Characterization of Novel Kidney-Specific Delivery System Using an Alkylglucoside Vector

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ABSTRACT

The alkylglucoside vector has been demonstrated to be a kidney-specific drug delivery system via cell surface-specific binding sites. In the present study, we examined the targeting efficiency of this vector derivatized with several types of ligand to determine the efficacy and limitations of this system. The tissue uptake clearance in the kidney (CLuptake, kidney) of alkylglucoside-acetylated poly-L-lysine conjugates (Glc-S-C8-APL) with a mol. wt. of 4,500, 17,000, or 41,000 was greater than that accounted for by glomerular filtration and was reduced by addition of n-octyl-thioglucoside, which has an affinity for alkylglycoside binding sites. The mol. wt. distribution, assessed by gel filtration high-performance liquid chromatography, of the radioactivity associated with the kidney after intravenous administration of Glc-S-C8-APL41000 was shifted to a lower mol. wt. range compared with the authentic compound. Both the CLuptake, kidney and specific binding of Glc-S-C8-APL, fractionated based on mol. wt., to kidney membrane fractions was increased. These results suggest that the target efficiency of this vector depends on the size of the ligand that it delivers. Both the CLuptake, kidney and specific binding to kidney membranes of an alkylglucoside-tyrosine conjugate (Glc-S-C8-Tyr) with an acidic charge was much lower than that of Glc-S-C8-APL with cationic and neutral charges, suggesting that the anionic moiety could reduce the renal targeting efficiency. Thus, the targeting efficacy of the alkylglucoside vector seems to depend on, at least, the size and charge of the ligand that it delivers.

A number of methods have been proposed and are currently under investigation for developing tissue-specific targeting vectors for several types of the drugs (Meijer and van der Sluijs, 1989; Kato and Sugiyama, 1997). Polypeptide ligands, antibodies, viruses, fatty acids, and sugar moieties have all been used as vector systems. Drug delivery targeted to "sugar-recognizing" receptors such as asialoglycoprotein and mannose receptors has been demonstrated to lead to the highly efficient delivery of various types of molecules, including low molecular weight therapeutic agents, proteins, and genes to the liver (Spanjer et al., 1985; Nishikawa et al., 1995; Takakura and Hashida, 1996; Wu et al., 1997; Zanta et al., 1997). In addition, several methods have been shown to deliver therapeutic agents to the kidney. These include a delivery system using reabsorption systems expressed on the brush-border membrane for macromolecular proteins and peptides after they have undergone glomerular filtration (Franssen et al., 1992; Haas et al., 1993, 1997). The prodrug system that is activated by kidney-specific enzymes has also been investigated (Elfarra et al., 1995; Kearney, 1996). However, compared with liver-targeting vectors, only a limited amount of information is available on systems that target the kidneys.

Suzuki et al. (1999b) recently found that arginine-vasopressin (AVP), when modified by linking it to a sugar via an octamethylene group, exhibits renal-selective and efficient association in rats. Further investigations revealed that 1) the alkylglucoside structure (Glc-S-C8-, Fig. 1) is necessary for recognition by the kidney, and n-octyl-thioglucoside (OTG, Fig. 1) is also distributed specifically to the kidney; 2) the affinity of an alkylglucoside vector for its specific binding site on the kidney membrane depends on the type of sugar moieties involved, the length of the alkyl chain, the structure of the peptide, and type of linkage between the sugar and alkyl chain; 3) the distribution of alkylglucoside derivatives to the kidney is much higher than can be explained by glomerular filtration alone, and saturation of such distribution occurs over a similar concentration range to that found for binding to the membrane, suggesting that specific binding site(s) are involved in the renal distribution (Suzuki et al., 1999a,c). Actually, the cross-linking of 125I-labeled alkylglycoside to the renal plasma membrane revealed the presence of a number of binding sites that were specific to the membrane.

ABBREVIATIONS: AVP, arginine-vasopressin; OTG, n-octyl-thioglucoside; APL, acylated poly-L-lysine; PBS, phosphate-buffered saline; CLuptake, kidney uptake clearance; TFA, trifluoroacetic acid; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; BLMV, basolateral membrane vesicle; BBMV, basolateral brush-border membrane vesicle; GFR, glomerular filtration rate; fu, unbound fraction.
alkyl-thio-glucoside (Glc-S-C8-)

\[
\begin{align*}
R: & \quad \text{O} \quad S-(\text{CH}_2)_8^* \\
\text{(A) n-octyl-thioglucoaidide (OTG)} & \quad \text{R-H} \\
\text{(B) Glc-S-C8-AVP} & \quad \text{R-CO-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH}_2 \\
\text{(C) Glc-S-C8-Ala-VP} & \quad \text{R-CO-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Ala-Gly-NH}_2 \\
\text{(D) Glc-S-C8-APLs} & \quad \text{R-CO-Cys-Tyr-NH-(CH}_2)_2^*\text{NH-CO-R} \\
\text{(E) Glc-S-C8-Tyr-Neutral} & \quad \text{R-CO-Tyr-NH-(CH}_2)_2^*\text{CH}_3 \\
\text{(F) Glc-S-C8-Tyr-Acid} & \quad \text{R-CO-Tyr-NH-(CH}_2)_2^*\text{COO}^- \\
\text{(G) Glc-S-C8-Tyr-Base} & \quad \text{R-CO-Tyr-NH-(CH}_2)_2^*\text{NH}_3^+ \\
\end{align*}
\]

Fig. 1. Structures of the alkylglucoside vector and its derivatives.

