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Novel Guanidine-Containing Molecular Transporters Based on Lactose Scaffolds: Lipophilicity Effect on the Intracellular Organellar Selectivity

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Abstract: We have synthesized two lactose-based molecular transporters, each containing seven guanidine residues attached to the lactose scaffold through ω-aminocarboxylate linker chains of two different lengths, and have examined their cellular uptakes and intracellular and organellar localizations in HeLa cells, as well as their tissue distributions in mice. Both molecular transporters showed higher cellular uptake efficiencies than Arg8, and wide tissue distributions including the brain. Mitochondrial localization is of special interest because of its potential relevance to "mitochondrial diseases". Interestingly, it has been found that the intracellular localization sites of the G7 molecular transporters—namely either mitochondria or lysosomes and endocytic vesicles—are largely determined by the linker chain lengths, or their associated lipophilicities.

Keywords: blood–brain barrier · drug delivery · lipophilicity · organelle selectivity · tissue selectivity

Introduction

Biomembrane systems pose formidable physical barriers for the trafficking of foreign molecules such as therapeutic and diagnostic agents. Tightly controlled cellular membranes allow entry into the cell and organelles only to those foreign molecules with appropriate ranges of molecular size, polarity, and charge. Potential drug molecules have to overcome these physical barriers in order to reach their target tissues, cells, and organelles for their desired therapeutic effects. A number of physical and chemical methods by which to accomplish these difficult challenges have been proposed.

In nature, a variety of peptides called cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) have been discovered to have the ability to cross the cell membranes; they include HIV-1 Tat, *Drosophila Antennapedia* peptides, and Kaposi's sarcoma fibroblast growth factor membrane translocating sequence.[1] Among these peptides, the Tat peptide—consisting of nine basic amino acids (RKKRRQRRR)—and its arginine-rich peptide analogues have been most extensively studied in terms of uptake mechanism[2] and potential applications in delivering various cargoes including small organic molecules,[3] proteins, nucleotides, and genes.[4] A number of research groups have also endeavored to develop synthetic molecular transporters in attempts to improve upon the Tat peptide and arginine-rich peptides (e.g., stability to endogenous peptidases, in vivo efficacy, long-term safety) by using peptide mimetics such as oligoarginine peptoids, guanidine-containing oligocarbamates, β-peptides, dendrimers, and other backbones.[5]

We, too, have been exploring novel synthetic molecular transporters, which consist of multiple units of guanidine residues attached to a carbohydrate-like scaffold through linkers based on linear or branched alkyl chains of variable length, together with additional functional groups reserved for possible cargo and probe conjugation.[6–8] G8 (eight guanidine residues) molecular transporters based on dimeric inositol scaffolds showed excellent cellular uptake properties, similar to or better than those of arginine oligomers (R8), in several cell lines including simian kidney COS-7, mouse

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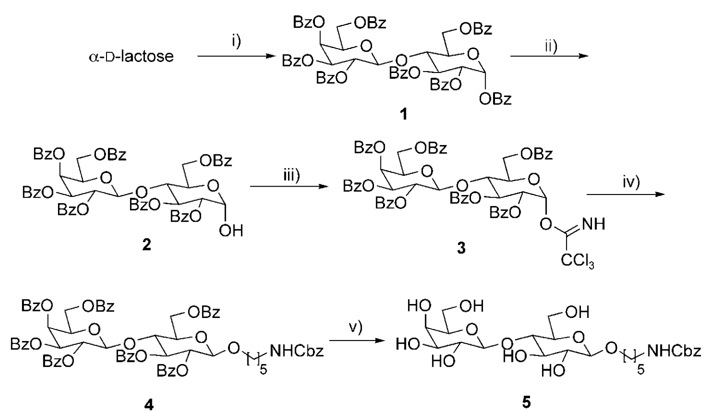
macrophage RAW264.7, and HeLa at 37°C after 5 min exposure. The transporter with the longer linker chain displayed enhanced uptake, although it was not clear whether this is due to increased hydrophobicity or higher flexibility for the interaction with cell membrane. Intracellular localization studies in the presence of tetramethylrhodamine-labeled transferrin and Tat peptide, MitoTracker and LysoTracker, as well as the mouse tissue distribution pattern, indicated that they were quite distinct from Tat and R8 peptides. The dimeric-inositol-based transporter and its doxorubicin conjugate were found to cross the blood–brain barriers efficiently.[6]

We have also prepared a series of G8 molecular transporters based on a sorbitol scaffold and branched linkers, and have examined their uptake properties. They showed internalization efficiencies somewhat superior to those of R8, but displayed highly selective co-localization with MitoTracker Red after 1 h at 37°C in HeLa and CD34^{+} stem cell-like KG1a leukemia cells. This is in stark contrast with the observation that in HeLa cells the dimeric-inositol-based (G8) carriers were widely distributed in the cytosol. Their mouse tissue distributions were also interesting in the sense that better distribution was noted in the heart muscle and brain sections than in any other tissues examined. It was suggested that the mitochondrial selectivity and the better tissue distribution might be related to the higher energy metabolism in these organs.[7] The mitochondrial concentrations of the sorbitol-based transporters were deemed very significant, since mitochondrial abnormalities have been increasingly observed in a wide variety of degenerative neural diseases such as familial amyotrophic lateral sclerosis (ALS, Lou Gehrigs' Disease), Hungtington's diseases, and Alzheimer's disease, as well as in apoptosis and aging,[9–13] and there is currently a dearth of viable mitochondrial transporters.[14–16] These results suggested to us that the structural diversity in terms of the scaffold and linker component and pattern might be profitably exploited not only for cellular uptake, but also for organelle (mitochondria in particular) and tissue selectivity in the design of synthetic molecular transporters. Here we report the synthesis and cellular translocation properties of novel lactose-based transporters, in which two transporters differing only in their linker chain lengths show different organelle selectivity.

Results and Discussion

Lactose (4-*O*-β-D-galactopyranosyl-D-glucopyranose), together with sucrose, maltose, and cellobiose, is one of the most common disaccharides and so is readily available. It occurs in mammalian milk; hence its trivial name of milk sugar. In designing the lactose-based molecular transporters we noticed a general trend that as the number of guanidine residue increases, cellular uptake becomes more efficient and cytotoxicity increases. We thus envisaged that the anomeric hydroxyl group of lactose might be reserved for possible cargo or fluorescent marker conjugation, and the remaining seven hydroxyl groups used for the attachment of guanidine functionalities through linear linker chains of varying lengths. Lactose was thus perbenzoylated with benzoyl chloride and pyridine at RT to afford **1** (Scheme 1),[17] and the anomeric benzoyl group was selectively removed by treatment with gaseous NH_3 in methanolic THF to give **2**. Compound **2** was activated to the corresponding trichloroacetimidate **3** with CCl_3CN and DBU, and subsequently glycosylated with HO-$(CH_2)_5$-NHCbz/TMSOTf to give **4** in good yield.[18,19] β-Anomeric stereochemistry was expected for compound **4** on the basis of literature precedents, and was confirmed by its ^{1}H NMR spectrum ($J = 8.0$ Hz). The remaining benzoyl protecting groups in **4** were removed with NaOMe in MeOH at reflux to provide **5** in good yield.



Scheme 1. i) BzCl, pyridine, RT, 8 h, 97%; ii) NH_3 (gas), MeOH/THF 3:7, RT, 24 h, 75%; iii) CCl_3CN, DBU, 0°C, 3 h, CH_2Cl_2, 92%; iv) HO-$(CH_2)_5$-NHCbz, 0°C, 1.5 h, TMSOTf, CH_2Cl_2, 80%; v) NaOMe, MeOH, reflux, 3 h, 97%.

