Bioorganic & Medicinal Chemistry 19 (2011) 2494–2500

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# New and more efficient multivalent glyco-ligands for asialoglycoprotein receptor of mammalian hepatocytes

Reiko T. Lee[a], Mei-Hui Wang[b], Wuu-Jyh Lin[b,*], Yuan C. Lee[a,*]

[a] Department of Biology, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218, USA
[b] Institute of Nuclear Energy Research, Taoyuan 325, Taiwan, ROC

ARTICLE INFO

*Article history:*
Received 5 January 2011
Revised 4 March 2011
Accepted 11 March 2011
Available online 15 March 2011

*Keywords:*
Liver-targeting
Tri-valent glyco-ligand
Hexa-valent glyco-ligand
Hepatocytes
Endocytosis

ABSTRACT

New multi-valent, carbohydrate ligands that contain terminal *N*-acetylgalactosamine (GalNAc) or lactose (Lac) were prepared using a nitrilotriacetic acid (NTA) derivative of L-lysine as scaffold. Tri-valent structures were prepared by attaching an ω-amino glycoside of GalNAc or Lac to each of the three carboxyl groups of $N^{\varepsilon}$-protected $N^{\alpha}$-dicarboxymethyl-L-lysine. In addition, a hexa-valent lactoside was synthesized by attaching $N^{\varepsilon}$-deprotected trivalent lactoside to each of the carboxyl group of $N^{\alpha}$-(trifluoroacetamido)hexanoyl L-aspartic acid. Tri-valent GalNAc glycosides and the hexa-valent lactoside had high affinity (dissociation constants approaching nM) for rat hepatocytes. The hexa-valent lactoside, after de-$N^{\varepsilon}$-protection, was modified with a chelator, diethylenetriaminepentaacetic acid (DTPA), through which a fluorescent or radioactive tag, such as europium or indium, can be firmly attached. Intravenous infusion of $^{111}$Indium-tagged hexa-valent lactoside to rats and mice resulted in nearly exclusive accumulation of radioactivity in the liver.

## 1. Introduction

Liver is an important organ for homeostasis that produces many important biomolecules, including acute-phase proteins. Liver is also unique in that it is endowed with a powerful carbohydrate-based receptor, asialoglycoprotein receptor also known as the Ashwell–Morell receptor (AMR), that is highly specific for galactose- and *N*-acetylgalactosamine-terminated glycans, and is present in large numbers on mammalian hepatocyte cell surface.[1] Their efficacy, however, depends on the presence of at least three terminal Gal or GalNAc residues with proper spatial arrangement.[2] When terminal Gal residues are suitably arranged in space, di- and tri-valent Gal-terminated glycans can manifest affinity as high as ca. µM and nM (as $K_D$ values), respectively.[2] Based on the chemical and physical information of high-affinity natural ligands for AMR, we constructed tri-valent GalNAc-containing glycopeptides, YEE(ahGalNAc)$_3$[3] and YDD(GahGalNAc)$_3$[4] (D, E, G, and Y are single letter abbreviations for aspartic acid, glutamic acid, glycine, and tyronine, respectively. For structures, see Fig. 1), utilizing three carboxylic acid groups of EE (γ-glutamyl-glutamic acid, γ-Glu-Glu) and DD (β-aspartyl-aspartic acid, β-Asp-Asp) as the attachment sites for amino-terminated GalNAc glycosides. These tri-valent GalNAc glycosides manifested strong binding affinity to AMR ($K_D$ <10 nM). Merwin et al.[5] used $^{125}$I-labeled YEE(ahGalNAc)$_3$ linked to polylysine complexed with the luciferase gene to demonstrate specific hepatic uptake. Cryoautoradiography showed that the iodinated glycopeptide complex was preferentially taken up by hepatocytes rather than by non-parenchymal cells. The same GalNAc ligand, YEE(ahGalNAc)$_3$, was also successfully utilized for hepatocyte-targeted delivery of antisense material to mask hepatic mRNA or genomic DNA.[6] The examination of biodistribution of $^{125}$I-YEE(ahGalNAc)$_3$-labeled material showed that it accumulated specifically in hepatocytes.[7]

In this paper, we report synthesis of new multivalent glyco-ligands based on a single amino acid, lysine, as scaffold. One of the new ligands has affinity for hepatic AMR higher than YEE(ahGalNAc)$_3$ and YDD(GahGalNAc)$_3$. In addition, we prepared lactose (Lac)-containing, hexa-valent ligand, which proved to be a powerful ligand for AMR that is effective in liver targeting.

## 2. Results and discussion

### 2.1. Syntheses of multi-valent glycosides

Tri-valent GalNAc glycosides for liver-targeting in the past used dipeptides, γ-glutamyl-glutamic acid (EE) and β-aspartyl-aspartic

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*Abbreviations:* ah, aminohexyl; AMR, Ashwell–Morell receptor; ASOR, asialo-orosomucoid; DCM, dicarboxymethyl; DIEA, diisopropylethylamine; DTPA, diethylenetriaminepentaacetic acid; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Lac, lactose; NTA, nitrilotriacetic acid; SPECT, single photon emission computed tomography; TFA, trifluroacetyl or trifluroacetic acid; TLC, thin layer chromatography; YDD, L-tyrosyl-γ-L-glutamyl-L-glutamic acid; YEE, L-tyrosyl-β-L-aspartyl-L-aspartic acid; Z, benzyloxycarbonyl.

