Design for Cell-Specific Targeting of Proteins Utilizing Sugar-Recognition Mechanism: Effect of Molecular Weight of Proteins on Targeting Efficiency

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Hepatic targeting of proteins utilizing the sugar-recognition mechanism was investigated in mice after intravenous injection. Five proteins with different molecular weights, i.e., bovine γ-globulins (IgG), bovine serum albumin (BSA), recombinant human superoxide dismutase (SOD), soybean trypsin inhibitor (STI), and chicken egg white lysozyme (LZM), were modified with 2-imino-2-methoxyethyl 1-thiogalactoside to obtain galactosylated proteins (Gal-IgG, Gal-BSA, Gal-SOD, Gal-STI, and Gal-LZM). The numbers of galactose residues were 38, 20, 11, 6, and 5 for Gal-IgG, Gal-BSA, Gal-SOD, Gal-STI, and Gal-LZM, respectively. All galactosylated proteins were dose-dependently taken up by the liver and the relative amount accumulated in the liver was decreased with an increase of the administered dose. At low doses (0.05 and 0.1 mg/kg), Gal-IgG, Gal-BSA, and Gal-SOD could be taken up by the liver up to more than 70–80% of dose within 10 min after intravenous injection, but the maximum amounts accumulated in the liver were approximately 40 and 30% of the dose for Gal-STI and Gal-LZM, respectively. Pharmacokinetic analysis revealed that the hepatic uptake clearance (CL_{liver}) was quite different around the molecular weight of 32 kDa and correlated with the amount delivered to the liver; Gal-IgG, Gal-BSA, and Gal-SOD has a large CL_{liver} that is close to the hepatic plasma flow rate (85 ml/hr), whereas those of Gal-STI and Gal-LZM were approximately 10 ml/hr at low doses. As for the total amount accumulated in the liver, high glomerular filtration rate of Gal-STI and Gal-LZM was also shown to cause insufficient delivery to the liver apart from being caused by their low CL_{liver}.

KEY WORDS: protein targeting; sugar recognition; pharmacokinetics; molecular weight; liver.

INTRODUCTION

Protein drugs can be made therapeutically more efficient by adequately altering their inherent *in vivo* distribution characteristics, and we have investigated the possibility of improving body distributions through diverse chemical modifications (1–7). To achieve the selective targeting to desired sites, a homing device having a high affinity to the target cell should be introduced in the protein molecule. As well as antibody/antigen interaction, some carbohydrates are known to selectively interact with corresponding receptors on the surface of some cells (8,9). The asialoglycoprotein receptor on hepatocytes and the mannose receptor on several macrophages strictly recognize compounds with terminal galactose and mannose residues, respectively, and are widely used in the selective delivery of drugs to such cells (10–12).

We have previously demonstrated that bovine serum albumin (BSA) and recombinant human superoxide dismutase (SOD) could be selectively delivered to liver parenchymal and non-parenchymal cells by direct attachment of galactose and mannose moieties, respectively (5,7). In addition, SOD derivatives with galactose or mannose exhibited superior inhibitory effects to native SOD or SOD-polysaccharide conjugates against the hepatic ischemia/reperfusion injury (13). However, there are no systematic studies on the applicability of glycosylation techniques in targeting of protein drugs with different physicochemical and biological characteristics. Among various physicochemical properties, the molecular weight of proteins was found to greatly affect the *in vivo* distribution characteristics of proteins administered by intravenous injection (14). Since most protein drugs produced by genetic recombinant technique such as interferons (15–25 kDa), interleukin 2 (15 kDa), and erythropoietin (30 kDa) have molecular weights smaller than BSA and SOD, it is necessary to study the effect of galactosylation combined with the molecular weight of the proteins on the efficiency of targeting. For this purpose, we considered the total disposition of galactosylated proteins in relation to their basic physicochemical characteristics and affinities for the galactose-recognizing receptors in the liver.

In this paper, the hepatic targeting efficiency of galactosylated proteins with various molecular weights after intravenous injection were examined in mice. Pharmacokinetic analyses were performed to elucidate the underlying mechanisms determining the *in vivo* distribution of these galactosylated proteins.