Experimental Procedures

Materials. Thioglucoaidide derivatives were synthesized by Meiji Seika (Kanagawa, Japan) as follows: the carboxyl residue of the 3-tyrosine was conjugated with an amino residue of ethylendiamine, and the other amino residue of the conjugate was condensed with carboxy residue of 9-11-thio-3-3-glucopyranosylmononanoic acid synthesized by the reported method (Suzuki et al., 1999b) and the obtained conjugate was named compound X. The acylation of poly-l-lysines with mol. wt. of 1,000 to 4,000, 4,000 to 15,000, and 15,000 to 30,000 was performed by a reported method (Gonsho et al., 1994). The carboxyl terminal of each of the three kinds of APL was condensed with the amino residue of compound X and the obtained conjugates were named Glc-S-C8-APL4500, Glc-S-C8-APL17000, and Glc-S-C8-APL41000. The number in these abbreviations represents the mean mol. wt., assuming that all the lysine residues in the poly-l-lysines were acylated. Glc-S-C8-Tyr-Base, Glc-S-C8-Tyr-Neutral, and Glc-S-C8-Tyr-Acid were synthesized as follows: the carboxyl residue of 9-11-thio-3-3-glucopyranosylmononanoic acid was conjugated with the amino residue of 3-tyrosine (Glc-S-C8-Tyr). Glc-S-C8-Tyr-Base, Glc-S-C8-Tyr-Neutral, and Glc-S-C8-Tyr-Acid were obtained by conjugating carboxyl residue of Glc-S-C8-Tyr with the amino residue of ethylendiamine, propylamine, and -alanine, respectively. Glc-S-C8-Ala-VP and Glc-S-C8-AVP were synthesized as described previously (Suzuki et al., 1999b). Na[125I] (98.5 mCi/ml) was purchased from Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK). [3H]Inulin was purchased from PerkinElmer Life Science Products (Boston, MA). Soluene 350 and HONIC Flour were purchased from Packard Instrument Co. (Dowegs Grove, IL). Sep-Pak(C18) was purchased from Waters (Milford, MA). OTG was purchased from Wako (Osaka, Japan). Centrifree and Microcon were purchased from Millipore Corporation (Bedford, MA).

Animals. Male Sprague-Dawley rats were purchased from Nihon Ikagaku (Tokyo, Japan) and used at 6 to 7 weeks of age. Food and water were available ad libitum. The studies reported in this article have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

125I-Labeling of Alkylglucoside Derivatives. Alkylglucoside derivatives were labeling by the chloramine-T method. Briefly, 20 μl of alkylglucoside derivative in PBS (50 nM) was mixed with 50 μl of 0.5 M phosphate buffer, pH 7.5 and then 1 μl of Na[125I] was added. Following this, 10 μl of chloramine-T (2 mg/ml dissolved in PBS) was added to the reaction mixture and mixed using a vortex mixer for 30 s. After mixing, 50 μl of Na2S2O5 (2.5 mg/ml in PBS) and 10 μl of potassium iodide (100 mg/ml in PBS) were added to stop the labeling. To exclude free Na[125I], the reaction mixture was applied to a Centrifree and Microcon were purchased from Millipore Corporation (Bedford, MA). OTG was purchased from Wako (Osaka, Japan). Centrifree and Microcon were purchased from Millipore Corporation (Bedford, MA).

Binding to Rat Kidney Membrane. Kidney membrane was prepared from 10 rats by the centrifugation method (Stassen et al., 1982), mixed with PBS, pH 7.4, containing 125I-labeled ligand and 0.1% BSA, and incubated for 1 h on ice at a concentration of 1 mg of protein/ml (Suzuki et al., 1999b). After centrifugation (15,000g, 10 min at 4°C), the supernatant was removed and the radioactivity in the supernatant and precipitate was measured by gamma counter. Data were fitted to the following equation using the MULTI program (Yamaoka et al., 1981).

\[
C_b = B_{\text{max}} C_l/(K_b + C_l) + C_i + \alpha C_l
\]

where \(C_b\) is the ligand bound to the membrane (pmol/mg of protein), \(C_l\) is the unbound ligand concentration (pmol/ml), \(B_{\text{max}}\) is the maximum binding capacity (pmol/mg of protein), \(K_b\) is the dissociation constant (nM), and \(\alpha\) is the proportional constant of nonspecific binding.
In the inhibition study both $^{125}I$-Glc-S-C8-APL (2 nM) and unla-
beled Glc-S-C8-APL (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 μM) were incubated with kidney membrane for 1 h on ice
in 0.1% BSA/PBS, pH 7.4 (Suzuki et al., 1999b). The inhibition constant
($K_i$) (nM) was determined by fitting the data to the following equation:

$$C_b/C_t = B_{max}/(K_i(1 + I/K_i) + C_i) + \alpha$$

where $I$ represents the inhibitor concentration, and $B_{max}$, $K_i$, and $\alpha$
were fixed as the values obtained from a Scatchard analysis.