* Corresponding authors. Tel.: +886 3 4711400x7008; fax: +886 3 4711416 (W.-J.L.); tel.: +1 410 516 7041; fax: +1 410 516 8716 (Y.C.L.).

*E-mail addresses:* wjlin@iner.gov.tw (W.-J. Lin), yclee@jhu.edu (Y.C. Lee).

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doi:10.1016/j.bmc.2011.03.027

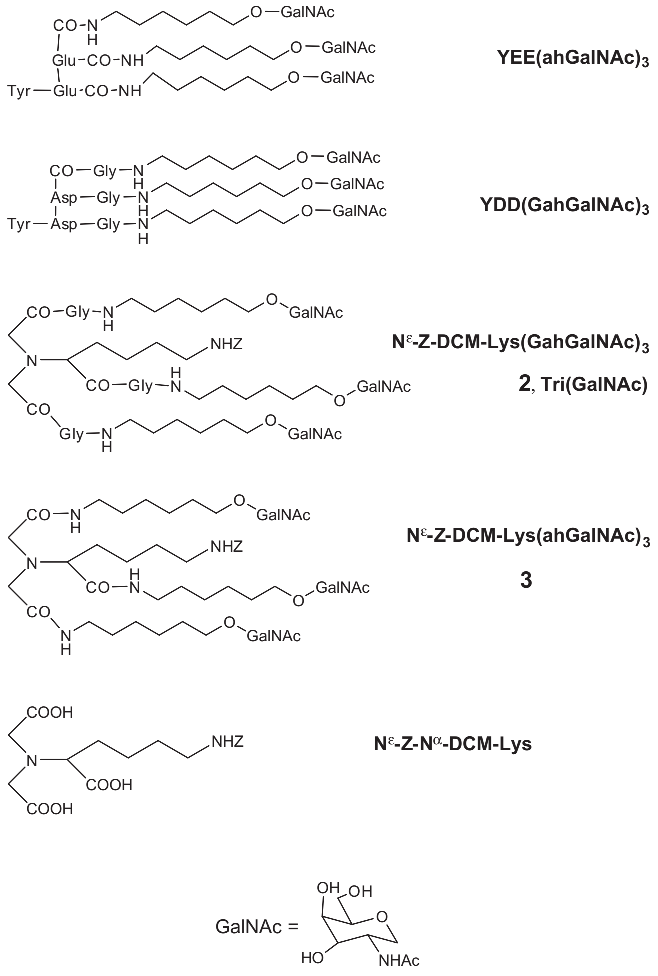

**Figure 1.** GalNAc-containing trivalent structures.

acid (DD), as scaffold.[3,4] In the present communication, we used a single amino acid, lysine, as scaffold. The new scaffold could, not only be prepared less expensively, but also was easier to use because of the better solubility. In order to attach glycosides having amino-terminated aglycon to the scaffold, Lys was first converted to an NTA (nitrilotriacetic acid) derivative[24] by a facile procedure of carboxymethylation of the α-amino group of Lys.[10] Conjugation of glycosides to this scaffold, $N^{\varepsilon}$-Z-$N^{\alpha}$-DCM-Lys (Fig. 1), by the DCC/1-OH-Bt method in non-aqueous solvents proceeded smoothly to produce desired tri-valent glycosides. We used a large excess of glycosides over the carboxyl group (50% excess for GalNAc glycosides and 80–90% excess for lactoside) in order to force the tri-valent conjugation to completion. Although tri-valent glycosides can be prepared using smaller excess of glycosides over the Lys scaffold, forcing the conjugation to completion helped in the purification of the tri-valent products by gel filtration, in that di- and mono-conjugated products were virtually absent.

It is a well-known fact that AMR of all mammalian species thus far examined binds GalNAc much better than Gal and lactose. For this reason, whereas tri-valent GalNAc glycosides have been shown to be useful ligands for liver targeting via AMR, a tri-valent lactoside is expected to be a much poorer ligand. Since AMR on the hepatocyte surface is known to be densely clustered[15] we reasoned that connecting two molecules of trivalent lactoside by a short linker should improve the affinity tremendously. For this reason, we synthesized a hexa-valent lactoside, HexaLac (Fig. 2), by attaching tri-valent lactoside molecule to each of the two carboxyl groups of Asp. The α-amino group of Asp will be a convenient handle for attaching a fluorescent or other tag in future. Construction of HexaLac again uses DCC/1-OH-Bt method, which proceeded smoothly requiring only 10% excess of tri-valent lactoside for each carboxyl group.

### 2.2. Binding affinity of various multi-valent glycosides to rat hepatocyte cell surface

The hepatocyte binding assay was carried out at 4° in order to prevent internalization of the surface-bound ligands. In the past, we have used inhibition assays with $^{125}$I-ASOR as a high affinity ligand to compare a number of potential ligands side by side, using hepatocyte suspension,[11] isolated liver membrane preparations,[12] and solubilized AMR.[13] In the present study, we chose intact, isolated hepatocytes, as this would be closest to the in vivo liver system.