MATERIALS AND METHODS

Chemicals

Bovine γ-globulins (corn fraction II; IgG; 150 kDa), bovine serum albumin (fraction V; BSA; 67 kDa), soybean trypsin inhibitor (STI; 20 kDa), and chicken egg white lysozyme (LZM; 14 kDa) were obtained from Sigma, St. Louis, USA. Recombinant human superoxide dismutase (111-Ser; SOD; 32 kDa) was kindly supplied by Asahi Kasei, Tokyo, Japan. β-D-galactose was obtained from Nacalai Tesque, Kyoto, Japan. ^{111}Indium chloride was supplied by Nihon Medi-physics, Co., Takarazuka, Japan. All other chemicals were of the finest grade available.

Synthesis of galactosylated proteins

Galactose was coupled to proteins according to the method of Lee et al. (15) as described previously (7). The galactose content and the number of galactose residues on each protein were determined by anthrone-sulfuric acid method. The percentage of modified amino groups with ga-

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lactose moieties was estimated by measuring the free amino groups with 2, 4, 6-trinitrobenzene-sulfonic acid using glycine as a standard (16). Molecular weights of galactosylated proteins were determined by size-exclusion high-performance liquid chromatography (LC-6A, Shimadzu, Japan) using a Shim-pack Diol-300 column. For animal experiments, native and galactosylated proteins were radiolabeled with ^{111}In (17) as described previously (7).

In vivo distribution experiments

Male ddY mice (25–28 g) were purchased from the Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan. Mice received 0.05, 0.1, 1, 10, or 20 mg/kg dose of ^{111}In-labeled each galactosylated protein or 1 mg/kg dose of each native protein in saline by tail vein injection. At adequate periods after injection, blood was collected from the vena cava to obtain plasma sample. Liver, kidney, spleen, heart, lung, and muscle were excised, rinsed with saline, weighed, and subjected to assay. ^{111}In-radioactivity was counted with a well-type NaI-scintillation counter (ARC-500, Aloka Co., Tokyo). Contamination of plasma in each tissue sample was corrected using the distribution data of ^{111}In-labeled BSA at 10 min after intravenous injection under the assumption that BSA was not taken up by tissues at that time.

Calculation of organ uptake clearance

The tissue distribution was evaluated using an organ uptake clearance (CL_{org}) described previously (18). In the early period after injection, the efflux of the ^{111}In radioactivity from the organ is considered to be negligible (19) since the degradation products of the ^{111}In-labeled proteins cannot easily pass through the biological membranes (20). With the assumption described above, CL_{org} can be calculated by dividing the amount of radioactivity in the organ at an appropriate interval of time by the area under the plasma concentration-time curves (AUC) up to the same time point. When the tissue uptake process followed non-linear kinetics and CL_{org} was not constant, the calculated CL_{org} values would represent an average value for the overall experimental period.

RESULTS

Characterization of galactosylated proteins

Table I summarizes the physicochemical properties of synthesized galactosylated proteins. Galactosylation of each protein slightly increased its molecular weight in size-exclusion chromatography determination but no aggregates were observed. We used derivatives with a relatively identical degree of galactosylation as regards the content of galactose in each protein (4.4 ~ 6.0 w/w %); the number of galactose residues were 38, 20, 11, 6, and 5 galactose residues for Gal-IgG, Gal-BSA, Gal-SOD, Gal-STI, and Gal-LZM, respectively.

Tissue distribution of ^{111}In-labeled native proteins

Figure 1 shows the plasma concentration- and liver accumulation-time courses of native proteins at a dose of 1 mg/kg after intravenous injection in mice. The elimination profiles of proteins from plasma were highly dependent on their molecular weights, which was in accordance with our previous study (14). IgG and BSA were retained in plasma for an extremely long period compared with low-molecular weight proteins, SOD, STI, and LZM. Hepatic uptake was almost negligible in all native proteins. The difference in the plasma concentration patterns of proteins was explained by the extent of glomerular filtration which was represented by amounts recovered in the kidney and urine. Figure 2 shows the recovery of radioactivities in the kidney and urine at 1 hr for SOD, STI, and LZM and at 24 hr for BSA and IgG after intravenous injection in mice. More than 80% of radioactivities were recovered in the kidney and urine after the administration of SOD, STI, and LZM, whereas those of IgG and BSA were less than 5%.

Table I Physicochemical characteristics of synthesized galactosylated proteins

Compounds	Apparent[a] molecular weight	Modified[b] NH_2-groups (%)	Number[c] of galactose (mol/mol protein)	Galactose[c] content (w/w %)
Gal-IgG	159000	69.0	38.4	4.40
Gal-BSA	68300	45.6	20.2	5.14
Gal-SOD	33600	61.2	10.8	5.74
Gal-STI	21300	78.9	6.04	5.12
Gal-LZM	15200	72.9	4.82	6.03

[a] The apparent molecular weights of galactosylated proteins were determined by size exclusion chromatography.
[b] The modified NH_2-groups were determined by TNBS method.
[c] The number of galactose residues and galactose content were determined by anthrone-sulfuric acid method.

