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Exploring Guanidinoglycoside Molecular Transporters

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in
Chemistry

by

Andrew Dix

Committee in Charge:
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Professor Jeffrey Esko
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2011
The Dissertation of Andrew Dix is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego
2011
DEDICATION

True
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ABSTRACT OF THE DISSERTATION

Properties of Guanidinoglycosides as Molecular Transporters

by

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Doctor of Philosophy

University of California, San Diego, 2011

Professor Yitzhak Tor, Chair

Guanidinium-rich molecular transporters have been shown to deliver otherwise non-permeable biologically relevant cargo into cells. While many such transporters have been reported, the studies reported here focus on guanidinoglycosides, which have been shown to permeate the cell-membrane
in a heparan sulfate-dependent manner. In attempt to promote a cooperative interaction with cell-surface heparan sulfate, dimeric guanidinoglycosides were synthesized and studied for their cellular uptake properties in various Chinese hamster ovary (CHO) cell lines. Flow cytometry studies revealed that conjugates prepared with the monomeric compounds showed reduced uptake in mutant cells expressing heparan sulfate chains with altered patterns of sulfation, whereas the dimeric constructs could distinguish the various mutants and maintain high levels of uptake. These findings suggest that uptake depends on the valency of the transporter and the sulfate content on the cell surface receptors, thereby providing reagents that could distinguish cell types based on the composition of their cell surface heparan sulfate proteoglycans.

To further investigate the viability of these transporters, their potential to escape endosomal vesicles and diffuse into the cytosol was examined using a FRET-based cellular assay in HeLa cells. Findings indicate that guanidinoglycosides appear to enter the cytosol at around half the rate of polyarginine. Interestingly, confocal microscopy studies indicate co-localization of the two different transporters.
CHAPTER 1: INTRODUCTION.

Typically, large polar molecules such as proteins demonstrate poor bioavailability, and cell membrane permeability is focused on sufficiently non-polar molecules. In 1988, however, a paradigm changing discovery demonstrated that a protein could permeate the cell membrane in a receptor-independent fashion.\textsuperscript{1,2} The HIV-TAT protein, part of the HIV-machinery, was the first protein reported to accomplish this, violating the dogma that polar molecules do not cross the cell membrane. Additionally, the Drosophila homeoprotein antennapedia transcription protein\textsuperscript{3-5} and the Herpes simplex virus\textsuperscript{6} expressed similar structural motifs, and were later found to be cell permeable as well.\textsuperscript{7} In exploring this novel biological phenomenon, these full length proteins were conjugated to various otherwise non-permeable cargo, and shown to facilitate intracellular delivery.\textsuperscript{8,9} The wide range of cargo utilized suggest that there are no size restrictions in the transport process.\textsuperscript{10}

Further studies into the specific requirements for cell permeability revealed that only the peptide transduction domain (PTD) was required. This was demonstrated using short amino acid sequences cleaved from the full length cell-penetrating proteins (CPPs).\textsuperscript{11} It was later revealed that the peptidic backbone was non-essential, leading to investigations of the specific amino acid functionality that characterize the PTD.\textsuperscript{12-14} These studies demonstrated that, although the highly-basic PTD was made of various amino acids, arginine — specifically its guanidinium functional group — was
responsible for the observed translocation properties. This is believed to facilitate recognition, interaction, and subsequent internalization of the guanidinium-containing construct.

Guanidinium Functional Group: Multiple Layers of Multivalency

The guanidinium functional group is seemingly unique in its ability to facilitate cellular uptake. In studies comparing peptides comprised of polyammonium and poly-guanidinium, only guanidinylated peptides demonstrated cellular uptake, indicating that charge alone is insufficient to promote uptake (Figure 1.1).\textsuperscript{15,16} In a subsequent study, the Wender laboratory observed that increased methylation, resulting in decreased hydrogen bonding ability, reduced activity to less than 80\% of the parent oligo-guanidine (Figure 1.2).\textsuperscript{7,15} Hydrogen bonding, specifically the unique ability of guanidinium to form bidentate hydrogen bonds with compatible functionality at the cellular surface, is believed to be responsible for its cellular uptake properties (Figure 1.3). The multivalent hydrogen-bonding ability of guanidinium function groups sets it apart from its ammonium analogue.
Figure 1.1. A) Poly-ammonium and B) poly-guanidinium transporter scaffolds.

Figure 1.2. Successive methylation limits the ability of the guanidinium functional group to serve as a hydrogen bond donor significantly decreasing cellular uptake.\textsuperscript{7,15}
Figure 1.3. An example of bidentate hydrogen bonding between the charged guanidinium functional group and a complementary sulfate or phosphate.

The multivalent hydrogen-bonding required to facilitate cellular uptake is further characterized by the degree of guanidinium modification. This second layer of multivalency determines the ability of the transporter to interact with the cellular surface. Studies have shown that, overall, increased guanidinylation leads to increased cellular uptake. However, the degree of guanidinylation alone is not sufficient to identify the better transporter, as different scaffolds have been shown to demonstrate distinct behavior.\textsuperscript{17,18}

**Multivalency and Cooperativity**

Multivalency refers to the binding interactions of two or more ligands, utilizing simultaneous and identical binding modes, with one target.\textsuperscript{21} The presentation of multiple binding sites for a specific target is also considered multivalency. The valency of a system is defined by the number of ligands presented on the structural scaffold. For example, a scaffold presenting two ligands (a dimeric ligand) is considered divalent and a trimeric ligand would be
trivalent. The binding interactions of a multivalent event can be referred to as cooperativity. This phenomenon is commonly observed in nature, characterized by the ability to enhance binding ability while increasing specificity.\textsuperscript{22,23} Lectins, carbohydrate binding proteins, are a prime example of nature utilizing multivalency. For example, the target protein P-selectin glycoprotein ligand-1 (PSGL-1) and its substrate P- and L-selectin, utilize a combination of tetrasaccaride modifications along with incorporation of multiple tyrosine sulfate moieties to increase its binding affinity by several orders of magnitude.\textsuperscript{23} In utilizing multivalency, specifically the increased incorporations of tyrosine and tetrasaccaride modifications, the overall binding affinity shifts from low affinity ($K_d \approx 1\text{mM}$) to a significant physiologically relevant affinity ($K_d \approx 1\mu\text{M}$).

The increased affinity caused by multivalency, referred to as avidity, is a result of combined binding enthalpy.\textsuperscript{21} Each individual binding event, between one ligand and one target, has a specific enthalpy. In a multivalent system, these contributions are combined and are proportional to the degrees of possible binding. For example, a divalent system has roughly twice the enthalpy of the governing monovalent interaction. An additional governing element of multivalency is the difference in binding association versus dissociation. Analogous to the chelate effect, the decreased rate of dissociation of a multivalent system can be orders of magnitude different from its monovalent counterpart.\textsuperscript{24} These properties allow for poor binding ligands to cooperatively combine, generating increased specificity towards their target.
This increased specificity is a result of the additive effects of increased binding enthalpy along with a decrease in dissociation.

Cooperativity and multivalent interactions are being elucidated as a standard paradigm in biological systems. Gene regulation, antibodies, virus and bacterial adhesion to cells, binding of oxygen to hemoglobin, and the chemistry of carbohydrate-binding proteins are a few examples of system interactions that are governed by multivalent principles. In addition to these known systems, chemists and biologists are implementing this strategy to optimize binding and recognition and to explore the versatility of known systems.

Recently, Deyev and Lebedenko explored the impacts of multivalency on the antibody targeting of tumors. Small recombinant antibodies, in spite of their high-affinity, present only a modest retention time on the target antigen. However, when converted into a multivalent format, decreased dissociation rates and increases in affinity were observed. This is a classic example of utilizing multivalency to increase and optimize a known interaction. Another noteworthy study was performed by Sisu et al. Here, dimeric and trimeric constructs of ganglioside GM1, a potent monovalent inhibitor of a cholera toxin, were investigated to explore the role of valency in the system. The multivalent ligands show a modest increase in avidity over their monovalent parent, but the multivalent inhibitors induced aggregation and the formation of space-filling networks. This undesired aggregation forms when the valency of the ligand does not match the valency of the target. This observation is of
particular interest as it implies that there is an optimal degree of valency governing a system.

**Internalization Mechanisms and Endosomal Trapping**

Cells have numerous pathways with which they can absorb nutrients and other necessary molecules. Endocytosis engulfs large highly polar molecules that cannot sufficiently diffuse through the membrane. Several specific types of endocytosis have been observed, two of the most prominent are clathrin-mediated endocytosis and macropinocytosis (Figure 1.4). While both processes are characterized by vesicle formation, Clathrin-mediated endocytosis is a receptor-mediated process whereas macropinocytosis is a nonspecific process. The vesicles formed in macropinocytosis are also significantly larger, containing large amounts of extracellular fluid and molecules. Following internalization, the vesicles are recruited into the endocytotic pathway of the cells.
Figure 1.4. Depiction of various proposed cellular entry pathways, specifically highlighting heparan sulfate-mediated endocytosis and pinocytosis.34

The cell surface is rich with bivalent anions, such as sulfates, phosphates, and carboxylates, that are complementary to the guanidinium cation. Coordination of the ionic species is suggested to convert the polar cationic nature of the transporter to a lapidated ion pair complex enabling diffusion through the membrane (Figure 1.3).35 It is proposed that increased hydrogen-bonding, specifically increased guanidinylation, enhances this interaction and the ability to permeate the cellular membrane.
A universal internalization mechanism for guanidinium-rich molecular transporters has not been demonstrated, and many aspects of it remain unclear (Figure 1.4). The Chung laboratory recently proposed that each class of transporter may utilize distinct pathways, or to different degrees multiple pathways. For example, evidence suggests that certain cell surface glycosaminoglycans, which are highly anionic and believed to interact with the cationic transporter, are involved. The extent with which these glycans are involved, however, remains under continuous debate. Other laboratories have reported that cellular uptake of their transporters is independent of glycosaminoglycans, and that macropinocytosis is the primary entry pathway.

Regardless of the internalization mechanism, data indicate that the transporters are internalized via vesicle formation. This observation is conserved among various transporter scaffolds, indicated by the high degree of endosomal trapping. These endocytotic vesicles remain encapsulated by the cellular membrane. While data have demonstrated that the transporters can present in the cytosol, it is unclear to what extent and by what mechanism this occurs. A prominent hypothesis for endosomal release/escape is the formation of bidentate hydrogen bonds with counter anions bearing hydrophobic character, facilitating a decrease in overall polarity wherein the complex can eventually permeate the membrane.
Commonly Used Techniques: Flow Cytometry and Confocal Microscopy

Flow cytometry is a technique in which populations of cells are analyzed based on health, phenotype, and morphology. Using the principle of hydrodynamic focusing, the instruments laser treats one cell at a time, capturing the emitted photons for analysis. This occurs at a rate of hundreds of cells per second. The unique feature of a flow cytometer is its ability to measure fluorescence per cell. This is key for cellular uptake studies, where flow cytometry is used to determine the internalization of reportable fluorophores (Figure 1.5). While powerful, this analysis can only report on the overall internalization and/or binding of the fluorescently labeled construct.