Two modifications were made in the present inhibition assay: (1) Eu-DTPA-labeled ASOR was used instead of $^{125}$I-ASOR; (2) rat primary hepatocytes on collagen-coated surface were used instead of hepatocyte suspension. Although the sensitivity of detection of Eu fluorescence is comparable to that of $^{125}$I-radioactivity,[14] the former lacks the versatility of $^{125}$I that allows measurement to be made with wet cell pellet in a plastic tube or radioactive counts trapped on filter discs. After testing the use of Eu fluorescence in the cell suspension method, using either centrifugation through an oil layer[11] or filtration using MultiScreen 96-well filters of 0.45 or 1 μm pore (Millipore, Bedford, MA) to collect hepatocytes, we came to a conclusion that the method using the surface-immobilized cells would be simpler for the measurement of Eu fluorescence than the centrifugation or filtration method. In order to compare four inhibitors on a same 24-well hepatocyte plate, single point determinations were made for inhibitors at concentrations spanning 5 decades (0.1 M to 1 μM). Experiments were repeated, however, at least twice to assure the reproducibility of the results. Typical inhibition curves are shown in Figure 3. For the purpose of direct comparison with the previously reported trivalent GalNAc glycosides, YEE(ah-GalNAc)$_3$[3] and YDD(G-ah-GalNAc)$_3$[4] (Fig. 1) were also included in the test for direct comparison. The IC$_{50}$ values obtained are listed in Table 1. The IC$_{50}$ values of YEE(ah-GalNAc)$_3$ and YDD (G-ah-GalNAc)$_3$ in the present assay were 10 and 20 nM, respectively, as compared to 8 nM for both compounds determined in the past by inhibition assay using rat hepatocyte suspension and $^{125}$I-ASOR as reference ligand, indicating that the IC$_{50}$ values obtained by the both methods are comparable. Table 1 also suggests that $N^{\varepsilon}$-Z-DCM-Lys(GahGalNAc)$_3$ (**2**, Fig. 1) has at least 10-fold higher affinity than all other GalNAc-containing trivalent ligands.

### 2.3. Whole animal experiments with $^{111}$In-labeled HexaLac

The efficiency of loading of $^{111}$In to DTPA-HexaLac was >98% as determined by radio-HPLC and was stable for 72 h. The specific activity of $^{111}$In-HexaLac (Fig. 2) was $2.5 \times 10^{10}$ Bq/mg. The SPECT/CT image showed the radioactivity was accumulated specifically in the liver (Fig. 4). There is virtually no uptake in the other organs. This specific accumulation of radioactivity in the liver was ablated in the presence of a powerful competitor, bovine asialofetuin. Distribution of $^{111}$In in various organs of mice 15 min after iv injection of $^{111}$In-HexaLac is shown in Figure 5, which again showed the almost exclusive accumulation of radioactivity in the liver. These data indicated that $^{111}$In-HexaLac possesses a highly specific liver targeting property, which makes HexaLac structure to be useful as liver-specific drug-delivery vehicle that would enhances the therapeutic effect of drugs and reduces the unwanted side effects.