Compound **5** was then exhaustively acylated with ω-*N*-Boc-aminocarboxylic acids [BocNH-$(CH_2)_n$-CO_2H, where $n = 5$ and 7] in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) in DMF for 48 h at RT to provide **6** and **7** (Scheme 2) in good yields after silica gel column chromatography. The *N*-Boc protecting groups of **6** and **7** were removed by treatment with gaseous HCl in EtOAc to give **8** and **9**, which were subjected to guanidinylation with *N*,*N'*-diBoc-*N''*-trifluoromethanesulfonylguanidine and Et_3N in aqueous dioxane to yield compounds **10** and **11** in moderate yields after column purification. Removal of the *N*-Cbz protecting groups in **10** and **11** with H_2 (35 psi) over Pd/C (10%) afforded the free amines **12** and **13**, which on treatment with FITC-I (FITC = fluorescein isothiocyanate) yielded **14** and **15** after purification. Compounds **14** and **15** were treated with gaseous HCl in EtOAc to remove the *N*-Boc protecting groups of the guanidine units. The thus obtained target compounds **16** and **17** were rigorously purified by reversed-phase MPLC and thoroughly characterized by ^{1}H NMR spectroscopy and MALDI-TOF mass spectral analyses.

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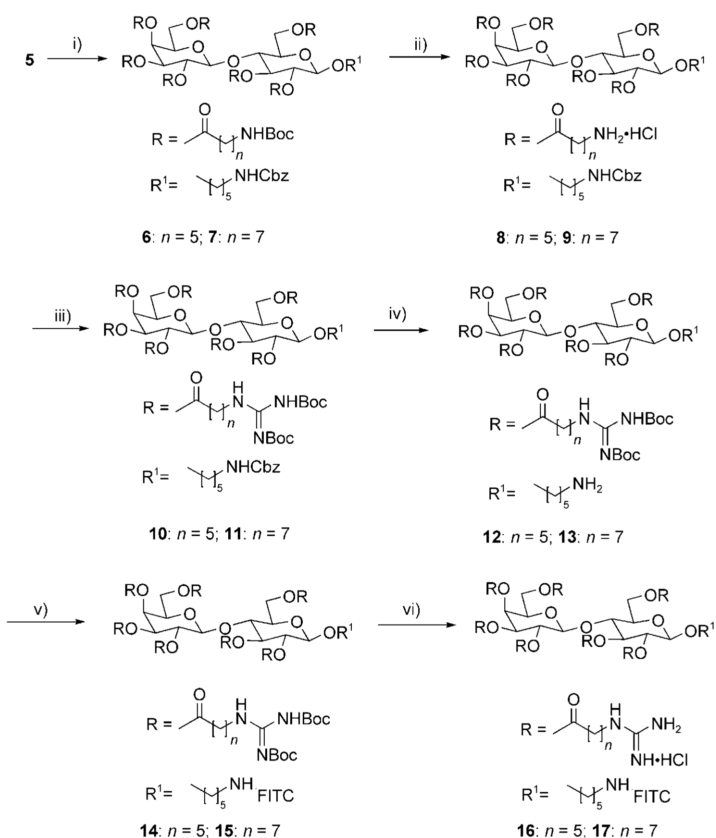

Scheme 2. i) EDC, DMAP, *N*-Boc-protected aminohexanoic acid ($n=5$), ω-aminooctanoic acid ($n=7$), DMF, RT, 2 d, 82 & 92%; ii) HCl (gas), EtOAc, RT, 2 h, quant.; iii) *N*,*N'*-diBoc-*N''*-triflylguanidine, Et_3N, dioxane/H_2O 5:1, RT, 3 d, 61 & 73%; iv) 10% Pd/C, H_2 (35 psi), MeOH/CH_2Cl_2 9:1, 3.5 h, quant. & 90%; v) FITC-I, Et_3N, THF, abs. EtOH, RT, 24 h, 63 & 63%; vi) HCl (gas), EtOAc, 24 h, 70 & 73%.

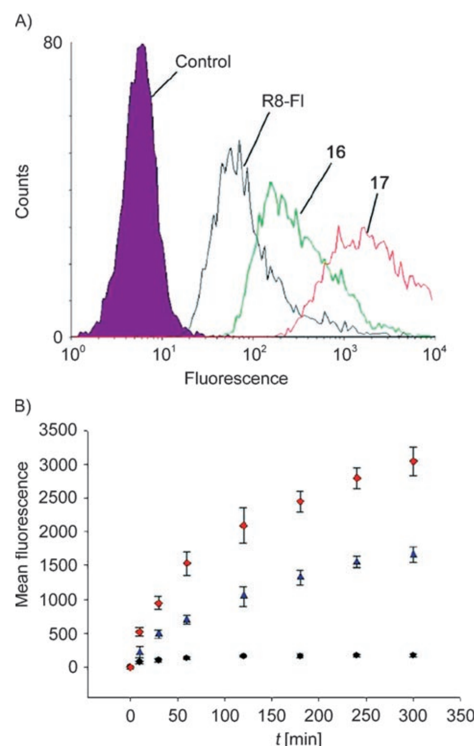

Figure 1. Cellular uptake studies of lactose-based transporters **16** and **17** by FACS analysis. A) FACS analysis of take-up of compounds **16** and **17** (1 μM each) by HeLa cells. The cells were incubated with compounds in serum-free DMEM at 37°C for 1 h and analyzed for their average fluorescence intensity. B) Kinetics study of the amounts of compounds internalized by the cells. The cells were incubated at 37°C for the indicated times and analyzed by FACS; ●: R8-Fl, ▲: **16**, ◆: **17**.

Preliminary evaluations of the synthetic G7 transporters for their uptake properties were carried out by confocal laser scanning microscopy (CLSM) in HeLa cells without fixing and comparison with results obtained with FITC-labeled arginine octamer (R8-Fl) as the reference standard. After incubation of the cells with the transporters (10 μM) for 15 min at 37°C, cells were found to be substantially marked with fluorescence. More detailed fluorescence-activated cell-sorter (FACS) studies were performed for both transporters (Figure 1). The R8 has commonly been used as the main representative among the peptide transporters that show efficient internalization. From Figure 1 it is clear that both **16** and **17** were internalized very efficiently and that their fluorescence intensities internalized at 1 μM concentration were substantially higher than that of R8-Fl.

To investigate the intracellular localization of these transporters, we stained each transporter with specific cellular organelle markers. Thus, when HeLa cells were incubated with **16** (1 μM) at 37°C for 1 h in the presence of Mito-tracker Red 580 (a mitochondria marker), considerable localization in mitochondria was observed. However, when the cells were similarly treated with **16** in the presence of Lyso-tracker Red DND 99 (a lysosome marker) or Transferrin-TxRed (a clathrin-mediated endocytic vesicle marker), it did not show discernible co-localization (Figure 2A). Next, HeLa cells were incubated with compound **17** in the presence of the same cellular organelle markers for 1 h at 37°C. Unlike **16**, compound **17** was not located in mitochondria. Instead it showed a punctuate pattern and partial localization with endocytic vesicles and lysosomes (Figure 2B).

We next examined the tissue distributions of compound **16** and **17** in mice (Figure 3). Compounds **16** and **17** (HCl salts, 82 $mg\,kg^{-1}$) were dissolved in sterile distilled water and injected intraperitoneally (i.p.) into 8-week-old mice (C57BL/6). After 20 min, the treated mice were perfused with paraformaldehyde (4%) in phosphate buffer solution (PBS; pH 7.4), and the organs—including the heart, spleen, liver, kidneys, lungs, and brain—were incubated overnight in sucrose solution in PBS (0.5 M). Placed in cryoprotectant, they were cut into 15 μm sections with the aid of a cryostat and transferred to coated glass slides. After drying at 37°C, the sections were washed with PBS, treated with Triton X-100 (0.3%) for 15 min at RT, and analyzed under an Axioplan2 fluorescence imaging microscope.