In a typical flow cytometry experiment, cells are incubated with a fluorescently-labeled transporter. After incubation, the cells are washed to remove any non-bound or external material. To distinguish between internalized material from what is bound to the external surface of the cell, proteases such as trypsin can be used to cleave cell surface proteins and proteoglycans. Conversely, EDTA solutions can be used to leave the membrane-bound material intact. The cellular samples are then injected into the flow cytometer and analyzed based on the degree of increased fluorescence from the labeled transporter (Figure 1.5). First, a dot plot is generated characterizing the cells based on morphology (Figure 1.6). This enables a visualization of the cell population. Gating, a process of limiting the analysis to a specific subset of the overall data population, can be used. The advantage of gating is the ability to rule out data from damaged or unhealthy
cells, as well as to distinguish between multiple cell types. A histogram is then generated detailing the cell counts versus the degree of fluorescent signal (Figure 1.7). The histograms can be statistically analyzed for a mean value, which can be plotted to compare multiple samples. Figure 1.7, for example, is a histogram of a series of Chinese hamster ovary (CHO) cell samples that have been treated with increasing concentrations of a fluorescently-labeled transporter. The untreated cells, designated by the shaded red curve, serve as a negative control. A shift, from left to right, occurs in samples containing increased fluorescent signal.

**Figure 1.5.** Schematic of flow cytometer analysis. The sample of cells is injected into the instrument wherein the flow rate analyzes each cell individually at a rate of hundreds per second. The laser analyzes the cell based on the light that emerges from each cell as it passes through the instrument.
Figure 1.6. Dot plot generated during flow cytometry analysis. The cells are plotted and represented by a heat map. Gating selects for the highest density of cell. Here, the gate is represented by the black oval.

Figure 1.7. A histogram generated by flow cytometry analysis. The y-axis describes the normalized sample population and the x-axis describes the amount of signal in each sample. Here, untreated cells are represented by the filled in red curve. Successive curves represent increased internalization of the fluorophore-labeled transporter.

The second commonly employed technique used to analyze cellular uptake is confocal microscopy. A confocal microscope is a modified
fluorescent microscope that uses point illumination and a pinhole in front of the detector to block out-of-focus light. This modification distinguishes it from traditional fluorescent microscopes in its increased optical resolution. However, the increase in resolution is achieved at the cost of overall sensitivity. With respect to molecular transporters, confocal microscopy is used to photograph cells and describe the internalization of fluorescently-labeled compounds. This enables a snapshot visualization of complex internalization events. Used in conjugation with intracellular markers, this technique can be extremely powerful. Instruments will have several channels in which it can excite a fluorophore and monitor its emission, as opposed to a table-top fluorometer.

In a typical confocal experiment, cells are exposed to fluorescently-labeled molecules. The cells are directly visualized through the microscope, or first washed, treated, and re-plated. Figure 1.8 is an example of a confocal image, in which HeLa cells were treated with a fluorescently-labeled transporter.
Figure 1.8. Confocal microscopy images of HeLa cells. A) Digital contrast image (DIC), and B) an overlay of the DIC and the fluorescence (red) of the labeled transporter.

Overview of Non-Peptidic, Carbohydrate-Based Molecular Transporters

While HIV-TAT and poly-arginine dominate the cell penetrating peptide field (Figure 1.9), their implementation is not without drawbacks. Peptide-based molecular transporters suffer from innate toxicity and degradation by peptidases. To circumvent this, several laboratories have investigated installing guanidinium functionality onto various non-peptidic scaffolds, such as dendrimers and carbohydrates. These transporters avoid the traditional peptide-backbone and fall into a broader class of carriers known as guanidinium-rich molecular transporters (GRTs). The following highlights several key contributions from laboratories investigating non-peptidic, carbohydrate-based, guanidinium-rich molecular transporters, specifically those from the Chung and Tor laboratories.
Motivated by the ability of the guanidinium functional group to enable cellular uptake of CPP’s, Chung and coworkers sought to install guanidinium moiety onto a novel, benign scaffold. Having identified the carbohydrate inositol as non-toxic and while maintaining a high density of functional groups, the authors proceeded to incorporate guanidine containing ω-amino acids (Figure 1.10). Using confocal microscopy, the authors compared the cellular uptake properties of the inositol derivatives to those of polyarginine (Arg8). In the cell lines examined (simian kidney COS-7, mouse microphage RAW264.7, and HeLa cells), the fluorescently-labeled inositol transporters internalized roughly 2-fold more efficiently than their Arg8 counterparts. It was also
observed that the derivatives containing longer linkers, five methylene chains versus seven, exhibited higher uptake efficiencies. In flow cytometry-based kinetic experiments, the authors demonstrated that the cellular uptake of the \( \text{Arg}_8 \) transporter plateaued after 3 hours, while the inositol derivates displayed progressively increasing uptake even after 6 hours. Chung and coworkers further noted that linker flexibility of their inositol derivatives linkers likely enables increased cell membrane interactions and enhance uptake.\(^{53}\) This hypothesis was supported by their observation that inositol and \( \text{Arg}_8 \) transporters exhibited contrasting intracellular localization.

\[ \text{Figure 1.10. Inositol-based conjugate (scyllo-dimer). Note that the authors also synthesized and examined the myo dimer and found it to be less active. } n = 3, 5 \text{ or } 7 \]

To further characterize their inositol transporter, Chung and coworkers explored biodistribution in mouse models. The biodistribution of HIV-TAT and arginine-based transporters has been well documented in the literature, having been shown to distribute uniformly through the liver, kidney, spleen and heart.\(^{54}\) Interestingly, when similar experiments were performed with the inositol-based transporters, significantly higher distribution was observed in
heart, lung, and brain tissues.\textsuperscript{55} In a follow-up experiment, the authors confirmed that their transporter did indeed present in the brain and passage through the blood-brain barrier. This study was expanded to include a doxorubicin-inositol conjugate, wherein the authors again observed localization in the brain tissue. This finding promotes the concept of tissue-targeting through molecular transporters. Additionally, this is the first report utilizing a guanidinium-rich molecular transporter, which successfully crosses the blood-brain barrier.

Chung and coworkers went on to develop a second generation carbohydrate-based transporter, utilizing a sorbitol core and bis-guanidine linkers (Figure 1.11).\textsuperscript{56} The translocation, or cellular uptake properties, of this transporter are similar to those of the previous inositol derivatives. However, the sorbitol-based transporters were shown to co-localize with a mitochondria-specific dye (MitoTracker Red) in HeLa and CD34\textsuperscript{+} stem cell-like KG1a leukemia cells, demonstrating a high level of organelle specificity. The tissue distribution of the sorbitol derivatives improved the biodistribution of its predecessor, showing high concentrations in lung and brain tissues. The authors comment that this is likely due to the high levels of mitochondria in these tissues.\textsuperscript{17}
In efforts to further expand their library of transporters and investigate intracellular localization and tissue targeting, the Chung laboratory have built transporters on lactose\textsuperscript{57} and sucrose\textsuperscript{19} scaffolds (Figure 1.12). Initially, they explored the effect of linker length, by conjugating both cores to their guanidinium functionality though linkers bearing a 5 or 7 methylene chain. In both systems, the transporters bearing the 5-methylene linker were observed to co-localize in mitochondria, similar to their predecessors. However, studies on the 7-methylene linked transporters revealed punctuate formation and partial localization in endosytic vesicles and endosomes. Interestingly, the lactose-based transporter studies showed prominent distributions in the brain, liver, and spleen tissues independent of the linker length.\textsuperscript{57} The authors note that while the overall distribution pattern of their reported transporters (inositol, sorbitol, and lactose) may differ, all demonstrate an ability to cross the blood-brain barrier.
In the sucrose-based transporters, the authors observed that the fluorophore impacted the localization properties. The Alexa-fluor labeled conjugate did not co-localize with the mitochondrial tracker, whereas both the fluorescein (FITC) and tetramethyl rhodamine (TRITC) labeled conjugates did. The authors go on to hypothesize that this is due to variations in lipophilicity, supported by noting a similar trend in reversed phase HPLC retention times. Notably however, all sucrose labeled fluorophore transporters demonstrated similar tissue distributions in the brain, heart, liver, lung, kidney, and spleen.

**Guanidinoglycosides**

Guanidinoglycosides, generated from the parent aminoglycosides wherein the amino groups are chemically converted into guanidinium groups (Figure 1.13), were initially developed to increase binding specificity toward RNA, specifically the HIV Rev Response Element (RRE). Further studies by Tor and co-workers demonstrated that these guanidinoglycosides exhibited
a 10–20 fold increase in cellular uptake compared to their parent aminoglycosides.\textsuperscript{60} In particular, guanidinoneomycin, having six guanidine-modifications, was distinctly more effective at entering the cell than its lesser guanidinylated counterpart, guanidinotobramycin.

![Figure 1.13](image_url)

*Figure 1.13. Neomycin, guanidinoneomycin, and bodipy-guanidinoneomycin.*

To explore the requirements for internalization of guanidinoglycosides, the Tor laboratory generated biotinylated compounds and screened the resulting streptavidin conjugates against a variety of cell lines (Figure 1.14). Using HeLa, CHO, and several CHO mutants deficient in specific glycosaminoglycans (GAGs), the authors were able to systematically address the role or need for several prominent cell surface glycosaminoglycans in the cellular uptake pathway.\textsuperscript{18,61,62} It was observed that at nanomolar
concentrations, the cellular uptake of the guanidinoneomycin conjugate was dependent on heparan sulfate, a ubiquitously expressed cell surface glycosaminoglycan (Figure 1.15). The relevance of which was accentuated by the observation that a similar Arg₉ conjugate was not fully inhibited by the lack of heparan sulfate, and was able to maintain moderate levels of uptake. Additionally, the guanidinoglycoside conjugates were shown to outperform the Arg₉ in cellular uptake studies. The authors went on to demonstrate that cellular uptake of Arg₉ is inhibited by guanidinoneomycin, suggesting that these distinct transporters share a common internalization pathway.

![Figure 1.14. Biotin guanidinoneomycin and Arg₉ conjugates.](image)

In the same study, the authors exemplified the delivery potential of guanidinoglycosides. Using streptavidin conjugated to a non-permeable toxin,
saporin, the resulting guanidinoneomycin conjugates were able to promote cell
death.\textsuperscript{18} Expanding on the observed heparan sulfate dependence, cell death
was not observed in mutant cell lines absent of the heparan sulfate
glycosaminoglycan. Indeed, the authors demonstrated a unique dependence
on heparan sulfate for cellular uptake and that guanidinoglycosides could
sufficiently deliver biologically active cargo into the cell.

![Chemical Structure](image)

\textbf{Figure 1.15.} Octasaccaride segment of heparan sulfate chondroitin sulfate.

