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Journal

ChemBioChem, 15(5)

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Publication Date

2014-03-21

DOI

10.1002/cbic.201300606

Peer reviewed

Published in final edited form as:
Chembiochem. 2014 March 21; 15(5): 676–680. doi:10.1002/cbic.201300606.

Macromolecular Uptake of Alkyl Chain-Modified Guanidinoglycoside Molecular Transporters

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Keywords

Guanidinium-rich transporter; cellular uptake; heparan sulfate; membrane; proteoglycans

Guanidinoglycosides, a family of cellular transporters capable of delivering high MW biopolymers, have previously been shown to display high selectivity to cell surface heparan sulfate proteoglycans and promote their clustering. Here the internalization mechanism of amphiphilic guanidinoglycoside derivatives is investigated by cell surface FRET studies. Unexpectedly, although their heparan sulfate selectivity is maintained, their cellular uptake does not appear to involve clustering of cell surface proteoglycans. This suggests a distinct uptake mechanism when compared to the parent guanidinoglycoside-based carriers.

Cell-penetrating peptides (CPP) and guanidinium-rich transporters serve as intracellular delivery vehicles for biologically relevant macromolecules, such as peptides, proteins, and nucleic acids. Extensive research has demonstrated their use as research tools and their potential pharmaceutical applications.[1–4] The mechanistic understanding of the cellular uptake and internalization of these transporter molecules remains complex since multiple mechanisms are likely to operate, depending on the specific transporter and cell types. Uptake mediated by specific receptors appears inconsistent with the structural diversity of the guanidinium-based transporters reported to date. Several reports favor endocytosis-based mechanisms, but the internalization mechanism remains controversial.[5]

Positively charged peptides have been proposed to electrostatically interact with membrane phospholipids and with negatively charged cell surface proteoglycans,[6] which decorate the surface of virtually every mammalian cell. These abundant biopolymers consist of one or more glycosaminoglycan chains covalently attached to a core protein,[7,8] and are

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Supporting information for this article is available.

categorized based on the nature of the glycosaminoglycan composition (heparan sulfate, chondroitin sulfate/dermatan sulfate, or keratan sulfate). Among them, heparan sulfate proteoglycans (HSPGs) are of particular significance as they are involved in numerous processes including binding to diverse ligands, which can be internalized via a non-clathrin mediated pathway and delivered to lysosomes.[9]

Over the past decade we have demonstrated that guanidinoglycosides, synthetic carriers made by converting the ammonium groups of aminoglycoside antibiotics into guanidinium groups, can effectively transport macromolecules into cells.[10–14] Their cellular delivery takes place at nanomolar concentrations and depends exclusively on HSPGs, which distinguishes them from other widely used CPPs, such as Tat-related peptides and oligoarginines.[11] Furthermore, we have recently shown that HSPG aggregation is a pivotal step for endocytic entry into cells by guanidinoglycoside-based molecular transporters.[14] We hypothesized that modifying the guanidinoneomycin core with a long alkyl chain could alter the uptake process by promoting clustering of the transporter molecules on the cell surface thereby impacting HSPG aggregation. In this contribution, we probe the cellular uptake of streptavidin as a model proteinaceous cargo using new amphiphilic transporters **3**–**7** in which the guanidinoneomycin core is modified with a single alkyl chain of varying lengths (Scheme 1). We observe enhanced cell surface binding and improved cellular uptake, when compared to the pentaguanidinylated neomycin carrier without alkyl groups (**2**, Scheme 1). These superior features depend on the length of the hydrophobic chain. A mechanistic investigation involving cell surface FRET studies suggests an unexpected entry pathway and points to a possible uptake mechanism.

