Guanidinylated Neomycin Delivers Large, Bioactive Cargo into Cells through a Heparan Sulfate-dependent Pathway

Lev Elson-Schwab *, Omai B. Garner †¶, Manuela Schuksz *§, Brett E. Crawford *‡, Jeffrey D. Esko *§¶, and Yitzhak Tor *‡²

From the Departments of *‡Chemistry and Biochemistry and *¶Cellular and Molecular Medicine, Glycobiology Research and Training Center, *§Biomedical Sciences Graduate Program, University of California, San Diego, La Jolla, California 92093 and *‡²Zacharon Pharmaceuticals, Inc., La Jolla, California 92037

Facilitating the uptake of molecules into living cells is of substantial interest for basic research and drug delivery applications. Arginine-rich peptides have been shown to facilitate uptake of high molecular mass cargos into cells, but the mechanism of uptake is complex and may involve multiple receptors. In this report, we show that a derivative of the aminoglycoside antibiotic neomycin, in which all of the ammonium groups have been converted to guanidinium groups, can carry large (>300 kDa) bioactive molecules across cell membranes. Delivery occurs at nanomolar transporter concentrations and under these conditions depends entirely on cell surface heparan sulfate; (ii) in aqueous solution, guanidinium moieties represent the critical feature responsible for efficient cell membrane permeability. In fact, guanidinium-containing peptoids (9) and β-peptides exhibit useful cell uptake properties (10). Short polyproline-based helices appended with guanidinium groups (11), highly branched guanidinium-rich dendritic oligomers (12), and heterocyclic guanidinium vectors also serve as cell transporters (13).

Advances in genomics and proteomics have identified high molecular mass biomolecules and their analogs as potential therapeutic agents. The ability of a cell to take up a high molecular mass drug and to release it into the cytoplasm in an active form represents, however, a major obstacle for the development of these agents. For biological macromolecules effective delivery entails minimal exposure to conditions that may denature or otherwise disrupt activity. Numerous approaches for the physical control of drug localization and release significantly improve the pharmacokinetic features of bioactive molecules, but they typically do not address the inherent challenge of transport of the therapeutic agent across cell membranes. Delivery procedures based on passive diffusion encounter problems due to charged groups, and carriers that exploit endogenous membrane transporters limit the size of potential drug candidates.

Certain polybasic proteins have been shown to enhance the cellular uptake of biomolecules (1). Over the past 15 years, tremendous progress has been made in advancing the basic science, applications, and preclinical evaluation of these and other cationic cell transduction domains (2, 3). In 1988, the human immunodeficiency virus 1 Tat protein was shown to cross lipid bilayers and enter the nucleus (4, 5). Subsequently, numerous other naturally occurring and chimeric peptides have been found to exhibit efficient translocation properties. A database search, inspired by Tat, identified a number of membrane-permeable peptides that contain clustered arginine residues (6, 7). Further exploration of stereochemistry and composition identified p-Tat and Arg₉, as competent transporters (6, 7). Additionally, significant activity was observed for branched arginine-rich oligomers (8). These observations suggested that the presence of guanidinium moieties represents the critical feature responsible for efficient cell membrane permeability. In fact, guanidinium-containing peptoids (9) and β-peptides exhibit useful cell uptake properties (10). Short polyproline-based helices appended with guanidinium groups (11), highly branched guanidinium-rich dendritic oligomers (12), and heterocyclic guanidinium vectors also serve as cell transporters (13).

The mechanistic understanding of the cellular uptake and internalization of arginine-rich peptides and their analogs has yet to be fully elucidated. Endocytosis-based mechanisms have been both supported and questioned, but some of the early studies may have suffered from artifacts generated by cell fixation. Electrostatic interactions of the positively charged peptides with membrane phospholipids have been proposed as the first step in the transduction process (14–17). An alternative model that has recently been gaining support is the interaction of the positively charged peptides with negatively charged cell surface proteoglycan receptors (18–20).