Tissue distribution of ^{111}In-labeled galactosylated proteins

At doses lower than 1 mg/kg, Gal-IgG and Gal-BSA were efficiently taken up by the liver to an extent of more than 80% of the dose within 10 min, irrespective of the dose (Fig. 3). The plasma concentration rapidly decreased reflecting their rapid accumulation in the liver. At doses higher than 1 mg/kg, the increase in the administered dose decreased the rate and the extent of their hepatic uptake, and consequently delayed their plasma elimination. Any other tissues sampled and urine contained no significant radioactivity during the experimental period. Gal-SOD also accumulated in the liver up to 70–80% of the dose at doses lower than 1 mg/kg and the amounts taken up by the liver were decreased in correspondence with the increase of the dose. However, at doses higher than 10 mg/kg, the amounts recovered in the liver were much smaller than those of Gal-IgG and Gal-BSA. Gal-STI and Gal-LZM were taken up by the liver but the amounts accumulated in the liver were smaller than those of the other three galactosylated proteins at all doses and the maximum liver accumulation was only 40 and 30% for Gal-STI and Gal-LZM, respectively.

In the cases of Gal-SOD, Gal-STI, and Gal-LZM, significant amounts of radioactivity were observed in the kidney and urine especially at higher doses, and the ratio of the

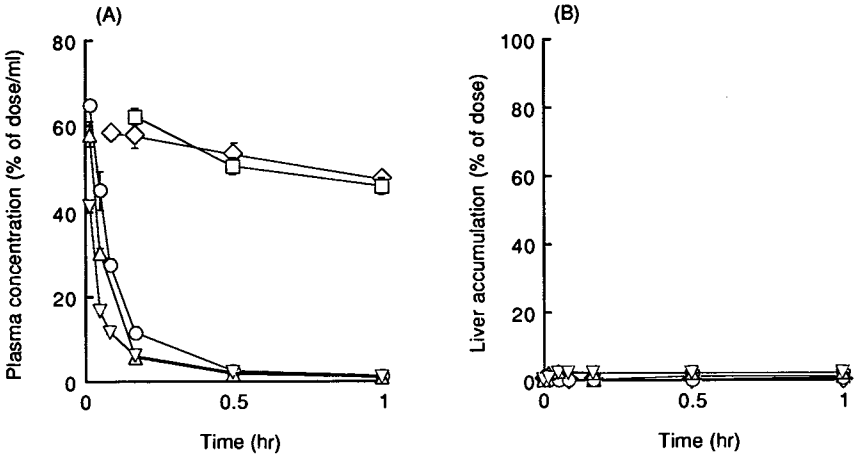

Fig. 1 Plasma concentration (A)- and liver accumulation (B)- time courses of ^{111}In-labeled native proteins after intravenous injection in mice at a dose of 1 mg/kg. Results are expressed as the mean ± S.D. of three mice. (◇) IgG; (□) BSA; (○) SOD; (△) STI; (▽) LZM.

amount excreted in urine and that present in the kidney (urine/kidney) increased with an increase in the dose (data not shown). Little radioactivity was counted in any other tissues for all galactosylated proteins.

Calculation of pharmacokinetic parameters

Pharmacokinetic parameters of native and galactosylated proteins were calculated from the experimental data to reveal factors affecting the different efficiency of hepatic targeting of galactosylated proteins. IgG and BSA have large AUCs and small total-body clearances reflecting their long retention in plasma (Fig. 1). The total-body clearances of SOD, STI, and LZM were larger than those of IgG and BSA, and slightly increased with a decrease in their molecular weights. All native proteins have hepatic clearances (CL_{liver}) less than 0.5 ml/hr and the difference in their total-body clearances is explained by urinary clearance (CL_{urine}) and kidney uptake clearance (CL_{kidney}).