The new transporter molecules, containing five guanidinium groups and one alkyl chain, were synthesized as outlined in Scheme S1. Key intermediates are shown in Scheme 2. To regioselectively introduce the alkyl group into the guanidinoneomycin core, a partially guanidinylated neomycin derivative of which one amino group remained intact was first prepared. Considering that the 3-amino group on the 2-deoxystreptamine core of neomycin is the least basic and nucleophilic out of the 6 amines,[15] we rationalized that very mild guanidinylation conditions would yield the partially guanidinylated product leaving this group intact. Therefore, the previously reported azido-neomycin **8** was treated with a limiting amount of *N*,*N*′-di-tert-butoxycarbonyl-*N*″-triflylguanidine[16] (5.5 eq) for 7 days at ambient temperature to afford partially guanidinylated **9** in moderate yield (Scheme S1; Scheme 2). This orthogonally functionalized intermediate can be independently extended by an azide/alkyne cycloaddition or by an acylating reaction. Subsequent 1,3-dipolar cycloaddition of **9** with a propargylamide-extended biotin, followed by deprotection using trifluoroacetic acid yielded compound **2** (Scheme 1). As a key control carrier, the structure of compound **2** was confirmed by extensive 2D NMR analyses (COSY, TOCSY, HSQC, HMBC, Figures S1–S5). Next, alkyl groups were introduced to the biotinylated intermediate **10** via an acylation reaction with the corresponding acyl chloride to give the fully protected carriers **11**, **12** and **13** (shown as a general structure, Scheme 2). Alternatively, acylation of compound **9** with the suitable acyl chloride and further 1,3-dipolar cycloadition, led to fully protected compounds **16** and **17** (Scheme 2). Treatment with trifluoroacetic acid yielded the alkyl chain containing transporters **3**–**7** (Scheme S1, Scheme 1). For comparison, a fully

guanidinylated reference, compound **1**, containing six guanidinium groups, was also prepared.[14]

To test if the synthetic new derivatives serve as HSPG-dependent cellular transporters, fluorescent streptavidin-phycoerythrin-Cy5 (ST-PECy5) was used as a model macromolecular payload. First, binding to the surface of Chinese hamster ovary (CHO) cells was measured by flow cytometry as reported previously.[14] Two distinct cell lines were used: wild-type and CHO-derived mutant cells (pgsA-745). The latter produce less than 2% of the wild-type heparan sulfate chains.[17] By mixing ST-PECy5 and compounds **1**–**7** at a 1:5 ratio for 20 min at room temperature, tetravalent conjugates were obtained. The conjugates were then diluted with cell culture medium to a final concentration of 2 nM. Mutant and wild-type cells were incubated with the fluorescent conjugates for 30 min at 4°C. Under these conditions, binding occurs but uptake does not.[11] After harvesting the cells using EDTA and evaluating the binding level using FACS, significantly higher values were observed for the wild-type cells compared to pgsA-745 cells for all the compounds tested, illustrating the HSPG-selectivity of the new transporters (Figure 1A). The binding of compound **2**, which contains only 5 guanidinium groups, was lower than that of compound **1**, showing that the number of guanidinium groups on the transporter plays an important role in HSPG binding, confirming our earlier observations.[12] Interestingly, transporters **3**–**7**, where an alkyl chain is installed on the five guanidinium-containing core compound **2**, exhibited unexpectedly and significantly improved binding, while retaining their HSPG-dependency.

To investigate the cellular uptake of compounds **1**–**7**, cells were incubated with the ST-PECy5 conjugates for 1 h at 37°C, washed, harvested with trypsin/EDTA, and analyzed by flow cytometry. Under these conditions, all cell-surface-bound material is detached, and the fluorescence readout represents the level of internalized conjugates.[13] As shown in Figure 1B, uptake of the tetravalent conjugates was significantly lower in the pgsA mutant cells than in the wild-type, demonstrating that the cellular uptake remains HSPG-dependent for all the compounds tested. The uptake of compound **2** was lower than that of compound **1**, which is consistent with the binding result discussed above. A short alkyl group installed on compound **2** (i.e., pentyl, compound **3**) had no effect on uptake, contrary to its improved binding. On the other hand, longer alkyl chains (i.e., decyl and dodecyl, compound **4** and **5**, respectively) improved the cellular uptake of the fluorescent high molecular weight cargo. Thus, alkyl chains above a certain critical length show beneficial effect both on cellular binding and uptake. Further elongation of the alkyl group (i.e., tetradecyl and octadecyl, compounds **6** and **7**, respectively) yielded diminished cellular uptake compared to that observed for **4** and **5**.[18]