Tissue Distribution of Alkylglucoside Derivatives. The fem-
oral artery was cannulated under light anesthesia with diethyl ether.
$^{125}I$-Labeled ligand was dissolved at a concentration of 1
nmol/ml in PBS alone or PBS containing OTG (final concentration
2.5, 5, 10, or μmol/ml) and administered via the tail vein at a dose of
1 ml/kg. Blood was collected at 0.5, 1, 1.5, 2, 3, 4, and 5 min and then
quickly centrifuged to obtain plasma. Rats were sacrificed at 5 min
and all or a portion of the tissues (liver, kidney, small intestine,
skeletal muscle, lung, and spleen) were removed and rinsed with
saline. The radioactivity was determined by gamma counter. The tissue
uptake clearance ($CL_{uptake}$) was calculated with the following equation:

$$CL_{uptake} = X_{5\text{ min}}/AUC_{\text{0} - 5\text{ min}}$$

where $X_{5\text{ min}}$ (pmol/g of tissue) and $AUC_{\text{0} - 5\text{ min}}$ (pmol · min/ml) are
the amount of ligand in the tissue at 5 min and the area under the
plasma concentration-time curve from 0 to 5 min, respectively.

Mol. Wt. Distribution of Radioactivity Associated with Kid-
ney after Glc-S-C8-APL Administration. $^{125}I$-Glc-S-C8-
APL17000 or $^{125}I$-Glc-S-C8-APL41000 (1 nmol/ml) dissolved in PBS
or PBS containing OTG (10 μmol/ml) was administered via the tail
vein. The kidney was removed at 5 or 90 min and homogenized after
adding a 3-fold volume of PBS. This 25% homogenate was mixed
with a 4-fold volume of CH$_3$CN and, after centrifugation, the super-
natant was evaporated and the residue dissolved in mobile phase
through a 0.22-
membrane filter (pore size 0.45 μm; Millipore Corporation), rapidly
filtered, and washed twice with 4 ml of ice-cold 0.1% BSA/PBS. The radioactivity in the filter was measured by gamma counter.

Results

Specific Binding of Glc-S-C8-APLs to Rat Kidney Membrane. To characterize the mol. wt. dependence of the targeting efficiency of the alkylglycoside system for the kidney, we first synthesized Glc-S-C8-APLs from poly-$\alpha$-lysines with three different mol. wt. distributions (Fig. 1). Gel filtration HPLC analysis revealed that the mean mol. wt. of Glc-
S-C8-APL45000, Glc-S-C8-APL17000, and Glc-S-C8-
APL41000 assessed using polyethylene oxide as a mol. wt.
marker was 6,920, 11,200, and 28,200, respectively.

The binding of $^{125}I$-Glc-S-C8-APL45000, Glc-S-C8-
APL17000, and Glc-S-C8-APL41000 to rat kidney membrane exhibited both saturable and nonsaturable components (Fig. 2) and was much lower than that of $^{125}I$-Glc-S-C8-APL (mol.
wt. ~1500). The $K_d$ of Glc-S-C8-APL was in the nanomolar range, whereas the $K_d$ of Glc-S-C8-APLs was in the micro-
molar range (Table 1). The specific binding ($B_{max}/K_i$) of Glc-
S-C8-APLs was 11 to 23 times lower than that of Glc-S-C8-
APL (Table 1). The specific binding ($B_{max}/K_i$) of Glc-
S-C8-APLs was comparable with their respective $K_d$.

![Fig. 2. Scatchard plot representing the specific binding of Glc-S-C8-APL and Glc-S-C8-APLs to rat kidney membrane. Various concentrations of $^{125}I$-Glc-S-C8-APL (○), Glc-S-C8-APL45000 (□), 17000 (△), and 41000 (●) were incubated with kidney membrane (1 mg/ml) at 4°C for 1 h. The straight line represents the fitted line. Each value represents the mean ± S.E. of triplicate experiments in two membrane preparations. The inset represents the enlarged scale.](Image)
values (Table 1). Glc-S-C8-AVP also inhibited the binding of these three compounds (Fig. 3B), and the obtained $K_i$ value ($13-28$ nM) was comparable with the $K_d$ of Glc-S-C8-AVP (Table 1).

**Tissue Distribution of Glc-S-C8-APLs.** $^{125}$I-Glc-S-C8-APL4500, $^{125}$I-Glc-S-C8-APL17000, and $^{125}$I-Glc-S-C8-APL41000 were administered intravenously to rats, and the CL uptake in each tissue was examined (Fig. 4). The CL uptake of each ligand was highest in the kidney compared with other tissues (Fig. 4). As a control experiment, the CL uptake of $[^{14}$C$]$inulin, an extracellular and glomerular filtration rate (GFR) marker, was also examined. The CL uptake of $[^{14}$C$]$inulin was $0.0148 \pm 0.0025$, $0.0658 \pm 0.0011$, $0.713 \pm 0.122$, $0.0334 \pm 0.0065$, $0.0201 \pm 0.0060$, and $0.0277 \pm 0.0061$ ml/min/g of tissue in muscle, small intestine, kidney, lung, spleen, and liver (mean $\pm$ S.E., $n=3$). Thus, the CL uptake of $^{125}$I-Glc-S-C8-APLs in tissues other than the kidney was almost comparable with that of $[^{14}$C$]$inulin.