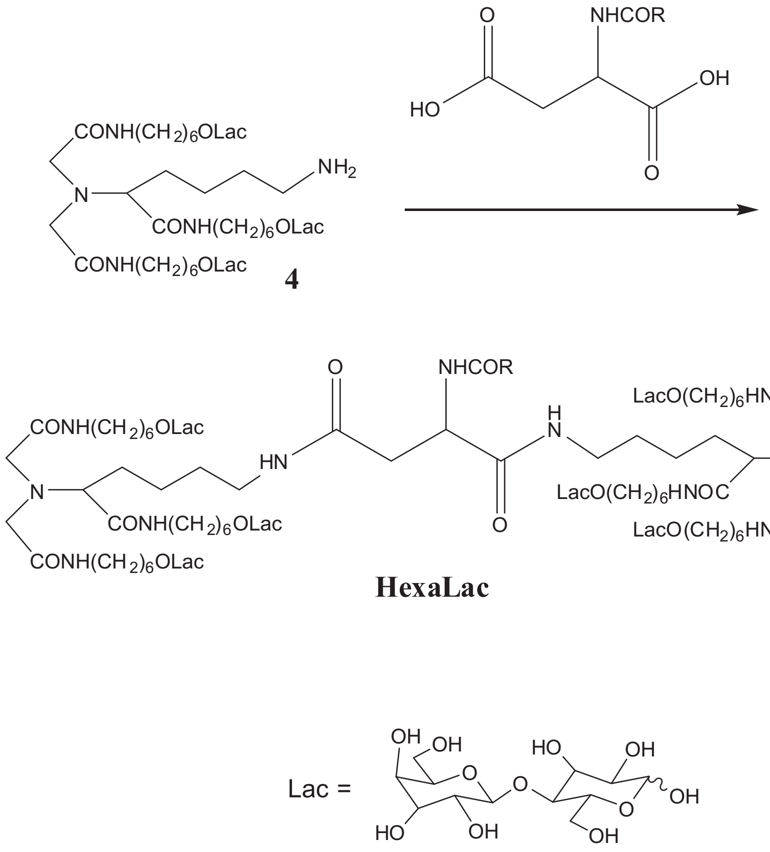

**Figure 2.** Formation of HexaLac from trivalent Lac (**4**).

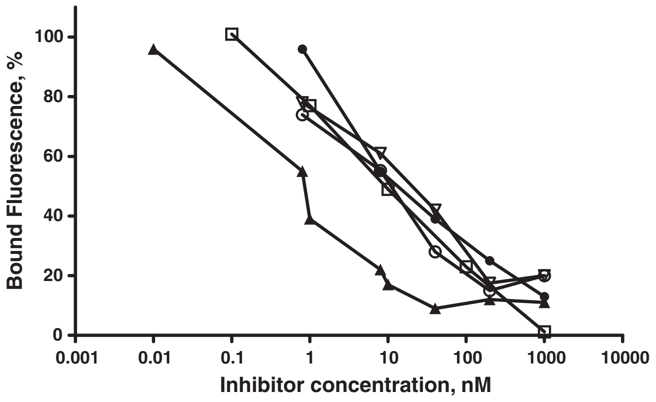

**Figure 3.** Inhibition curves of five multivalent glycol-ligands generated using plated rat hepatocytes as described in Methods: ●, $N^{\varepsilon}$-Z-$N^{\alpha}$-DCM-Lys(ah-GalNAc)$_3$ (**3**); ▲, $N^{\varepsilon}$-Z-$N^{\alpha}$-DCM-Lys(G-ah-GalNAc)$_3$ (**2**); ○, YEE(ah-GalNAc)$_3$; ▼, YDD(G-G-ah-GalNAc)$_3$; and □, 'HexaLac'.

**Table 1**
Characterization of cluster glycosides

| Compounds | Molecular mass | | $IC_{50}$ (nM) |
|---|---|---|---|
| | Calcd | Found[a] | |
| $N^{\varepsilon}$-Z-$N^{\alpha}$DCM-Lys(ah-GalNAc)$_3$ (**3**) | 1303.47 | 1303.7 | 12 |
| $N^{\varepsilon}$-Z-$N^{\alpha}$DCM-Lys(G-ah-GalNAc)$_3$ (**2**), tri(GalNAc) | 1474.63 | 1474.7 | 1 |
| $N^{\varepsilon}$-Z-$N^{\alpha}$DCM-Lys(ah-Lac)$_3$ (**4**) | 1666.23 | 1666.8 | >1000 |
| TFA-AH-Asp[Lys(ahLac)$_3$]$_2$, (**5**, HexaLac) | 3371.5 | 3371.0 | 9 |
| YEE(ahGalNAc)$_3$[b] | | Not analyzed | 10 |
| YDD(G-ahGalNAc)$_3$[c] | | Not analyzed | 20 |

[a] By ESI.
[b] See Ref. 3.
[c] See Ref. 4.

## 3. Conclusions

Compounds containing NTA(Nitrilotriacetic acid)-group have been frequently used for immobilized metal affinity chromatography (IMAC), such as for capture of His$_6$-tagged proteins with Ni-complexed NTA materials. One of the efficient methods for preparation of NTA structure is by di-carboxymethylating the α-amino group of lysine.[16,17] Compared to our earlier trivalent glyco-ligands using dipeptides, γ-Glu-Glu or β-Asp-Asp, as scaffold, our new strategy of using a lysine-based NTA-structure for sugar conjugation was simpler and more cost effective, and produced glyco-ligands of equal to or higher affinity to AMR than the previously synthesized structures.

Of the three newly synthesized high-affinity ligands, that is, compound **2**, tri(GalNAc), compound **3**, and HexaLac, compound **2** had the highest affinity, outperforming our previously reported YEE- and YDD-derivatives. As expected from the fact of lactose being much less strongly bound by AMR than GalNAc, tri-valent lactoside was indeed much weaker ligand than the GalNAc counterparts, having $IC_{50}$ value higher than μM (Table 1). However, conjoining of two trivalent lactoside molecules produced a hexavalent lactoside (HexaLac) which possessed $IC_{50}$ in the nM range. After modifying HexaLac with DTPA and radiolabelling with $^{111}$In, it was shown to possess excellent liver-targeting activity by both the whole-body micro-SPET/CT imaging and by the $^{111}$In distribution among major organs. Thus NTA-based multivalent glyco-

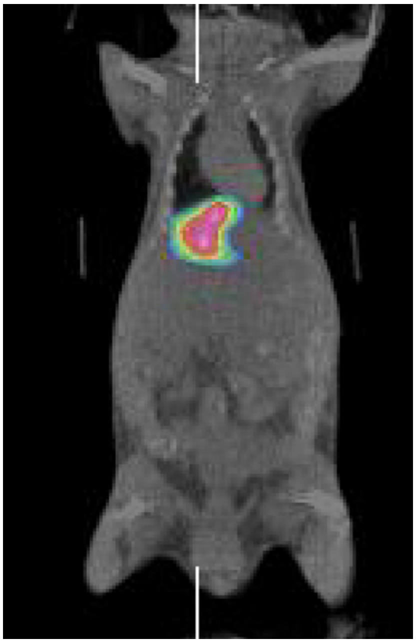

**Figure 4.** Biodistribution of 'HexaLac' in Balb/c mice studied with the single-photon emission computed tomography (SPECT)/computed tomography (CT) after iv injection of $^{111}$In-HexaLac.