While a universal uptake pathway has not been established in the field
of guanidinium-rich transporters, the guanidinoglycosides have demonstrated
a singular involvement and specificity. The authors continue to circumvent
problematic areas of the field as they take advantage of a chief failing of these
molecular transporters – endosomal/lysosomal trapping. Using an \textit{N}-
hydroxysuccinimide (NHS) ester of guanidinoneomycin (Figure 1.16), lysine
residues of various enzymes were labeled, which allowed enzyme entry into
cells via endosomal vesicles.\textsuperscript{64} While this phenomenon is not unique, it was
novel in utilizing the trapping effect to deliver therapeutically relevant enzyme
to these vesicles to treat for lysosomal storage diseases. The authors were
able to demonstrate that active enzyme, \(\beta\)-\textit{D}-glucuronidase and \(\alpha\)-\textit{L}-
iduronidase, could be delivered to deficient cells and reconstituting normal levels of activity. The use of currently employed therapeutic agents as positive controls highlighted the true successes of this technique, as the guanidinoneomycin-enzyme conjugates are significantly more active, indicated by the lower EC$_{50}$ values.$^{64}$

Figure 1.16. Guanidinoneomycin-NHS.

**Personal Contributions to Guanidinoglycoside Research**

Having established dependence between heparan sulfate and guanidinoglycosides, we aimed to explore the underlying principles behind this interaction. Specifically, can the heparan sulfate-specificity of the guanidinoglycoside cellular uptake mechanism be enhanced? In addressing this, the authors sought to utilize cooperativity. A common phenomenon in biology, cooperativity enables an individual substrate unit to overcome its low binding affinity by means of additive affinity of multiple binders. The overall effect is increased avidity toward the target. This is particularly common in carbohydrate chemistry.$^{23}$ We sought to utilize a similar strategy to enhance
interactions. As heparan sulfate has inherently multivalent potential, complementary multivalency was installed on the guanidinoglycosides transporter. We propose that this enhanced valency would promote interactions 1) along a given heparan sulfate chain, 2) across multiple heparan sulfate chains on a given core protein, and 3) between heparan sulfate chains on adjacent or nearby core proteins (Figure 1.17).

Using dimeric guanidinoglycoside-streptavidin conjugates (Figure 1.18), the authors probed the ability to elicit a cooperative response from heparan sulfate, which is inherently multivalent. Furthermore, several CHO mutant cell lines were utilized, each with specific deficiencies in the pattern of sulfation of heparan sulfate. It was observed that the heparan sulfate dependence was maintained for all conjugates examined, and that the cellular uptake of the monomeric carriers were significantly diminished in cells expressing “poor quality” heparan sulfate. Interestingly, the dimeric conjugates were able to overcome deficient heparan sulfate and maintain high levels of uptake. A 5-10 fold cooperative response was observed in CHO cell mutants that express altered heparan sulfate. The observation of glycan-
sulfate effects might have boarder significance, as it appears to be an additional variable to consider in the evaluation of molecular transporters.

Figure 1.18. Monomeric and dimeric guanidinoneomycin-Biotin.

**Outlook**

Overall, the fields of cell-penetrating peptides and guanidinium-rich transporters have produced many advances. However, the ultimate goal to successfully deliver therapeutics to human patients has yet to be realized. Indeed, the observation of transporter based tissue localization and the utilization of that specificity to selectively deliver organelle-based cargo is an
exciting advancement. This will help transport targeted drug therapies in a very positive direction.

References:


7. It is worth noting that only the Drosophila homeoprotein antennapedia transcription protein is beneficial, being a transcriptional regulator as well as being involved in intracellular trafficking. HIV TAT and the Herpes simplex virus are however malicious with respect to the host organism.


16. *Polyamines have however been utilized successfully in lipople克斯 to facilitate cellular delivery.*


34. Figure modified from Professor Jeffrey Esko.


CHAPTER 2: Cooperative, Heparan Sulfate-Dependent Cellular Uptake of Dimeric Guanidinoglycosides

Abstract. Oligoarginine and guanidinium-rich molecular transporters have been shown to facilitate the intracellular delivery of a diverse range of biologically relevant cargos. Several such transporters have been suggested to interact with cell-surface heparan sulfate proteoglycans as part of their cell-entry pathway. Unlike for other guanidinium-rich transporters, the cellular uptake of guanidinoglycosides at nanomolar concentrations is exclusively heparan sulfate dependent. As distinct cells differ in their expression levels and/or the composition of cell-surface heparan sulfate proteoglycans, one might be able to exploit such differences to selectively target certain cell types. To systematically investigate the nature of their cell-surface interactions, monomeric and dimeric guanidinoglycosides were synthesized by using neomycin, paromomycin, and tobramycin as scaffolds. These transporters differ in the number and 3D arrangement of their guanidinium groups. Their cellular uptake was measured by flow cytometry in wild-type and mutant Chinese hamster ovary cells after the corresponding fluorescent streptavidin–phycoerythrin-Cy5 conjugates had been generated. All derivatives showed negligible uptake in mutant cells lacking heparan sulfate. Decreasing the number of guanidinium groups diminished uptake, but the three dimensional arrangement of these groups was less important for cellular delivery. Whereas conjugates prepared with the monomeric carriers showed significantly reduced uptake in mutant cells expressing heparan
sulfate chains with altered patterns of sulfation, conjugates prepared with the
dimeric guanidinoglycosides could overcome this deficiency and maintain high
levels of uptake in such deficient cells. This finding suggests that cellular uptake
depends on the valency of the transporter and both the content and arrangement
of the sulfate groups on the cell-surface receptors. Competition studies with
chemically desulfated or carboxy-reduced heparin derivatives corroborated these
observations. Taken together, these findings show that increasing the valency of
the transporters retains heparan sulfate specificity and provides reagents that
could distinguish different cell types based on the specific composition of their
cell-surface heparan sulfate proteoglycans.
Introduction

High-molecular-weight biomolecules such as certain proteins and nucleic acids display therapeutic potential.\textsuperscript{1–4} Their limited cellular uptake, however, hampers their utility and has prompted the development of diverse delivery technologies including viromes,\textsuperscript{5} liposomes,\textsuperscript{6} lipoplexes,\textsuperscript{7} and molecular transporters.\textsuperscript{8} The latter largely rely on arginine-containing peptides\textsuperscript{9} and the installation of guanidinium groups on diverse multifunctional scaffolds derived from peptides,\textsuperscript{10} peptoids,\textsuperscript{11} carbohydrates,\textsuperscript{12} and dendrimers.\textsuperscript{13} These guanidinium-rich transporters have been demonstrated to effectively deliver otherwise nonpermeating cargos.\textsuperscript{2, 14–16} Their mechanism of cell entry is, however, not fully understood. Multiple uptake pathways are likely to operate, including clathrin-mediated endocytosis and macropinocytosis.\textsuperscript{17–27}

The association of guanidinium-rich transporters with heparan sulfate has been observed and suggests a significant role for this abundant cell-surface glycosaminoglycan in either the recognition or internalization of these carriers.\textsuperscript{3, 28–31} The prominent arginine-rich oligomers (e.g., Arg\textsubscript{9}) display cellular uptake, albeit less effectively, in heparan sulfate-deficient cell lines, thus indicating the contribution of heparan sulfate-independent entry pathways.\textsuperscript{25, 32} In this respect, guanidinylated aminoglycosides or guanidinoglycosides, a family of synthetic derivatives in which all the ammonium groups of aminoglycoside antibiotics were converted into guanidinium groups, stand out.\textsuperscript{33–34} At low carrier concentrations, their uptake
is exclusively heparan sulfate-dependent; this suggests unique cell surface interactions with these multivalent cell-surface receptors.\textsuperscript{32}

Heparan sulfate proteoglycans are expressed by virtually all multicellular organisms and play essential roles in human physiology.\textsuperscript{35} Extending from a core protein, heparan sulfate glycosaminoglycans protrude into the extracellular matrix, coating the cell. Heparan sulfate itself is a linear polymer comprised of repeating disaccharide units of glucosamine and uronic acid, which are heterogeneously N- and O-sulfated (Scheme 2.1). While the overarching structure is conserved, individual heparan sulfate chains maintain a high level of diversity, differing in chain length, the extent of sulfation, and the degree of epimerization.\textsuperscript{36} Furthermore, distinct cells differ in their expression levels and/or composition of cell-surface heparan sulfate proteoglycans.\textsuperscript{35} This might allow one to exploit differences in this proteoglycan landscape for selective targeting by guanidinoglycosides, provided that their cell-surface interactions are better understood.
Scheme 2.1. Representative octasaccharide segments as they are expressed in the cell lines utilized. Heparan sulfate from A) wild-type CHO-K1 cells, B) pgsE cells, and C) pgsF cells. D) Chondroitin sulfate from pgsD cells, as these mutants do not express heparan sulfate. All the negatively charged moieties are highlighted in bold. Note that although only a octasaccharide segment is schematically depicted, full-length heparan sulfate is heterogeneous and typically 40–60 saccharide units long.

In this report we explore the impact and significance of three key parameters on cellular uptake: 1) The number and spatial distribution of guanidinium groups on guanidinoglycosides, 2) the degree and pattern of sulfate groups on cell-surface glycosaminoglycans, and 3) potential cooperativity governed by the guanidinoglycoside scaffold, being either monomeric or dimeric. We have synthesized both monomeric and dimeric guanidinoglycoside carriers, derived from three different aminoglycosides (Scheme 2.2). Their uptake was evaluated in five unique Chinese hamster ovary (CHO) cell lines, each differing in its expression of heparan sulfate, with the aim of deciphering the nature of heparan sulfate–guanidinoglycoside
interactions and the possibility of exploiting their specificity to increase the efficacy and versatility of the guanidinoglycoside transporter. Our findings illustrate the significance of the number of guanidinium groups on uptake, although their spatial distribution plays a relatively minor role. Importantly, the dimeric guanidinoglycoside derivatives maintain considerably higher levels of cellular uptake than the monomeric carriers in cells expressing poorly sulfated heparan sulfate chains.

Scheme 2.2. The guanidinoglycoside scaffolds in both monomeric and dimeric forms, compounds 1–6.

Results and Discussion

To explore the subtle features of the heparan sulfate-selective uptake of guanidinoglycosides, we evaluated the impact of the number and arrangement of guanidinium groups in both monomeric and dimeric guanidinoglycoside constructs on their cellular uptake. The use of cell lines expressing
biosynthetically altered heparan sulfate, in addition to cell lines that do not express it at all, facilitated the systematic investigation of the fundamental interactions between this important cell-surface charged biopolymer and these unique carriers.

**Carrier design.** To explore differences between monomeric and dimeric guanidinoglycosides, structurally related, flexible, and water soluble monomeric and dimeric linkers were prepared (Scheme 2.3A). Bifunctional poly(ethylene glycol) (PEG) chains incorporating an azide and an amine were designed to facilitate the "clicking" of a guanidinoglycoside and the coupling of biotin, respectively. Conjugation of the biotinylated guanidinoglycoside to a fluorescently labeled streptavidin enabled comparative flow cytometry measurements. Note that under these conditions the monomeric guanidinoglycoside constructs become tetravalent and the dimeric constructs yield octavalent conjugates, as four molecules of biotin bind to a single streptavidin.