To estimate the contribution of glomerular filtration to the renal CL uptake of Glc-S-C8-APLs, the plasma unbound fraction ($f_u$) was determined. The $f_u$ of Glc-S-C8-APL4500, Glc-S-C8-APL17000, and Glc-S-C8-APL41000 was $0.432 \pm 0.02$, $0.458 \pm 0.06$, and $0.483 \pm 0.12$, respectively. The GFR was assessed as the CL uptake of inulin, and the contribution of glomerular filtration was estimated as $f_u$GFR, which is shown in Fig. 5. The CL uptake of Glc-S-C8-APLs and Glc-S-C8-AVP was higher than their corresponding $f_u$GFR values, although the $f_u$GFR was near to the CL uptake for larger molecules (Glc-S-C8-APL17000 and Glc-S-C8-APL41000) (Fig. 5).

Coadministration of OTG, which also binds to the specific binding site for alkylglycoside, reduced the CL uptake of Glc-S-C8-APLs and Glc-S-C8-AVP (Fig. 5). The CL uptake of these compounds at an OTG of $10 \mu$mol/kg was almost comparable with their respective $f_u$GFR values (Fig. 5).

**Mol. Wt. Distribution of Radioactivity in Kidney after Injection of Glc-S-C8-APLs.** When $^{125}$I-Glc-S-C8-APL41000 was administered intravenously, the radioactivity in the kidney at 5 min after injection exhibited a smaller mol. wt. distribution than authentic $^{125}$I-Glc-S-C8-APL41000 and $^{125}$I-Glc-S-C8-APL17000 (Fig. 6A). The mol. wt. distribution of radioactivity at 90 min after injection was lower than that at 5 min (Fig. 6B). When OTG was coadministered, the radioactivity was reduced to a greater extent than in the absence of OTG (Fig. 6B).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$B_{max}$ (pmol/mg of protein)</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}/K_d$ (ml/mg of protein)</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-S-C8-AVP</td>
<td>20.1</td>
<td>28.7</td>
<td>0.700</td>
<td></td>
</tr>
<tr>
<td>Glc-S-C8-APL4500</td>
<td>86.0</td>
<td>1320</td>
<td>0.0692</td>
<td>1360</td>
</tr>
<tr>
<td>Glc-S-C8-APL17000</td>
<td>78.4</td>
<td>662</td>
<td>0.118</td>
<td>693</td>
</tr>
<tr>
<td>Glc-S-C8-APL41000</td>
<td>34.2</td>
<td>906</td>
<td>0.0577</td>
<td>1290</td>
</tr>
</tbody>
</table>

$^a$ Inhibition constant of Glc-S-C8-APLs for the binding of $^{125}$I-Glc-S-C8-AVP to rat kidney membrane fraction.

$^b$ Inhibition constant of Glc-S-C8-AVP for the binding of $^{125}$I-Glc-S-C8-APLs to rat kidney membrane fraction.

**Fig. 3.** Mutual inhibition between Glc-S-C8-AVP and Glc-S-C8-APLs in terms of specific binding to rat kidney membrane. A, inhibitory effect of Glc-S-C8-APL4500 ( ), 17000 ( ), and 41000 ( ) on the binding of Glc-S-C8-AVP. Each value represents the mean $\pm$ S.E. of triplicate experiments in two membrane preparations. B, inhibitory effect of Glc-S-C8-AVP on the binding of Glc-S-C8-APL4500 ( ), 17000 ( ), and 41000 ( ). Each value represents the mean $\pm$ S.E. of triplicate experiments in two membrane preparations.

**Fig. 4.** Tissue CL uptake of $^{125}$I-Glc-S-C8 derivatives. Alkylglycoside derivatives (1 nmol/kg) were administered intravenously, and the time-profile of the plasma concentrations was followed. At 5 min after administration, tissues were removed and counted. Each value represents the mean $\pm$ S.E. of four to eight rats.

**Fig. 5.** Inhibitory effect of OTG on renal uptake of $^{125}$I-Glc-S-C8 derivatives. $^{125}$I-Glc-S-C8-AVP (A), $^{125}$I-Glc-S-C8-APL4500 (B), 17000 (C), or 41000 (D) at 1 nmol/kg were simultaneously administered with OTG at the different doses shown. The dotted line represents the contribution of glomerular filtration, which was assessed as the $f_u$GFR. Each value represents the mean $\pm$ S.E. of four to eight rats.
each fraction was examined (Table 2). In each case, the CL uptake of fractionated Glc-S-C8-APLs was higher for the lower mol. wt. fraction than for the higher mol. wt. fraction, whereas the $f_u$ of each did not differ by much (Table 2). The contribution of binding and/or uptake from the blood side, assessed as the CL uptake $- f_u$ GFR, also depended on the mol. wt. of Glc-S-C8-APLs (Table 2).

The binding of the Glc-S-C8-APLs fractionated in this way to kidney membranes was examined (Table 3). The specific binding, assessed by subtracting the binding in the presence of excess unlabeled Glc-S-C8-AVP from that in its absence, was relatively higher for the fractionated Glc-S-C8-APLs with a lower mol. wt., whereas no specific binding of Glc-S-C8-APLs with a mol. wt. higher than 13,900 was found (Table 3).

