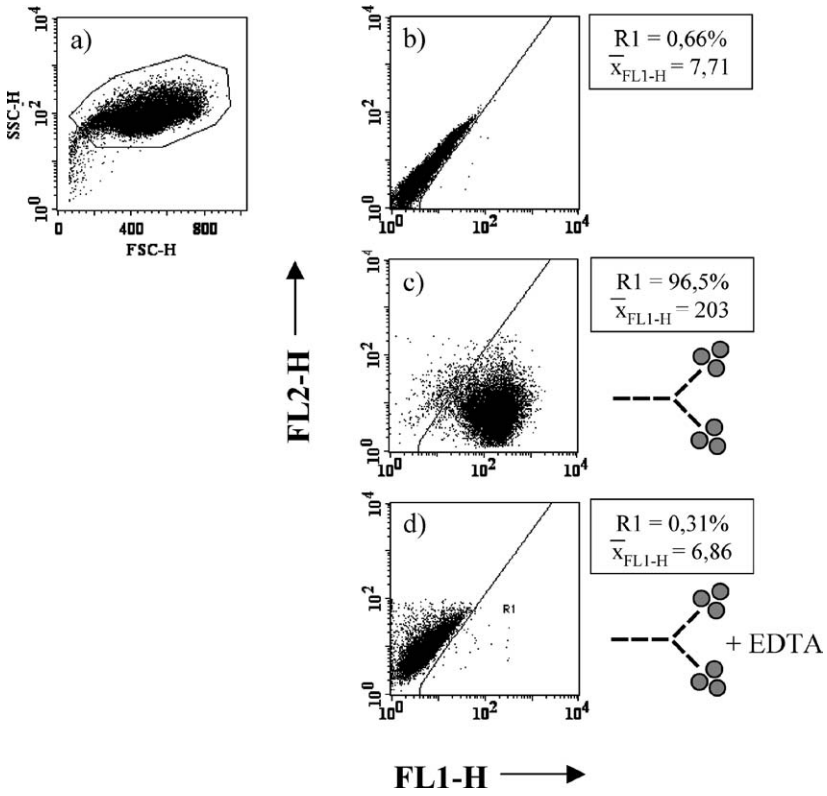

Figure 7. Receptor mediated uptake of streptavidin functionalized with ligand **11c.** Alexa Fluor 488-labeled streptavidin functionalized with ligand **11c** was incubated with ASGPr expressing HepG2 cells for 1,5 h and uptake monitored by FACS analysis. Diagram (a) represents the HepG2 cell population gated for analysis, plotted as a function of forward scatter (linear x-axis) and side scatter (logarithmic y-axis). Diagram (b)–(d) shows the green fluorescence intensity (FL1-H) of the gated cells on x-axis, plotted against red fluorescence intensity (FL2-H) on the y-axis. Both FL1-H and FL2-H are in logarithmic scale. Gate "R1" in diagram (b)–(d) defines the population of cells that has internalized functionalized streptavidin. The shift in green fluorescence intensity is proportional to the amount of internalized material and represented by the arithmetic mean of the green fluorescence intensity "x". Diagram (b)–(d) shows the green fluorescence intensity of (b) untreated cells, (c) cells treated with streptavidin functionalized with ligand **11c** and (d) cells treated with streptavidin functionalized with ligand **11c** followed by treatment with 5 mM EDTA to remove calcium. Treatment with EDTA abolished uptake in accordance with ASGPr mediated uptake.

and binding studies were performed as described. Streptavidin functionalized with ligand **11c**, displaying six GalNAc units, resulted in uptake in almost 90% of the cells. Furthermore the positive cells displayed a considerably lager shift in fluorescence intensity compared to corresponding cells incubated with streptavidin functionalized with ligands **7i** or **9i**, containing two or three GalNAc units (Figure 9). This indicated enhanced uptake efficiency with increasing number of displayed GalNAc units.

However, the ligand **11c** differs from ligands **7i** or **9i**, not only in number of displayed GalNAc units, but also in the number of ether linkers that separate the trimeric GalNAc units from the biotin anchor. Ligand **11c** contains four more linkers than ligand **7i** and **9i**, meaning that the two trimeric GalNAc units of ligand **11c** have a potentially higher accessibility to interact strongly with the receptor. To be able to value the importance of this linker length and accessibility for efficient uptake, ligand **10i** was tested, which contain one trimeric GalNAc unit, analogous to ligand **9i**, but separated with five linkers from the biotin anchor, analogous to ligand **11c**. Another set of uptake experiments was carried out as described above, to compare the uptake efficiency of ligand **7i**, **9i**, **10i** and **11c** (Figure 10). Ligand **10i**, containing five linkers, mediated uptake in 80% of the cells with a larger shift in fluorescence intensity compared to its shorter-spaced counterpart ligand **9i**. The improved flexibility and accessibility of ligand **10i** consequently influenced uptake efficiency markedly, while increasing the number of GalNAc units per ligand above three (ligand **11c**), provided only a minor contribution to the overall affinity. In all experiments fluorescent streptavidin alone, or functionalized with the di-lysine-spacer backbone without sugar units, resulted in no or very low uptake as expected.

Discussion

In order to adapt and optimize the Bioplex vector system for specific gene delivery to hepatocytes, we have evaluated the structure-function relationship for synthetic biotin-labelled

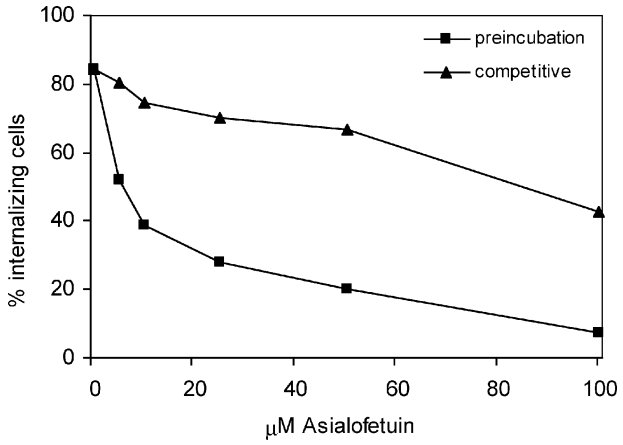

Figure 8. Competitive binding assay for streptaividin functionalized with ligand 11c in presence of different concentrations of the natural ligand asialofetuin. One representative experiment is shown where 0,1 μM of functionalized sterptavidin was used. Filled squares represents preincubation with asialofetuin for 1,5 h before washing and addition of functionalized streptavidin. Filled triangles represent simultaneous incubation with functionalized streptavidin and asialofetuin. Uptake was abolished only when the cells were preincubated with 1000 times excess of asialofetuin to saturate the receptors previous to addition of functionalized streptavidin. Simultaneous incubation with 1000 times excess of asialofetuin reduced uptake by 50%, indicating receptor affinities of ligand **11c** considerably higher than the affinity of the natural ligand asialofetuin.