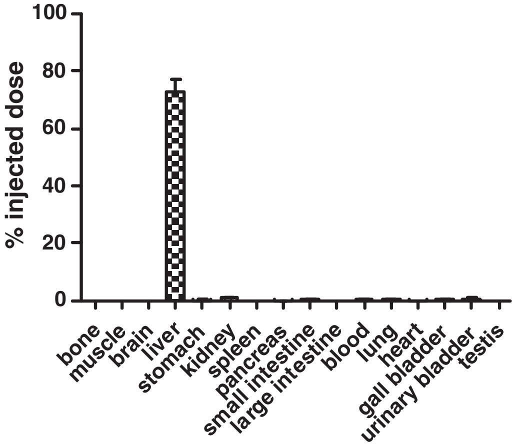

**Figure 5.** Biodistribution of $^{111}$In-HexaLac in various organs of Balb/c mice after intravenous injection.[25]

ligands promise to be powerful liver-specific carrier for diagnostic and therapeutic agents.

## 4. Experimental

### 4.1. Materials

$N^{\varepsilon}$-Benzyloxycarbonyl-L-lysine ($N^{\varepsilon}$-Z-Lys), *N*-benzyloxycarbonyl glycine (Z-Gly), and dibenzylester of L-aspartic acid *p*-tosylate were purchased from Advanced ChemTech (Louisville, KY). Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (1-OH-Bt), DTPA (diethylenetriaminepentaacetic acid), its dianhydride, and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] were from Sigma-Aldrich (St. Louis, MO). 6-Aminohexyl β-glycosides of GalNAc and lactose (ahGalNAc and ahLac, respectively)[8] and 6-trifluroacetamido-hexanoic acid (TFA-AHA)[18] were prepared as described previously. Orosomucoid (α1-acid glycoprotein) was desialylated to produce asialo-orosomucoid (ASOR) as described.[13] Rat primary hepatocytes on collagen-coated, 24-well plates and hepatocyte basal medium (HBM) were purchased from Lonza (Walkersville, MD).

### 4.2. General methods

Thin-layer chromatography (TLC) on Silica Gel 60 layer with F254, coated on aluminum sheet (EMD Chemicals), was used routinely to monitor reaction progress and column elution profile. UV-absorbing material was detected under a UV-lamp, and carbohydrate-containing material was visualized by spraying TLC plate with 15% sulfuric acid in 50% ethanol, and heating it on a hot plate until the carbohydrate material charred. Unmasked amino group was visualized by spraying with 0.2% ninhydrin solution in 95% ethanol, and heating on a hotplate briefly. The trifluoroacetyl or benzyloxycarbonyl masked amino group was detected by first spraying the TLC plate with 0.2 M NaOH, heating it briefly, then spraying with glacial acetic acid followed by ninhydrin, and heating the plate again. Protein was assayed using a micro BCA (bicinchoninic acid) assay.[19]

For the determination of Eu-fluorescence, samples in the wells of a 96-well plate were incubated with 200 μL of Eu-enhancing solution[20] for 20 min, and then counted using the time-resolved mode with Eu fluorescence setting in a microplate fluorometer (Wallac Victor 1460 or Perkin-Elmer Envision).[21,22] $^1$H NMR spectra were measured using a Bruker 300 and 400 MHz ultrashield, and Varian 300 MHz. Molecular mass of various trivalent and hexa-valent glycosides was determined by ESI-MS, using a Finnigan LCQ.

For the DCC-dependent amide linkage formation, the reaction was carried out in DMF, DMSO, or a mixture of the two, depending on the solubility of the reactants, using equimolar amounts of amino- and carboxy-containing compounds. 1-OH-Bt was in the equimolar amount and DCC was in 15% excess of the carboxyl group. After stirring the reaction mixture overnight at room temperature, the precipitate (dicyclohexyl urea) was filtered off, and the filtrate was evaporated. The residue was purified by gel filtration through a column of Sephadex G-15 or G-25 (2.5 × 140 cm) equilibrated in and eluted with 0.1 M acetic acid. The Sephadex G-15 column was used for all tri-valent products, and the G-25 column was used for the hexa-valent product. Removal of N-benzyloxy group was by hydrogenolysis using a Brown hydrogenator, in either 80–95% ethanol or 60% acetic acid, depending on the solubility of the compounds. Catalyst, 10% Pd on carbon, was used in 10% of the compound to be hydrogenated on the weight basis.

### 4.3. Synthesis of Asp derivative as a connector

In order to join two molecules of trivalent lactoside to produce a hexa-valent ligand, we chose an aspartic acid derivative as a connector, since Asp has two carboxyl groups separate by only two carbons, and it has an amino group that can be used for linkage of fluorescent or radioactive probe. In order to make the amino group more accessible, N-protected 6-aminohexanoyl group was attached to the amino group of dibenzyl ester of Asp. One millimole each of dibenzyl ester of L-Asp *p*-tosylate and 6-*N*-(trifluroacetyl)aminohexanoic acid (TFA-AHA) were reacted using the DCC/1-OH-Bt method in DMF with DIEA (1 mmol) to neutralize the tosylate. The product was purified by silica gel column chromatography using toluene–ethyl acetate as solvent. Fractions that contained dibenzyl ester of *N*-(trifluroacetamidohexanoyl)aspartic acid [TFA-AHA-Asp(OBn)$_2$, **1**] (TLC in toluene–ethyl acetate, 1:1; $R_f = 0.46$) were combined and evaporated to yield a white solid in 89% yield; mp, 112–113°. $^1$H NMR in methanol-$d_4$ showed the presence of two benzyl groups and 5 side chain methylene groups

[$\sigma$ 2.9–1.3 ppm]. Methylene group of Asp was at 3.25 ppm, but the methine H appeared to be buried under HOD peak at 4.8 ppm.