Recently, we described a new family of synthetic RNA ligands, coined “guanidinoglycosides” (Fig. 1), in which the amino groups of naturally occurring aminoglycoside antibiotics were converted to guanidinium groups. These compounds exhibit high affinity and selectivity for RNA targets that are naturally recognized by Arg-rich domains (21). Guanidinoglycosides also display cellular uptake properties (22). Here, we explore the cellular requirement for uptake, as well as the delivery potential of guanidinoglycosides. We demonstrate that (i) the cellular binding and uptake of guanidinomycin at low concentration depends exclusively on heparan sulfate; (ii) in

---

* This work was supported by National Institutes of Health Grants CA11227 and GM33063 (to J. D. E.) and AI47673 and GM77471 (to Y. T.), Cancer Cell Biology Training Grant CA67754 (to M. S.), and National Research Service Award Individual Fellowship SF31 AI05891602 (to O. G.). The UCSD Neuroscience Microscopy Shared Facility was supported by National Institutes of Health Grant NS047101. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Schemes S1 and S2 and supplemental data.

‡ To whom correspondence may be addressed. E-mail: jesko@ucsd.edu.

§ To whom correspondence may be addressed. E-mail: ytor@ucsd.edu.
Guanidinylated Neomycin Uses HS-dependent Pathway

contrast, the uptake of arginine-rich peptides in the same concentration range follows both heparan sulfate-dependent and independent pathways; (iii) guanidinoneomycin will transport high molecular mass and bioactive cargo into cells at low concentration in a completely proteoglycan-dependent manner; and (iv) effective guanidinoneomycin-mediated delivery can be achieved with little or no cellular toxicity.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells (CHO-K1) (ATCC CCL-61) and Lec 2 (ATCC CRL-1736) were obtained from the American Type Culture Collection (Rockville, MD). Mutants pgsA745 and pgsG224 were described previously (23, 24). All cell lines were grown under an atmosphere of 5% CO2 in 24-well plates. All cultures were grown under an atmosphere of 5% CO2 in complete growth medium containing 10% fetal bovine serum, 100 μg/ml of streptomycin sulfate, and 100 units/ml of penicillin G.

Guanidinylated Neomycin—Synthesis of guanidinoneomycin-biotin, neomycin-biotin, and Arg9-biotin—Synthesis of guanidinoneomycin-biotin and neomycin-biotin is described in the supporting information. Arg9-biotin and Arg9-BODIPY were synthesized using standard Fmoc (N-(1H-benzotriazole-1-yl)-O-(1-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) chemistry as described in the supporting information.

Inhibition Experiments—Wild-type CHO cells were grown to confluence on 6-well tissue culture plates, harvested with 10 mM EDTA (37 °C, 10 min), washed with phosphate-buffered saline (PBS), and incubated in suspension with biotinylated FGF-2 (10 ng/ml) (25) in F12 medium for 1 h at 37 °C in the presence of increasing concentrations of guanidinoneomycin (100 nM to 1.8 mM). The cells were then stained with streptavidin-PE-Cy5 (BD Biosciences) for 20 min, washed three times with PBS, and analyzed by flow cytometry. Cells were also incubated in F12 medium containing 0.5 μM Arg9-BODIPY and increasing concentrations of guanidinoneomycin (1–300 nM) for 1 h at 37 °C and analyzed by flow cytometry.

Preparation of Fluorescently Tagged Guanidinoneomycin-Biotin, Neomycin-Biotin, and Arg9-Biotin and Cell Uptake Studies—Biotinylated compounds were stored in water at −20 °C. After thawing at room temperature, compounds were diluted into F12 medium to 1 μM. To this solution, streptavidinylated saporin in complete growth medium was added to the cells and incubated at 37 °C for 24 h. After washing with PBS, cells were incubated with the fluorescently tagged guanidinoneomycin-, neomycin-, or Arg9-biotin for 1 h at 37 °C under an atmosphere of 5% CO2. Cells were washed with PBS, released with EDTA, and analyzed by flow cytometry.