Figure 4 summarizes the CL_{liver} of galactosylated proteins as functions of an administered dose and molecular weight of proteins. Galactosylation of proteins increased CL_{liver} without changing the tissue uptake clearances in any other organs except for kidney and urine. At doses lower than 0.1 mg/kg, hepatic clearances of Gal-IgG, Gal-BSA, and Gal-SOD were large and comparable to the hepatic plasma flow rate (85 ml/hr for 25 ~ 28 g weight-mouse) (21). On the contrary, the CL_{liver} of Gal-STI and Gal-LZM was approximately 10 ml/hr. Hepatic clearance of all galactosylated proteins decreased with an increase in administered doses higher than 0.1 mg/kg, indicating that the hepatic uptake process is saturable (Fig. 4 and 5).

In the cases of Gal-IgG and Gal-BSA, no significant and dose-dependent clearance was observed except for CL_{liver} (data not shown). On the other hand, other three galactosylated proteins showed a high CL_{urine} and CL_{kidney} and the sum of CL_{urine} and CL_{kidney} was identical to that of the particular native protein irrespective of the administered dose (Fig. 5).

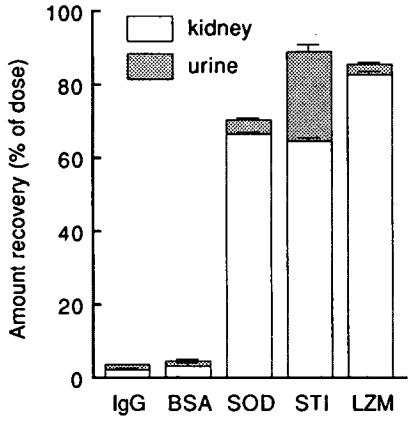

Fig. 2 Recovery of radioactivity in the kidney and urine of ^{111}In-labeled native proteins at 24 hr for IgG and BSA and 1 hr for SOD, STI, and LZM after intravenous injection. Results are expressed as the mean ± S.D. of three mice.

DISCUSSION

We investigated the *in vivo* disposition patterns of several galactosylated proteins with different molecular weights to clarify the effect of molecular weights of proteins on their hepatic targeting. Proteins and their derivatives were radiolabeled with ^{111}In using DTPA anhydride (17) to determine their *in vivo* distribution patterns without considering the degradation or efflux from the tissues (19,20). To clarify the factors affecting the hepatic targeting, we applied a pharmacokinetic approach to the interpretation of the distribution data.

In every case we tested, the introduction of galactose moieties to proteins led to an increased uptake by the liver, probably by liver parenchymal cells via the asialoglycoprotein receptor. The amounts accumulated in the liver, however, differed among these galactosylated proteins. At doses lower than 0.1 mg/kg, Gal-IgG, Gal-BSA, and Gal-SOD were effectively delivered to the liver compared with Gal-STI and Gal-LZM. Pharmacokinetic analysis revealed that the difference in hepatic targeting efficiency can be attributed to their