**Synthesis of guanidinoglycoside conjugates.** By using previously reported procedures, neomycin B (9) was Boc-protected and treated with 2,4,6-triisopropylbenzenesulfonyl chloride (TIBS-Cl) to selectively activate the primary hydroxymethyl group (Scheme 2.3 B). Treatment with methanolic ammonia provided the aminomethyl derivative (10), which was coupled to hexynoic acid under standard peptide coupling conditions to give 11. The Boc
groups were removed with trifluoroacetic acid, and the resulting modified
aminoglycoside was treated with $N,N'$-di-tert-butoxycarbonyl-$N'$-triflylguanidine
to yield the protected guanidinoglycoside (12). The alkyne-linked 12 was
conjugated to linker 7 or 8 by using Cul-mediated “click chemistry” and
subsequently treated with trifluoroacetic acid to give the corresponding
monomeric (1) or dimeric (2) construct as a guanidinium–trifluoroacetic acid
(TFA) salt. The guanidinoparomomycin (3, 4) and guanidinotobramycin (5, 6)
derivatives were prepared in a similar manner (Scheme A1.4 and A1.5).
Scheme 2.3. Synthesis of biotinylated monomeric (1) and dimeric (2) guanidinoneomycin carriers.  

a) Boc₂O, NEt₃, DMF, H₂O (72%); b) TIBS-Cl, pyridine (75%); NH₃, MeOH, 80°C (91%); 
d) hex-5-ynoic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 4-dimethylaminopyridine, N,N-diisopropylethylamine, CH₂Cl₂ (84%); 
e) TFA, triisopropylsilane (TIPS), CH₂Cl₂ (94%); f) (BocNH)₂(C=NTf), NEt₃, MeOH, H₂O (73%); 
g) 7, CuI, Cs₂CO₃, CH₂Cl₂, CH₃CN (80%); h) TFA, TIPS, CH₂Cl₂ (88%); i) 8, CuI, Cs₂CO₃, CH₂Cl₂, CH₃CN (75%); 
j) TFA, TIPS, CH₂Cl₂ (72%).
Selection of cell lines with varied heparan sulfate expression. Chinese hamster ovary cells (CHO-K1) expressing natural levels of heparan sulfate and specific mutants were utilized to investigate the effect of heparan sulfate sulfation patterns on cellular uptake (Scheme 2.1). Several well-behaved CHO mutants, including pgsA, pgsD, pgsE, and pgsF, were utilized to systemically evaluate the role of sulfation levels in the recognition/uptake of guanidino glycosides. Two undersulfated mutants, pgsE and pgsF, each deficient in specific enzymes that catalyze sulfation of the heparan sulfate oligosaccharide during its biosynthesis were employed. The pgsE mutants lack GlcNAc Ndeacetylase/N-sulfotransferase, and this results in poor N-sulfation and a significantly decreased level of overall sulfation (Scheme 2.1 B). Mutant pgsF cells are deficient in uronyl-2-O-sulfotransferase; this results in under expression of 2-O sulfation and a slightly increased level of 6-O sulfation (Scheme 2.1 C). Comparatively, the pgsE mutant cells are less sulfated than then the pgsF mutants. The two remaining cell lines serve as important controls, as neither pgsA nor pgsD expresses heparan sulfate. The pgsD mutant cells, however, do express elevated levels of chondroitin sulfate (Scheme 2.1 D), a related, highly charged proteoglycan that differs from heparan sulfate primarily in its uniform b-linked oligosaccharide chains.

Quantifying cellular uptake. The biotinylated guanidino glycosides were conjugated to streptavidin-PE-Cy5 and diluted into F-12 medium. Cells were
incubated in a solution of the corresponding monomeric or dimeric transporter for 2 h at 37°C and were then washed, detached by using trypsin/EDTA, and analyzed by using flow cytometry. Under these conditions, cell-surface-bound material is entirely cleaved, and the FACS signal represents internalization of the conjugates. For any given cell line, the mean fluorescence intensity was plotted against the concentration of the transporter. Figure 2.1 highlights the uptake data for the monomeric and dimeric constructs, while Figure 2.2 summarizes the data at 100 nm carrier concentration.

\textbf{Figure 2.1.} Cellular uptake of A) monomeric and B) dimeric constructs. i) Comparing the uptake of guanidinoneomycin (○), guanidinoparomomycin (▲), and guanidinotobramycin (□) into CHO-K1 (WT) cells. The cellular uptake of ii) guanidinoneomycin, iii) guanidinoparomomycin, and iv) guanidinotobramycin against WT cells (□), pgsA (▼), pgsD (▲), pgsE (◊), and pgsF (○) cell lines.\textsuperscript{52-53}
Effect of number and distribution of guanidinium groups. Cellular internalization was dependent on the number of guanidinium groups on the guanidinoglycoside scaffolds. For both the monomeric and dimeric constructs, the uptake of guanidinoneomycin (1/2) was 30% higher than that of guanidinoparomomycin (3/4) and guanidinotobramycin (5/6; Figure 2.1i). This has been observed with other guanidinium-containing transporters.\textsuperscript{54–55} Additionally, the guanidinoparomomycin and guanidinotobramycin conjugates for both the monomeric and dimeric constructs (compounds 3/5 and 4/6, respectively) demonstrated similar uptake behavior in all cell lines (Figure 2.1). As both guanidinoglycoside scaffolds contain five guanidinium groups, this observation suggests that the 3D architecture of the guanidinium groups plays a limited role in cellular uptake. The inability of the cells, and more specifically the cell-surface glycans, to distinguish between the different arrangements
suggests that the binding/recognition inherent to the uptake process could be quite plastic. We note, however, that guanidinoglycosides, in contrast to linear guanidinium-rich carriers such as HIV TAT and poly-Arg, possess a high density of charged groups, and that the subtle differences in the architecture of these transporters might have been overshadowed by the inherent tetrameric nature of the streptavidin core.

**Significance of glycan sulfation levels.** Heparan sulfate-deficient pgsA and pgsD mutant cells showed poor uptake (<5%) compared to the uptake in wild-type CHO cells (Figure 2.1ii–iv). This observation confirms the heparan sulfate-dependent nature of guanidinoglycoside cellular uptake observed before when using different constructs and demonstrates that this is an inherent trait of the guanidinoglycoside scaffold and is not linker or construct dependent. For the monomeric constructs (Figure 2.1 A), uptake in the undersulfated pgsE and pgsF cells was reduced to <20% of that observed in the wild-type cells. This behavior was not observed for the dimeric constructs (Figure 2.1 B), as high uptake levels, between 50 and 75% of the uptake in wild-type cells, were observed in both pgsE and pgsF mutant cells. These trends were observed at all concentrations tested and suggest that there is a significant relationship between glycan sulfation and either the level of guanidinylation or the carrier valency.
Dimeric constructs illicit cooperative responses. Minimal internalization was observed at transporter concentrations lower than 50 nm (particularly for the guanidinoparomomycin and guanidinotobramycin derivatives), thus suggesting a switch-like or cooperative mechanism (Figure 2.1). Possibly, the enhanced uptake observed as concentration increases is due to the aggregation of proteoglycan receptors on the cell surface and/or the activation of endocytosis. This suggests that a critical concentration of the transporter might be necessary to induce effective uptake. The multivalent and heterogeneous nature of heparan sulfate makes it difficult to determine the underlying mechanism. Interestingly, this cooperative-like effect was accentuated in the dimeric constructs, consistent with the idea that the guanidinoglycosidic ligand induces clustering of heparan sulfate proteoglycans. In wild-type cells, all the dimeric constructs showed 2.5-fold enhanced uptake over the corresponding monomeric carriers, while a fivefold increase was observed in the undersulfated cell lines pgsE and pgsF at 100 nm concentration (Figure 2.2). For the guanidinoneomycin constructs (1 and 2), a tenfold increase in uptake of the dimeric construct over monomeric was observed in pgsF cells at the same concentration (Figure 2.2). This trend demonstrates that the dimeric constructs are increasingly able to overcome undersulfation and maintain high levels of cellular uptake. Likely, this behavior is due to the apparent increase in avidity of the dimeric constructs, enabling cooperative cell-surface binding. The modest increase in uptake of the dimers in wild-type cells compared to their monomeric counterparts speaks to
the efficiency of the guanidinoglycoside-mediated uptake mechanism. A guanidinoglycoside monomer appears to enter the cell with high efficiency, negating the contribution from the second arm of the dimeric constructs when heparan sulfate is abundant. This is reinforced by the increased response in the undersulfated cells, where the poor sulfation is insufficient to facilitate adequate uptake of the monomeric carriers. In this case, the dimeric constructs demonstrate a chelate-like effect that enables increased interactions between the carrier and cellsurface glycans, thus facilitating effective uptake.

**Competition experiments on model oligomeric glycans.** To further assess the significance of glycan sulfation levels and support the observations described above, the cellular uptake of the guanidinoneomycin constructs, compounds 1 and 2, was evaluated in the presence of competing model glycans derived from heparin. In this fashion, coloring of the results due to subtle variations between mutant cell lines, in addition to the specific glycosaminoglycan modification described above, can be eliminated. Specifically, heparin, de 2-O sulfated heparin, de 6-O sulfated heparin, de O-sulfated heparin, N-desulfo-N-acetylated heparin, and heparin containing reduced uronic acids were tested (Scheme 2.4). Dermatan sulfate, an analogue of chondroitin sulfate which is over expressed in pgsD cells, was also explored as a competitor.\(^{36, 48}\) Wild-type cells were treated with increasing concentrations of the competing glycans, along with a fixed
concentration of the guanidinoneomycin conjugates, 1 and 2, and the cellular uptake was quantified by flow cytometry, as outlined above. The mean fluorescence intensity was plotted against the concentration of the glycans utilized (Figure 2.3), and IC\textsubscript{50} values were extracted (Table 2.1).

![Scheme 2.4](image)

**Scheme 2.4.** Structures of glycans used for competition studies. All glycans were derived from heparin (HP) or dermatan sulfate (DS), each differing in its sulfation pattern as well as other key features. A)–F) Modified heparin derivatives, and G) dermatan sulfate. Note that full-length glycans were utilized (typically 12 kDa, ca. 40–60 saccharide units), although only a hexasaccharide segment is depicted.\textsuperscript{36}
Figure 2.3. Normalized inhibition curves for A) monomeric guanidinoneomycin 1 and B) dimeric guanidinoneomycin 2. Key: heparin A (■), de 2-O-sulfated heparin B (▲), de 6-O-sulfated heparin C (▼), de 6-O-sulfated heparin D (♦), N-acetylated heparin E (●), carboxy-reduced heparin F (●), and dermatan sulfate G (*). See Scheme 4 for the structures of glycans A–G.52

Table 2.1: IC\textsubscript{50} values [mg/mL] glycan competitors that inhibit the uptake of monomeric (1) and dimeric (2) guanidinoneomycin. See Scheme 1.4 for structures of Glycans A – G.