Microscopy—Cells were cultured on Lab-Tek chambered coverglass slides (Electron Microscopy Sciences) in F12 medium. After washing with PBS, cells were incubated for 1 h in 1 ml of 60 nM guanidinoneomycin coupled to streptavidin-Alexa-488 (Molecular Probes) at 37 °C. Guanidinoneomycin-A488 was prepared by incubating 1 μM guanidinoneomycin-biotin with 6 μg of streptavidin-Alexa-488 in 1 ml of medium. Hoechst 33342 (2 μg/ml; Molecular Probes) was added to cells for the last 20 min of incubation. Cells were washed three times with F12 medium before live cell imaging. Microscopic images were acquired on an Olympus IX70 DeltaVision Spectris Image Deconvolution system, equipped with a temperature and atmospherically controlled stage. Images were deconvolved (10 cycles) using SoftWoRx Explorer Suite software.

Saporin Delivery—A conjugate of saporin and biotinylated guanidinoneomycin was prepared by mixing streptavidylated saporin (Advanced Targeting Systems) with biotinylated compound in 1:4 ratio. Wild-type CHO and pgsA cells were incubated with guanidinoneomycin-biotin, guanidinoneomycin-biotin and saporin or the conjugate of guanidinoneomycin-biotin and streptavidylated saporin in complete growth medium for 4 days at 37 °C. CellTiter-Blue (Promega) was added to the medium and cells were incubated for an additional 4 h to measure viability.

RESULTS

Uptake of Guanidinoneomycin Depends on Heparan Sulfate—Neomycin is a member of a family of aminoglycoside antibiotics that inhibit protein synthesis in bacteria (26). Conversion of the amino groups to guanidinium groups alters the properties of the glycoside, allowing it to interact with cell surface heparan sulfate. To study its interactions with cells, we synthesized biotinylated guanidinoneomycin, as well as biotinylated neomycin and biotinylated Arg9 for comparison (Fig. 1). Biotinylation facilitates conjugation of the carriers to fluorophores and its versatility allows for the preparation and testing of a variety of analogs in different assays.

Fluorescent streptavidin-phycerythrin-cychrome (streptavidin-PE-Cy5) conjugates of biotinylated guanidinoneomycin, neomycin, and Arg9 were incubated with CHO cells and uptake was measured by flow cytometry. As described below, these measurements reflect both binding and internalization of the conjugates but are referred to as “uptake” for the sake of clarity. Uptake of the fluorescent guanidinoneomycin conjugate occurred at concentrations as low as 10 nM and proportionately increased up to a concentration of 1 μM, the highest concentration tested (Fig. 2, upper panels, blue lines). Cells also took up the neomycin conjugate but much less efficiently than the guanidinylated derivative (middle panel, blue lines). Uptake of the Arg9 peptide occurred in a more complex manner, exhibiting two classes of receptors expressed by different cells in the population (lower panel, blue lines).

Incubation with heparin at concentrations as low as 100 ng/ml blocked uptake of the fluorescent guanidinoneomycin conjugate (Fig. 3), suggesting a high affinity of the compound for the negatively charged residues in heparin. These data also suggested the possibility that cell surface heparan sulfate proteoglycans might represent one class of receptors that mediate

3 The abbreviations used are: CHO, Chinese hamster ovary; FGF, fibroblast growth factor; PE, phycoerythrin; PBS, phosphate-buffered saline.
binding and uptake. To test this idea, the fluorescent guanidino-neomycin conjugate was incubated with pgsA cells, a mutant that makes <2% of the wild-type level of chondroitin sulfate and heparan sulfate chains (Table 1) (23). Guanidino-neomycin uptake in pgsA cells was barely detectable up to concentrations of 100 nM and was over 20-fold lower than that observed with wild-type cells, even at 1 μM (Fig. 2, upper panels, green lines). At higher concentrations, a second, glycosaminoglycan-independent mode of uptake began to emerge. The same trend was observed for the fluorescently tagged neomycin, with internal-
ization being more efficient in wild-type cells than pgsA cells (Fig. 2, middle panels, green lines). The signal from pgsA cells was not affected by trypsin, indicating interactions with a non-proteinaceous receptor.