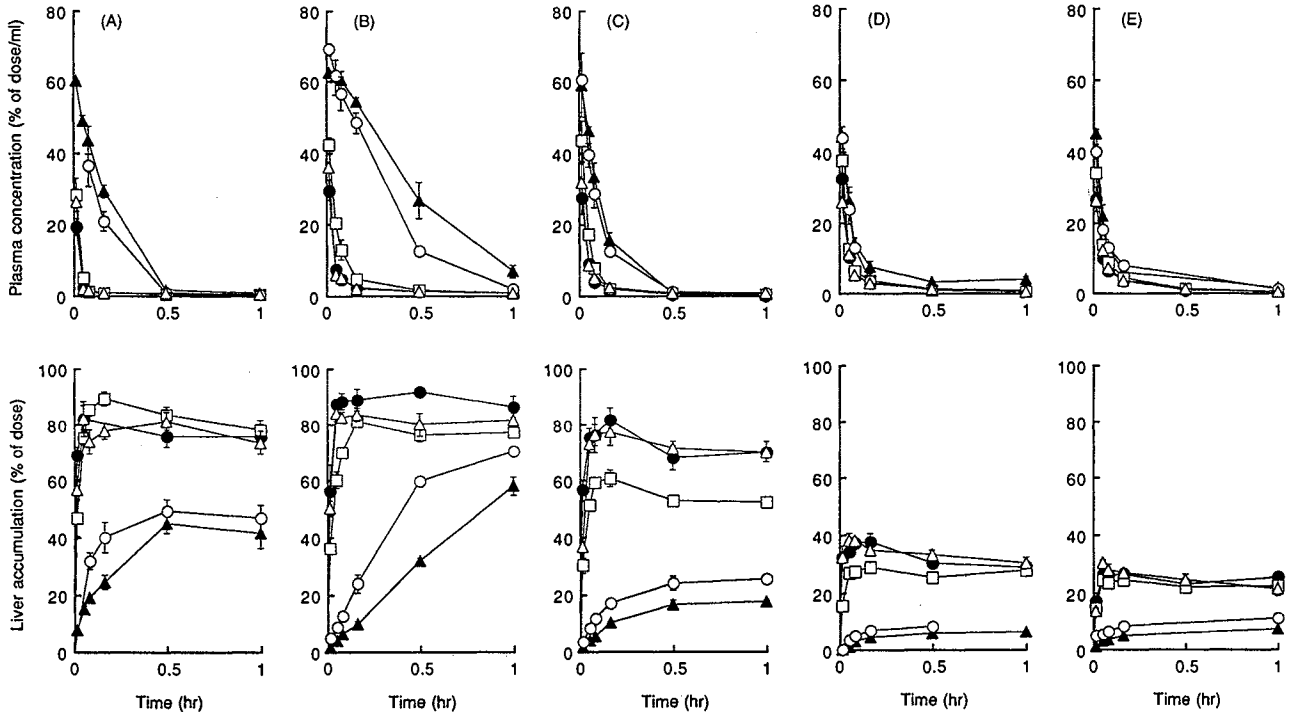

Fig. 3 Plasma concentration (upper panel)- and liver accumulation (lower panel)- time courses of ^{111}In-labeled Gal-IgG (A), Gal-BSA (B), Gal-SOD (C), Gal-STI (D), and Gal-LZM (E) after intravenous injection in mice at doses of 0.05 (●), 0.1 (△), 1 (□), 10 (○), or 20 (▲) mg/kg. Results are expressed as the mean ± S.D. of three mice.

CL_{liver}. There was a large difference between the hepatic clearances of Gal-SOD and those of Gal-STI, especially at doses lower than 1 mg/kg. However, when the dose was increased and the uptake of these galactosylated proteins by the liver became saturated, the difference in CL_{liver} was much less pronounced. The affinity of synthesized glycosylated proteins to carbohydrate receptors depends on the numbers, the clustering, and the geometric organization of sugars (22–24). In this study, the numbers of galactose residues per protein molecule was varied in order to use derivatives having the same galactose contents (between 4.4–6.0%). The small numbers of galactose residues on Gal-STI and Gal-LZM very likely cause the inefficient hepatic delivery probably due to the low affinity to the receptor. Further modification with galactose can not be achieved since STI and LZM have the small numbers of amino groups. We recently confirmed the importance of the total numbers or the density of galactose moieties studying the *in vivo* distribution properties of Gal-SODs with different extents of modification (unpublished data).

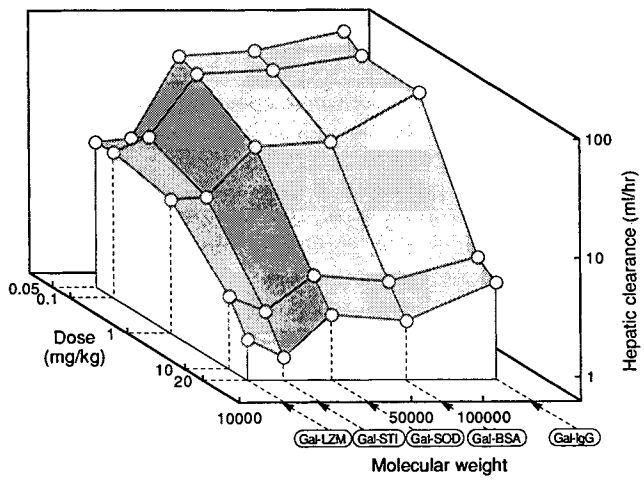

Fig. 4 Effects of dose and molecular weight on hepatic clearances (CL_{liver}) of ^{111}In-labeled galactosylated proteins. The CL_{liver} was calculated by dividing the amount of each derivative in the liver by the area under the plasma concentration-time curve (AUC). Circles represent the CL_{liver} of each galactosylated protein at an each dose from 0.05 to 20 mg/kg.