**Localization of Glc-S-C8-APLs in Kidney.** Both semimicroautoradiography (Fig. 7; Table 4) and microautoradiography (Fig. 8) were performed for the analysis of intrarenal localization. Radioactivity was found in the kidney cortex after the injection of $^{125}$I-Glc-S-C8-4500, $^{125}$I-Glc-S-C8-41000, and $^{125}$I-Glc-S-C8-AVP alone (Fig. 7, A, C, and E), whereas this radioactivity fell when OTG was coadministered (Fig. 7, B and D). The density of radioactivity was quantified and this is shown in Table 4. The accumulation of radioactivity was mainly found in the cortex after the injection of $^{125}$I-Glc-S-C8-AVP, whereas, after injection of Glc-S-C8-4500 and Glc-S-C8-41000, it was also found in the inner and outer medulla to a lesser extent (Table 4). The reduction after coadministration of OTG was found mainly in the cortex, whereas the effect of OTG was not so obvious in the inner or outer medulla (Table 4).

The microautoradiography studies showed that $^{125}$I-Glc-S-C8-AVP was mainly distributed to the proximal tubules (Fig. 8E), whereas the distribution of $^{125}$I-Glc-S-C8-APL4500 was found in proximal tubules and around the glomeruli and distal tubules (Fig. 8, A and B). Glc-S-C8-APL41000 was distributed in the proximal and distal tubules in the cortex (Fig. 8, C and D) and the collecting tube in the medulla (data not shown).

To confirm the localization of the binding site for alkylglycosides, the binding of $^{125}$I-Glc-S-C8-AVP to BBMV and BLMV was examined (Fig. 9). The binding to BLMV was higher than that to BBMV and was reduced in the presence of excess unlabeled Glc-S-C8-AVP (Fig. 9).

**TABLE 2**

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Mol. wt.</th>
<th>CL uptake</th>
<th>$f_u$</th>
<th>Specific Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-S-C8-APL17000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High mol. wt. fraction</td>
<td>16,600–33,300</td>
<td>0.449 ± 0.06</td>
<td>0.518 ± 0.08</td>
<td>0.0931 ± 0.134</td>
</tr>
<tr>
<td>Middle mol. wt. fraction</td>
<td>9,810–11,700</td>
<td>0.674 ± 0.183</td>
<td>0.491 ± 0.07</td>
<td>0.324 ± 0.219</td>
</tr>
<tr>
<td>Low mol. wt. fraction</td>
<td>4,100–8,240</td>
<td>0.746 ± 0.05</td>
<td>0.563 ± 0.03</td>
<td>0.338 ± 0.132</td>
</tr>
<tr>
<td>Glc-S-C8-APL41000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High mol. wt. fraction</td>
<td>39,700–113,000</td>
<td>0.175 ± 0.011</td>
<td>0.519 ± 0.05</td>
<td>N.D.</td>
</tr>
<tr>
<td>Low mol. wt. fraction</td>
<td>4,100–8,240</td>
<td>1.11 ± 0.088</td>
<td>0.590 ± 0.05</td>
<td>0.689 ± 0.196</td>
</tr>
</tbody>
</table>

N.D., not determined.

* Fractionated ligand derived either from Glc-S-C8-APL17000 or Glc-S-C8-APL41000 was intravenously administered, and the tissue uptake clearance in kidney was determined.

* Molecular weight determined by gel filtration HPLC.

* Tissue uptake clearance in kidney (mean ± S.E. of three rats).

* CL uptake of alkylglycoside derivatives subtracted by $f_u$ × CL uptake of inulin (0.713 ± 0.122 ml/min/g of kidney).
TABLE 3
Specific binding of fractionated ligands derived from Glc-S-C8-APL17000 to rat kidney membrane
Fractionated ligands (2 nM) derived from Glc-S-C8-APL17000 was incubated with rat kidney membrane and the binding was determined.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Elution Timea</th>
<th>Mol. wt.b</th>
<th>Bindingc</th>
<th>Bindinge (+1 μM Glc-S-AVP)</th>
<th>Specifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22–24</td>
<td>16,600–33,300</td>
<td>0.891 ± 0.081</td>
<td>0.916 ± 0.103</td>
<td>N.D.</td>
</tr>
<tr>
<td>2</td>
<td>24–24.5</td>
<td>13,900–16,600</td>
<td>0.647 ± 0.046</td>
<td>0.594 ± 0.030</td>
<td>0.074 ± 0.060</td>
</tr>
<tr>
<td>3</td>
<td>24.5–25</td>
<td>11,700–13,900</td>
<td>0.589 ± 0.005</td>
<td>0.540 ± 0.010</td>
<td>0.049 ± 0.011</td>
</tr>
<tr>
<td>4</td>
<td>25–25.5</td>
<td>9,810–11,700</td>
<td>0.607 ± 0.023</td>
<td>0.442 ± 0.045*</td>
<td>0.165 ± 0.050</td>
</tr>
<tr>
<td>5</td>
<td>25.5–26</td>
<td>8,240–9,810</td>
<td>0.864 ± 0.049</td>
<td>0.504 ± 0.049*</td>
<td>0.361 ± 0.070</td>
</tr>
<tr>
<td>6</td>
<td>26–28</td>
<td>4,100–5,240</td>
<td>0.785 ± 0.010</td>
<td>0.368 ± 0.035*</td>
<td>0.416 ± 0.041</td>
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<tr>
<td>7</td>
<td>28–30</td>
<td>2,040–4,100</td>
<td>0.972 ± 0.044</td>
<td>0.514 ± 0.061*</td>
<td>0.458 ± 0.076</td>
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N.D., not determined.
a Elution time in gel filtration HPLC.
b Molecular weight determined by gel filtration HPLC.
c Binding at 2 nM of the fractionated ligand alone.
d Binding at 2 nM of the fractionated ligand in the presence of 1 μM Glc-S-C8-AVP.
e Specific binding was assessed as the binding in the presence of 1 μM Glc-S-C8-AVP subtracted from that in its absence.