A detailed cellular uptake analysis was therefore done with compounds **1**–**5**. As shown in Figure 2, the cellular uptake was found to be dose-dependent, increasing at higher concentrations. Compound **2** showed the lowest uptake throughout the concentrations tested, while compound **5** showed similar level of uptake to that of **1**. The uptake remained exclusively dependent on HSPG as the selectivity between wild-type and pgsA-745 cells (Figure 2) was retained even at the highest concentration tested (10 nM of conjugate).

Finally, to investigate the impact of HSPG aggregation on cellular uptake, a cell-surface FRET study using equimolar mixture of two different fluorescent streptavidins, streptavidin-phycoerythrin (ST-PE; λ_{max}=496 nm, λ_{em} = 575 nm) and streptavidin-Cy5 (ST-Cy5; λ_{max}=649 nm, λ_{em} = 666 nm), instead of ST-PECy5, was performed as previously reported (Figure 3).[14] Aggregation of these two photophysically distinct streptavidins produces a FRET signal, which can be independently monitored using FACS (FL3-H signal). The conjugate made from the fully guanidinylated carrier **1** and a mixture of fluorescent streptavidins exhibited FRET signal in a dose-dependent manner, and the signal was quite evident at 10 nM of ST-PE (or ST-Cy5, Figures 3A, 3B, S7), supporting the clustering of streptavidin on the cell surface. The extent of FRET signal intensity at 10 nM of ST-PE (or ST-Cy5) is similar to that observed for ST-PECy5 conjugated to carrier **1** (Figure 3C).

The dose-dependent increase of FRET signal exhibited by compound **1** conjugated to the fluorescent streptavidins indicates that the signal intensity correlates with the degree of cellular uptake, suggesting that the FRET signal intensity represents the degree of HSPG aggregation. On the other hand, compound **2** and alkyl chain derivatives **3**–**5** showed negligible FRET signal (Figures 3A, 3B, S7). Since these transporters have one less guanidinium group compared to compound **1** (and therefore four less in the tetravalent streptavidin conjugate), this observation indicates that valency has a significant impact on HSPG clustering. This notion is consistent with the lower uptake of **2** or **3** compared to that of **1**. For longer alkyl chain derivatives such as **5**, however, the negligible level of HSPG clustering by itself appears contradictory to the high cellular uptake observed, which is comparable to that of **1** (Figures 1B, 2). This apparent inconsistency is also found for binding of **5**, shown as FL2-H signal intensity, which is less than half than that observed for **1** at the highest concentration (Figure 3D). Taken together, these observations suggest that the cellular uptake of derivatives with longer alkyl chain is not as critically dependent on HSPG clustering, as observed with **1**.

Taking into account the above observations, we conclude that: a) alkyl chains directly interact with the cell membrane thus assisting with cellular uptake and b) the interaction with HSPG has a synergistic effect on alkyl chain-assisted internalization. The latter conclusion is drawn from the results showing that the cellular uptake by the long alkyl chain transporters in the wild-type cells is more than 20-fold higher than that in the mutant cells (Figures 1B, 2), while the former is consistent with our findings that uptake improvement is higher for derivatives with longer alkyl chains. This is notable specially at higher concentrations (Figure 2). We exclude the possibility that the long alkyl chains play a role in forming large particles, which might contribute to enhanced cellular uptake, based on dual angle light scattering. The conjugates prepared from compounds **1**–**5** and streptavidin showed hydrodynamic diameter similar to streptavidin itself, suggesting that aggregated particles did not form (Table S1).