<table>
<thead>
<tr>
<th>Glycan\textsuperscript{a}</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin (A)</td>
<td>0.33 ± 0.1</td>
<td>0.11 ± 0.07</td>
</tr>
<tr>
<td>de 2-O-sulfate (B)</td>
<td>6.3 ± 3.5</td>
<td>0.94 ± 0.8</td>
</tr>
<tr>
<td>de 6-O-sulfate (C)</td>
<td>&gt; 100</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>de O-sulfate (D)</td>
<td>&gt; 100</td>
<td>6.5 ± 2</td>
</tr>
<tr>
<td>N-acetyl (E)</td>
<td>&gt; 100</td>
<td>11 ± 10.5</td>
</tr>
<tr>
<td>carboxyl reduced (F)</td>
<td>&gt; 100</td>
<td>3.2 ± 3</td>
</tr>
<tr>
<td>Dermatan sulfate, DS (G)</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Glycan structures follow the order of the table (A–G).
Reaffirming observed cooperativity. Inhibition of guanidinoglycoside uptake with native heparin elicited the most pronounced inhibitory response (Figure 2.3) and highlighted a threefold increase of sensitivity of the dimeric over the monomeric construct. This appears intuitive, as heparin contains the highest sulfation levels of the competing glycans, and mirrors the modest cooperative affect observed for cellular uptake in wild-type cells. For monomeric guanidinoneomycin 1, only heparin and de 2-O sulfate heparin showed inhibition at the tested concentrations.57 For dimeric guanidinoneomycin 2, inhibition was observed for all the heparin derivatives. Dermatan sulfate failed to inhibit the cellular uptake of either construct. The inhibition response from undersulfated heparin glycans demonstrated a dramatic tenfold difference in efficiency between constructs. These observations parallel the uptake data, which showed that the dimeric scaffold increases the ability of the guanidinoglycoside to interact with undersulfated cell-surface glycans. Notably, the observed three- to tenfold cooperative effect between the monomeric and dimeric guanidinoneomycin constructs was similar for both the uptake and the competition experiments. Additionally, the competition experiment enabled quantification of the binding interactions between the conjugates and the model glycans, as well as between the monomeric and dimeric constructs.

Not all charge is created equal. The IC_{50} values obtained from the competition study indicate that all the charged functional groups of the
glycosaminoglycans are important for binding/recognition toward guanidinoglycosides. However, as indicated by the high level of inhibition maintained for de 2-O sulfate heparin (Figure 2.3), some positions and functional groups might be more vital than others. This is likely the result of the overall structure and presentation of these charged groups. Additionally, dermatan sulfate displays no significant inhibition, further supporting that there is little to no interaction between this glycan and guanidinoglycosides, thereby corroborating and complementing the lack of uptake observed in the pgsD mutant cells, which over express an analogous glycan. As the sulfation levels of heparan sulfate and dermatan/chondroitin sulfate are similar, our observations suggest that the interactions governing this highly selective recognition process are more complicated than strict electrostatic interactions. Moreover, these glycans differ significantly in their incorporations of a versus β glycosidic linkages, thus suggesting that the folded 3D structures of the glycans, and not the transporter scaffold, are of prominence in governing the uptake process of guanidinoglycosides. This hypothesis could explain the perceived disparity in the field of guanidinium-rich transports, as different carriers have shown distinct degrees of involvement by these glycans during the cellular uptake process.

Conclusion

Taken together, the data demonstrate that the cellular uptake of monomeric guanidinoglycoside constructs is limited in cells expressing
undersulfated heparan sulfate, whereas the additional cell-surface binding ability of the dimeric guanidinoglycoside transporters enables them to overcome these deficiencies. This trend suggests that by manipulating the valency of the transporter, one can discern the “quality” of cell-surface heparan sulfate chains. Additionally, the concept that glycan sulfation affects overall uptake might have universal significance, as it suggests an additional variable when evaluating the behavior of molecular transporters. This implies that the cell lines utilized in uptake experiments can have a profound effect on the outcome, as different cell lines express varying amounts of heparan sulfate proteoglycans. These findings highlight the versatility and promise of a guanidinoglycoside based molecular transporter, as well as the increased applicability of targeting cell-surface heparan sulfate as a gateway into the cell.

**Experimental Section**

**Materials.** Unless otherwise specified, materials obtained from commercial suppliers were used without further purification. Neomycin, paromomycin, and tobramycin were purchased as their sulfate salts from Sigma–Aldrich and were converted into the corresponding neutral form by being passed through DOWEX MONOSPHERE 550 Å (OH) anion-exchange resin. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. The heparin derivatives utilized in the competition study were purchased from Neoparin (Alameda, CA, USA). PBS (Dulbecco’s phosphate buffered saline), HBSS (Hanks’ balanced salt solution), and F-12K Media were purchased from
Invitrogen. FACS buffer (Isotonic solution 0.85% w/v, phosphate buffered, pH 7.1–7.3) and streptavidin–PE-Cy5 were purchased from BD Biosciences (San Jos, CA, USA). Trypsin/EDTA was purchased from VWR (Mediatech, Manassas, VA, USA). Costar 3524 (Corning) 24-well plates were used.

**Instrumentation.** NMR spectra were recorded on either a Varian Mercury 400 MHz or 500 MHz spectrometer. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility by utilizing either an LCQDECA (Finnigan) ESI with a quadrupole ion trap or an MAT900XL (ThermoFinnigan) FAB double-focusing mass spectrometer. Reversed-phase HPLC (Vydac C18 column) purification and analysis were carried out on an Agilent 1200 series instrument. Flow-cytometry studies were performed on a BD FACSCalibur, with excitation at 635 nm and emission monitored at 670 nm.

**Synthesis.** Detailed synthetic procedures and characterization data are described in the Supporting Information (See Appendix 1).

**Quantifying cellular uptake.** The guanidinoglycoside derivatives were dissolved in HBSS and treated with fluorescently labeled streptavidin (ST–PE-Cy5) in a 10:1 molar ratio. After 15 min, the unbound biotinylated-guanidinoglycoside conjugates were removed by using a desalting spin column (Amicon Ultra-4 Centrifugal Filter with a 10 KDa threshold; Millipore) to
leave only the guanidinoglycoside–streptavidin conjugate in the column. The purified conjugates were diluted into medium to form 10, 25, 50, 100, 150, and 200 nm solutions. The medium used in these experiments was treated with 10% fetal bovine serum (FBS).

150000 cells were counted by using a hemocytometer for each of the cell lines examined and transferred to 24-well plates, and incubated in medium (300 mL; with a 1% solution of penicillin/streptomycin and 10% FBS) overnight at 37°C. The cells were then washed with PBS and treated with a solution of the corresponding conjugate (150 mL; F-12 medium containing 10% FBS) and incubated for 2 h. Following this, the cells were washed twice with PBS to remove any remaining extracellular conjugates. The cells were then detached with trypsin/EDTA (50 mL), diluted with medium (50 mL) and FACS buffer (200 mL), and analyzed by flow cytometry. Cellular uptake was quantified by the mean fluorescence intensity; the crude data were interpreted by using FlowJo v8.8.6 wherein the median value was determined and later plotted and further analyzed by using GraphPad Prism v5.0a.

**Competition study.** WT CHO-K1 cells (50 000) were counted by using a hemocytometer, seeded onto 48-well plates, and incubated in medium (300 mL; 1% solution of penicillin/streptomycin and 10% FBS) overnight at 37°C. The cells were then washed with PBS and treated with a solution (75 mL medium with 10% FBS) of the various glycans. A solution of the guanidinoglycoside conjugates (1.5 nm, 75mL medium with 10% FBS) was added to each well. (Note, the conjugates were prepared and purified in the
same way as for the quantification study.) The cells were then incubated for 2 h, at which point they were washed twice with PBS and detached by using trypsin/EDTA (30 mL). The lifted cells were diluted with medium (30 mL) and FACS buffer (200 mL) and analyzed by flow cytometry.

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**References**


40. See Supporting Information for additional synthetic schemes and characterization data.


49. The pgsA mutants lack the xylosyltransferase that initiates the two major sulfated polysaccharides in CHO cells, heparan sulfate and chondroitin/dermatan sulfate. Thus, both classes of glycosaminoglycan are missing. The cells also make the sialylated oligosaccharides found on glycoproteins (Asn- and Ser-linked) and glycolipids, and a small amount of sulfated glycolipid, but the charge density of these relatively small glycans is quite different from that of the heavily sulfated, uronic acid containing glycosaminoglycans. Based on the flow-cytometry data shown in the paper, these other glycans do not, apparently, bind GNeo.
50. The pgsD mutants lack both N-acetylglucosaminyltransferase and glucuronosyltransferase, enzyme activates that are required for the polymerization of heparan sulfate chains.


52. See the Supporting Information for additional graphics.

53. Note that, in some cases, the symbol size is larger than the error for a given data point.


CHAPTER 3: Quantifying Cytosolic Delivery of Guanidinium-based Molecular Transporters

Abstract: Fluorescent conjugates of Guanidinoneomycin and D-Arg₉ were synthesized and studied to discern their ability to localize in the cytosol of live cells. Using a cell-based FRET assay, kinetic data was extrapolated and quantified. The data demonstrates that although these two transporters maintain similar overall cellular uptake, the rate at which they appear in the cytosol is quite distinct. Furthermore, we are able to quantify the transporter delivered specifically to the cytosol.
Introduction

Since the discovery that guanidinylation can lead to increased cellular uptake, numerous guanidinium based molecular transporters (GRTs) have been developed on an array of scaffolds.\textsuperscript{1-4} While initial attempts in the field were aimed at deciphering a single model of internalization and shared delivery properties, it is growing increasingly clear that each class of transporters maintains its own distinct delivery profile.\textsuperscript{5} Typically, studies revolve around overall cellular uptake as determined by flow cytometry, or intracellular delivery monitored by microscopy.\textsuperscript{6} However, it is difficult to visualize and characterize cytosolic information or kinetic data of uptake in live cells. Transporter localization and kinetic information is crucial for the growing effort to utilize these types of molecules as therapeutics, as most intracellular therapeutics require cytosolic delivery.\textsuperscript{7,8}

Literature data suggest that while GRTs can deliver cargo to the cytosol, though the specific mechanism in which this occurs is unknown,\textsuperscript{9} endosomal trapping is a prominent issue preventing these molecules from being useful for therapeutic drug delivery.\textsuperscript{8} It is unclear, however, how much and at what rate the cargo reaches its targeted destination.\textsuperscript{10,11} While current methods exist to evaluate this information, they suffer a lack of sensitivity or an inability to observe data in real time.\textsuperscript{12-15} In this report, we analyze and quantify the rate of cytosolic-delivery of these two transporters, using confocal microscopy and a cell-based FRET assay. Specifically, D-Arg\textsubscript{9} (r\textsubscript{9}) and
guanidinoneomycin (Gneo, Figure 3.1) are investigated for their ability to present into the cytosol.