The Tat peptides have been reported to show wide distributions in the liver, kidneys, lungs, heart muscle, and spleen, whereas the dimeric-inositol transporters have been shown to be distributed predominantly in the heart, lung, and brain

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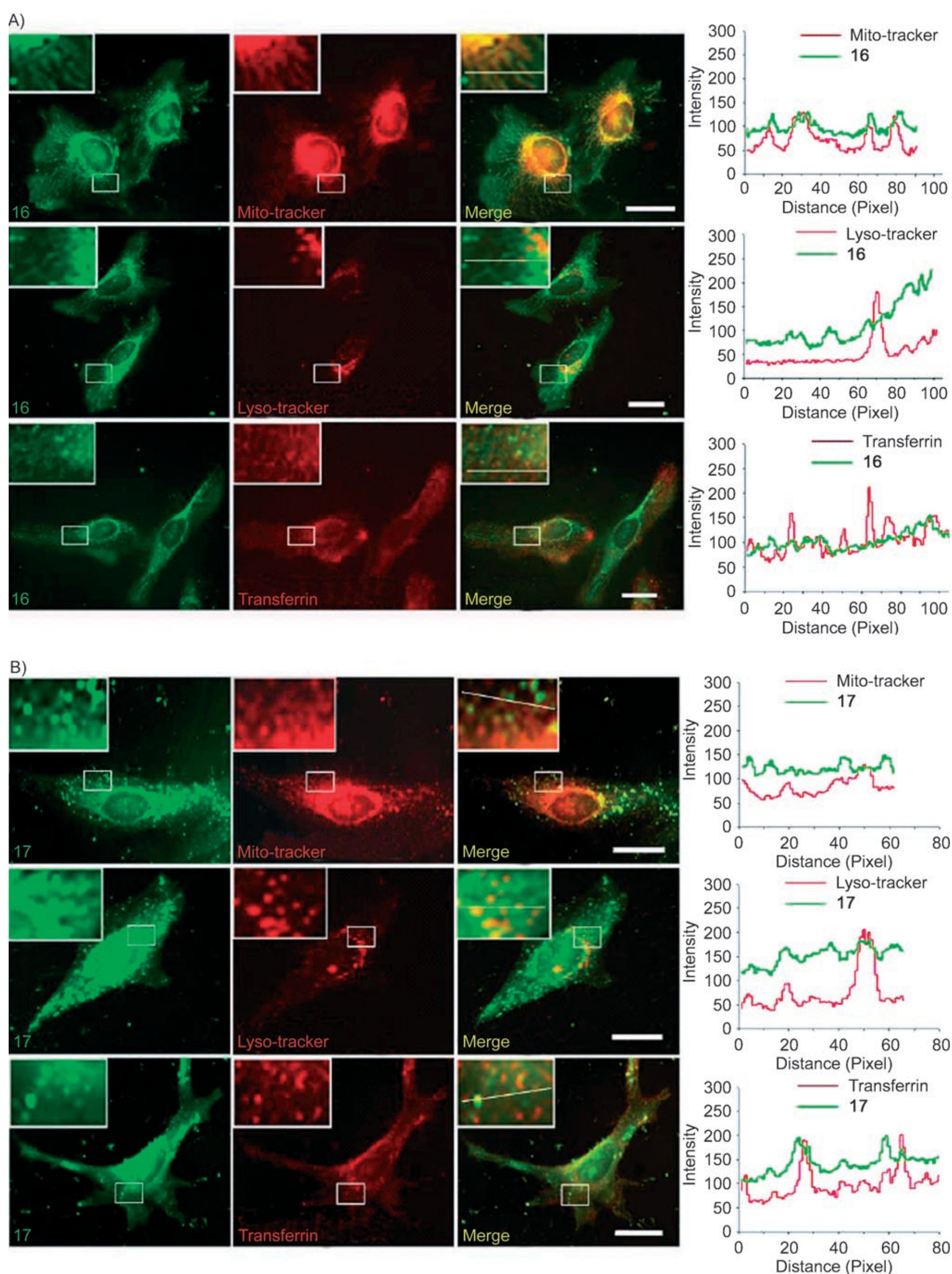

Figure 2. Intracellular localization studies of transporters **16** and **17**. A) Fluorescence microscope images of compound **16** and its localization. The HeLa cells were treated with **16** (green fluorescence from FITC, 1 μM) and with mitochondria (Mito-tracker, 50 nM), lysosome (Lyso-tracker, 50 nM), and endocytic vesicle (Transferrin-TxRed, 20 μg mL^{-1}) markers at 37 °C for 1 h. Compound **16** was significantly localized in mitochondria, but not in other regions. B) No co-localized signal from **17** (green fluorescence from FITC, 1 μμM) with Mito-tracker is seen; **17** is seen partially targeted to lysosome and endocytic vesicle. High-magnification views (insets) of the region enclosed by rectangle are shown. Scale bars: 20 μm

tissues, and the sorbitol transporter in heart and brain sections. In the cases of the lactose transporters **16** and **17**, both showed more pronounced distributions in the brain, liver, and spleen sections. It is quite clear that all three types of transporters we have studied so far (dimeric inositol, sorbitol, and lactose scaffolds) readily cross the blood–brain barrier, but also that they show somewhat variant tissue selectivity depending on their molecular structures.

Conclusion

In summary, we have thus far examined three different types of carbohydrate-based molecular transporters, with a particular interest in the intracellular and tissue selectivity. In terms of the intracellular localization, 1) the dimeric-inositol-based transporters (G8 and linear chain) showed wide distribution in the cytosol, 2) the sorbitol-based transporters

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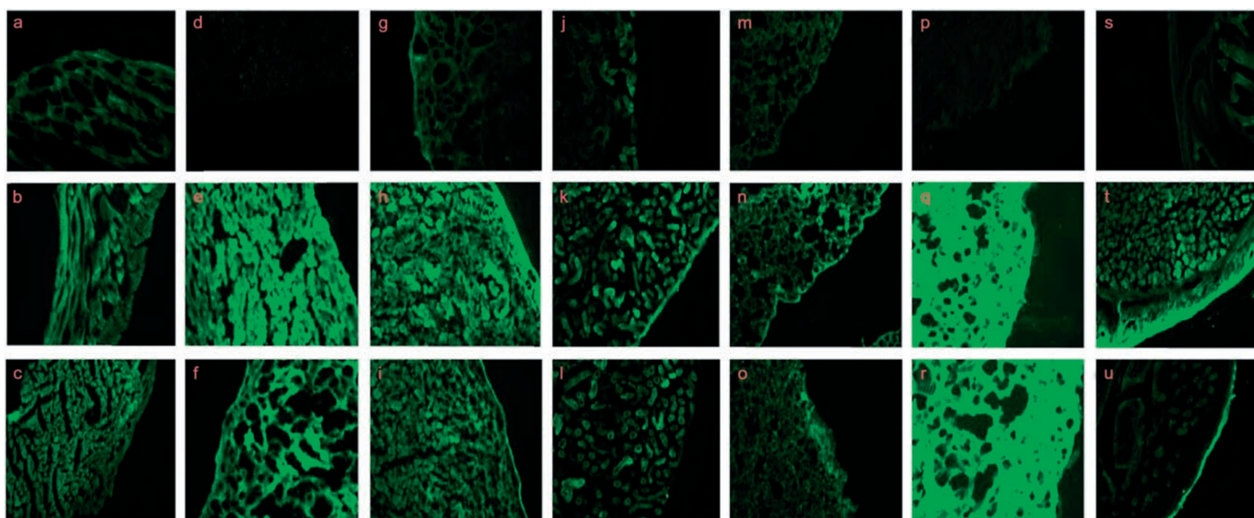

Figure 3. Distribution of **16** (middle) and **17** (bottom) (HCl salts) in mouse tissues (top: control). Fluorescence micrographs of: a)–c) heart, d)–f) spleen, g)–i) liver, j)–l) kidney, m)–o) lung, p)–r) brain, and s)–u) stomach tissue sections, isolated from mice 20 min after i.p. injection. Exposure times (ms): a)–c) 1000, d)–f) 1000, g)–i) 2500, j)–l) 2500, m)–o) 5000, p)–r) 10000, and s)–u) 2000. $\lambda_{max}=488$ nm (green fluorescence from FITC).