The dependency of cellular internalization on the alkyl chain length has also been reported for acylated CPP analogs[19–24] and amphiphilic aminoglycosides.[25–26] Additionally, the impact of hydrophobicity on uptake has been documented for systems including polymers[27], amphiphiles[28], and oligoarginine carriers with different counterions.[29] We hypothesize that the hydrophobic alkyl chains assist uptake via hydrophobic interactions,

which ultimately impact membrane curvature. This explains the effective cellular uptake of long alkyl chain transporters, which appears to proceed without HSPG clustering. This proposed pathway is distinct from the HSPG aggregation-dependent cellular uptake observed for **1**, the parent carrier.[14] Our findings illuminate yet another intriguing facet of guanidinium-rich transporters and their complex cellular uptake. The information provided here may be useful for designing transporter molecules with superior or altered cellular internalization efficiency.

Experimental Section

Cell culture

Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61), and pgsA-745 cells were prepared as previously reported.[17, 30] All cells were grown under an atmosphere of 5% CO_2 in air and 100% relative humidity in Dulbecco's Modified Eagle's Medium (DMEM, low glucose, Life Technologies) supplemented with 10% (v/v) fetal bovine serum, 100 μg/mL of streptomycin sulfate, and 100 Units/mL of penicillin G.

Preparation of conjugates and cellular binding/uptake studies

In a typical experiment, transporter molecules (0.75 μM) were incubated with ST-PECy5 (0.15 μM, BD Biosciences) for 20 min at ambient temperature and then diluted with ice-chilled DMEM cell culture medium (final ST-PECy5 concentration of 2 nM).

Wild-type and mutant pgsA cells were seeded onto 24-well tissue culture plates (100,000 cells/well) and grown for 24 h to achieve 80% confluence. For binding studies, cells were treated with the diluted conjugate solutions (300 μL) after removal of the medium and incubated for 30 min at 4°C. Cells were washed with ice-chilled phosphate buffered saline (PBS, 300 μL) twice, detached with Versene (EDTA, 100 μL, Life Technologies), diluted with PBS containing 5% BSA and analyzed by flow cytometry. For uptake experiments, cells were incubated for 1 h at 37°C under an atmosphere of 5% CO_2. Work-up procedure was the same as in the binding studies, except that trypsin/EDTA (50 μL, 37°C, 3 min) was used to release the cells and remove any cell surface bound streptavidin conjugates.

FRET studies

In a typical experiment, conjugate solution made from transporter molecule and 1:1 mixture of ST-PE and ST-Cy5 (transporter molecule:ST-PE:ST-Cy5 = 10:1:1) was diluted with DMEM cell culture medium. After incubating for 30 min at 4°C, cells were washed with PBS twice, detached with Versene, diluted with PBS containing 5% BSA and analyzed by flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the National Institutes of Health for support (GM077471 to Y.T. and J.D.E.). We thank the Gianneschi laboratory (Chemistry and Biochemistry, UCSD) for help with DLS measurements, Dr. Tong (Cellular Molecular Medicine, UCSD) for help with cell culture and uptake experiments and Dr. Su (Mass Spectrometry Facility, Chemistry and Biochemistry, UCSD) for help with sample analysis.

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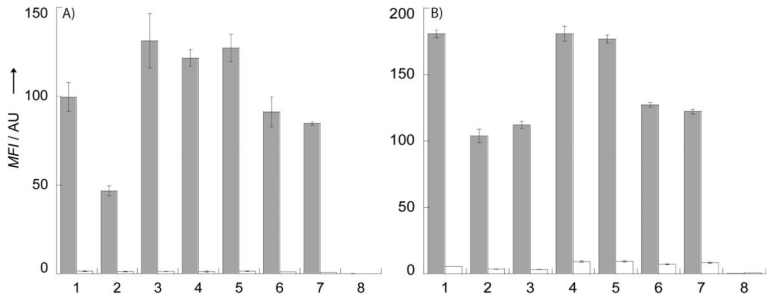


Figure 1.