Whereas the uptake of guanidinoneomycin was strongly dependent on cell surface glycosaminoglycans, fluorescently labeled Arg₉ exhibited multiple modes of uptake in pgsA cells even at low concentrations (Fig. 2, lower panels, green lines). Multiple populations of cells exhibiting differential binding or uptake capacity were observed in pgsA cells and in wild-type cells. These data suggest that the internalization of Arg₉ follows both glycosaminoglycan-dependent and -independent pathways. Analysis of the mean fluorescence values showed that uptake of the compounds occurred in proportion to concentration, but did not saturate (Fig. 2b). Because of this, no further experiments to measure affinity were attempted.

To further study the uptake of guanidinoglycosides, other mutant CHO cells were examined (Table 1, Fig. 3). pgsG cells, mutants lacking all glycosaminoglycans due to a deficiency in glucuronyltransferase I (24), showed a reduction in binding and uptake similar to pgsA cells. Reintroduction of the gene for glucuronosyltransferase I (pgsG/H₁₁₀₀₁/GlcATI) restored binding and uptake, demonstrating their dependence on glycosaminoglycans. Cells selectively lacking heparan sulfate (pgsD) (27) also exhibited dramatically reduced binding and uptake of fluorescent guanidinoneomycin. Because pgsD cells express higher than normal levels of chondroitin sulfate on the cell surface, these findings demonstrate the specificity of binding and uptake for heparan sulfate. Examination of Lec2 cells, which lack sialic acid residues, excluded participation of sialylated glycoproteins and glycolipids (data not shown).

To test the dependence of guanidinoglycoside uptake on heparan sulfate in other cells, we incubated human HeLa ovarian carcinoma cells with fluorescent guanidinoneomycin. A robust signal was obtained, and treatment of the cells with heparin lyases reduced uptake (Fig. 3). The extent of reduction was not as great by enzymatic treatment as by genetic inactivation of heparan sulfate formation in CHO cells, presumably due to incomplete digestion of heparan sulfate chains. Similar results were also obtained for STO mouse fibroblast cells (data not shown), indicating that heparan sulfate on other cells can also mediate uptake of guanidinoneomycin.

To distinguish between cell surface binding and internalization, wild-type CHO cells were incubated with the fluorescent guanidinoneomycin conjugate at 4 °C, where only surface binding occurs. The extent of labeling at 4 °C was reduced by ~6-fold compared with incubations performed at 37 °C (Fig. 4, inset), suggesting that ~85% of the fluorescence signal at 37 °C was due to internalization. Binding at 4 °C was sensitive to tryp-

![FIGURE 3. Binding and uptake of guanidinoneomycin by CHO cells and mutants.](image)

**TABLE 1**

| Strain     | Glycan alteration | Enzymatic defect         |
|------------|-------------------|--------------------------|
| Wild-type CHO | Normal            | None                     |
| CHO pgsA    | Glycosaminoglycan-deficient | Xylosyltransferase (XTII) (23) |
| CHO pgsG    | Glycosaminoglycan-deficient | Glucuronosyltransferase I (GlcATI) (24) |
| CHO pgsD    | Heparan sulfate-deficient   | Copolymerase (EXT1) (27)   |
| CHO Lec2    | Sialic acid deficient     | CMP-sialic acid transporter (28) |
| HeLa        | Normal              | None                     |

![FIGURE 4. CHO cell binding of guanidinoneomycin at 4 °C.](image)
Guadinidinylated Neomycin Uses HS-dependent Pathway

sin and heparinase treatment, consistent with binding to membrane proteoglycans. Incubation of pgsA cells showed that at low concentrations binding was entirely dependent on expression of glycosaminoglycans. At higher concentrations, a second class of binding sites was detected that did not show saturability (Fig. 4). Binding to wild-type cells at higher concentrations represents the sum of both classes of binding sites. Subtraction of the fluorescent values obtained from the mutant (pink line) from those obtained from the wild-type (blue line) yielded a binding curve (green line) that presumably reflects the contribution of the proteoglycans (Fig. 4).