Renal Distribution and Specific Binding to Kidney Membranes of Glc-S-C8-Derivatives with Different Charge Moieties. To characterize the effect of the charge on the ligand derivatized with alkylglucoside vector, we examined the renal distribution and specific binding to kidney membranes of Glc-S-C8-Tyr-Base, Glc-S-C8-Tyr-Neutral, and Glc-S-C8-Tyr-Acid (Table 5; Fig. 10). We also examined Glc-S-C8-Ala-VP, which has a neutral amino acid, alanine, instead of the cationic one, arginine, in Glc-S-C8-AVP (Table 5; Fig. 10). The Cluptake of Glc-S-C8-Tyr-Base and Glc-S-C8-Tyr-Neutral was higher than that of Glc-S-C8-Tyr-Acid, whereas the Cluptake in the kidney of Glc-S-C8-Tyr-Acid was much lower than that of others (Table 5). The specific binding (Bmax/Kd) of Glc-S-C8-Tyr-Acid was much lower than that of Glc-S-C8-Tyr-Base and Glc-S-C8-Tyr-Neutral. The Bmax/Kd of Glc-S-C8-Tyr-Base and Glc-S-C8-Tyr-Neutral was similar as that of Glc-S-C8-VP and Glc-S-C8-Ala-VP (Table 5). Glc-S-C8-Tyr-Base, Glc-S-C8-Tyr-Neutral, Glc-S-C8-Tyr-Acid, and Glc-S-C8-Ala-VP inhibited the binding of Glc-S-C8-AVP, whereas Glc-S-C8-AVP also inhibited the binding of these four compounds (Table 5), the Ki of each inhibitor not being very different from the Kd of its own binding (Table 5).

Discussion
Kidney is one of the major organs involved in maintaining homeostasis in the body and, therefore, could be a target for various types of drugs. We previously found that the kidney-specific distribution of AVP could be modified by some sugar moieties via octamethylene (Suzuki et al., 1999c). Further studies revealed that the alkylglucoside structure is necessary for recognition by the kidney (Suzuki et al., 1999b). To develop this structural recognition as a kidney-targeting vector for therapeutic agents, it is important to clarify the target efficiency and limitations of the compounds derivatized with alkylglucosides. As far as these limitations were concerned, the present study focused on two aspects, molecular size and charge.

To examine the molecular size dependence in the distribution to the kidney, we synthesized Glc-S-C8-APLs from poly-L-lysines with three ranges of mol. wt. To minimize the effect of the positive charge on poly-L-lysines, these compounds were acylated before alkylglycoside conjugation. Because this vector system is recognized by the binding sites on renal membranes (Suzuki et al., 1999b,c), we first examined their specific binding to the kidney membrane fractions (Figs. 2 A–E).
and 3; Table 1). The results obtained suggest that Glc-S-C8-APL4500, Glc-S-C8-APL17000, and Glc-S-C8-APL41000 share the same binding site with Glc-S-C8-AVP because 1) mutual inhibition was observed (Fig. 3), 2) the obtained $K_i$ values for these four compounds were comparable with their respective $K_d$ values (Table 1), and 3) the $B_{\text{max}}$ value was within 4 times the difference between these compounds (Table 1). In addition, the $CL_{\text{uptake}}$ of Glc-S-C8-APL4500, Glc-S-C8-APL17000, and Glc-S-C8-APL41000 was much larger in the kidney, whereas in other organs it was similar to that of inulin, suggesting the kidney-specific distribution of these compounds. Considering that 1) the $CL_{\text{uptake}}$ in kidney was higher than the $f_u$GFR of the three compounds and 2) OTG inhibits such renal distribution of these compounds (Fig. 5), a mechanism other than GFR appears to be involved in such renal distribution of Glc-S-C8-APLs.

However, it should be noted that the affinity ($1/K_d$) of Glc-S-C8-APLs was 20 to 50 times lower than that of Glc-S-C8-AVP (Table 1), and that the difference between the $CL_{\text{uptake}}$ and the $f_u$GFR was not as marked for Glc-S-C8-APL41000 (Fig. 4). These results suggest that the greater molecular size of Glc-S-C8-APLs may, at least to some extent, hinder the association of these compounds in the kidney. In addition, because these Glc-S-C8-APLs consists of a variety molecular sizes due to the crudeness of the poly-L-lysines used to synthesize Glc-S-C8-APLs, it may be that the apparent specific binding and/or renal distribution represents that

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<th>C</th>
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A, Glc-S-C8-APL4500 1 nmol/kg; B, Glc-S-C8-APL4500 1 nmol/kg + OTG 10 μmol/kg; C, Glc-S-C8-APL41000 1 nmol/kg; D, Glc-S-C8-APL41000 1 nmol/kg + OTG 10 μmol/kg; and E, Glc-S-C8-AVP 1 nmol/kg.