Cellular binding (A) and uptake (B) for conjugates of ST-PE-Cy5 and transporter molecules. MFI is the mean fluorescence intensity measured by FACS. Binding and uptake were studied with either wild-type cells (gray) or pgsA-745 cells (white). For the binding study, cells were incubated with conjugates made from 2 nM ST-PE-Cy5 and 10 nM transporter molecule for 30 min at 4°C. For the uptake study, incubation was done for 1 h at 37°C: (1) compound **1**, (2) compound **2**, (3) compound **3**, (4) compound **4**, (5) compound **5**, (6) compound **6**, (7) compound **7**, (8) without compound.

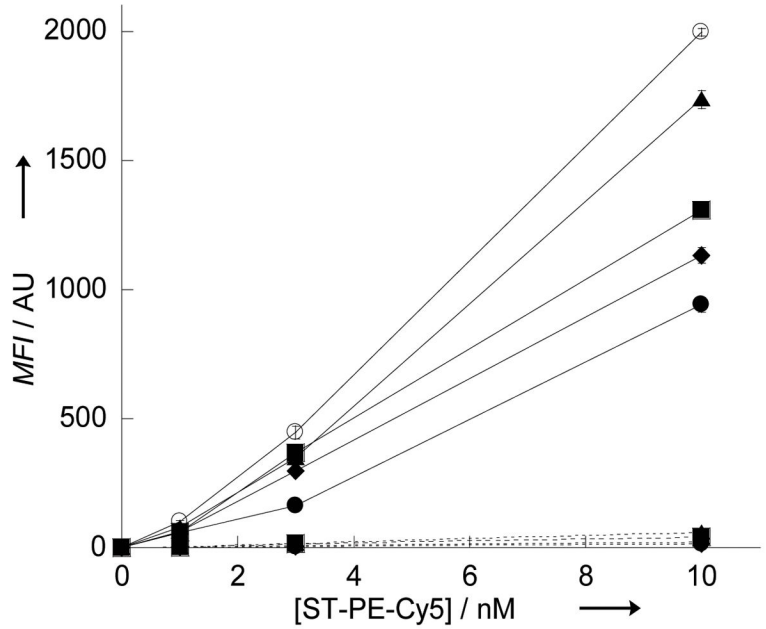


Figure 2.

Dose-response of ST-PECy5 on cellular uptake by wild-type and mutant pgsA-745 cells (solid and dashed lines respectively). MFI is the mean fluorescence intensity measured by FACS. Compound **1** (open circles), compound **2** (solid circles), compound **3** (diamonds), compound **4** (squares) and compound **5** (triangles).