**Guadinidinoneomycin Inhibition of FGF-2 and Arg9 Binding to Cell Surface Heparan Sulfate**—The binding and uptake studies described above predicted that guadinidinoneomycin would inhibit binding of ligands that are known to interact with heparan sulfate, such as basic fibroblast growth factor (FGF-2) (29, 30). Prior studies have shown that biotinylated FGF-2 will bind to wild-type CHO cells in a heparan sulfate-dependent manner (24). When mixed with increasing concentrations of guadinidinoneomycin, binding was inhibited, with an IC50 value of ~20 μM (Fig. 5a). In contrast, neomycin, the parent aminoglycoside, did not inhibit binding of FGF-2, which is consistent with its reduced affinity for heparan sulfate (data not shown). Guadinidinoneomycin also blocked fluorescent-Arg9 binding and uptake (Fig. 5b). However, inhibition of Arg9 was incomplete, saturating at the same level of fluorescence intensity as observed when fluorescent Arg9 was incubated with pgsA cells (Fig. 5b, inset). These data show that Arg9 and guadinidinoneomycin bind to a common set of glycosaminoglycan-dependent sites and support the idea that Arg9 also has one or more glycosaminoglycan-independent mechanisms of uptake.

**Guadinidinoneomycin Internalization and Cytoplasmic Delivery of Cargo**—To study uptake of guadinidinoneomycin in live cells, wild-type and glycosaminoglycan-deficient pgsA cells were incubated with a conjugate prepared from guadinidinoneomycin-biotin and streptavidin-Alexa-488. Deconvolution fluorescence microscopy demonstrated uptake into punctate vesicles (Fig. 6a), whereas uptake was not observed in pgsA cells (Fig. 6b). Inclusion of heparin (50 μg/ml) in the incubation medium completely abolished uptake in wild-type cells (data not shown), but washing the cells with heparin (350 μg/ml) after incubation with the fluorescent guanidinoglycoside conjugate had little effect on vesicle fluorescence, consistent with the idea that the punctate structures were intracellular. With longer incubation, more diffuse cytoplasmic staining was observed as well (data not shown).

To probe the mechanism of uptake, wild-type cells were incubated with the fluorescent guadinidinoneomycin conjugate in the presence of sucrose, which has been shown to inhibit clathrin-mediated endocytosis through dissociation of the clathrin lattice (31), and amiloride, which specifically blocks macropinocytosis through inhibition of Na+/H+ exchange (23, 32). Cells treated with sucrose showed a marked decrease in internalization of guadinidinoneomycin, whereas amiloride had no effect (Fig. 6c). These data indicate that guadinidinoneomycin is likely internalized into cells via clathrin-dependent endocytosis, consistent with other studies indicating that proteoglycans undergo constitutive internalization (33).

These findings suggested that much of the internalized fluorescent guadinidinoneomycin was present in endocytic vesicles or lysosomes but with time some will appear in the cytoplasm. To examine whether guadinidinoneomycin could deliver large cargo into the cytoplasm, streptavidinylated saporin was conjugated to guadinidinoneomycin-biotin. Saporin, a Type I ribosome-inactivating toxin from Saponaria officinalis seeds, does not kill cells due to lack of cell surface receptors (34). However, conjugation of saporin to a ligand for which receptors exist leads to cell death (34). As shown in Fig. 7, the guadinidinoneomycin-saporin complex killed wild-type CHO cells with an LD50 of ~2

![FIGURE 5. Inhibition of FGF and Arg9 binding by guadinidinoneomycin.](image)

a, wild-type CHO cells were incubated with biotinylated FGF-2 (10 ng/ml) for 1 h in the presence of increasing concentrations of guadinidinoneomycin. Cells were then stained with streptavidin-phycocyanin-cychrome and analyzed by flow cytometry. Guadinidinoneomycin inhibited FGF-2 binding to cells with an IC50 of ~20 μM. b, wild-type and pgsA cells were incubated with 0.5 mM Arg9, BODIPY and increasing concentrations of guanidinoneomycin. After 1 h the cells were analyzed by flow cytometry. Guadinidinoneomycin is able to partially block Arg9 binding to the surface of cells. The signal saturates once it reaches that of pgsA cells incubated with the fluorescent peptide. Inset, relative fluorescence of untreated wild-type cells (a), wild-type cells treated with fluorescent Arg9 (b), pgsA cells treated with Arg9 (c), and wild-type cells treated with Arg9 and 1 μM (d) or 300 μM guadinidinoneomycin (e).