ligands **7i**, **9i**, **10i**, and **11c**, designed for specific interaction with the hepatic lectin ASGPr. The synthetic pathways are summarized in Figures 1–6. The carboxylic acid **5** was prepared (Figure 1) by a modified [31,14] literature procedure and used in two principally different ways as a key mono-GalNAc synthon: a) direct acylation (Figures 3 and 6) of diamine **7f** or hexaamine **11a** gave, after deprotections, the di-GalNAc compound **7i** and the hexa-GalNAc compound **11c** in reasonable (25–46%) yields. b) Acylation (Figure 4) of triamines **8d** or **8g** gave the tri-GalNAc carboxylic acid intermediate **8f** in acceptable yields for both routes, but the liquid-phase reaction (with **8g**) was easier to scale up since the coupling consumed fewer molar equivalents of **5**. The obtained intermediate **8f** was then used (Figure 5) to acylate solid-phase bound amines **7d** or **7h** to give, after cleavage of protecting groups, the tri-GalNAc compounds **9i** and **10i**, respectively. The low (3–5%) yield of **10i** was caused by incomplete spacer solid-phase couplings and incomplete product separation on gel filtration of the product mixture (only a few relatively pure fractions were selected and used). Thus, the target biotin-labelled compounds were synthesized, demonstrating the feasability of solid-phase methodology in appropriate cases, and paving the road for later similar syntheses of GalNAc-containing PNA-constructs.

The synthesized biotin-labeled constructs **7i**, **9i**, **10i**, and **11c** were bound to Alexa Fluor 488 labeled streptavidin, and the interaction with hepatocytes was studied. The labeled streptavidin provides not only a convenient and pH stable marker for following the functional properties of each ligand. It also provides information on the ability of each ligand to bring in large molecules of at least 60 kDa size into hepatocytes through the receptor mediated pathway, and thereby mimicking their intended use in the Bioplex vector system.

We have shown in this study that all synthesized ligands containing GalNAc were able to mediate uptake of functionalized streptavidin into HepG2 cells to different extent. In agreement with earlier reports, uptake efficiency increased with number of displayed GalNAc units per ligand [2,12]. Ligands containing two and three GalNAc-units (**7i** and **9i**) showed a moderate but increasing uptake compared to ligand **11c** containing two trimeric GalNAc units. The ligand **11c** showed a markedly increased uptake efficacy both in number of internalizing cells as well as in increased amount of material taken up per each cell. Since the carbohydrate binding subunits of the ASGPr are known to cluster in coated pits on the cell surface in a rigid and lattice-like configuration [21,23] we anticipated that ligands displaying multiples of the trimeric GalNAc unit would display a higher affinity and uptake efficiency. The observed increase in uptake efficiency for ligand **11c** was however not exclusively due to the multiple GalNAc units. In contrast to ligand **7i** and **9i**, the five ether-linkers separating the biotin label and each trimeric GalNAc unit in ligand **11c** provides improved flexibility and accessibility for the sugar units to interact strongly with the binding sites of the receptor and contributes to high affinity. This was confirmed by comparing uptake mediated by ligand **9i** and ligand **10i**, containing one trimeric GalNAc unit each, but differing in spacing from the biotin label with one or five linkers, respectively. The uptake efficiency proved to be markedly higher for ligand **10i** than for its shorter-spaced counterpart. Despite lower number of GalNAc units of ligand **10i**, its uptake efficiency was surprisingly close to the uptake efficiency of ligand **11c**, containing twice as many GalNAc units. Consequently, proper spacing of the trimeric GalNAc units to provide accessibility is most important for high affinity interactions with the receptor, while adding an additional trimeric GalNAc unit provides a minor contribution to the affinity.

Uptake mediated by the synthesized ligands was truly dependent on the ASGPr receptor pathway, since addition of EDTA to remove calcium ions completely abolished uptake as expected for a lectin receptor. Furthermore, incubation of HepG2 cells with ligand at 4°C only, resulted in no uptake indicating that uptake is mediated through an active transport mechanism. In addition, binding assays performed with cells not expressing the ASGPr (Neura 2A cells) showed no uptake of functionalized streptavidin as expected. Also the competitive binding studies, where the natural ligand asialofetuin were pre-incubated with the cells to allow saturation of the binding sites of the receptors, blocked the uptake and indicated receptor dependent uptake. In contrast, the co-incubation with asialofetuin and ligand could not reduce uptake with more than