![Figure 3.1. Structures of A) r_{9}, and B) guanidinoneomycin. \( R = \text{Cy5, FlAsH, or TAMRA.} \) Guanidine groups are highlighted in blue.](image)

**Results and Discussion**

Utilizing orthogonal dyes, confocal microscopy studies were performed using TAMRA-Gneo and Cy5-r_{9} (Figure 3.2) to determine their independent and combined distribution inside live cells. In agreement with previous reports, puncta formation is largely prevalent for both transporters. The guanidinoneomycin-TAMRA conjugate displays significantly more punctate localization than its r_{9}-Cy5 counterpart at similar concentrations. Interestingly, the transporters appear to co-localize. Based on these images, however, it is difficult to discern how much of the transporters are present in the cytosol.
Figure 3.2. Confocal microscopy images highlighting co-localization and internalization of guanidinoneomycin-TAMRA and Arg9-Cy5. A) DIC image, B) 0.75 µM transporters, C) DIC image, D) 2.0 µM transporters. Images are taken after 60 minutes.

To expand our ability to characterize intracellular events, a cell-based FRET assay described by Tsien and co-workers was used to determine and quantify the rate at which the transporters localize into the cytosol.16,17 The assay utilizes bi-arsenical fluorescein (FIAsH)-labeled conjugates that, only upon internalization and exposure to the cytosol, bind to the tetracysteine tag expressed on cyan-fluorescent protein (CFP-4Cys).18-21 FIAsH conjugates binding to CFP-4Cys results in the formation of a reportable FRET pair, where CFP acts as the FRET donor and FIAsH as the FRET acceptor.18 By
monitoring the rate at which the fluorescence changes over time, kinetic information can be extrapolated and quantified in live cells.\textsuperscript{22} The assay, however, cannot take into account which pathway the transporters use to gain entry to the cell and eventually present in the cytosol.

FIAsH-labeled conjugates of Gneo and r\textsubscript{9} were synthesized and exposed to CFP-4Cys-transfected HeLa cells.\textsuperscript{16,20,23} The initial rates, in pM/min, were obtained and plotted in Figure 3.\textsuperscript{3,23,24} The r\textsubscript{9} conjugate demonstrates a 2–4 fold improved rate of cytosolic delivery compared to Gneo. It is worth noting that at concentrations of 5\textmu{M} and above the r\textsubscript{9} conjugates were found to be toxic, whereas the Gneo conjugates were not.\textsuperscript{8,25} The cytosolic rate of r\textsubscript{9} uptake appears to retard and become constant, this is perhaps due to a maximum rate of endocytosis or transporter release. Gneo, however, continues to increase linearly in this range.
The data suggests that while both transporters internalize similarly, their rate of release into the cytosol is quite distinct. Indeed, it appears that Gneo suffers from endosomal trapping more than r₉. The tendency of Gneo to be trapped has been reported and is hypothesized to be linked to its selective affinity toward heparan sulfate glycosaminoglycans.⁶⁶,⁶⁷ Arginine-rich transporters, however, do not share this selectivity.⁶⁵

**Conclusion**

Overall, the data support the trend that various guanidinylated scaffolds have distinct, but not entirely unique, delivery profiles. While r₉ displays superior cytosolic delivery to guanidinoneomycin, it can alternatively be stated
that guanidinoneomycin is a superior vector for delivery to endosomal/lysosomal vesicles. This technique has already been utilized for therapeutic treatment of lysosomal storage diseases.27

Experimental Section

See Appendix 3 for supporting information containing characterization of compounds, schematics, additional figures, and experimental details.

Acknowledgement

Chapter 3 is currently being prepared for submission for publication. The dissertation author was the primary author. Co-authors include Sujata R. Emani, Dr Yitzhak Tor, and Dr Roger Y. Tsien.

References


22. FlaSh binds to the tetra–cystein label of CFP in a stochiometric fashion causing a decrease in the flourecence emission of CFP. This change can be monitored over time to give kinetic information.

23. See Appendix 2

24. Calibration was done using a series of known CPF concentrations.


CHAPTER 4: Synthesis of molecules to study RNA interactions

The ribosomal decoding site, also known as the A-site, ensures high fidelity in protein synthesis by appraising codon–anticodon matching.\textsuperscript{1–3} Numerous naturally occurring potent antibiotics, particularly the aminoglycosides family, have evolved to meddle with this precise monitoring and corrupt bacterial protein production.\textsuperscript{4–8} Specifically, the aminoglycosides bind a small loop within the 16S rRNA and interfere with the conformational flexibility of A1492 and A1493, two key adenine residues (Figure 4.1a).\textsuperscript{9–12} The aminoglycosides stabilize an RNA conformation similar to the one induced by the cognate acyl-tRNA–mRNA complex, causing the ribosome to lose its ability to distinguish between correct and incorrect anticodon–codon hybrids.\textsuperscript{13–18}

The A-site, the Achilles heel of the bacterial ribosome, has remained one of the most attractive targets for the discovery and development of new antibiotics.\textsuperscript{19–25} A number of tools have been developed to assess ligand binding to this unique RNA site.\textsuperscript{26,27} Fluorescent A-site constructs, which contain emissive and responsive nucleoside analogs, such as 2-aminopurine at positions 1492 or 1493, have shown great promise.\textsuperscript{28–31} Their fluorescence response is, however, antibiotic-dependent.\textsuperscript{28,32} To overcome this drawback and devise a robust analysis and discovery platform for A-site binders, we have envisioned an approach where detection of antibiotic binding is not
dependent on changes in the environment of a single fluorophore, but rather on the interaction between two chromophores acting as a Förster Resonance Energy Transfer (FRET) pair (Figure 4.1b). In this fashion, we hypothesized, one could follow direct binding of appropriately labeled antibiotics and their displacement by competing binders with “FRET accuracy” without relying on a fluorescent nucleobase as the sole sensing moiety (Figure 4.1b).

**Figure 4.1**: (a) The binding of aminoglycosides to the bacterial A-site impacts the placement and dynamics of the unpaired A1492 and 1493 residues. (b) By replacing one of the nucleosides in the A-site with an isosteric emissive nucleoside analog as a donor (D) and tagging the antibiotics with an appropriate acceptor (A), binding and displacement events can be accurately monitored using FRET.

To realize such a system, we have relied on two key features: (a) one of the native nucleobases in the A-site, proximal to the binding site, but not part of it, were replaced with an emissive isomorphic nucleobase analog acting as a FRET donor, and (b) aminoglycosides, the cognate binders of the A-site, had to be labeled with an appropriate FRET acceptor in positions that are not essential for RNA binding (Figure 4.1b). By monitoring the interactions of
ligands and their RNA targets based on distance and location, convoluting factors such as modes of binding can be eliminated when studying binding and displacement. Herein, the syntheses of fluorescently-labeled aminoglycosides are described and their relation to the overall success of the binding assay.\textsuperscript{34}

**Results and Discussion**

The isomorphic fluorescent nucleobase 1, and its corresponding phosphoramidite, were synthesized for their incorporation into the A-site oligomer 2 (Figure 4.2).\textsuperscript{34} To complement the incorporated nucleobase as a FRET donor, an appropriate FRET acceptor had to be identified and conjugated onto aminoglycoside binders. The coumarin family of fluorophores was identified to be an appropriate match as they are commercially available and contain varied photophysical properties. Specifically, 7-(diethylamin)coumarin-3-carboxylic acid was utilized. Figure 4.3 highlights the viability of the FRET pair, demonstrating the high level of spectral overlap, wherein the emission of 2 overlaps with the excitation of the coumarin acceptor.
Figure 4.2. Structure of the modified fluorescent nucleoside and the modified A-Site construct utilized in the FRET-assay (2: $\lambda_{\text{ex}}$ 320 nm and $\lambda_{\text{em}}$ 395 nm).

Figure 4.3: Normalized absorption (---) and emission (—) spectra of 2 (black) and 5 (red) in water.

**Synthesis of coumarin-labeled aminoglycosides.**

7-(diethylamin)coumarin-3-carboxylic acid was identified to be a sufficient FRET acceptor and was conjugated to neomycin through amide bond formation at the 5’ primary hydroxymethyl group of the ribose. Previous studies have determined that this position is not required for binding to the A-
site.\textsuperscript{35} Using published procedures, neomycin was Boc-protected and the 5’ primary hydroxymethyl was activated by 2,4,6-triisopropylbenzensulfonyl chloride (TIBS-Cl).\textsuperscript{36} Treatment with methanolic ammonia provided the aminomethyl product (3). Using standard peptide coupling conditions, the newly installed amine was coupled to the benzoic carboxylic acid moiety of the coumarin. The resulting Boc-protected (4, Scheme 4.1), coumarin-labeled neomycin compound was treated with trifluoroacetic acid (TFA) to remove the protecting groups yielding 5 ($\lambda_{\text{ex}}$ 400 nm and $\lambda_{\text{em}}$ 473 nm). The incorporated coumarin label sufficiently maintained the desired photophysical properties and was able to serve as a viable FRET acceptor for the binding assay (Figure 4.3).

\begin{center}
\begin{tikzpicture}
\node (1) at (0,0) {3};
\node (2) at (2,0) {4};
\node (3) at (4,0) {5};
\draw (1) -- (2) -- (3);
\end{tikzpicture}
\end{center}

\textbf{Scheme 4.1.} Synthesis of neomycin-coumarin. (a) 7-(diethylamino)coumarin-3-carboxylic acid, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide, 4-dimethylaminopyridine, iPr$_2$EtN, Cl$_2$CH$_2$, 84%. (b) Trifluoroacetic acid, triisopropylsilane, Cl$_2$CH$_2$, 82%.

To add versatility to the assay, a tobramycin-coumarin 6 was synthesized using similar procedures (Figure 4.4, Scheme A4.1). As
tobramycin maintains a lower binding affinity to the A-site than neomycin, this compound may be more readily displaced by weaker A-site binders.

**Figure 4.4.** Coumarin-labeled tobramycin.

**Expansion of FRET assay to discern between two distinct RNA targets.**

Having successfully established the initial assay, we sought out a straightforward approach to expand the FRET-concept to determine the selectivity traits between two discrete RNA targets, the human and bacterial A-sites\(^{37}\) as a proof of this concept (Figure 4.5). Here, we disclose the design and implementation of a FRET-based, three-component assembly that facilitates a rapid determination of the relative affinity of any given binder to the eukaryotic versus prokaryotic decoding sites in a single experiment.\(^{38}\)
To accomplish this task, we have relied on the following components: 1) A “placeholder” aminoglycoside with modest affinity to both the prokaryotic and eukaryotic A-sites, labeled with a small non-perturbing fluorophore (marked F2) at a position that is not essential for rRNA binding; 2) A bacterial 16S A-site RNA construct modified with an isomorphic, emissive nucleoside analog (labeled F1) at a position proximal to the binding site, but not part of it; and 3) A human 18S A-site rRNA construct labeled at its terminus with a third fluorophore (designated F3). To generate unique spectral signatures for each binding event, the following photophysical conditions had to be met: 1) The isomorphic fluorescent probe on the bacterial A-site construct (F1) had to exclusively serve as a FRET donor to the fluorophore placed on the aminoglycoside antibiotic (F2); and 2) The latter, in turn, had to specifically serve as a FRET donor for the terminal fluorophore on the human A-site (F3). The spectroscopic properties of the system are described in Figure 4.6.
Figure 4.6: Structures of F1 (blue), F2 (black), and F3 (red) along with their normalized absorption (---) and emission spectra (—) in water.