Fig. 8. Microautoradiography for the distribution of $^{125}$I-labeled alkylglucoside derivatives to rat kidney cortex. Kidney slices at 5 min after intravenous administration of $^{125}$I-Glc-S-C8-APL4500 (A and B), $^{125}$I-Glc-S-C8-APL41000 (C and D) or $^{125}$I-Glc-S-C8-AVP (E) without (A, C, and E) or with (B and D) OTG (10 μmol/kg). RC, renal corpuscle; PT, proximal tube; DT, distal tube. Original magnification, 400×.

Table 4

Localization of radioactivity in rat kidney assessed in semimicroautoradiography

$^{125}$I-Labeled alkylglucoside derivatives were administered in rat via tail vein. Five minutes after administration, kidney was removed and the freeze-dried section prepared by microtome was contact to imaging plate for 1 h. Density of radioactivity in autoradiographic images was analyzed by Bio imaging analyzer BAS-2000. Radioactive intensity was represented as PSL/mm$^2$ normalized by dose of radioactivity.

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A, Glc-S-C8-APL4500 1 nmol/kg; B, Glc-S-C8-APL4500 1 nmol/kg + OTG 10 μmol/kg; C, Glc-S-C8-APL41000 1 nmol/kg; D, Glc-S-C8-APL41000 1 nmol/kg + OTG 10 μmol/kg; and E, Glc-S-C8-AVP 1 nmol/kg.

Fig. 9. Binding of $^{125}$I-Glc-S-C8-AVP to BLMV and BBMV prepared from rat kidney cortex. BLMV and BBMV (12.5 μg) were incubated with $^{125}$I-Glc-S-C8-AVP (2 nM) in the absence and presence of 1.25 μM Glc-S-C8-AVP. Binding was determined by the rapid filtration technique. Each value represents the mean ± S.E. of triplicate experiments.

Fig. 8. Microautoradiography for the distribution of $^{125}$I-labeled alkylglucoside derivatives to rat kidney cortex. Kidney slices at 5 min after intravenous administration of $^{125}$I-Glc-S-C8-APL4500 (A and B), $^{125}$I-Glc-S-C8-APL41000 (C and D) or $^{125}$I-Glc-S-C8-AVP (E) without (A, C, and E) or with (B and D) OTG (10 μmol/kg). RC, renal corpuscle; PT, proximal tube; DT, distal tube. Original magnification, 400×.
radioactivity after the injection of $^{125}$I-Glc-S-C8-APL41000, there was relatively less radioactivity compared with the also examined (Fig. 6B). When OTG was coadministered, the involvement of alkylglycoside binding sites on the kidney membranes in such renal distribution, the effect of OTG was reduced by OTG, was also found in the same region after the addition, the location of the radioactivity, which can be related to the cortex for $^{125}$I-Glc-S-C8-AVP, whereas $^{125}$I-Glc-S-C8-APL4500 than $^{125}$I-Glc-S-C8-APL41000 (Table 4). This of only the smaller size fraction of the Glc-S-C8-APLs. Therefore, we next attempted to identify the mol. wt. size distribution of the radioactivity actually associated in the kidney after the injection of $^{125}$I-Glc-S-C8-APLs (Fig. 6). The size distribution at 5 min after the injection of Glc-S-C8-APL41000 exhibited a lower mol. wt. compared with authentic Glc-S-C8-APL41000 (Fig. 6A), indicating that the Glc-S-C8-APLs actually distributed to the kidney are smaller molecules compared with those injected. In addition, a similar mol. wt. distribution was found in case of Glc-S-C8-APL17000 (Fig. 6C). Therefore, the smaller molecules could be preferentially targeted to the kidney, although we cannot rule out the possibility that the larger molecules may be rapidly degraded into smaller ones during the 5-min period after injection. The mol. wt. distribution of radioactivity at 90 min was different from that at 5 min (Fig. 6B), indicating the possible fragmentation of Glc-S-C8-APL41000. To estimate the involvement of alkylglycoside binding sites on the kidney membranes in such renal distribution, the effect of OTG was also examined (Fig. 6B). When OTG was coadministered, there was relatively less radioactivity compared with the radioactivity after the injection of $^{125}$I-Glc-S-C8-APL41000 alone (Fig. 6B). This was compatible with the hypothesis that the smaller sized molecules are preferentially recognized by the binding sites.

To more directly examine the mol. wt. dependence in the kidney targeting efficiency, $^{125}$I-Glc-S-C8-APL41000 and $^{125}$I-Glc-S-C8-APL17000 were further fractionated into $^{125}$I-Glc-S-C8-APLs with a more limited size distribution (Tables 2 and 3). Both the specific portion of the CLuptake (Table 2) and the specific binding to the kidney membrane (Table 3) were relatively smaller for the fractionated $^{125}$I-Glc-S-C8-APLs of a larger size, suggesting that the kidney targeting is highly dependent on the molecular size of the derivatives containing the alkylglycoside moieties. However, even after fractionation, the $^{125}$I-Glc-S-C8-APLs will still exhibit a degree of variation in their mol. wt. Therefore, the present analysis cannot conclusively determine the exact size limitation of kidney targeting. Considering the CLuptake and binding of fractionated $^{125}$I-Glc-S-C8-APLs (Tables 2 and 3), the renal distribution of Glc-S-C8-APLs with a mol. wt. higher than 10,000 seemed to be greatly impaired, compared with those with a lower mol. wt.