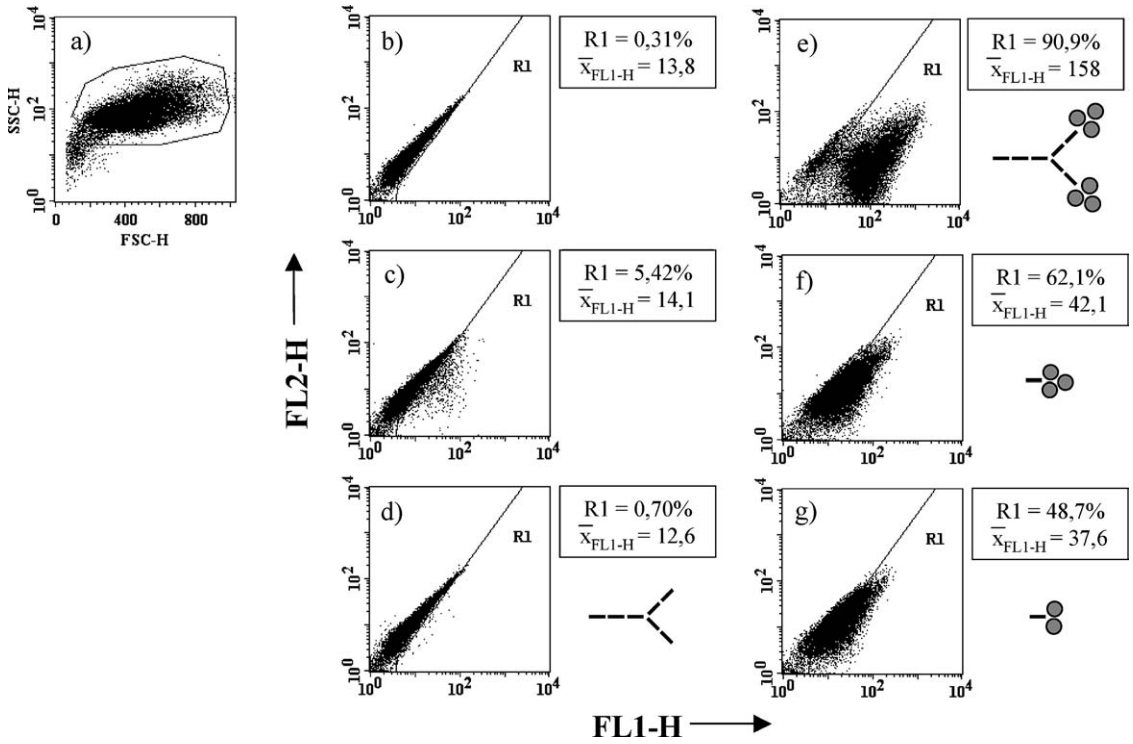

Figure 9. FACS analysis of uptake efficiency to evaluate its dependency on the number of displayed sugar units. Alexa Fluor 488-labeled streptavidin was functionalized with each of ligand **7i**, **9i** and **11c**, containing two to six GalNAc units, incubated with HepG2 cells for 1,5 h and uptake was monitored by FACS analysis. Diagram (a)–(h) are presented as described in Figure 7. Diagram (b)–(g) shows the green fluorescence intensity of (b) untreated cells, (c) cells treated with non-functionalized streptavidin only, (d) cells treated with streptavidin functionalized with **11a** (which is the hexameric derivative **11c** without sugar units), (e) cells treated with streptavidin functionalized with ligand **11c** (f) cells treated with streptavidin functionalized with ligand **9i** and (g) cells treated with streptavidin functionalized with ligand **7i**. Ligand **11c,** containing two trimeric GalNAc units, showed a markedly increased uptake both in number of internalizing cells and amount of material taken up per cell, while ligands of two and three (**7i** and **9i**) sugars showed a moderate uptake only.

50% even at 1000-fold excess of asialofetuin. This suggests that our synthesized ligand is taken up through receptor dependent interactions with affinities considerably higher than the affinity of the natural ligand asialofetuin.

Consequently, in this study the ligand **11c**, containing duplicate trimeric GalNAc units and five ether linkers, proved to be the best ligand for delivering large molecules into hepatocytes with high efficiency. Still, the more complex procedure required for synthesizing this ligand, and its subsequently lower gain, turns the focus to the simpler, but still highly efficient ligand **10i**, containing a single trimeric GalNAc and five linkers. This ligand provides an attractive candidate for future incorporation as targeting function in the Bioplex vector system.

Materials and methods

Materials

Alexa Fluor 488-labeled streptavidin was from Molecular Probes (Leiden, Netherlands), asialofetuin, Fmoc-PAL resin (100–200 mesh, 1% crosslinked), 8-fluorenylmethoxycarbonylamino-3,6-dioxaoctanoic acid, N(α),N(ε)-di-Fmoc-L-Lysine and N(α)-Fmoc-N(ε)-biotinyl-L-Lysine, Di-L-lysine dihydrochloride and trifluoroacetic acid (TFA) were from Sigma-Aldrich (Sweden). Fmoc-Lys-(Mtt)-Wang resin (100–200 mesh), and benzotriazol-1-yloxytris(pyrrolidino)phosphoniumhexafluorophophate (PyBOP) was from Merck Biosciences Ltd (Beeston/Nottingham) and N-acetyl-D-galactosamine was from Senn Chemicals (Dielsdorf, Switzerland). Trimethylsilyl trifluoromethanesulfonate was from Acros organics, Netherlands, and compound **11a** was synthesized on special order by Neosystems, Inc (Strasbourg, France). *O*-Succinimidyl-1,3-dimethyl-1,3-trimethyleneuronium tetrafluoroborate (TSDU) was freshly prepared [32] immediately before use.

General methods, synthesis

Reactions were monitored by TLC on silica gel 60 F_{254} (Merck) glass plates, using detection with UV light and/or charring with 5% H_2SO_4 or AMC-solution (ammonium molybdate, cerium (IV) sulfate, 10% sulfuric acid [5: 0.1: 100, w/w/v]). Column chromatography was performed on silica gel (Matrex Silica Si, 60 Å, 35–70 μm or Normasil, 40–63 μm, Prolabo, VWR