![FIGURE 6. Visualization of guadinidinoneomycin uptake in CHO cells.](image)

a, wild-type cells were incubated with a conjugate of biotinylated guadinidinoneomycin and streptavidinylated Alexa-488 for 1 h at 37 °C. An overlay of the 4′,6-diamidino-2-phenylindole nuclear stain (blue) and fluorescent guadinidinoneomycin conjugate (green) shows internalization in punctate vesicular structures. When the same experiment was performed with heparan/chondroitin sulfate-deficient pgsA cells (b), no cell-associated guanidinoglycoside fluorescence was observed. c, incubation of wild-type cells with 0.4 M sucrose inhibited uptake, whereas incubation with 5 mM amiloride had no effect.
Guanidinylated Neomycin Uses HS-dependent Pathway

FIGURE 7. Guanidinoneomycin can efficiently deliver large, bioactive cargo into the cell in a heparan/chondroitin sulfate-dependent manner. Various combinations of guanidinoneomycin, saporin, and streptavidinylated saporin (~130 kDa) were added to cells. After 4 days, the number of viable cells was estimated using CellTiter assay (“Experimental Procedures”), where the emission at 575 nm corresponds to the relative number of viable cells. Guanidinoneomycin-biotin does not display cell lysis activity on wild-type (black) or pgsA (pink) cells up to the highest concentration examined (84 nM). Little to no cell death was observed in both wild-type (blue) and pgsA (gray) cells when incubated with a mixture of non-streptavidinylated saporin and guanidinoneomycin-biotin. Cell toxicity was observed when wild-type cells were incubated with guanidinoneomycin conjugated to saporin through biotin-streptavidin (red) with an LD₅₀ of ~2 nM. pgsA cells were relatively resistant to the guanidinoglycoside-toxin conjugate (green).

No cell toxicity was observed for unconjugated guanidinoneomycin-biotin or for free saporin at concentrations up to 100 nM. Mutant pgsA cells were resistant to toxin within this range of concentration but succumbed at higher concentrations, similarly to cells treated with neomycin-saporin or unconjugated saporin at high concentration. Taken together, these data show that guanidinoneomycin can deliver at very low concentrations large, bioactive cargo into the cytoplasm in a heparan sulfate-dependent manner.

DISCUSSION

Cationic transduction domains, such as the Arg-rich Tat peptide, have been demonstrated to effectively cross lipid bilayers and enter cells (7). Importantly, such relatively short peptides have also been shown to facilitate the uptake of diverse molecular cargos, from small molecules to oligonucleotides and proteins. These observations support the notion that such molecular transporting vehicles can eventually be used to facilitate cellular delivery of impermeable therapeutic agents. A natural peptidic backbone (or sequence) is unnecessary for delivery, because several guaninium-containing derivatives have been shown to function in a similar manner to Arg-rich peptides. Here, we have evaluated the cell surface requirements for the uptake of guanidinoneomycin, a carbohydrate-based, non-oligomeric guaninium-rich derivative of the naturally occurring aminoglycoside antibiotic. Like their oligo-arginine counterparts, guanidinoneomycin can deliver high molecular mass cargos, but with much greater selectivity for cell surface heparan sulfate. Thus, guanidinylated glycosides such as guanidinoneomycin may provide the opportunity to develop cell-selective delivery tools, exploiting the differences in proteoglycan expression among different cell types (35).