(G8 and branched chain) showed high selectivity toward mitochondria, and 3) one of the lactose-based transporters (**16**: G7, linear chain, C6: hexanoic acid linker) with shorter linker chains was found to target mitochondria, whereas the other one (**17**: G7, linear chain, C8: octanoic acid linker) with longer linker chains was found to be distributed in the endocytic vesicles and endosomes. It is difficult at the moment to pinpoint what structural factors have the major impact on the organellar selectivity. Although it is likely that the charge (number of guanidine moieties), the scaffold structures with their associated spatial arrays, the chain flexibility and lipophilicity collectively play important roles affecting both the organellar affinity and the uptake mechanism, in the present cases at least, it is the chain length (more likely due to lipophilicity than flexibility) that determines the organellar selectivity. Further studies directed towards elucidation of these structure/organellar selectivity relationships of the guanidine-containing transporters are clearly warranted and in progress.

Experimental Section

General methods: All non-hydrolytic reactions were carried out in oven-dried glassware under dry argon or nitrogen. All commercial chemicals were used as received except for solvents, which were purified and dried by standard methods prior to use. Analytical TLC was performed on Merck 60 F254 silica gel plates (0.25 mm thickness), analytical reversed-phase TLC on Merck RP-8 F254s plates, and visualization was accomplished with the aid of UV light (254 nm and 365 nm) and/or by spraying with a 5% solution of phosphomolybdic acid or ninhydrin, followed by charring with a heat gun. Column chromatography was performed on Merck 60 silica gel (70–230 or 230–400 mesh), whereas MPLC was performed on Fluka 100 C8-reversed phase silica gel. Melting points were determined on a Thomas–Hoover MP apparatus and are uncorrected. NMR spectra were recorded on Bruker DPX 300 (^{1}H NMR at 300 MHz; ^{13}C NMR at 75 MHz) and Bruker DRX 500 (^{1}H NMR at 500 MHz; ^{13}C NMR at 125 MHz) spectrometers. Tetramethysilane was used as reference, and the chemical shifts are reported in ppm and the coupling constants in Hz. Analytical HPLC was performed on an Agilent 1100-HPLC Chemstation with a Zorbax SB-C8 analytical column (5 μm, 4.6 mm ID × 25 cm). Low-resolution mass spectra were determined on a Micromass Platform ll instrument (EI and FAB). High-resolution mass spectra were done on a JMS-700 instrument, and MALDI-TOF mass spectra on a Voyager-DE STR system at the Korea Basic Science Support Center. The standard extractive workup procedure consisted of pouring into a large amount of water, extracting thoroughly with the organic solvent indicated, washing the combined extract successively with water and brine, drying the extract over anhydrous Na_2SO_4 or $MgSO_4$, and evaporating the solvent.

2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-benzoyl-α-D-glucopyranose (1): Benzoyl chloride (7.73 mL, 66.6 mmol) was added dropwise at 0 °C to a solution of α-lactose (2.3 g, 6.38 mmol) in dry pyridine (40 mL). The mixture was stirred for 8 h at RT, and cold water (10 mL) was added to quench the reaction. The reaction mixture was diluted with CH_2Cl_2 (100 mL), and the organic phase was washed first with HCl solution (1 N, 2 × 20 mL) and then with sat. $NaHCO_3$ solution to neutrality. The extract was dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product as a yellow oil, which was purified on silica gel (CH_2Cl_2/CH_3OH 20:1) to give **1** as a white solid (7.67 g, 97.2%). M.p. 118–120 °C (lit.:[9] 119–120 °C); ^{1}H NMR ($CDCl_3$): δ = 3.73–3.86 (m, 2H; H-4 & H-5′), 3.94 (t, J = 6.7 Hz, 1H; H-5), 4.32–4.47 (m, 2H; H-6′), 4.60 (s, 2H; H-6), 4.99 (d, J = 7.9 Hz, 1H; H-1′), 5.42 (dd, J = 10.3, 3.3 Hz, 1H; H-3′), 5.66 (dd, J = 10.3, 3.7 Hz, 1H; H-2), 5.78–5.84 (m, 2H; H-2′ & H-4′), 6.25 (t, J = 9.9 Hz, 1H; H-3), 6.79 (d, J = 3.7 Hz, 1H; H-1), 7.20–8.16 ppm (m, 40H; aromatic); ^{13}C NMR ($CDCl_3$): δ = 61.55, 62.36, 68.06, 70.51, 70.78, 70.60, 71.76, 72.0, 72.52, 76.13, 90.39, 101.71, 128.64, 128.74, 128.79, 128.99, 129.16, 129.41, 130.07, 130.16, 130.19, 130.23, 130.39, 130.45, 133.66, 133.80, 133.89, 134.22, 164.88, 165.33, 165.65, 165.86, 165.96, 166.20 ppm.

2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-D-glucopyranose (2): Compound **1** (0.50 g, 0.42 mmol) was dissolved in methanol/THF (3:7), and NH_3 gas was passed through the solution for 30 min at 0 °C. The solution was stirred at RT for 24 h and was then concentrated to afford the crude product as a yellow oil, which was chromatographed on silica gel (EA/hex 1:2) to provide compound **2** (0.34 gm, 75%) as a white, foamy solid. ^{1}H NMR ($CDCl_3$): δ = 3.73–3.86 (m, 2H; H-4 & H-5′), 4.07–4.11 (m, 1H; H-5′α & H-5′β), 4.23–4.37 (m, 1H; H-6′), 4.44–4.56 (m, 1H; H-6), 4.90 (d, J = 8.0 Hz, 1H; H-1′β), 4.94 (d, J =

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7.9 Hz, 1H; H-1′α), 5.26 (dd, $J=10.2$ Hz, 3.5 Hz, 1H; H-3′α & H-3′β), 5.34 (d, $J=1.7$ Hz, 1H; H-1β), 5.41 (dd, $J=10.3$ Hz, 3.1 Hz, 1H; H-2α & H-2β), 5.64 (d, $J=2.5$ Hz, 1H; H-1α), 5.70–5.76 (m, 1H; H-4′α & H-2′α), 5.81 (t, $J=9.0$ Hz, 1H; H-3β), 6.18 (t, $J=9.5$ Hz, 1H; H-3α), 7.16–7.98 ppm (m, 35H; aromatic); ^{13}C NMR ($CDCl_3$): $\delta=61.58$, 62.81, 62.97, 68.78, 70.40, 70.60, 71.76, 72.19, 72.29, 72.56, 72.89, 73.62, 74.33, 76.35, 76.47, 77.74, 90.71, 101.45, 128.70, 128.86, 129.02, 129.21, 129.80, 129.89, 130.01, 130.07, 130.16, 130.30, 130.36, 130.40, 133.54, 133.74, 133.85, 133.99, 134.28, 165.32, 165.71, 165.77, 165.88, 165.93, 166.08, 166.43, 166.57, 167.02 ppm; MS (FAB): *m*/*z*: calcd for $C_{61}H_{50}O_{18}Na$: 1093.29 $[M+Na]^+$; found: 1093.27.