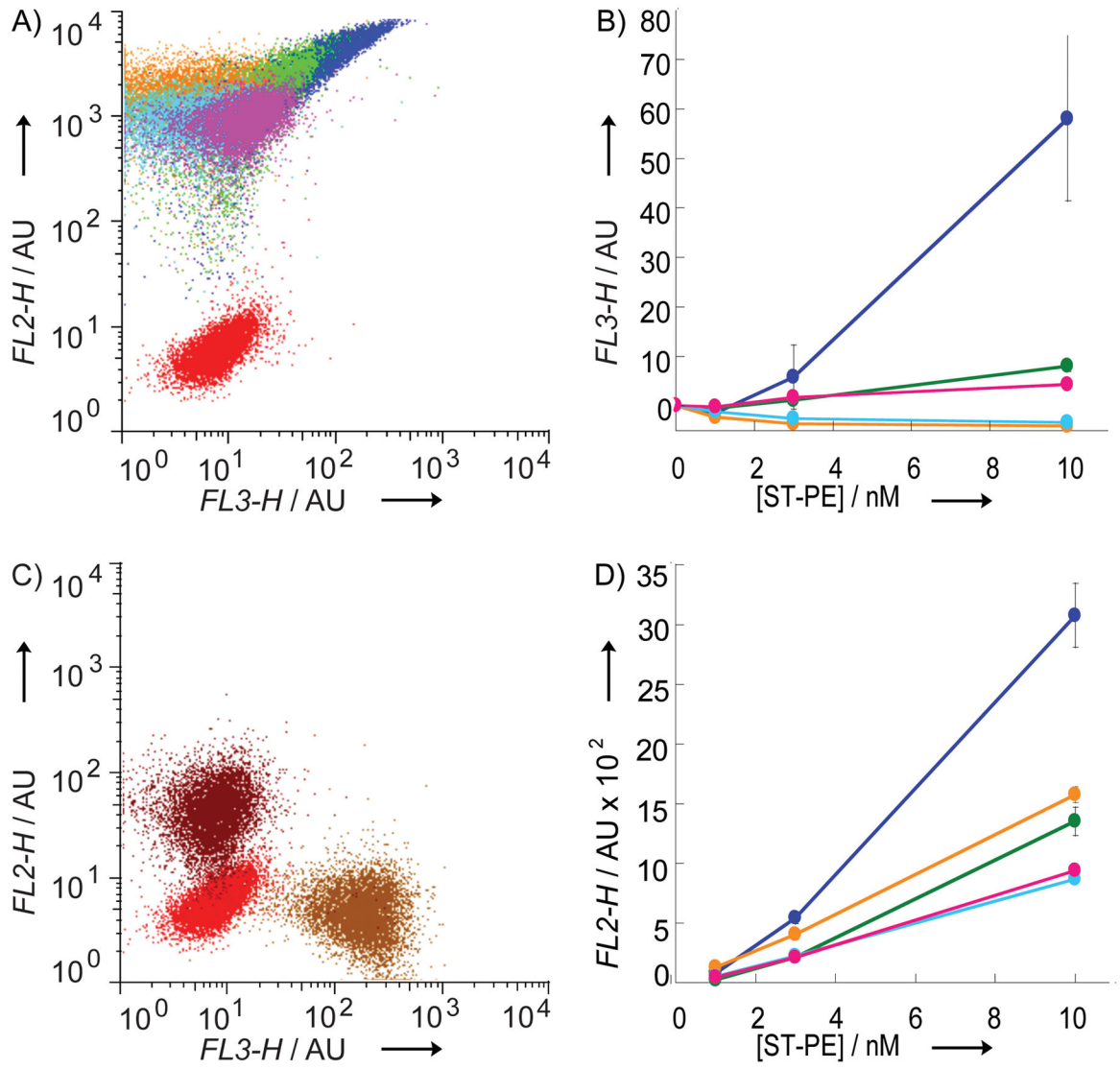
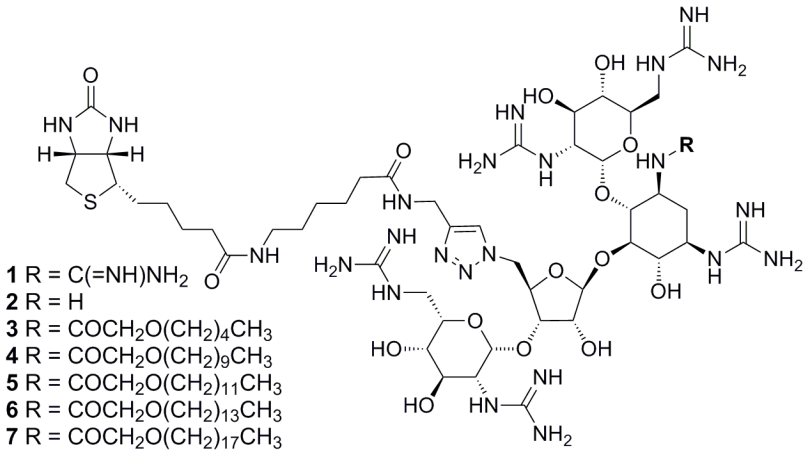
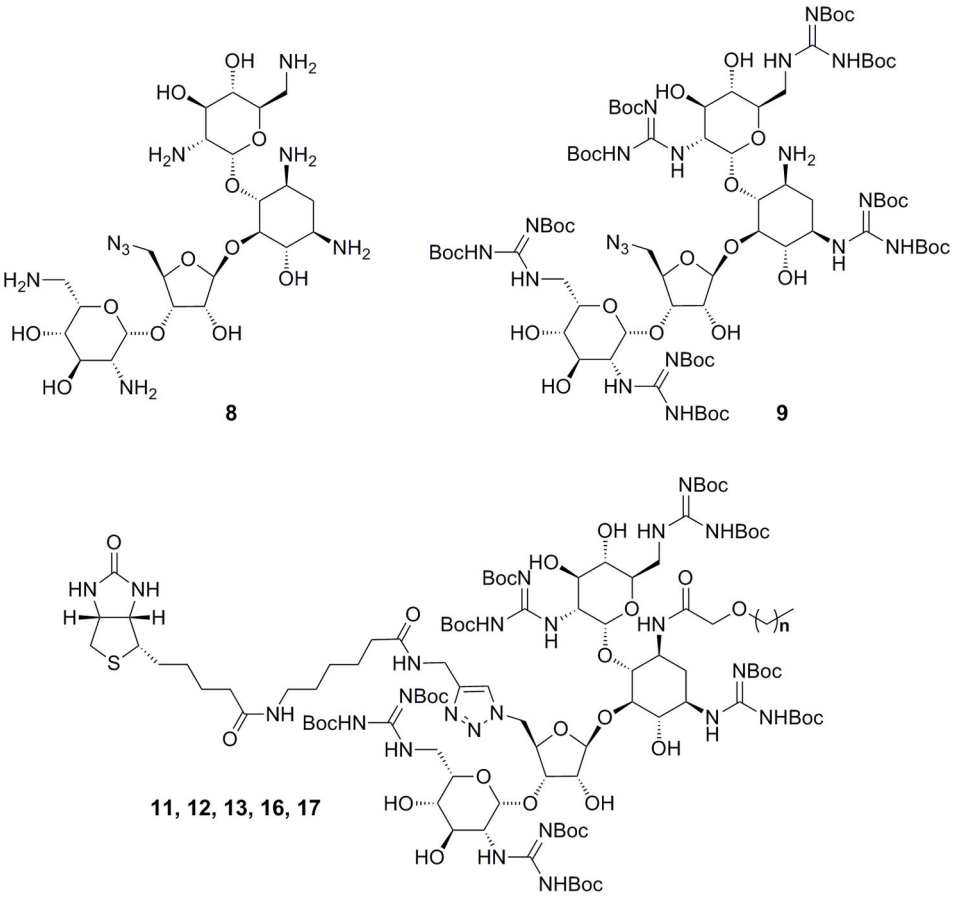


Figure 3.

Intermolecular FRET result using ST-PE and ST-Cy5. Cells were incubated at 4°C for 30 min with conjugates of carriers and ST-PE/ST-Cy5 mixture. Carrier:ST-PE:ST-Cy5 was set as 10:1:1 molar ratio. Cells were detached by Versene and analyzed by FACS. FL2-H and FL3-H represent the fluorescent intensity of PE (λem = 560 nm) and PE/Cy5 FRET complex (λem = 670 nm), respectively. Conjugates of 10 nM ST-PE (ST-Cy5) with compound **1** (blue), compound **2** (green), compound **3** (tangerine), compound **4** (cyan), compound **5** (magenta) and only cells (red) (A). Dose-response of ST-PE (ST-Cy5) on mean fluorescent intensity of FRET signal FL3-H (B), color code as in (A). Conjugate of compound **1** and 10 nM of either ST-PE (marroon) or ST-PECy5 (brown) and only cells (red) (C). Dose-response of ST-PE (ST-Cy5) on mean fluorescent intensity of FL2-H (D), color code as in (A).



Scheme 1.
Synthesized transporter molecules



Scheme 2.
Key synthetic intermediates