Hydrogenolysis of **1** in 95% ethanol produced a single ninhydrin-positive product, TFA-AHA-Asp, **1′**, in quantitative yield. $^1$H NMR showed that the benzyl signals have disappeared, and the correct number of side-chain methylene groups were present between $\sigma$ 2.78 and 1.35 ppm. The methylene group of Asp seemed to be buried under the methanol peak at 3.24 ppm, whereas the methine signal was visible at 4.7 ppm. Comparison of $^{13}$C NMR of **1** and **1′** against TFA-AHA showed that both **1** and **1′** had, in addition to two extra carboxylic acid signals at around 174 ppm, a methylene signal at 37.00 and 36.92 ppm, and methine signal at 50.42 and 50.23 ppm, respectively. In addition, **1** had two benzyl signals that were slightly different from each other (e.g., benzyl methylene signals at 67.69 and 68.26 ppm). All aromatic C signals, other than the alkyl attachment site, were located around 129.3 ppm.

### 4.4. Synthesis of multi-valent glycosides

Syntheses of 6-(trifluroacetamido)hexyl β-glycosides of GalNAc and lactose, ahGalNAc and ahLac, were by the well-established methods.[8,9] To make a longer glycoside of GalNAc, first Z-Gly was attached to ahGalNAc using the DCC/1-OH-Bt method in DMF and then the Z-group of the product was removed by hydrogenolysis to yield (6-*N*-glycyl)-aminohexyl β-GalNAc (GahGalNAc).[4] The product was crystallized from hot 95% ethanol. The NTA derivative of lysine, to be used as scaffold for attachment of sugars, was prepared by bromoacetylation of $N^{\varepsilon}$-Z-L-Lys[10] which produced $N^{\varepsilon}$-Z-$N^{\alpha}$-dicarboxylmethyl derivative of lysine ($N^{\varepsilon}$-Z-$N^{\alpha}$-DCM-Lys, see Fig. 1). To this scaffold (0.1 mmol) was conjugated ahGalNAc (0.45 mmol), GahGalNAc (0.45 mmol), or ahLac (0.57 mmol), using the DCC/1-OH-Bt method in either DMF for GalNAc derivatives and DMF-DMSO mixture for lactoside. In order to ensure complete sugar conjugation, GalNAc glycosides were used in 50% excess for each carboxyl group of $N^{\varepsilon}$-Z-$N^{\alpha}$-DCM-Lys, whereas, in the case of ahLac, it was necessary to use 80–90% excess over each carboxyl group. Eluted fractions from the Sephadex G-15 column showed, by TLC using ethyl acetate–acetic acid–water (3:2:1), that the tri-valent product ($R_f$ = 0.048 for **2**, 0.058 for **3**, and 0.02 for tri-valent lactoside) eluted well ahead of excess ω-amino glycoside with little under sugar-derivatized products being produced under such reaction conditions. Yield of **3**, **2**, and tri-valent lactoside on the basis of $N^{\varepsilon}$-Z-$N^{\alpha}$-DCM-Lys was 70%, 99%, and 30%, respectively. The de-N-protected trivalent GalNAc derivatives and tri-valent lactoside (**4**) were obtained in quantitative yield by hydrogenolysis.

A hexa-valent lactoside (HexaLac, **5**, Fig. 2) was prepared by attaching two molecules of **4** (142 μmol) to TFA-AHA-Asp (60 μmol) by the DCC/1-OH-Bt method in DMSO. The fractionation of reaction mixture on the Sephadex G-25 column yielded HexaLac (earliest eluting material, $R_f$ = 0.18 in 80% ethanol) in 69% yield.

All multi-valent products appeared pure by the TLC examination in the respective solvent system, and ESI-MS examination indicated that all had the dominant peak of the expected molecular mass. No incompletely glycoside-conjugated products, namely mono- and di-valent products, were detected in any of the tri-valent compounds. The MS of the HexaLac showed no peak for the starting tri-valent lactoside, **4**, and only a negligible amount of Asp derivative with a single tri-valent lactoside attached was detected.

$^1$H and $^{13}$C NMR data for all the multi-valent products are summarized below. $^1$H NMR spectra of tri-valent GalNAc derivatives, **2** and **3**, showed the following: With aromatic signals ($\sigma$ = 7.393 for **2** and 7.351 for **3**) set as 5H atoms, benzyl methylene signals at 5.08 ppm and 5.03 ppm were 2 H atoms each for both **2** and **3**. Both had 3 H atoms for anomeric signals at 4.42 and 4.35 ppm. Methyl signals for **2** (2.013 ppm) and for **3** (1.959 ppm) had 8.87 and 8.8 H atoms, respectively. Between $\sigma$ of 3.9 and 3.0 ppm, where 6 sugar Hs and some low-field methylene and a methine signals are located, there were 43 (calcd = 41) and 37 (calcd = 35) hydrogens for **2** and **3**, respectively. In the high-field methylene region ($\sigma$ = 1.57–1.23) were 31 Hs for both **2** and **3** (calcd = 32).