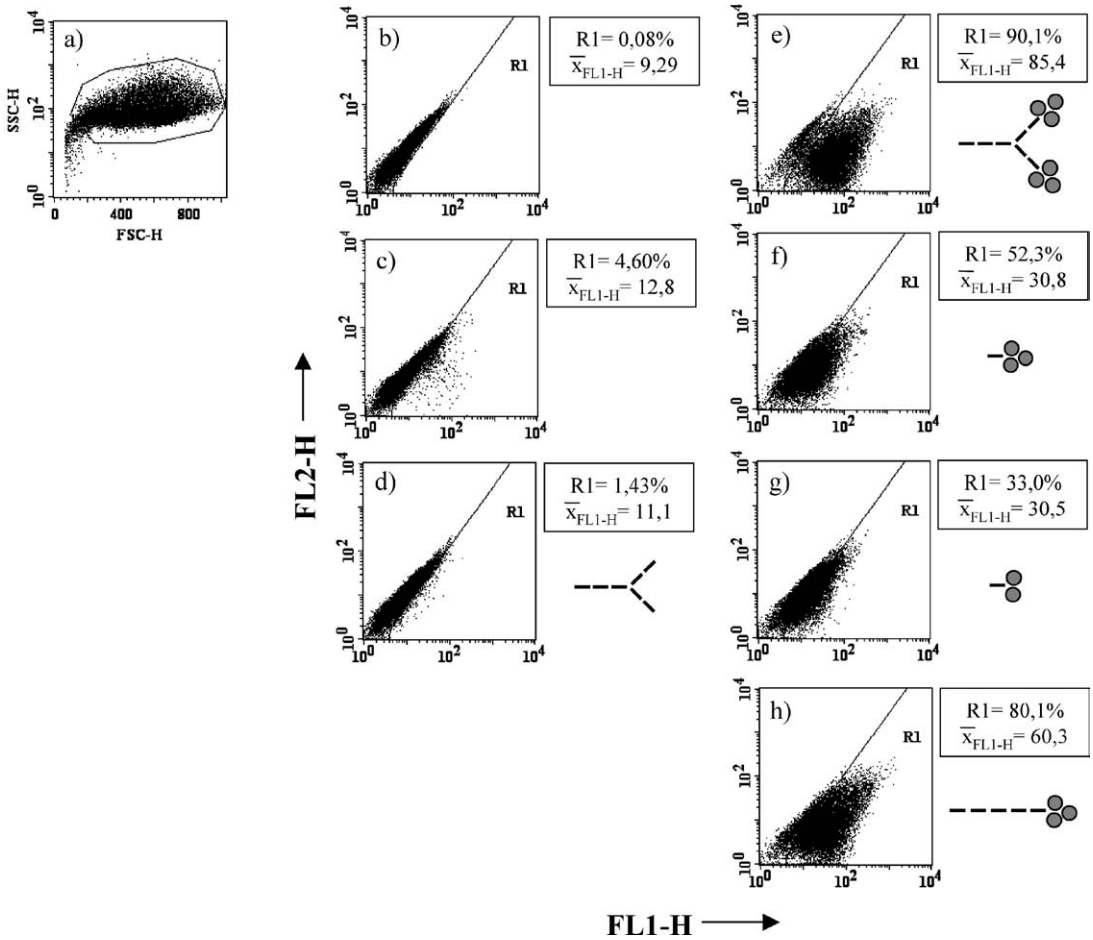

Figure 10. FACS analysis of uptake efficiency to evaluate its dependency on the spacing between the sugar units and the biotin. Alexa Fluor 488-labeled streptavidin was functionalized with each of ligand **7i**, **9i**, **10i and 11c**, incubated with HepG2 cells for 1,5 h and uptake was monitored by FACS analysis. Diagram (a)–(h) are presented as described in Figure 7. Diagram (b)–(h) shows the green fluorescence intensity of (b) untreated cells, (c) cells treated with non-functionalized streptavidin only, (d) cells treated with streptavidin functionalized with **11a**, (e) cells treated with streptavidin functionalized with ligand **11c** (f) cells treated with streptavidin functionalized with ligand **9i**, (g) cells treated with streptavidin functionalized with ligand **7i** and (h) cells treated with streptavidin functionalized with ligand **10i**. The uptake efficiency of the longer spaced ligand **10i** proved to be markedly higher than its shorter-spaced counterpart ligand **9i**. Despite lower number of GalNAc units of ligand **10i** compared to ligand **11c**, its uptake efficiency was surprisingly close to the uptake efficiency of ligand **11c**, indicating strong dependency on proper spacing and accessibility for high affinity interactions with the receptor.

International), and size-exclusion chromatography on Biogel P-2 or P-4. Solvents were evaporated under reduced pressure at <40°C (water bath). Powdered 4 Å molecular sieves (Fluka) were dried under reduced pressure overnight at 300°C before use. NMR spectra were recorded at 30°C with Bruker or Varian 400 MHz spectrometers, using $CDCl_3$ (internal $CHCl_3$ δ_H 7.260 ppm, δ_C 77.0 ppm), D_2O (internal acetone δ_H 2.185 ppm, δ_C 30.3 ppm), DMSO-d_6 (internal DMSO δ_H 2.474 ppm, δC 39.0 ppm) or CD_3OD (internal CH_3OH δ_H 3.31 ppm, δC 49.2 ppm). Chemical shifts are reported in parts per million (ppm, δ). All ^{1}H assignments were based on 2D experiments. Only selected NMR data are reported. In the NMR assignments, greek letters denote lysine atoms, and arabic numbers denote biotin (italic) or galactose atoms (normal). ESI positive- and negative-ion mass spectra were recorded on an Esquire LC ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI ion source. Positive ion MALDI-TOF mass spectra were recorded on a Bruker Reflex III time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) using 2′, 4′, 6′ -trihydroxyacetophenone (THAP) or 2,5-dihydroxybenzoic acid (DHB) as matrix.

Solid phase synthesis was carried out manually using Fmoc-PAL resin unless otherwise stated. The agitation was performed by rotating the sample in a closed reaction vessel. The resin was swelled with dichloromethane for 30 min and washed with N,N-dimethylformamide (DMF) (5 × 1 min). The resin was then exposed to the following general cycle:

1	20% Piperidine in DMF	3 ×10 min
2	DMF	5 × 1 min
3	Isopropanol	5 × 1 min
4	DMF	5 × 1 min
5	Carboxylic acid, PyBOP and DIPEA	———
6	DMF	5 × 1 min
7	Isopropanol	5 × 1 min
8	DMF	5 × 1 min

Kaiser ninhydrin tests were performed after step 3 and 7. The Fmoc group was deprotected using 20% piperidine in DMF. Before the product was cleaved from the resin it was washed with diethyl ether (5 × 1 min) and dried by suction filtration for 1 h. Then TFA-water 95:5 was added and the mixture was agitated for 3 h. The resin was then removed by filtration and washed twice with TFA-water 95:5. The filtrate was concentrated and triturated with diethyl ether. The precipitate was decanted, dissolved in water and lyophilized.

Synthesis of specific compounds

Benzyl 8-hydroxy-3,6-dioxaoctanoate (1)

A solution of diethylene glycol (20 g, 182 mmol) in dry DMF (120 mL) was mixed with sodium hydride (80% in oil, 3.6 g, 120 mmol) and then bromoacetic acid (8.34 g, 60 mmol) in dry DMF (15 mL) was added, and the mixture was swirled by hand, since it became very thick on addition of sodium hydride (the mixture became less thick again after addition of the acid). The mix was stirred at 80°C for 60 min, then it was cooled to RT and benzyl bromide (9.9 g, 58 mmol) was added. After 60 min at 80°C, the mixture was partitioning between ethyl acetate (100 mL) and aq. 1M HCl (500 mL). The aqueous layer was saturated with KCl (30–40 g) and after-washed with 2 × 200 mL ethyl acetate. The combined organic layers were concentrated (normal vacuum, then pump vacuum, residue 10.5 g) and purified by silica gel chromatography (ethyl acetate as eluant) to give **1** (2.8 g, 11.0 mmol, 19%). NMR data were very similar to those reported for the ethyl ester [31].