1-*O*-[2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-D-glucopyranosyl] trichloroacetimidate (3): Trichloroacetonitrile (20 mL) and DBU (0.002 mL, 0.012 mmol) were added to a solution of **2** (0.24 g, 0.25 mmol) in dry CH_2Cl_2 (5 mL). The reaction mixture was stirred for 3 h and concentrated in vacuo. Flash chromatography (hexane/EtOAc 3:1) provided **3** (0.25 g, 92%) as a colorless foam. ^{1}H NMR ($CDCl_3$): $\delta=3.72$–3.89 (m, 3H; H-5′, H-6a′& H-6b′), 4.32 (m, 2H; H-4 & H-5), 4.55 (m, 2H; H-6a & H-6b), 4.94 (d, $J=7.3$ Hz, 1H; H-1′), 5.38 (d, $J=10.2$ Hz, 1H; H-3′), 5.54 (d, $J=10.1$ Hz, 1H; H-2), 5.72 (m, 2H; H-2′ & H-4′), 6.16 (t, $J=9.9$ Hz, 1H; H-3), 6.71 (d, $J=3.6$ Hz, 1H; H-1α), 7.19–8.03 (m, 35H; aromatic), 8.56 ppm (s, 1H; NH); ^{13}C NMR ($CDCl_3$): $\delta=61.39$, 62.32, 67.90, 70.39, 70.70, 70.92, 71.54, 71.77, 71.81, 72.36, 72.48, 73.25, 73.47, 74.11, 74.42, 76.06, 91.09, 93.49, 101.60, 101.72, 128.69, 128.76, 128.86, 129.01, 129.13, 129.26, 129.80, 129.86, 129.98, 130.04, 130.08, 130.15, 130.17, 130.35, 130.39, 130.54, 131.06, 133.71, 133.82, 133.98, 134.28, 161.08, 165.28, 165.64, 165.89, 165.95, 166.16 ppm; MS (FAB): *m*/*z*: calcd for $C_{63}H_{50}Cl_3NO_{18}Na$: 1236.20 $[M+Na]^+$; found: 1236.22.

5-(Benzyloxycarbonylamino)pentyl 2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-D-glucopyranoside (4): A mixture of trichloroacetimidate **3** (0.25 g, 0.21 mmol) and 5-(benzyloxycarbonylamino)pentanol (0.054 g, 0.23 mmol) in dry CH_2Cl_2 (5 mL) was stirred for 1 h under N_2. It was then cooled to −10 °C, and neat TMSOTf (0.004 mL, 0.023 mmol) was added. After stirring for 0.5 h at −10 °C, the reaction was terminated by addition of solid $NaHCO_3$ (100 mg). The mixture was diluted with CH_2Cl_2, washed with sat. aq $NaHCO_3$, dried, and concentrated. Chromatography (EtOAc/petroleum ether 2:3) of the crude product on silica gel afforded **4** (0.21 g, 80%) as a foamy solid. $[\alpha]_D^{18}=26.66$ ($c=1.15$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=1.30$–1.55 (m, 6H), 2.94 (m, 2H), 3.44–3.48 (m, 1H), 3.77–3.87 (m, 4H; H-6a′, H-6b′, H5, *C*H-glycosyl), 3.95 (t, $J=6.5$ Hz, 1H; H-5′), 4.29 (t, $J=9.5$ Hz, 1H; H-4), 4.51–4.67 (m, 2H; H-6a, H-6b), 4.70 (d, $J=8.0$ Hz, 1H; H-1), 4.93 (d, $J=7.5$ Hz, 1H; H-1′), 5.10 (s, 2H; CH_2-Ph), 5.43 (dd, $J=10.5$, 3.5 Hz, 1H; H-3′), 5.48 (dd, $J=9.5$, 8.0 Hz, 1H; H-2), 5.74–5.78 (m, 2H; H-2′, H-4′), 5.84 (t, $J=9.5$ Hz, 1H; H-3), 7.16–8.06 ppm (m, 40H; aromatic) [chemical shift assignments are made by ^{1}H–^{1}H COSY]; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=22.99$, 28.89, 29.36, 29.69, 40.82, 61.17, 62.46, 66.49, 67.65, 69.89, 70.03, 71.49, 71.83, 71.94, 72.96, 73.12, 76.14, 101.01, 101.18, 128.04, 128.25, 128.40, 128.49, 128.52, 128.56, 128.59, 128.63, 128.71, 128.79, 128.94, 129.47, 129.51, 129.59, 129.63, 129.67, 129.70, 129.74, 130.00, 133.15, 133.24, 133.38, 133.52, 136.79, 156.30, 163.36, 164.85, 165.172, 165.25, 165.41, 165.45, 165.59, 165.87 ppm; MS (FAB): *m*/*z*: calcd for $C_{74}H_{67}NO_{20}Na$: 1312.42 $[M+Na]^+$; found: 1312.57.

5-(Benzyloxycarbonylamino)pentyl β-D-galactopyranosyl-(1→4)-D-glucopyranoside (5): NaOMe (0.04 mL, 0.17 mmol, 25% *w*/*v*) was added to a solution of **4** (200 mg, 0.44 mmol) in MeOH (15 mL), and the solution was heated at reflux for 3 h. After cooling, the reaction mixture was filtered through acidic resin. The filtrate was concentrated, and the crude product was washed with EtOAc in *n*-hexane (5%) to remove the byproduct, methyl benzoate. The washed product was dried under vacuum and recrystallized from abs. EtOH to afford **5** as a white solid (88 mg, 97%). M.p. 180–182 °C (from EtOH); $[\alpha]_D^{18}=-4.77$ ($c=0.965$ in H_2O); ^{1}H NMR (500 MHz, CD_3OD): $\delta=1.39$–1.67 (m, 6H), 3.14 (t, $J=7.0$ Hz, 2H), 3.26 (t, $J=8.5$ Hz, 1H), 3.32 (m, 1H), 3.40–3.43 (m, 1H), 3.49–3.62 (m, 5H), 3.71–3.74 (m, 1H), 3.78–3.93 (m, 5H), 4.30 (d, $J=8.0$ Hz, 1H), 4.38 (d, $J=8.0$ Hz, 1H), 5.08 (s, 2H), 7.36 ppm (m, 5H; aromatic); ^{13}C NMR (CD_3OD): $\delta=24.41$, 30.51, 30.76, 41.87, 62.07, 62.64, 67.45, 70.46, 70.83, 72.72, 74.92, 74.98, 76.59, 76.63, 77.25, 80.78, 104.39, 105.25, 128.92, 129.08, 129.60 ppm; MS (FAB): *m*/*z*: calcd for $C_{25}H_{39}NO_{13}Na$: 584.23 $[M+Na]^+$; found: 584.40.

5-(Benzyloxycarbonylamino)pentyl 2,3,4,6-tetra-*O*-(6-Boc-aminohexanoyl)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(6-Boc-aminohexanoyl)-D-glucopyranoside (6): A solution of **5** (60 mg, 0.10 mmol), *N*-Boc-6-aminohexanoic acid (494 mg, 2.13 mmol), EDC (408 mg, 2.13 mmol), and DMAP (18.4 mg, 0.15 mmol) in DMF (6.5 mL) was stirred at RT for 48 h under N_2, diluted with EtOAc, and washed several times with sat. $NaHCO_3$, water, and then brine. The organic phase was dried and concentrated to give the crude product, which was purified by column chromatography on silica gel to afford **6** (180 mg, 82%) as a white, sticky solid. ^{1}H NMR ($CDCl_3$): $\delta=1.20$–1.61 (m, 111H), 2.11–2.34 (m, 14H), 3.06–3.16 (m, 14H), 3.39 (m, 1H), 3.51 (m, 1H), 3.67–3.85 (m, 2H), 4.03 (m, 2H), 4.42 (m, 2H), 4.82 (m, 6H), 4.91 (dd, $J=9.0$, 3.0 Hz, 1H), 5.06 (s, 2H), 5.01–5.06 (m, 1H), 5.15 (t, $J=9.3$ Hz, 1H), 5.29–5.37 (m, 1H), 7.29–7.33 ppm (m, 5H); ^{13}C NMR ($CDCl_3$): $\delta=23.41$, 24.50, 24.62, 24.72, 24.80, 24.87, 24.99, 25.12, 26.61, 27.73, 28.82, 29.39, 29.93, 30.09, 33.89, 34.03, 34.14, 34.26, 34.37, 34.52, 40.74, 41.27, 66.75, 68.99, 69.34, 69.93, 70.09, 70.96, 71.20, 71.41, 71.82, 71.92, 72.14, 72.71, 72.82, 73.08, 73.49, 79.37, 100.93, 101.33, 102.28, 128.41, 128.46, 128.85, 137.10, 156.42, 156.86, 172.43, 172.48, 172.56, 172.65, 172.79, 172.94, 173.26, 173.42, 173.53 ppm; MS (MALDI-TOF): *m*/*z*: calcd for $C_{102}H_{172}N_8NaO_{34}$: 2077.19 $[M+Na]^+$; found: 2077.26.