$^{13}$C NMR spectra of **2** and **3** showed that: (1) **2** had four types of carbonyl carbons at 175.11, 174.97, 171.45 and 158 ppm, whereas **3** had three types at 175.24, 174.06 and 159.2 ppm. Signals around175 ppm belong to nitriloacetyl group and around 159 ppm to benzyloxycarbonyl group. Signals at 174.06 and 174.97 ppm are from GalNAc carbonyl groups and those around 171 ppm should belong to the glycyl carbonyl groups. (2) Aromatic C signals were similar for both compounds, with three peaks located between 129.7 and 128.24 ppm. (3) Anomeric C signals for **2** and **3** were at 102.24 and 102.64 ppm, respectively. (4) Methyl signal was at 53.10 and 53.51 ppm for **2** and **3** (note: this signal is absent in the spectra of lactose-containing multivalent structures). (5) Six oxygen-linked C signals for both compounds were between 76 and 61.56 ppm (5 peaks discernible). (6) Eight peaks belonging to methylene carbons in high field (29.66–22.01 ppm) were visible (calcd = 9). (7) One type of methylene and a methine carbons were located between 40.03 and 40.17 ppm, and compound **2** had an extra signal in this region at 42.92 ppm, which should belong to methylene of glycyl group. The combined $^1$H and $^{13}$C NMR results suggest both compounds **2** and **3** contain all the expected elements in correct proportions and no extraneous material.

$^1$H NMR of tri-valent lactoside in $D_2O$: With the methylene signal of benzyl group ($\sigma$ = 4.94) set as 2, the number of H in aromatic signal ($\sigma$ = 7.25) was 4.7 and that of anomeric signal (2 anomeric hydrogens for each lactose unit) was 5.4. The mid range ($\sigma$ = 3.82–2.96), where all the remaining lactose H and some H atoms of methylene groups of aglycon and lysine side chain are located, contained signals for 55 H atoms (calculated number = 53), and the high-field region for most of methylene groups ($\sigma$ = 1.445–1.066) contained signal worth 30 H atoms (calculated number = 33). $^1$H NMR of HexaLac in $D_2O$: HexaLac is composed of 6 aminohexyl lactosyl residues, 2 dicarboxymethylated lysyl residues and 1 TFA-aminohexanoylated aspartyl residue. Setting the number of H in the methylene signal of aspartyl group ($\sigma$ = 2.554–2.426, m) as 2, anomeric signal of lactose, mid section of spectra (all the remaining lactose H signals and some low-field methylene and methine signals; $\sigma$ = 3.83–2.99), and high-field methylene signals ($\sigma$ = 1.46–1.19) were 11.5, 116.2 and 66.5 H atoms, respectively. The calculated H numbers are 12, 115 and 66 for the same three regions, showing excellent agreement of experimental values with the projected values.

$^{13}$C-NMR data of tri-valent lactoside and HexaLac: Similar to the tri-valent GalNAc glycosides, the tri-valent lactoside had carbonyl signals from nitriloacetic acid group and benzyloxycarbonyl group, but lacked the carbonyl group of GalNAc. The same triad of aromatic C around 128–129 ppm was present, as well as a much smaller signal at 136.7 ppm that belongs to the C attached to methylene group. There were two anomeric signals of lactose at 103.1 and 102.2 ppm. As expected, between 78.6 and 60.3 ppm, there were many more C signals than seen in the GalNAc counterparts; 12 peaks (2 of which were almost totally overlapping) in this region represent 10 C from lactose and 2 other O-linked carbons. Only 6 peaks were discernible in the high-field methylene region of $\sigma$ = 28.9–23, where nine carbons should be located, as in the GalNAc counterparts.

$^{13}$C NMR spectrum of HexaLac is quite similar to that of the tri-valent lactoside, with a few extra signals and a few missing signals. Carbonyl signals were at 176.5, 174.3, 173.3, 172.2 and 171.4 ppm, representing two from the aspartyl group and one from aminohexanoyl group, in addition to the carbonyls present in the tri-valent lactoside. The carbonyl signal of trifluoroacetyl group was not visible, as in the case of TFA-AHA. There were two extra signals between 39 and 35 ppm. These are likely signals from aspartic methylene and methine carbons. In the region of 140–127 ppm, the entire aromatic signal is missing, as well as benzyl C signal at 158–159 ppm. Thus, combined $^{1}$H and $^{13}$C NMR data are in good agreement with the structures of tri-valent lactoside and HexaLac.

The trivalent GalNAc derivatives were de-N-protected by hydrogenolysis, and HexaLac was de-N-protected in 10% TEA in 10% ethanol, and leaving the mixture at room temperature overnight, followed by thorough evaporation. The amino-exposed tri-valent GalNAc derivative and HexaLac were reacted with 5-fold excess of DTPA dianhydride in 0.2 M sodium borate buffer (pH 8.5), with NaOH added as needed to maintain the pH slightly alkaline. DTPA derivatives were purified with the Sephadex G-15 column (for the GalNAc derivative) or with the Sephadex G-25 column (for HexaLac). The DTPA-conjugated products no longer moved on TLC plate using ethyl acetate–acetic acid–water (2:1:1) or 80% ethanol, and eluted well ahead of free DTPA (see Section 4.5).