Benzyl 8-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl)-3,6-dioxaoctanoate (4)

D-galactosamine pentaacetate **(2**, 3.5 g, 9.05 mmol, obtained from N-acetyl-D-galactosamine by acetylation with 1:2 acetic anhydride/pyridine) was dissolved in dichloromethane (35 mL) and trimethylsilyl trifluoromethanesulfonate (4.2 mL, 22 mmol) was added. The mixture was stirred at 50°C for 16 h under anhydrous conditions, then triethylamine (1.75 mL) was added at 0°C. The solution was washed with saturated. aq. $NaHCO_3$ and water, dried and concentrated to give crude **3**. This material, 4 Å molecular sieves (2.5 g), and **1** (3.5 g, 13.76 mmol) were dissolved in dichloromethane (50 mL) and stirred for 30 min. Trimethylsilyl trifluoromethanesulfonate (875 μL, 4.5 mmol) was added and the mixture was stirred overnight at RT. Triethylamine (875 μL) was added while cooling in a ice-water bath and the mixture was then filtered. The filtrate was diluted with dichloromethane and washed with water, 1 M $NaHCO_3$ and water, dried and evaporated. The crude mixture was purified by silica gel column chromatography (ethyl acetate, then ethyl acetate-acetic acid-methanol-water 60:3:3:2) to give **4** (4.15 g, 7.11 mmol, 79% from **2**). ^{1}H NMR ($CDCl_3$): δ 7.36 (m,5H, $PhCH_2$), 6.82 (d, 1H, $J = 9.8$ Hz, NH), 5.25–5.16 (m, 3H, $PhCH_2$, H-4), 4.98 (m, 2H, H-1, H-3), 4.35–4.24 (m, 2H, H-2, H-6a), 4.18–4.12 (m, 3H, H-6b, CH_2), 2.15, (3, 3H, OAc), 2.05 (s, 3H, OAc), 1.95 (s, 3H, OAc), 1.94 (s, 3H, OAc).

8-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl)-3,6-dioxaoctanoic acid (5)

Compound **4** (4.15 g, 7.11 mmol) was dissolved in ethyl acetate (50 mL) and a slurry of 10% Pd/C (2.0 g) in ethyl acetate (50 mL) was added. The mixture was flushed with nitrogen and then with hydrogen and thereafter stirred overnight at R.T under a hydrogen atmosphere. The solution was filtered, the filtrate concentrated, and the residue was purified by column chromatography (dichloromethane, then a gradient from 2-100% MeOH in dichloromethane). Appropriate fractions were pooled, concentrated, and lyophilized from benzene to give **5** (2.12 g, 4.30 mmol, 60%). ^{1}H NMR (DMSO-d_6): δ 8.04 (d, 1H, $J = 9.1$ Hz, NH), 5.20 (d, 1H, $J = 3.4$ Hz, H-4), 4.98 (dd, 1H, $J = 3.4$ Hz, H-3), 4.61 (d, 1H, $J = 8.6$ Hz, H-1), 4.05-3.96 (m, 3H, H-5, H-6 a, H-6b), 2.09 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.87 (s, 3H, OAc), 1.77 (s, 3H, NHAc). ^{13}C NMR (DMSO-d_6): δ 171.5 (CO_2H), 169.4 (OAc), 169.3 (OAc), 169.0 (OAc), 100.4 (C-1), 70.0 (C-3), 69.3 (C-5), 68.8 (CH_2), 67.6 (CH_2), 66.2 (C-4), 61.0 (C-6), 48.8 (C-2), 22.2 (NHAc), 20.0 (OAc), 19.9 (OAc). ESI-MS (positive- ion mode): $[M + Na]^+ = 516.2$, ESI-MS (negative- ion mode): $[M + H]^- = 491.8$.

Compound **6e**

Fmoc-PAL resin (167 mg, 0.07–0.13 mmol) was swelled with dichloromethane (3 mL) for 30 min and the Fmoc group was deprotected using 20% piperidine in DMF (2 mL). N(α), N(ε)-di-Fmoc-L-Lysine (295 mg, 0.5 mmol) and PyBOP (260 mg, 0.5 mmol) were dissolved in DMF (2 mL) and DIPEA (175 μL, 129 mg, 1.0 mmol) was added. The solution was then added to the resin and the mixture was agitated for 1.5 h. The resin (**6a**) was washed with DMF (5 × 3 mL), isopropanol (5 × 3 mL) and DMF (5 × 3 mL), whereafter the Fmoc groups were cleaved off with 20% piperidine in DMF to give resin **6b**. A mixture of **5** (400 mg, 0.8 mmol) and PyBOP (416 mg, 0.8 mmol) in DMF (3 mL), containing DIPEA (350 μL, 258 mg, 2.0 mmol) was added to the resin. After agitation overnight, the resin (**6c**) was washed with DMF (5 × 3 mL), isopropanol (5 × 3 mL) and diethyl ether (5 × 3 mL) and dried by suction filtration for 1 h.

TFA-water 95:5 was then added and the resin was agitated for 3 h. The resin was removed by filtration and washed twice with TFA-water 95:5. The filtrate was concentrated and triturated with diethyl ether (3 mL). The precipitate was decanted, dissolved in water and lyophilized to give crude **6d** (64 mg, 0.058 mmol). The material was dissolved in dry methanol (1 mL) and 0.5 M sodium methoxide (100 μL) was added. The mixture was stirred for 4 h and was then neutralized with acetic acid (50 μL). The crude product was concentrated and purified on a Biogel P-2 column, using water as eluant, to give **6e** (17 mg, 0.020 mmol, 15-29%). 1H NMR (D_2O): δ 4.46 (d, 2H, $J = 8.6$ Hz, H-1, H-1′), 4.29 (dd, 1H, $J = 5.6$ Hz, 9.1 Hz, alfa), 3.91 (m, 4H, H-4, H-4′, H-2, H-2′), 3.22 (t, 2H, $J = 7.1$ Hz, ε), 2.00, (s, 3H, NHAc), 1.99 (s, 3H, NHAc), 1.88–1.68 (m, 2H, β), 1.53 (pentet, 2H, $J = 14.2$ Hz, 7.34 Hz, δ), 1.45–1.26 (m, 2H, γ). 13C NMR (D2O): δ 101.5 (C-1, C-1′), 53.1 (α), 38.6 (ε), 30.6 (β), 27.9 (δ), 22.5 (γ), 22.3 (NHAc). ESI-MS (positive- ion mode): [M+Na]+ = 866.5.