5-(Benzyloxycarbonylamino)pentyl 2,3,4,6-tetra-*O*-(8-Boc-aminooctanoyl)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(8-Boc-aminooctanoyl)-D-glucopyranoside (7): This compound was prepared similarly to **6**, from **5** (50 mg, 0.089 mmol), *N*-Boc-8-aminooctanoic acid (461 mg, 1.78 mmol), EDC (341 mg, 1.78 mmol), and DMAP (16.3 mg, 0.133 mmol). Colorless, sticky liquid (184 mg, 92%); ^{1}H NMR ($CDCl_3$): $\delta=1.20$–1.25 (m, 46H), 1.40 (m, 93H), 2.18–2.34 (m, 14H), 3.11 (m, 14H), 3.38–3.41 (m, 1H), 3.53 (m, 1H), 3.67–3.85 (m, 2H), 4.01–4.12 (m, 2H), 4.38–4.49 (m, 2H), 4.65 (m, 5H), 4.82 (t, $J=9.0$ Hz, 1H), 4.90 (dd, $J=9.0$, 3.0 Hz, 1H), 5.05 (s, 2H), 5.01–5.06 (m, 1H), 5.15 (t, $J=9.0$ Hz, 1H), 5.27–5.29 (m, 1H), 7.31 ppm (m, 5H); ^{13}C NMR ($CDCl_3$): $\delta=23.41$, 24.77, 25.06, 25.09, 25.15, 25.27, 27.01, 28.83, 29.37, 29.50, 29.98, 30.42, 34.20, 34.24, 34.30, 34.45, 40.95, 41.28, 60.77, 66.90, 69.32, 70.06, 71.12, 71.36, 71.81, 79.33, 101.01, 101.38, 128.42, 128.86, 137.11, 156.42, 156.85, 172.08, 178.53, 172.72, 172.92, 173.06, 173.35, 173.42 ppm; MS (MALDI-TOF): *m*/*z*: calcd for $C_{116}H_{200}N_8O_{34}Na$: 2272.41 $[M+Na]^+$; found: 2272.49.

5-(Benzyloxycarbonylamino)pentyl 2,3,4,6-tetra-*O*-(*N*,*N*″-bis-Boc-*N*″-aminohexanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(*N*,*N*″-bis-Boc-*N*″-aminohexanoylguanidine)-D-glucopyranoside (10): Compound **6** (200 mg, 0.097 mmol) was added at 0 °C to a saturated HCl (gas) solution of EtOAc (5 mL), and the solution was stirred for 3 h. The precipitate was separated and dried under vacuum to give compound **8** as a white HCl salt (132 mg, quant.). This material was directly used in the next step. ^{1}H NMR (CD_3OD): $\delta=1.42$–1.68 (m, 48H), 2.22–2.45 (m, 14H), 2.86–2.99 (m, 14H), 3.06–3.09 (m, 2H), 3.29–3.31 (m, 4H), 3.52–3.77 (m, 4H), 4.07–4.15 (m, 2H), 4.40–4.85 (m, 6H), 5.06 (m, 3H), 5.17 (m, 2H), 5.37–5.41 (m, 1H), 7.34–7.38 ppm (m, 5H); ^{13}C NMR (CD_3OD): $\delta=23.22$, 24.13, 24.21, 24.38, 24.48, 24.55, 24.74, 24.81, 25.91, 25.93, 26.11, 27.23, 27.29, 27.35, 29.12, 29.27, 29.53, 33.42, 33.55, 33.65, 33.85, 34.12, 39.62, 39.67, 40.74, 60.56, 60.92, 62.27, 66.31, 67.44, 67.71, 69.48, 69.71, 69.81, 70.64, 71.33, 71.49, 71.62, 72.05, 72.39, 72.79, 72.89, 73.20, 73.66, 76.59, 100.61, 100.87, 127.69, 127.99, 128.52, 137.50, 157.89, 171.99, 172.57, 172.65, 173.03, 173.39, 173.64 ppm; MS (MALDI-TOF): *m*/*z*: calcd for $C_{67}H_{117}N_8O_{20}$: 1353.84; found: 1353.86 $[M+H]^+$.

Et_3N (0.28 mL, 1.96 mmol) and *N*,*N*′-di-Boc-*N*″-trifluoromethanesulfonylguanidine (767 mg, 1.96 mmol) were sequentially added to a solution of **8** (132 mg, 0.098 mmol) in dioxane/water (5:1, 6 mL). The reaction mixture was stirred at RT for 3 days, concentrated, and diluted with EtOAc. The organic layer was washed with $NaHSO_4$ (1 N), saturated $NaHCO_3$, and brine, dried, and concentrated to give the crude product, which was purified on neutral alumina to afford **10** (181 mg, 61%) as a white, foamy solid. ^{1}H NMR ($CDCl_3$): $\delta=1.25$–1.67 (m, 174H), 2.12–2.39 (m, 14H), 3.15 (m, 1H), 3.38 (m, 15H), 3.51 (m, 2H), 3.68–3.83 (m, 3H), 4.01–4.04 (m, 3H), 4.41–4.48 (m, 3H), 4.82 (m, 1H), 4.93 (m, 1H), 5.02–5.05 (m,

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3H), 5.16 (m, 1H), 5.27–5.35 (m, 1H), 7.31 (m,5H), 8.27 (m, 7H), 11.47 ppm (m, 7H); ^{13}C NMR ($CDCl_3$): δ=23.41, 23.53, 24.54, 24.66, 24.79, 24.88, 24.95, 25.09, 26.72, 26.81, 28.23, 28.47, 28.71, 29.19, 29.53, 29.97, 33.95, 34.05, 34.25, 34.34, 34.43, 41.13, 53.82, 69.36, 70.03, 70.95, 71.35, 72.04, 73.08, 79.54, 83.37, 101.16, 101.46, 102.21, 128.39, 128.86, 153.69, 156.49, 164.02, 171.88, 172.24, 172.45, 172.70, 173.84, 173.00, 173.18 ppm.