In general, the *in vivo* distribution of macromolecules also depends on the molecular weight and overall electric charge of the molecules. Our finding show that the molecular weights of proteins largely determined the retention in blood circulation. This was anticipated since the glomerular filtration rate depends on the size of the proteins (14,25). Actually, SOD, STI, and LZM, that have a molecular weight less than 32 kDa, were rapidly eliminated from plasma by glomerular filtration. When we discuss the distribution characteristics using pharmacokinetic parameters, the sum of CL_{urine} and CL_{kidney} corresponds to the glomerular filtration rate since these proteins are hardly taken up by the kidney from the blood capillary side (25). Filtration did not change by modification with galactose moieties. Galactosylated proteins tended to be more easily excreted into urine rather than reabsorbed by the kidney compared with the native proteins. The reabsorption was also shown to be a saturable process since the ratio of CL_{urine} and CL_{kidney} (CL_{urine}/CL_{kidney}) of Gal-SOD, Gal-STI, and Gal-LZM was increased with the increase in the dose as observed in the perfusion study of several proteins (25). The abundant glomerular filtration rate

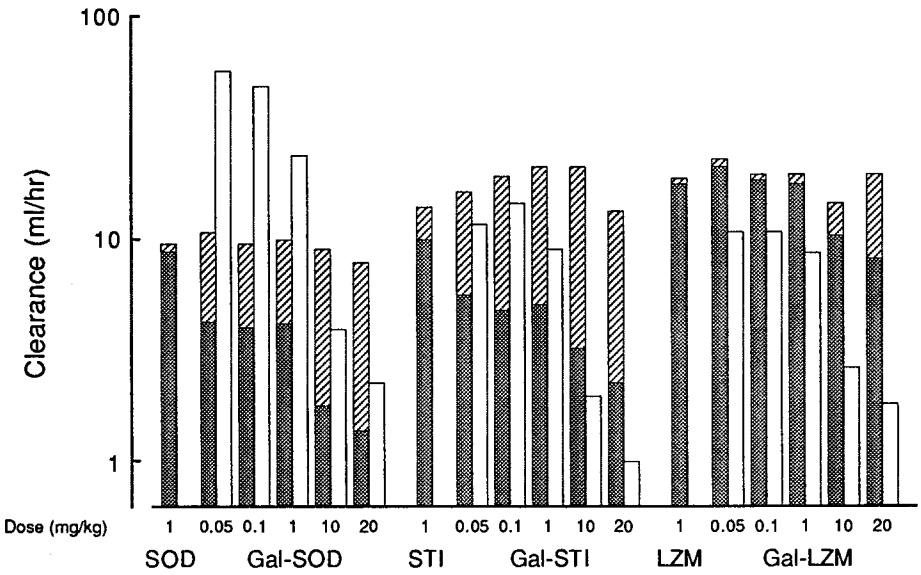

Fig. 5 Clearances of ^{111}In-labeled SOD, STI, LZM, and their galactosylated derivatives. Clearances were calculated by dividing the amount of each protein in urine (CL_{urine}; hatched bar), kidney (CL_{kidney}; closed bar), and liver (CL_{liver}; open bar) by the area under the plasma concentration-time curve (AUC).

is likely one of the reasons for the smaller amounts of Gal-STI and Gal-LZM delivered to the liver since the amount delivered to the liver can be determined by the balance of CL_{liver} and CL_{total} that highly depends on the glomerular filtration rate. A relatively high glomerular filtration rate of Gal-SOD (9.3 ± 1.0 ml/hr) led to the decrease in the accumulated amount in the liver at doses higher than 10 mg/kg since CL_{liver} decreased with an increase of the administered dose but the filtration rate was constant at the various doses studied. An approach to improve hepatic delivery of such glycosylated proteins with a molecular weight less than 32 kDa consists of covalent attachment of other macromolecules such as polyethylene glycol (PEG) or carboxymethyldextran (CMD) to reduce the glomerular filtration rate (1,2). Asialofetuin (26) and mannosylated and lactosylated BSA (6) modified with PEG retain their carbohydrate receptor binding potency. Modification with PEG or CMD would therefore be useful for hepatic targeting of the protein drugs with small molecular weights.

In conclusion, galactosylation of proteins larger than 32 kDa caused them to be effectively taken up by the liver parenchymal cells but saturation of uptake was observed at doses higher than 1 mg/kg. The insufficient amount of galactosylated proteins with low molecular weights like Gal-STI and Gal-LZM delivered to the liver was pharmacokinetically revealed to be due to both their low CL_{liver} and high glomerular filtration rate. Improvement of their hepatic delivery will be achieved by conjugating such proteins with other macromolecules to diminish the glomerular filtration or by attaching multi-antennary oligoglycosides to the proteins to increase the CL_{liver}.

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