Compound **7i**

The solid phase synthesis of **7i** was carried out according to the procedure for **6e** and the general cycle conditions. Fmoc-PAL resin (167 mg, 0.07–0.13 mmol) was swelled with dichloromethane (3 mL). After deprotection of the Fmoc groups the resin was coupled with N(α) -Fmoc-N(ε)-biotinyl-L-Lysine (0.5 mmol, 297 mg) using PyBOP (0.5 mmol, 260 mg) and DIPEA (1.0 mmol, 175 μL) in DMF (2 mL) for 3 h, to give resin **7a**. The Fmoc group was deprotected again (to give **7b**) and a mixture of 8-fluorenylmethoxycarbonylamino-3,6-dioxaoctanoic acid (0.5 mmol, 193 mg), PyBOP (0.5 mmol, 260 mg) and DIPEA (1.0 mmol, 175 μL) in DMF (2 mL) was added and the mixture was agitated for 3 h, which gave **7c**. The Fmoc group was removed (to give **7d**) and a solution of N(α), N(ε)-di-Fmoc-lysine (295 mg, 0.5 mmol), PyBOP (260 mg, 0.5 mmol) and DIPEA (175 μL, 129 mg, 1.0 mmol) in DMF (2 mL) was added and the mixture was agitated for 3h, which gave **7e**. The Fmoc groups were removed (to give **7f**) and **5** (400 mg, 0.8 mmol), PyBOP (416 mg, 0.8 mmol) and DIPEA (350 μL, 258 mg, 2.0 mmol) dissolved in DMF (3 mL) was added and the mixture was agitated overnight. The product **7h** was cleaved from the resin and the O-acetyl groups were deprotected according to the procedure for **6e**. The crude material was then purified on a Biogel P-2 column, using water as eluant, to give **7i** (44 mg, 0.033 mmol, 25–46%). ^{1}H NMR (D_2O): δ 4.57 (dd, 1H, $J = 4.9$ Hz, 7.8 Hz, *H*-4), 4.46 (d, 2H, $J = 8.6$ Hz, H-1′, H-1′), 4.38 (dd, 1H, $J = 4.7$ Hz, 7.8 Hz, *H*-3), 4.34–4.25 (m, 2H, α, α'), 3.91–3.83 (m, 4H, H-4, H-4′, H-2, H-2′), 3.28 (ddd, 1H, $J = 5.1$ Hz, 9.5 Hz, H-2), 3.21 (t, 2H, $J = 7.1$ Hz, ε), 3.15 (t, 2H, $J = 6.9$ Hz, ε'), 2.95 (dd, 1H, $J = 5.1$ Hz, 13.0 Hz, *H*-5*a*), 2.74 (d, 1H, $J = 13.0$ Hz, *H*-5*b*), 2.20 (t, 2H, $J = 7.1$ Hz, *H*-9), 2.00, (s, 3H, NHAc), 1.99 (s, 3H, NHAc). ^{13}C NMR (D_2O): δ 101.6 (C-1, C-1′), 67.9 (C-4, C-4′), 62.2 (*C*-3), 60.2 (*C*-4), 53.1 (α, α'), 39.7 (*C*-5), 39.0 (ε'), 38.7 (ε), 35.5 (*C*-9), 22.3 (NHAc). ESI-MS (positive- ion mode): $[M+Na]^+ = 1617.6$.

Compound **8f**

By solid-phase synthesis. Fmoc-Lys(Mtt)-Wang resin (**8a**, 139 mg, 0.1 mmol) was swelled in DCM (3 mL) and the Fmoc group was removed according to the general protocol to give **8b**. A mixture of N(α), N(ε)-di-Fmoc-lysine (295 mg, 0.5 mmol), PyBOP (260 mg, 0.5 mmol), and DIPEA (175 μL, 129 mg, 1.0 mmol) in DMF (2 mL) was then added to the resin and the mixture was agitated for 5 h to give **8c**. The 4-methyltrityl (Mtt) group was removed by washing with dichloromethane (5 × 3 mL) and then treatment with 1% TFA and 5% triisopropylsilane in dichloromethane (3 × 3 mL, each time for 15 min). The Fmoc groups were deprotected to give **8d** and then **5** (592 mg, 1.2 mmol), PyBOP (624 mg, 1.2 mmol) and DIPEA (525 μL, 3.0 mmol) in DMF (5 mL) was added and the mixture was agitated overnight to give **8e**. The material was cleaved from the resin according to general methods. The residue was diluted with water to 5 mL, and then slowly passed through a C-18 Isolute cartridge (3g, pre-washed with first 20 mL of methanol, then 100 mL of water). The cartridge was washed with 20 mL of water and then the material was eluted with 10–60% methanol to give **8f** (57 mg, 0.034 mmol, 34%), for spectral data see below.

By liquid-phase synthesis. Di-L-lysine dihydrochloride (204 mg, 0.59 mmol) was added to a solution of allyl alcohol (20 mL) and trimethylsilyl chloride (5 mL) and the mixture was stirred at 70°C until all material had dissolved (15 min–2 h). After concentration, **8g** (245 mg, 0.58 mmol, 98%) was obtained as a hygroscopic trihydrochloride salt, NMR (CD_3OD): ^{13}C, δ21.3, 22.6, 26.7 (2×), 30.2, 30.7 (CH_2), 39.0, 39.1 (CH_2NH_2), 52.5, 52.6 ($CHNH_2$), 65.7, 117.6, 132.0 (OAllyl), 169.1 (*C*OOAllyl), 171.4 (CO); ^{1}H, δ1.56 (m, 4H, CH_2), 1.72 (m, 4H, CH_2), 1.93 (m, 4H, CH_2), 2.95 (dd, 4H, CH_2NH$_2$), 4.06 (t, 1H, C*H*NH$_2$), 4.48 (dd, 1H, C*H*NH$_2$), 4.64 (dd, 2H, OAllyl), 5.24 (dd, 1H, OAllyl), 5.36 (dd, 1H, OAllyl), 5.94 (m, 1H, OAllyl).