5-(Benzyloxycarbonylamino)pentyl 2,3,4,6-tetra-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminooctanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminooctanoylguanidine)-D-glucopyranoside (11): Compound **9** was prepared similarly to compound **8**, from **7** (200 mg, 0.089 mmol) and a saturated HCl (gas) solution in EtOAc (5 mL) at 0°C. White HCl salt (137 mg, quant.); ^{1}H NMR (CD_3OD): δ=1.36–1.64 (m, 76H), 2.16–2.39 (m, 14H), 2.90 (m, 14H), 3.05–3.07 (m, 2H), 3.20–3.29 (m, 2H), 3.51 (m, 2H), 3.78 (m, 2H), 4.04–4.11 (m, 2H), 4.37–4.59 (m, 2H), 4.65–4.72 (m, 2H), 4.98–5.18 (m, 5H), 5.33–5.38 (m, 1H), 7.32 ppm (m, 5H); ^{13}C NMR (CD_3OD): δ=23.34, 24.71, 25.10, 25.28, 26.56, 26.63, 27.73, 27.80, 29.08, 29.25, 29.43, 29.66, 34.29, 34.45, 34.59, 40.26, 40.40, 40.84, 60.56, 61.53, 66.39, 67.55, 69.64, 70.19, 71.16, 71.85, 71.99, 73.13, 76.77, 101.01, 101.22, 127.74, 128.02, 128.57, 137.49, 157.89, 172.76, 172.82, 173.19, 173.53, 173.85 ppm; MS (MALDI-TOF): *m*/*z*: calcd for $C_{81}H_{145}N_8O_{20}$: 1550.06; found: 1550.05 $[M+H]^+$.

Compound **11** was prepared similarly to compound **10**, from **9** (166 mg, 0.107 mmol), *N*,*N*′-di-Boc-*N*′′-trifluoromethanesulfonylguanidine (838.2 mg, 2.14 mmol), and Et_3N (0.3 mL, 2.14 mmol). White, foamy solid (253 mg, 73%); ^{1}H NMR ($CDCl_3$): δ=1.28–1.50 (m, 202H), 2.09–2.32 (m, 14H), 3.11–3.13 (m, 2H), 3.35 (m, 15H), 3.53 (m, 1H), 3.67–3.82 (m, 3H), 4.02 (m, 3H), 4.40–4.48 (m, 3H), 4.80–4.92 (m, 2H), 5.01–5.04 (m, 3H), 5.16 (t, J=9.0 Hz, 1H), 5.27–5.29 (m, 1H), 7.31 (m, 5H), 8.26 (m, 7H), 11.45 ppm (m, 7H); ^{13}C NMR ($CDCl_3$): δ=23.41, 24.79, 25.06, 25.18, 25.23, 25.27, 27.15, 28.22, 28.37, 28.47, 28.70, 29.38, 29.99, 34.20, 34.29, 34.49, 41.32, 53.82, 60.77, 62.15, 66.84, 66.85, 69.25, 70.00, 71.12, 71.15, 71.83, 72.75, 73.00, 79.58, 83.38, 100.98, 101.00, 128.39, 128.86, 137.14, 153.69, 156.49, 164.02, 172.06, 172.48, 172.72, 172.90, 173.05, 173.26, 173.42 ppm.

5-Aminopentyl 2,3,4,6-tetra-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminohexanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminohexanoyl guanidine)-D-glucopyranoside (12): A solution of **10** (400 mg, 0.131 mmol) in a mixed solvent (CH_2Cl_2/MeOH 1:9, 30 mL) was hydrogenated (35 psi) over Pd/C (10%, 100 mg) at RT. After 3.5 h, the catalyst was filtered and the filtrate was concentrated to give **12** (270 mg, quant.) as an off-white, foamy solid. ^{1}H NMR ($CDCl_3$): δ=1.24–1.69 (m, 174H), 2.15–2.43 (m, 16H), 2.95–2.99 (m, 2H), 3.36–3.48 (m, 16H), 3.52–3.53 (m, 1H), 3.71–3.80 (m, 2H), 3.97–4.11 (m, 3H), 4.34–4.55 (m, 2H), 4.80–4.86 (m, 1H), 4.91–5.06 (m, 1H), 5.17–5.21 (m, 1H), 5.30–5.37 (m, 1H), 8.30–8.41 (m, 9H), 11.48 ppm (m, 7H); ^{13}C NMR ($CDCl_3$): δ=23.10, 24.12, 24.22, 24.32, 24.39, 24.52, 24.64, 26.25, 26.36, 27.89, 28.05, 28.28, 28.76, 31.20, 33.48, 33.78, 40.67, 61.16, 62.07, 66.53, 69.34, 70.56, 71.36, 71.80, 72.51, 72.90, 79.16, 79.93, 83.00, 100.62, 153.25, 156.07, 163.59, 171.92, 172.17, 172.28, 172.58, 172.84, 173.09 ppm.

5-Aminopentyl 2,3,4,6-tetra-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminooctanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminooctanoylguanidine)-D-glucopyranoside (13): Compound **13** was prepared similarly to compound **12**, from **11** (220 mg, 0.067 mmol). White, foamy solid (190 mg, 90%); ^{1}H NMR ($CDCl_3$): δ=1.30–1.54 (m, 202H), 2.11–2.37 (m, 16H), 2.92–3.04 (m, 2H), 3.34–3.38 (m, 16H), 3.52 (m, 2H), 3.69–3.85 (m, 2H), 3.98–4.10 (m, 3H), 4.35–4.55 (m, 3H), 4.80–4.86 (m, 1H), 4.91–5.06 (m, 1H), 5.17 (m, 1H), 5.31–5.39 (m, 1H), 8.29 (m, 9H), 11.35 ppm (m, 7H); ^{13}C NMR ($CDCl_3$): δ=23.48, 24.79, 25.05, 25.17, 25.29, 26.99, 27.12, 28.18, 28.36, 28.45, 28.68, 29.36, 30.07, 34.19, 34.28, 34.50, 41.43, 60.75, 62.14, 66.67, 69.30, 69.70, 71.06, 71.32, 71.85, 72.67, 73.21, 79.68, 79.88, 83.41, 100.45, 153.65, 156.42, 163.82, 172.02, 172.68, 172.89, 173.05, 173.25, 173.60 ppm.

5-Amino-(fluoresceinyl-5-thioureido)-pentyl 2,3,4,6-tetra-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminohexanoyl guanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminohexanoylguanidine)-D-glucopyranoside (14): Fluorescein 5-isothiocyanate (11.67 mg, 0.03 mmol) and triethylamine (10 μL, 0.07 mmol) were added to a solution of **12** (80 mg, 0.027 mmol) in a mixed solvent (THF and abs. ethanol 2:4, 6 mL). The reaction mixture was stirred in the dark for 24 h at RT and concentrated. The crude product was purified on silica gel to afford **14** (57 mg, 63%) as a light greenish-yellow, sticky mass. ^{1}H NMR ($CDCl_3$): δ=1.24–1.52 (m, 174H), 2.13–2.35 (m, 16H), 3.34–3.36 (m, 16H), 3.46–3.51 (m, 2H), 3.69–3.87 (m, 2H), 3.92–4.07 (m, 3H), 4.25–4.46 (m, 2H), 4.75–4.95 (m, 3H), 5.10–5.16 (m, 1H), 5.24–5.27 (m, 1H), 6.54–6.73 (m, 5H), 7.16–7.19 (m, 1H), 7.85 (m, 1H), 8.30–8.34 (m, 8H), 11.47 ppm (m, 7H); ^{13}C NMR ($CDCl_3$): δ=24.35, 24.47, 24.56, 24.78, 25.03, 25.94, 26.46, 26.58, 26.74, 28.41, 28.51, 28.60, 29.13, 30.03, 33.85, 34.18, 34.43, 34.55, 41.10, 41.20, 44.90, 45.12, 68.30, 71.20, 71.41, 79.58, 79.64, 79.73, 79.90, 79.95, 83.38, 83.47, 83.57, 83.70, 100.58, 102.06, 103.55, 126.24, 129.37, 153.20, 153.60, 156.48, 156.58, 163.73, 163.79, 163.89, 172.43, 172.59, 172.74, 173.34, 173.45, 177.10, 181.38 ppm.