The experiment is conceptually illustrated in Figure 4.7. When all three components, the fluorescently tagged “placeholder” and the two RNA constructs, are equilibrated together, the presence of the antibiotic on the 16S RNA can be visualized by selectively exciting F1, and monitoring the emission of F2. The fraction of the ligand bound to the 18S A-site can be visualized by selectively exciting F2 and detecting the emission of the acceptor F3. More importantly, when an unlabeled small-molecule is added to the mixture that is capable of displacing the tagged antibiotic from the 16S A-site, the acceptor emission F2 is lost, while the fluorescence of the donor nucleoside F1 is recovered. Accordingly, when the “placeholder” is displaced from the 18S A-site, the emission of F2 is recovered, and the sensitized emission of F3 is lost.
Based on the relative changes in these spectral signatures, the affinity and selectivity of any candidate antibiotic can be determined in a single cuvette.

**Figure 4.7**: Secondary structures for the 27-base RNA models of the 16S and 18S A-sites. U1406 of the 16S A-site is replaced with an isosteric emissive nucleoside analogue as a donor (F1); the place-holding molecule is tagged with an appropriate fluorophore (F2); the 18S A-site is tagged with an acceptor (F3) to match the labeled “place-holder” (F2). The affinity and selectivity of unlabeled small-molecules for either A-sites can be accurately monitored using FRET, as the place-holder is displaced.

The previously synthesized coumarin-labeled aminoglycosides were found to be poor “placeholders” as both 5 and 6 have high affinity towards both RNA targets. Kanamycin A, having only four amino groups, was identified as a viable “placeholder” with relatively low A-site affinity and proportional specificity between the two targets. Accordingly, 7 were synthesized to incorporate the necessary coumarin-label (7). In conjunction with this, a negative control (8) bearing no affinity to the A-site constructs, was synthesized to investigate the validity and specificity of the system (Figure 4.8).
Conclusion

In order to facilitate the realization of two FRET-based RNA binding assays, several coumarin-labeled molecules were synthesized. The generation of 5 and 6 provided the tools for a proof of concept by enabling enhanced detection of unlabeled compounds interacting with the Ribosomal decoding site (A-site). To expand concept of the assay, 7 and 8 were synthesized and enabled the implementation of an assay that can distinguish relative selectivity of unlabeled compounds between two discrete RNA targets.

Experimental Section

Materials. Unless otherwise specified, materials obtained from commercial suppliers were used without further purification. Neomycin, paromomycin, and tobramycin were purchased as their sulfate salts from Sigma–Aldrich and were converted into the corresponding neutral form by being passed through DOWEX MONOSPHERE 550 Å (OH) anion-exchange resin. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories.
**Instrumentation.** NMR spectra were recorded on either a Varian Mercury 400 MHz or 500 MHz spectrometer. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility by utilizing either an LCQDECA (Finnigan) ESI with a quadrupole ion trap or an MAT900XL (ThermoFinnigan) FAB double-focusing mass spectrometer. Reversed-phase HPLC (Vydac C18 column) purification and analysis were carried out on an Agilent 1200 series instrument.

**Synthesis**

**Boc6-protected coumarin-labeled-neomycin** (4). Anhydrous dichloromethane (300 μL), and 7-(diethylamino)coumarin-3-carboxylic acid (6.8 mg, 0.0263 mmol) were added to 3 (26.58 mg, 0.0219 mmol). To this, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (5.03 mg, 0.0262 mmol), N,N-diisopropylethylamine (8.62 μL, 0.048 mmol), and 4-(dimethylamino)pyridine (5.8 mg, 0.026 mmol) were added. The reaction was stirred for 18 h. The solvent was removed under reduced pressure and the resulting solid was dissolved in ethyl acetate and washed with water and brine. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (3% methanol in dichloromethane). Product: yellow powder (26.8 mg, 0.0184 mmol, 84% yield). 1H-NMR (400 MHz, CD3OD): 8.66 (s, 1H), 7.45 (d, J= 10.5 Hz, 1H), 6.81 (d, J = 9 Hz, 1H), 6.56 (s, 1H), 5.34 (s, 1H), 5.12 (s, 1H), 5.02 (s,
1H), 4.28 (s, 3H), 4.09 – 4.06 (m, 1H), 4.01 – 3.98 (m, 1H), 3.90 – 3.87 (m, 4H), 3.82 – 3.79 (m, 2H), 3.76 – 3.71 (m, 4H), 3.61 – 3.44 (m, 24H), 3.34 – 3.15 (m, 2H), 2.63 – 2.57 (m, 1H), 1.99 – 1.89 (2H), 1.46 – 1.38 (m, 54H), 1.37 (t, \( J = 5 \) Hz, 6H); 13C-NMR (100 MHz, CD3CN): 164.66, 163.53, 158.72, 158.21, 157.86, 157.62, 157.29, 156.55, 154.02, 149.22, 132.40, 111.34, 110.52, 109.87, 109.06, 101.22, 99.69, 97.06, 80.24, 79.90, 79, 81, 79.64, 79.56, 74.99, 73.82, 70.88, 68.43, 56.72, 53.13, 51.65, 45.70, 45.28, 42.54, 41.44, 28.69, 12.67, 12.21; ESI-MS calculated for C67H108N8O27 [M+Na]^+ 1479.72, found 1479.71.

**Coumarin-labeled-neomycin (5).** Anhydrous dichloromethane (2 mL) and triisopropylsilane (200 μL) were added to 4 (26.8 mg, 0.0184 mmol). To this, trifluoroacetic acid was added (2 mL) and the reaction was stirred at RT for 15 min. The reaction was diluted with toluene (5 mL) and the solvent was removed under reduced pressure. The resulting solid was dissolved in water and washed with dichloromethane. The aqueous layer was dried concentrated under reduced pressure and further purified by reverse phase HPLC using a gradient of 10 – 30% acetonitrile (0.1% TFA) in water (0.1% TFA) over 30 min, and eluted at 14.78 min. Product: yellow powder (21.7 mg, 0.0151 mmol, 82 % yield). 1H-NMR (400 MHz, D2O): 8.65 (s, 1H), 7.62 (d, \( J = 9.2 \) Hz, 1H), 6.92 (d, \( J = 9.2 \) Hz, 1H), 6.67 (s, 1H), 5.96 (s, 1H), 5.36, (s, 1H), 5.29 (s, 1H), 4.49 (t, \( J = 5.5 \) Hz, 1H), 4.39 – 4.35 (m, 2H), 4.28 (t, \( J = 5 \) Hz, 1H), 4.21 (t, \( J = 3.5 \) Hz, 1H), 3.99 – 3.95 (m, 2H), 3.89 – 3.83 (m, 2H), 3.79 (s, 2H), 3.64 (t, \( J = 9.5 \) Hz, 1H).
Hz, 1H), 3.59 (s, 1H), 3.56 – 3.51 (q, J₁ = 6.5 Hz, J₂ = 7.0 Hz, 4H), 3.33 (d, J = 4 Hz, 4H), 3.18 – 3.12 (m, 1H), 2.42 – 2.33 (m, 1H), 1.83 – 1.71 (m, 1H), 1.21 (t, J = 7 Hz, 6H); 13C-NMR (100 MHz, D2O): 166.19, 164.44, 163.17, 162.99 (J₁ = 27.8 Hz, J₂ = 58.4 Hz), 157.78, 154.03, 149.05, 131.83, 116.25 (J = 231 Hz, J₂ = 465 Hz), 115.08, 111.53, 110.43, 108.14, 106.39, 95.97, 95.44, 94.46, 79.69, 76.50, 73.24, 69.96, 67.52, 67.27, 50.7416, 48.49, 44.99, 40.32, 39.83, 11.44; ESI-MS calculated for C37H60N8O15 [M+2H]²⁺ 429.21, [M+H]^+ 857.43, and [M+Na]^+ 879.41, found 429.35, 857.43, and 879.59.

**Coumarin-labeled-tobramycin (6).** Anhydrous dichloromethane (2 mL) and triisopropylsilane (200 μL) were added to the Boc5-protected coumarin-labeled-tobramycin (37.2 mg, 0.0307 mmol). To this, trifluoroacetic acid was added (2 mL) and the reaction was stirred for 15 min. The reaction was diluted with toluene (5 mL) and the solvent was removed under reduced pressure. The resulting solid was dissolved in water and washed with dichloromethane. The aqueous layer was dried concentrated under reduced pressure and further purified by reverse phase HPLC (10 – 25% acetonitrile, 0.1% TFA) in water (0.1% TFA) over 22 min) and eluted at 20.6 min. Product: yellow powder (31.5 mg, 0.0264 mmol, 86% yield). 1H-NMR (400 MHz, D2O): 8.65 (s, 1H), 7.63 (d, J = 9.2 Hz, 1H), 6.93 (d, J = 9.2 Hz, 1H), 6.71 (s, 1H), 5.60 (d, J = 3.2 Hz, 1H), 5.03 (d, J = 3.2 Hz, 1H), 4.06 (t, J = 9.2 Hz, 1H), 3.97 (dd, J = 3.6 Hz, 6.4, 1H), 3.88 – 3.79 (m, 3H), 3.71 – 3.43 (m, 10H), 3.33(d, J = 12.4 Hz, 1H), 3.24 – 3.20 (m, 1H), 3.16 – 3.13 (m, 1H), 2.71 (dd, J = 8.8 Hz, 13.2,
1H), 2.50 – 2.47 (m, 1H), 2.16 – 2.12 (m, 1H), 1.89 – 1.84 (m, 2H), 1.20 (t, J = 6.8 Hz, 6H); 13C-NMR (100 MHz, D2O): 166.25, 164.23, 163.31 (J1 = 2.9 Hz, J2 = 5.5 Hz), 157.78, 153.91, 149.31, 132.19, 116.63 (J1 = 230 Hz, J2 = 469 Hz), 111.56, 108.43, 107.15, 101.44, 96.10, 84.50, 71.59, 68.17, 65.19, 54.81, 49.87, 48.88, 45.32, 29.58, 27.83, 11.81; ESI-MS calculated for C33H53N7O10 [M+H]+ 710.37 and [M+Na]+ 732.35, found 710.38 and 732.56.