Compound **8g** (70 mg, 0.17 mmol) was added to a pre-mixed (0°C, 30 min) solution of compound **5** (300 mg, 0.61 mmol), N-hydroxybenzotriazole (100 mg, 0.65 mmol) and N, N′-dicylcohexylcarbodiimide (150 mg, 0.73 mmol) in ethyl acetate (5 mL). After 2 h stirring at RT, the reaction mixture was filtered through a plug of Celite and silica gel, concentrated and purified on a silica gel column (dichloromethane → dichloromethane/methanol 10:1) yielding **8h** (235 mg, 0.14 mmol, 80%), NMR ($CDCl_3$): ^{13}C, δ 20.3, 22.5, 22.8, 24.7, 28.7, 28.8, 29.5, 31.8, 33.6, 38.1 (CH_2NH_2), 38.4 (CH_2NH_2), 50.6 (C-2), 52.2, 52.4 ($CHNH_2$), 61.4 (3x), 65.8 (OAllyl), 66.7 (3×), 68.3, 68.4, 69.9, 70.1, 70.4, 70.5, 70.8, 100.9 (C-1), 118.7 (OAllyl), 131.4 (OAllyl), 170.4, 170.4, 170.5, 170.6, 171.4, 171.5, 171.7, 172.1 (CO); ^{1}H, δ 1.35 (m, 4H, CH_2), 1.50 (m, 4H, CH_2), 1.66 (m, 4H, CH_2), 1.87, 1.88 (s, 9H), 1.94, 1.95 (s, 9H), 2.00, 2.01 (s, 9H), 2.10, 2.11 (s, 9H),

3.15-4.13 (m, 47H), 4.33-4.77 (m, 6H), 5.13-5.29 (m, 8H), 5.84 (m, 1H, OAllyl). MALDI-MS: 1762.7 $[M+Na]^+$, 1778.7 $[M+K]^+$ found: 1762.3 $[M+Na]^+$, 1778.2 $[M+K]^+$.

Tetrakis (triphenylphosphine)palladium (18 mg, 16 μmol) was added to a solution of **8h** (270 mg, 0.16 mmol) and morpholine (650 μL, approx. 50 equiv.) in tetrahydrofuran (27 mL). After 3 h, more catalyst (9 mg) was added and stirring was continued for 3 h. The mixture was filtered through a sandwich of filters (10 μm on top of a 5 μm filter pellet), concentrated and purified on a silica gel column (packed in dichloromethane, eluted with a gradient of up to 25% methanol in dichloromethane) yielding **8f** (180 mg, 0.11 mmol, 69%). NMR data ($CDCl_3$) for **8f**: ^{1}H, δ 7.50 (d, 1H, $J = 8.1$, NH-α), 7.46 (d, 1H, $J = 6.9$ Hz, NH-α'), 7.17 (2H, NH-ε, NH-ε'), 7.04 (d, 1H, $J = 9.05$ Hz, NH-Gal), 6.77 (d, 1H, $J = 8.8$ Hz, NH-Gal′), 6.69 (d, 1H, 8.8 Hz, NH-Gal″), 5.39-5.33 (m, 3H, H-4, H-4′, H-4″), 5.32-5.21 (m, 3H, H-3, H-3′, H-3″), 4.88 (d, 1H, $J = 8.3$ Hz, H-1), 4.82 (d, 1H, $J = 8.6$ Hz, H-1′), 4.78 (d, 1H, $J = 8.3$ Hz, H-1″), 4.55 (t, 1H, α), 4.36 (t, 1H, $J = 6.6$ Hz, α'), 2.15 (s, 9H, OAc), 2.05 (s, 9H, OAc), 2.00, (s, 9H, OAc), 1.96 (s, 3H, NHAc), 1.95 (s, 3H, NHAc), 1.93 (s, 3H, NHAc). ^{13}C, δ 101.0 (C-1), 100.9 (C-1″), 100.7 (C-1′), 70.3 (C-3″), 70.2 (C-3′), 70.0 (C-3), 66.6 (C-4, C-4′, C-4″), 22.9 (NHAc) ,20.5 (OAc). ESI-MS (positive- ion mode): $[M+Na]^+ = 1722.9$, ESI-MS (negative- ion mode): $[M+H]^- = 1698.8$.

Compound **9i**

This compound was synthesized similarly to **7f**, starting with Fmoc-PAL resin (16,7 mg, 7–13 μmol), and carrying on with an identical procedure up to compound **7d**. Then compound **8f** (51 mg, 3 μmol) was used in the coupling instead of N(α), N(ε)-di-Fmoc-lysine. The product **9f** was treated with 95% aq TFA to give **9h,** which was de-O-acetylated, purified (Biogel P-2), and lyophilized to give **9i** (3 mg, 1.65 μmol, 13–24%). ^{1}H NMR (D_2O): δ 4.60 (dd, 1H, $J = 5.3$ Hz, 7.9 Hz, $H-4$), 4.88 (d, 1H, $J = 8.3$ Hz, H-1), 4.50 (d, 3H, $J = 8.4$ Hz,H-1, H-1′, H-1″), 4.41 (dd, 1H, $J = 4.6$ Hz, 7.9 Hz, $H-3$), 4.38-4.22 (m, 3H, α, α', α''), 3.95-3.87 (m, 6H, H-4,H-4′, H-4″, H-2, H-2′, H-2″), 3.32 (ddd, 1H, $J = 5.1$ Hz, 9.5 Hz, $H-2$), 3.23 (m, 4H, ε, ε'), 3.18 (t, 2H, $J = 6.8$, ε''), 2.99 (dd, 1H, $J = 5.1$ Hz, 13.2 Hz, $H-5a$), 2.77 (d, 1H, $J = 13.0$ Hz, $H-5b$), 2.24 (t, 2H, $J = 7.2$ Hz, $H-9$), 2.03 (s, 9H, NHAc). ^{13}C NMR (D_2O): δ 101.8 (C-1, C-1′, C-1″), 68.2 (C-4, C-4′, C-4″), 62.4 ($C-3$), 60.6 ($C-4$), 55.9 ($C-2$), 54.2 (α''), 53.6 (α, α'), 40.0 ($C-5$), 39.3 (ε''), 39.1 (ε, ε'), 35.9 (C-9), 22.6 (NHAc). ESI-MS (positive- ion mode): $[M+Na]^+ = 1843.9$.