5-Amino-(fluoresceinyl-5-thioureido)pentyl 2,3,4,6-tetra-*O*-(*N*,*N*′′-bis-Boc-*N*′′-aminooctanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*–(*N*,*N*′′-bis-Boc-*N*′′-aminooctanoylguanidine)-D-glucopyranoside (15): Compound **15** was prepared similarly to compound **14**, from **12** (190 mg, 0.061 mmol). Light greenish-yellow, sticky mass (134 mg, 63%); ^{1}H NMR ($CDCl_3$): δ=1.24–1.52 (m, 202H), 2.15–2.35 (m, 16H), 3.34–3.36 (m, 16H), 3.48–3.54 (m, 2H), 3.69–3.87 (m, 2H), 4.02–4.07 (m, 3H), 4.41–4.46 (m, 2H), 4.81–4.94 (m, 2H), 5.00–5.04 (m, 1H), 5.16 (m, 1H), 5.29 (m, 1H), 6.57–6.77 (m, 5H), 7.10 (m, 1H), 7.96 (m, 1H), 8.32–8.34 (m, 8H), 11.41 ppm (m, 7H); ^{13}C NMR ($CDCl_3$): δ=23.34, 24.71, 25.10, 25.28, 26.56, 26.63, 27.73, 27.80, 29.08, 29.25, 29.43, 29.66, 34.29, 34.45, 34.59, 40.26, 40.40, 40.84, 60.56, 61.53, 66.39, 67.55, 69.64, 70.19, 71.16, 71.85, 71.99, 73.13, 76.77, 101.01, 101.22, 127.74, 128.02, 128.57, 137.49, 157.89, 172.76, 172.82, 173.19, 173.53, 173.85 ppm.

5-Amino-(fluoresceinyl-5-thioureido)-pentyl 2,3,4,6-tetra-*O*-(6-aminohexanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(6-aminohexanoylguanidine)-D-glucopyranoside·7 HCl (16): HCl(g)-saturated EtOAc (4 mL) was added at RT to a solution of **14** (30 mg, 0.009 mmol) in EtOAc (1 mL). After stirring for 24 h, the solution was concentrated, and the residue was washed with a mixture of diethyl ether and MeOH (20:1) to remove less polar impurities. The residue was dried and purified by MPLC on reversed-phase C-8 silica gel (H_2O/CH_3CN 1:1 to 1:2 with 0.1% TFA). The purified product was dissolved in deionized water, filtered through a PTGE syringe filter, and lyophilized to give **16** (13.7 mg, 70%) as a light greenish-yellow, foamy solid (HCl salt). Analytical HPLC (ZORBAX SB-C8): t_R=3.2 min [flow rate: 1 mL min^{-1}; UV: λ= 220 nm; CH_3CN (0.1%TFA)/H_2O (0.1%TFA) 40:60, purity 98+%]; ^{1}H NMR (500 MHz, CD_3OD): δ=1.42–1.49 (m, 16H), 1.63–1.71 (m, 32H), 2.24–2.27 (m, 2H), 2.37–2.50 (m, 14H), 3.21 (m, 16H), 3.60–3.72 (m, 2H), 3.78 (m, 1H), 3.85–3.88 (m, 1H), 4.12–4.32 (m, 4H), 4.46–4.59 (m, 1H), 4.70–4.78 (m, 1H), 4.88–5.06 (m, 1H), 5.15–5.24 (m, 2H), 5.40–5.44 (m, 1H), 6.77–6.97 (m, 6H), 7.25–7.26 (m, 1H), 7.86 (m, 1H), 8.37 ppm (m, 1H); UV (H_2O): λ_{max} (ε)=481 nm (17066 mol^{-1} m^{3}cm^{-1}); MS (MALDI-TOF): *m*/*z*: calcd for $C_{87}H_{136}N_{23}O_{23}S$: 1902.99 $[M+H]^+$; found: 1903.01.

5-Amino-(fluoresceinyl-5-thioureido)-pentyl 2,3,4,6-tetra-*O*-(8-aminooctanoylguanidine)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-(8-aminooctanoylguanidine)-D-glucopyranoside·7 HCl (17): Compound **17** was prepared similarly to compound **16**, from **15** (27.5 mg, 0.008 mmol) and HCl-saturated (gas) EtOAc (4 mL) at RT for 24 h. Light greenish-yellow, foamy solid (HCl salt, 14.7 mg, 73%). Analytical HPLC ((ZORBAX SB-C8): t_R=3.6 min [flow rate: 1 mL min^{-1}; UV: λ=220 nm; CH_3CN (0.1% TFA)/H_2O (0.1% TFA) 40:60, purity 95+%]; ^{1}H NMR (500 MHz, CD_3OD): δ=1.39–1.42 (m, 44H), 1.62–1.73 (m, 32H), 2.19–2.23 (m, 2H), 2.34–2.44 (m, 14H), 3.19–3.24 (m, 16H), 3.60–3.65 (m, 2H), 3.77 (m, 1H), 3.85–3.87 (m, 1H), 4.12–4.17 (m, 4H), 4.44–4.46 (m, 1H), 4.55–4.68 (m, 1H), 5.02–5.05 (m, 1H), 5.13–5.25 (m, 2H), 5.38 (m, 1H), 6.75–7.09 (m, 6H), 7.27–7.29 (m, 1H), 7.93–7.94 (m, 1H), 8.45 ppm (m, 1H); UV (H_2O): λ_{max} (ε)=482 nm (21000 mol^{-1} m^{3}cm^{-1}); MS (MALDI-TOF): *m*/*z*: calcd for $C_{101}H_{164}N_{23}O_{23}S$: 2099.21 $[M+H]^+$; found: 2099.14.

Bioassays

Cell culture: Human cervical cancer-derived HeLa cells were cultured as exponentially growing subconfluent monolayers on 100 mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplanted with fetal

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bovine serum (10% *v*/*v*) without antibiotics. A subculture was performed every 3–4 days. They were plated on poly-D-lysine-coated 18 mm glass coverslips.

Cell labeling and fluorescence microscopy imaging: HeLa cells were plated on poly-D-lysine-coated 18 mm glass cover slips and cultured for 48 h. After removal of the medium, the cells were washed twice with PBS. The cells were incubated with each compound (1 μM of **16**, 1 μM of **17**) and the mitochondria marker (50 nM Mito-tracker Red 580), lysosome marker (50 nM Lyso-tracker Red DND99), and endocytic vesicle marker (2020 gm L^{-1} transferring-TxRed) in serum-free DMEM at 37 °C for 1 h. After incubation, the cells were washed with PBS.

Images were obtained with an Olympus IX-71 inverted microscope (Olympus Optical, Tokyo, Japan) with a 40×, 1.0 N.A. oil lens and a CoolSNAP-Hq CCD camera (Roper Scientific, Tucson, AZ) driven by MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA) with a FITC and Tx-Red optimized filter set (Omega Optical, Brattleboro, VT). Light from a mercury lamp was shuttered with a VMM1 Uniblitz shutter (Vincent Associates, Rochester, NY). Analysis and quantification of data were performed with MetaMorph software.

Flow cytometry: 5×104 HeLa cells were plated into 12-well culture plate (SPL, Korea) and cultured for 48 h. After removal of the medium, the cells were incubated with compounds **16** and **17** in serum-free DMEM. After incubation, the cells were washed three times with PBS, and were then incubated with trypsin (0.02%, 500 μL) for 1 min at 37 °C. After addition of PBS (1 mL), the cells were centrifuged at 3000 rpm for 2 min. The cell pellet was resuspended and washed twice with PBS (1 mL), and finally resuspended in PBS (1 mL) for fluorescence analysis with an Epics-XL (Becktone Culter) flow cytometer, 488 nm laser excitation, and a 515–545 nm emission filter. Each sample was analyzed 5000 events×3 times. Analysis of data was performed with SigmaPlot 8.0 (Systat Software, Point Richmond, CA), and data were presented as means ± SEs.

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