A universal feature of cell transduction domains, independent of backbone structure, is the presence of a number of guaninium groups. Bearing a fixed positive charge, these groups can readily form charge-charge interactions with negatively charged groups present in macromolecules, such as phosphate groups in nucleic acids, sulfate and carboxyl groups in glycosaminoglycans, and polar head groups of acidic phospholipids enriched in the outer leaflet of the plasma membrane. The guanidinoglycosides bind more avidly than the corresponding aminoglycosides, presumably due to the higher basicity of the guaninium groups and their ability to form charged, paired hydrogen bonds with sulfate groups. Apparently, net charge plays a key role in efficacy, as cell transduction domains typically contain between 5 and 11 clustered guanidinium groups (3, 9, 22). The studies reported here also demonstrate that the three-dimensional distribution and density of guanidinium groups confer preferred interactions. Thus, exogenously supplied guanidinoneomycin preferentially interacts with heparan sulfate chains associated with cell surface proteoglycans, and not with other acidic glycans, such as chondroitin sulfate, which actually has a higher average charge density per unit length compared with heparan sulfate. Guanidinoneomycin can also bind to a second class of lower affinity receptors when added at higher concentrations. While the proteoglycan-dependent receptors became saturated at low micromolar concentrations of guanidinoneomycin, the binding to the third, non-heparan sulfate-dependent class of receptors did not plateau. This finding indicates that these receptors are abundant and may constitute a major part of the cell surface, such as the polar heads of the phospholipids (15). The ability to alter the number and spatial distribution of guanidinium groups on glycocide-based scaffolds may aid in the design of even more specific derivatives.

A major finding reported here is the use of guanidinoglycosides to facilitate the cytoplasmic delivery of bioactive cargo, such as streptavidinylated saporin (~130 kDa) and phycoerythrin (~300 kDa). The use of saporin as a probe of cytoplasmic delivery has several advantages, including greater sensitivity and the capacity to kill cells by inhibition of protein synthesis. The dependence of cytoplasmic delivery on heparan sulfate and its sensitivity to sucrose suggests that the guanidinoneomycin conjugates may bind to membrane proteoglycans and “piggy-back” into the cell during clathrin-dependent endocytosis. A portion of membrane proteoglycans undergoes constitutive internalization and degradation in lysosomes (33). Although it is tempting to speculate that the punctate structures labeled by fluorescent guanidinoneomycin represent a pool from which saporin complexes escape or are transported into the cytosol, further studies are needed to determine the actual compartment from which cytosolic cargo originates.

Guanidinoglycosides present several advantages over peptide/oligomer-based transport vehicles: 1) The mechanism of uptake and delivery of polyarginine appears to be more complicated, because both heparan sulfate-dependent and -independent pathways exist; 2) Non-peptidic and non-oligo-
meric structures may display enhanced in vivo stability; 3) Aminoglycoside-degrading enzymes, and by inference enzymes that degrade guanidinoglycosides, have not yet been described in animal cells, whereas multiple proteases exist that can degrade arginine-rich peptides; 4) Guanidinoglycosides may offer greater flexibility in conjugation chemistry as compared to peptide-based delivery agents; 5) The chemical synthesis of guanidinoglycosides allows for divergent synthesis of multiple conjugates; and 6) The use of cleavable linkers might further facilitate the delivery of small and large molecules and their release within the cytoplasm.

In summary, we have shown the capacity of guanidinoglycosides to deliver high molecular mass, bioactive cargos into cells. At low concentration, cellular uptake occurs exclusively by heparan sulfate-dependent receptors. This behavior may provide a window of opportunity to exploit differences in expression of cell surface proteoglycans for the development of more effective and selective cellular delivery vehicles.

Acknowledgment—We thank Arrate Mallabiabarrena for excellent assistance with deconvolution microscopy.