Compound **10i**

This compound was synthesized similarly to **9i** using Fmoc-PAL resin (12.5 mg, 5.3–9.8 μmol), but with repeated coupling cycles for 8-fluorenylmethoxycarbonylamino-3,6-dioxaoctanoic acid (total of five times). The de-O-acetylated material was purified on a Bio-Gel P4 column, and fractions were analyzed by MALDI-MS. Appropriate fractions were collected to give **10i** (0,6 mg, 0.25 μmol, 3–5%). MALDI-MS: $[M+Na]^+ = 2423.1$.

Compound **11b**

O-Succinimidyl-1,3-dimethyl-1,3-trimethyleneuronium tetrafluoroborate (TSDU) (11 mg, 35 μmol) was added at room temperature to a vial (1.5 mL) containing a solution of **5** (15 mg, 30 μmol) and triethylamine (5.5 μL, 39 μmol) in dimethylformamide (100 μL). After 10 min, compound **11a** (5.0 mg, 2.46 μmol in 100 μL 0.01M borate buffer, pH 8.5) was added to the active ester mixture and after additional 30 min, a MALDI-TOF experiment showed complete conversion of into a compound with the expected product molecular weight of **11b** (m/z 4903). The mixture was concentrated and co-evaporated (2 × toluene, 400 μL). The residue was dissolved in methanol (100 μL) and purified on a size-exclusion column (LH-20, 40 cm × 2 cm). Appropriate fractions were pooled and concentrated to give **11b** (7.5 mg, 1.54 μmol, 63%). MALDI-TOF: 4903 $[M+Na]^+$, 4919 $[M+K]^+$ found: 4900 $[M+Na]^+$, 4916 $[M+K]^+$.

Compound **11c**

Sodium methoxide in methanol (1M, 100 μL) was added to a solution of compound **11b** (7.0 mg, 1.44 μmol) in methanol (5 mL). The mixture was left stirring overnight and then neutralized with H^+ ion exchange resin. The resin was removed by filtration (washed with methanol and water) and concentrated and the residue purified on a size-exclusion column (Biogel P-2, 5 cm × 1.5 cm). Appropriate fractions were pooled, concentrated and lyophilized to give **11c** (3.2 mg, 0.77 μmol, 50%). MALDI-TOF: 4152 $[M+Na]^+$, 4168 $[M+K]^+$ found: 4152 $[M+Na]^+$, 4168 $[M+K]^+$.

Cell cultures

HepG2 cells (ATCC/LGC Promochem, London UK) were propagated as monolayers in 75cm^2 culture flasks at 37°C in an humidified atmosphere containing 5% CO_2, using DMEM with glutamax-1 media (Gibco, Invitrogen, CA, USA) supplemented with 10% fetal calf serum (FCS), 100 ug/ml gentamycin, and 2 mM L-glutamine. Cultures were routinely trypsinized and split 1:3 on every fourth day. Neura 2A and HeLa cells were propagated as monolayers in analogous manner, but routinely trypsinized and split 1:5 every third day.

Assay for receptor binding and uptake

Previous to binding assay, biotinylated ligand constructs (135 μM) were incubated with Alexa Fluor 488-labeled streptavidin (30 μM) in Tyrode buffer (containing 10 mM HEPES, 5.6 mM glucose, 10 mM KCl, 135 mM NaCl, 0.4 mM MgCl, 1.0 mM $CaCl_2$ and 0.1% BSA, pH 7.3) at 4°C over night at a molar ratio of 4.5:1 of biotinylated ligands to biotin binding sites.

Binding of biotinylated construct to streptavidin was confirmed by electrophoresis of the incubation mix on 15% PAGE-tris/Glycin gel, in 100 V for 1 h, followed by Western Blotting of the separated bands to a nitrocellulose filter. Unbound biotin-construct was detected by staining with horseradish peroxidase labeled streptavidin and bound proteins visualized using a Pierce Super Signal West Pico chemiluminescence detection kit (Pierce, Rockford, IL, USA).

Cells were seeded into sterile 6 wells plates (1.2×10^6/well) in complete DMEM media and cultured for 48 h at 37°C, reaching approximately 75% confluence. The culture media was removed, and after washing with Tyrode buffer the cells were incubated in ice cooled Tyrode-buffer on ice. Before adding the target-complex mix to cells, the mix was diluted 1:300 times in Tyrode buffer and put on ice. 50 pmol functionalized streptavidin was added to each well and incubated on ice for 1.5 h. Thereafter the cells were washed three times with ice-cooled Tyrode buffer to remove excess of unbound ligand complex, before adding preheated 37°C complete DMEM media and immediately incubating the cells in 37°C for 1 h to allow endocytosis of receptor bound ligand-complexes.

For inhibition of receptor mediated uptake an extra incubation step with 5mM EDTA in PBS was performed for 10 minutes on ice, before subsequent washing and incubation in 37°C as described above.

For competitive binding assay either of the two following setups were used; 5 μM–100 μM asialofetuin was incubated simultaneously with the functionalized streptavidin with the cells on ice for 1.5 h, as described above. Alternatively, cells were pre-incubated with 5 μM–100 μM asialofetuin on ice for 1.5 h, followed by washing to remove unbound asialofetuin before adding the functionalized streptavidin (above).

FACS analysis

Adherent cells were washed carefully in PBS and harvested with trypsin. Thereafter cells were washed again and finally suspended in PBS containing 3% FCS. Analyses were performed on a FACS-Calibur using the software Cell Quest (Bectron Dickinson, San Jose, CA, USA). The forward scatter and side scatter gate was set to include all viable cells. 15000 cells were counted for each sample and uptake was determined as increased intensity in green fluorescence at 488nm detected in the FL1 channel. The number of positive cells that had internalized functionalized streptavidin was defined by a gate in the FL2/FL1 window, which included all cells of higher FL1 fluorescence than the background fluorescence from cells incubated without functionalized streptavidin. The FL2 channel represents background fluorescence in red wavelength for the HepG2 cells.

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