### 4.5. Determination of DTPA and mono-conjugated DTPA derivatives

Reaction of DTPA dianhydride with amino-containing compounds in aqueous solution inevitably produces free DTPA. In order to locate DTPA-derivative and free DTPA in the column effluent, a facile assay method using TPTZ (2,4,6-tri-2-pyridyl-s-triazine) was devised. In this assay, DTPA is allowed to compete against TPTZ for the binding of ferrous ion. The TPTZ–ferrous sulfate solution described by Avigad[23] was diluted 2-fold to give $A_{593\text{nm}}$ of 0.95–1.0. A sample containing up to 0.2 μmol of DTPA or DTPA derivative was mixed with the diluted TPTZ solution (3 ml), incubated at 37° for 30 min, and $A_{593\text{nm}}$ was measured. The presence of DTPA or its derivative lowers the absorbance, which is approximately proportional to the DTPA concentration.

### 4.6. Competition assay for determination of binding affinity of various multi-valent glycosides to rat hepatocytes

The binding affinities of multivalent glyco-ligands **2**, **3**, **4**, and HexaLac to rat hepatocytes were determined by competitive assay, using Eu-labeled asialo-orosomucoid (Eu-ASOR) as the reference compound. The assay was carried out with a 24-well plate of rat hepatocyte monolayer half immersed in ice–water mixture. HBM in the well was removed by gentle suction, and replaced with 0.5 mL of five different concentrations of multivalent ligands (0.8 nM to 1 μM), followed by 0.5 mL of 10 nM of Eu-ASOR. All solutions were made in HBM with 5 mM calcium chloride. After gentle shaking for 1 h in the cold, the unbound ligand was removed and the wells were washed with HBM containing calcium chloride. Europium was stripped and solubilized from Eu-ASOR bound to hepatocytes by adding 0.3 mL of enhancement solution[20] in each well and swirling the plate gently for 20 min. An aliquot (200 μL) from each well of this solubilized Eu in a highly fluorogenic milieu was transferred into a 96-well plate and Eu-fluorescence was determined by time-resolved fluorometry. After subtracting the blank fluorescence (fluorescence obtained in the presence of 2 μM ASOR), the residual fluorescence values were converted to the % of fluorescence obtained in the absence of inhibitor. This fluorescence value at each inhibitor concentration was plotted on y axis with the inhibitor concentration in logarithmic scale on *x* axis. $IC_{50}$ (concentration at which 50% reduction in the bound fluorescence occurred) for each compound was determined from the respective curve.

### 4.7. Radiosynthesis

Labeling of DTPA-HexaLac with $^{111}$In for biodistribution study was according to the published method.[24] Briefly, DTPA-modified HexaLac or DTPA-modified tri(GalNAc) (0.012 nmol, each) was mixed with $1.85 \times 10^{7}$ Bq of $^{111}$In in 0.1 M citric acid (pH 2.1) for 30 min. The labeling efficiency was analyzed by silica gel impregnated glass fiber (ITLC-SG, Gelman) developed with 10 mM sodium acetate and confirmed with radio-HPLC.

### 4.8. Biodistribution of $^{111}$In-DTPA HexaLac in normal Balb/c mices

Four male mice, averaging 20 g, were sacrificed by cervical translocation 15 min after iv injection of 0.35 MBq/100 μL $^{111}$In-DTPA HexaLac. The chosen organs were excised out as whole organ when possible. The organs were rinsed with saline, blotted dry, weighed and then counted using Cobra II Auto-Gamma counter (Packard). The radioactivity in the organ was expressed as percentage of the injected dose (% ID).

### 4.9. Small animal microSPECT imaging

For the micro-SPECT/CT image of $^{111}$In-DTPA-HexaLac, each mouse received iv injection of 44 μCi of $^{111}$In-DTPA HexaLac. The mice were anesthetized with 1.5% isoflurane (positioned prone in the scanner) and 60-min image was obtained by micro-SPECT/CT 2 h after iv injection with urine squeezed out. The SPECT images and CT images were acquired using a micro-SPECT/CT scanner (X-SPECT/CT, Gamma Medica, Northridge, CA). The SPECT images were acquired using a parallel hole collimator, with the center of the field of view (FOV) focused on the abdomen of the mouse. The radius of rotation (ROR) was 1 cm with an FOV of 1.37 cm. The imaging was accomplished using 64 projections at 60 s per projection. The SPECT imaging were reconstructed to produce image sizes of 56 × 56 × 56 (pixels) with an image resolution of 1.2 mm. The CT images were also reconstructed in image sizes of 512 × 512 × 512 (pixels) with 0.15 mm image resolution.

## Acknowledgments

We thank Dr. H.-M. Yu and Mr. C.-Y. Chein for technical assistance in biodistribution study. We thank Mr. K. Mathos, E. Lloyd, and J.-T. Wang for recording of NMR spectra. The animal experiments approved by the Animal Care and Use Committee of the Institute of Nuclear Energy Research in Taiwan were also gratefully acknowledged.

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