REFERENCES

1. Ryser, H. J.-P. (1968) Science 159, 390–396
2. Dietz, G. P., and Bahr, M. (2004) Mol. Cell. Neurosci. 27, 85–131
3. Wadia, J. S., and Dowdy, S. F. (2005) Adv. Drug Deliv. Rev. 57, 579–596
4. Frankel, A. D., and Pabo, C. O. (1988) Cell 55, 1189–1193
5. Green, M., and Loewenstein, P. M. (1988) Science 241, 247–253
6. Futaki, S. (2005) J. Biol. Chem. 280, 15300–15306
7. Futaki, S. (2006) J. Am. Chem. Soc. 128, 3197–3201
8. Futaki, S., Nakase, I., Suzuki, T., Zhang, Y., and Sugiura, Y. (2002) Biochemistry 41, 7925–7930
9. Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13003–13008
10. Umezawa, N., Gelman, M. A., Haigis, M. C., Raines, R. T., and Gellman, S. H. (2002) J. Am. Chem. Soc. 124, 368–369
11. Fillon, Y. A., Anderson, J. P., and Chmielowski, J. (2005) J. Am. Chem. Soc. 127, 11798–11803
12. Chung, H. H., Harms, G., Seong, C. M., Choi, B. H., Min, C., Taulane, J. P., and Goodman, M. (2004) Biopolymers 76, 83–96
13. Fernández-Carneado, J., Van Gool, M., Martos, V., Castel, S., Prados, P., de Mendoza, J., and Giralt, E. (2005) J. Am. Chem. Soc. 127, 869–874
14. Rothbard, J. B., Jessop, T. C., and Wender, P. A. (2005) Adv. Drug Deliv. Rev. 57, 495–504
15. Rothbard, J. B., Jessop, T. C., Lewis, R. S., Murray, B. A., and Wender, P. A. (2004) J. Am. Chem. Soc. 126, 9506–9507
16. Caesar, C. E. B., Esbjourner, E. K., Lincoln, P., and Nordén, B. (2006) Biochemistry 45, 7682–7692
17. Hitz, T., Iren, R., Gardiner, J., Namoto, K., Walde, P., and Seebach, D. (2006) Biochemistry 45, 5817–5829
18. Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. (2001) J. Biol. Chem. 276, 3254–3261
19. Fuchs, S. M., and Raines, R. T. (2004) Biochemistry 43, 2438–2444
20. Richard, J. P., Melikov, K., Brooks, H., Prevot, P., Lebleu, B., and Chernomordik, L. V. (2005) J. Biol. Chem. 280, 15300–15306
21. Luedtke, N. W., Baker, T. J., Goodman, M., and Tor, Y. (2000) J. Am. Chem. Soc. 122, 12035–12036
22. Luedtke, N. W., Carmichael, P., and Tor, Y. (2003) J. Am. Chem. Soc. 125, 12374–12375
23. Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3197–3201
24. Bai, X. M., Wei, G., Sinha, A., and Esko, J. D. (1999) J. Biol. Chem. 274, 13017–13024
25. Wei, G., Bai, X., Sarkar, A. K., and Esko, J. D. (1999) J. Biol. Chem. 274, 7857–7864
26. Chambers, H. F. (2006) in Goodman & Gilman’s The Pharmacological Basis of Therapeutics (Brunton, L. L., Lazo, J. S., and Parker, K. L., eds) 11th Ed., pp. 1155–1171, McGraw-Hill, New York
27. Lidholt, K., Weinke, J. L., Kiser, C. S., Lugemwa, F. N., Bame, K. J., Cheifetz, S., Massaguo, J., Lindahl, U., and Esko, J. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2267–2271
28. Deutscher, S. L., Nuwayhid, N., Stanley, P., Briles, E. L., and Hirschberg, C. B. (1984) Cell 39, 295–299
29. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 841–848
30. Rapraeger, A. C., Krufta, A., and Olwin, B. B. (1991) Science 252, 1705–1708
31. Lamaze, C., and Schmid, S. L. (1995) Curr. Opin. Cell Biol. 7, 573–580
32. Kaplan, I. M., Wadia, J. S., and Dowdy, S. F. (2005) J. Controlled Release 102, 247–253
33. Williams, K. I., and Fuki, I. V. (1997) Curr. Top Microbiol. Immunol. 234, 57–61
34. Flavell, D. J. (1998) J. Biol. Chem. 273, 3856–3860
35. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471