To demonstrate that Glc-S-C8-APLs can be targeted to the alkylglycoside vector binding sites, we also attempted to examine their intrarenal distribution because the location of the alkylglycoside targeting site, assessed as the radioactivity after injection of Glc-S-C8-AVP, is the cortex, especially the proximal tubules (Suzuki et al., 1999b). Similar results were obtained for $^{125}$I-Glc-S-C8-AVP (Fig. 8E; Table 4). In addition, the location of the radioactivity, which can be reduced by OTG, was also found in the same region after the injection of $^{125}$I-Glc-S-C8-APL4500 and $^{125}$I-Glc-S-C8-APL41000 (Fig. 7; Table 4), and the density of radioactivity in the kidney cortex is higher after the injection of $^{125}$I-Glc-S-C8-APL4500 than $^{125}$I-Glc-S-C8-APL41000 (Table 4). This supports the hypothesis that Glc-S-C8-APLs share the same binding sites with Glc-S-C8-AVP with higher affinity for Glc-S-C8-APLs with a lower mol. wt. Considering that the CLuptake of Glc-S-C8-APLs was higher than the respective fGFR (Fig. 4), and that the binding of $^{125}$I-Glc-S-C8-AVP is much higher in BLMV than BBMV (Fig. 9), the binding sites for alkylglycoside should be localized on the basolateral side of kidney proximal tubules. However, it was noted that there was clear selective distribution to the cortex for $^{125}$I-Glc-S-C8-AVP, whereas $^{125}$I-Glc-S-C8-APL4500 and $^{125}$I-Glc-S-C8-APL14000 were also distributed to the outer and inner medulla (Table 4). Considering that
the contribution of GFR to the renal distribution seemed to be higher for Glc-S-C8-APLs (Fig. 4), such distribution to the medulla may reflect the radioactivity that undergoes glomerular filtration. The microautoradiography also showed that Glc-S-C8-APL4500 and Glc-S-C8-41000 in the kidney cortex were also localized in the distal tubules, and such distribution was more clearly defined in Glc-S-C8-APL41000 (Fig. 8). Because the affinity of Glc-S-C8-APL4500 and Glc-S-C8-41000 for the kidney membrane is much lower than that of Glc-S-C8-AVP (Table 1), nonspecific distribution, including glomerular filtration may be marked in the case of Glc-S-C8-APL4500 and Glc-S-C8-41000 compared with Glc-S-C8-AVP.

Of the five compounds examined in Table 5, only the acidic compound Glc-S-C8-Tyr-Acid exhibited a much lower affinity for kidney membrane than the others. As in the case of Glc-S-C8-APLs, mutual inhibition among Glc-S-C8-AVP and the other four compounds was found, and the $K_i$ values obtained were comparable with the $K_i$ of the binding of inhibitors, suggesting that these five compounds also share the same binding sites on the kidney membrane. Therefore, the acidic moiety may hinder the binding of alkylglycoside derivatives. This hypothesis is compatible with the present finding that the CLuptake of Glc-S-C8-Tyr-Acid in the kidney was lower than that of Glc-S-C8-Tyr-Base and Glc-S-C8-Tyr-Neutral (Fig. 10A). Additionally, the CLuptake of Glc-S-C8-Tyr-Base was smaller than that of Glc-S-C8-Tyr-Neutral (Fig. 10A), and that of Glc-S-C8-AVP was smaller than that of Glc-S-C8-Ala-VP (Fig. 10B). Such a difference (Glc-S-C8-Tyr-Base < Glc-S-C8-Tyr-Neutral, Glc-S-C8-AVP < Glc-S-C8-Ala-VP) was also found in the specific binding to the kidney membrane (Table 5). These results suggest that the basic moiety also hinders, to at least some extent, the renal targeting of the alkylglycoside, although further studies are needed before a final conclusion can be drawn. As far as tissues other than the kidney are concerned, the CLuptake of Glc-S-C8-oxytocin, a neutral peptide, both in the liver and small intestine (Suzuki et al., 1999b).

Although the mechanism for such extrarenal distribution is still unclear, some nonspecific mechanism affected by moieties other than the alkylglycoside are likely to be involved. The binding site for the alkylglycoside vector is located on the basolateral membrane (Fig. 9), whereas the Na+/glucose cotransporter, which is inhibited by OTG, is expressed on the brush-border membrane (Haase et al., 1990; Thorens, 1996). Therefore, involvement of this cotransporter in the renal targeting is unlikely. The kidney lectin that recognizes acidic sugars is expressed in many other organs and may not explain the kidney-specific distribution of alkylglycosides (Kojima et al., 1996). Watanabe et al. (2000) identified a 62-kDa protein expressed on the kidney membranes. Additional studies are needed to clarify the molecular mechanism involved in the renal distribution of the alkylglycoside vector.

In conclusion, the efficiency of kidney targeting by using the alkylglycoside vector depends both on the size and charge of the molecules of this vector to be more suitable for therapeutic agents with a lower mol. wt. and neutral charge.

**References**

dw Ther* **274**:1298–1304.


dw Ther Exp* **288**:67–64.

dw Exp Ther* **288**:888–897.


coeutical through the primate blood-brain barrier in vivo with a monoclonal anti


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