**Coumarin-labeled-kanamycin A (8).** Anhydrous dichloromethane (2 mL) and triisopropylsilane (200 μL) were added to Boc4-protected coumarin-labeled-kanamycin A (8.64 mg, 7.67 μmol). To this, trifluoroacetic acid was added (2 mL) and the reaction was stirred for 15 min. The reaction was diluted with toluene (5 mL) and the solvent was removed under reduced pressure. The resulting solid was dissolved in water and washed with dichloromethane. The aqueous layer was dried concentrated under reduced pressure and further purified by reverse phase HPLC, 15 – 26% acetonitrile (0.1% TFA) in water (0.1% TFA) over 16 min, and eluted at 13.2 min. Product: yellow powder (7.08 mg, 5.98 μmol, 72% yield). 1H-NMR (500 MHz, D2O): δ 8.67 (s, 1H), 7.65 (d, J = 9.3, 1H), 6.95 (dd, J = 2.4, 9.3, 1H), 6.72 (d, J = 2.0, 1H), 5.37 (d, J = 3.9, 1H), 5.10 (d, J = 3.5, 1H), 4.13-4.08 (m, 1H), 4.01 (dd, J = 3.9, 9.2, 1H), 3.91 (dd, J = 3, 14.4, 1H), 3.85 (t, J = 8.75, 1H), 3.79-3.48 (m, 22H), 3.36 (dd, J = 2.5, 13.2, 1H), 3.21 (q, J = 7.3, 14.9, 2H), 3.10 (dd, J = 6.6, 11.1, 1H), 2.94 (t, J = 9.8, 1H), 2.79 (dd, J = 9.3, 13.5, 1H), 2.50-2.45 (m, 1H), 1.88-1.80 (m, 1H), 1.29 (t, J = 7.3, 4H), 1.25 (t, J = 7.1, 6H); 13C-NMR (125 MHz, D2O): δ 166.22,
164.36, 163.68 (q, $J_1 = 42\ Hz$, $J_2 = 85.5$) 158.09, 153.21, 149.42, 132.70, 117.04 (q, $J_1 = 343.5\ Hz$, $J_2 = 694.5$), 112.85, 109.91, 101.49, 98.09, 96.55, 84.71, 77.20, 73.83, 72.24, 69.36, 68.72, 68.24, 55.39, 50.44, 49.04, 47.35, 46.62, 41.30, 40.82, 28.27, 12.27, 8.91; ESI-MS calculated for $C_{32}H_{50}N_6O_{13}$, [M+2H]$^{2+}$ 364.18, [M+H]$^+$ 727.35, and [M+Na]$^+$ 749.33, found 364.22, 727.14, and 749.29.

**Coumarin-labeled TRIS (8).** TRIS HLC (7.4 mg, 46.8 μM) and 7-(diethylamino)coumarin-3-carboxylic acid (10.2 mg, 39 μM) were dissolved into $N,N$-dimethylformamide (400 μL). $N,N$-diisopropylethylamine (15 μL, 85.9 μM) and 4-(dimethylamino)pyridine (0.57 mg, 46.8 μM) were added and the reaction was stirred at RT for 18 hr. The solvent was removed under reduced pressure and the crude solid was dissolved in water and purified by reverse phase HPLC, 20-39% acetonitrile (0.1% TFA) in water (0.1% TFA) over 19 min, and eluted at 17.58 min. Product: yellow powder (11.4 mg, 31.3 μMol, 67% yield). $^1$H-NMR (400 MHz, D$_2$O): $\delta$ 8.43 (s, 1H), 7.43 (d, $J = 8.8\ Hz$, 1H), 6.77 (d, $J = 8.8\ Hz$, 1H), 6.48 (s, 1H), 3.86 (s, 4H), 3.46 (m, 2H), 3.19 (q, $J = 7.6$, 14.6, 4H) 1.27 (t, $J = 7.2$, 6H), 1.19 (t, $J = 6.8$, 4H); ESI-MS calculated for $C_{18}H_{24}N_2O_6$ [M+H]$^+$ 365.17, and [M+Na]$^+$ 387.15, found 364.94 and 387.00.
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34. Note, compound 1 and construct 2 were synthesized by Yun Xie, who also performed all spectroscopy and titration studies. The dissertation author is responsible for the contributory synthesis of the labeled coumarin compounds highlighted in the chapter, as well as the amino-aminoglycosides examined in the published manuscript. For additional discussion on the nucleobase, and detail regarding experiments and synthesis, please see published manuscript: Xie, Y.; Dix, A. V., Tor, Y. *J Am. Chem. Soc.* **2009**, *131*, 48, 17605–17614.

35. A) According to crystal structures PDB 2ET4 and 1LC4, the positions of modification on the aminoglycosides are not involved in binding to the RNA. B) Wang, H.; Tor, Y. *J. Am. Chem. Soc.* **1997**, *119*, 8734-8735.


38. For additional detail on the experiments and synthesis, please see published manuscript: Xie, Y.; Dix, A. V., Tor, Y. *Chem Commun (Camb)*. **2010**, *46*, 5542–5544.
CHAPTER 5: Perspectives

The desire to achieve or improve upon cellular uptake did not originate with the discovery of the translocation ability of HIV TAT. The drug industry has been using cellular uptake/permeability as a parameter to evaluate potential targets for decades. It is estimated that 90% of investigated drug targets fail, and roughly 30% of those compounds suffer from poor aqueous solubility and/or insufficient delivery properties.\(^1\) Examples include taxol, doxorubicin, vancomycin, and siRNA.

Many approaches have been taken to compensate for poor cellular uptake. Liposomes and lipoplexes mimic the hydrophobic nature of the cellular membrane while encapsulating the desired cargo.\(^2,3\) These techniques have shown great success in the laboratory, but they suffer from difficulty in formulation and toxicity.\(^4,5\) Viral delivery, or virosomes, are similar, using hollowed virus particles to deliver cargo.\(^6\) These suffer from hepatotoxicity and poor efficiency.\(^4,5\) Molecular transporters, in which a carrier is conjugated to the drug cargo, have shown great promise.\(^7\) Limited formulation and toxicity concerns set this technique apart from others. Several drugs utilizing this method are currently in clinical trials. Research in the Tor laboratory focuses on a specific class of molecular transporters known as guanidinoglycosides.
Overview of Guanidinoglycosides through 2007

Guanidinoglycosides were initially developed as RNA binders, however they quickly found a significant role as molecular transporters. Like other highly guanidinylated molecules, they were significantly more cell permeable than their parent amino molecules. Additionally, they were able to act as molecular transporters, and ferry otherwise non-permeable molecules into cells. The complete dependence on heparan sulfate to enter the cell at low concentrations sets guanidinoglycoside molecular transporters apart from other similar constructs. Other transporters have been shown to utilize heparan sulfate and similar glycans but not exclusively, and are seemingly able to utilize additional pathways to maintain cellular uptake in the absence of such glycans.

Specific Research Aims

1. Heparan sulfate is inherently multivalent, having multiple chains present on a given core protein, and consisting of 40-100 saccharide units. The concept of multivalency facilitating increased avidity towards its target is a well known biological phenomenon. Can it be used in conjunction with guanidinoglycosides to increase their affinity for heparan sulfate, thereby increasing cellular uptake?
2. The formation of bidentate hydrogen bonds between the guanidinium moiety of guanidinoglycosides and the sulfates of heparan sulfate are likely key for recognition.\textsuperscript{1,13,16} Are all of the sulfate and carboxylate functional groups existing across heparan sulfate equally significant to guanidinoglycoside recognition and uptake? Essentially, do hydrogen-bonding and electrostatic interactions exclusively govern this interaction?

3. Endosomal trapping is a prominent issue in the field of guanidinium-rich molecular transporters.\textsuperscript{17} Guanidinoglycosides are not immune to this.\textsuperscript{18} To move forward in exploring the internalization potential of these transporters, more information is needed to quantify the amount of transporter that escapes from endosomal vesicles into the cytosol. This information is critical in directing the therapeutic potential of guanidinoglycosides, as desirable therapeutics such as siRNA require cytosolic delivery.\textsuperscript{7}

**Addressing Specific Aims in Collaboration with the Esko and Tsien Laboratories**

1. The effect of transporter valency was addressed by using a dimeric guanidinoglycoside conjugate and comparing cellular uptake to its monomeric counterpart.\textsuperscript{19} Though results in wild-type cells indicated an enhanced uptake proportional to stoichiometry, a pronounced 5–10 fold
cooperative effect was observed in the undersulfated mutant cell lines. The degree of cooperativity was consistent across each transporter scaffold examined, and was shown to be independent of total guanidinylation levels.

2. The effects of glycan sulfation levels on cellular uptake were evaluated in two distinct but complementary fashions. Mutant cell lines, expressing characteristic biosynthetically altered heparan sulfate, were utilized and studied using the streptavidin-based guanidinoglycosides. The monomeric constructs performed poorly in cells containing undersulfated heparan sulfate, indicating a sensitive balance between sulfation and the ability to facilitate cellular uptake. Conversely, the dimeric constructs were able overcome this barrier and maintain high levels of uptake, suggesting that the additional valency was able to facilitate increased avidity.

In the second study, model glycans were used as inhibitors of the cellular uptake of guanidinoneomycin. The model glycans were generated from modified heparin, a more homogeneous form of heparan sulfate, each with characteristic modifications. Loss of anionic functionality, as a whole, decreased affinity towards the guanidinoglycosides, as they were unable to inhibit cellular uptake. However, the study indicated that sulfation at the 2-O position on the glucosamine residue of heparan sulfate was less critical for recognition.
3. The ability of molecular transporters to present in the cytosol was addressed using a recently developed intracellular FRET assay.\textsuperscript{20,21} Here, it was demonstrated that guanidinoneomycin enters the cytosol at roughly half the rate of arginine ($r_9$).

**Concerns**

1. Unfortunately, the obtained data are not without limitation. Several of the experimental observations were handicapped by systematic constraints. The primary concern in dealing with the evaluation of the streptavidin-based guanidinoglycoside transporters was in the valency of the core itself. Is the generation of the streptavidin-constructs, a tetrameric “monomer” and octomeric “dimer,” necessary for the observed cooperativity? Additionally, can a further increase in valency, utilizing “trimers” for example, improve the transporters?

2. The lack of necessity of 2-O sulfation on heparan sulfate to facilitate cellular uptake of guanidinoglycosides indicates that there is much to learn about the structural requirements for recognition/cellular uptake.

3. The study into cytosolic delivery, however, was less straightforward. Foremost, the intracellular FRET experiments were based on a single guanidinoglycoside, where most of the current work being done utilized
a streptavidin core that generates a tetrameric system. The two molecular constructs behave differently. However, the two concepts were not effectively joined experimentally. More so, we were not able to conceive a system that generated a single FlAsH labeling of streptavidin. Additionally, while the assay utilized is indeed powerful, it lacks the ability to discern how the molecules enter into the cytosol. The release pathway of guanidinium-rich molecular transporters is still being investigated by the community and has yet to be elucidated. Of further concern is normalizing the data. Flow cytometry of analogous Cy5-labeled molecules demonstrated that $r_9$ internalizes at a higher level than guanidinoneomycin. Essentially, with more $r_9$ present inside the cell, does this affect the rate at which the transporter presents to the cytosol?

**Ongoing Questions**

While several accomplishments have been made, this work can be further explored in future experiments. With respect to the multivalency of the transporter, ongoing work is aimed to discover a threshold for this potential using controlled polymerization. Also, the structural requirements for heparan sulfate-guanidinoglycoside interactions are still unclear. Several other aspects may be addressed as well.

1. Why are guanidinoglycosides so unique in their specificity toward heparan sulfate? Studies have shown that demertan/chondroitin sulfate
is unable to facilitate cellular uptake of guanidinoglycosides.\textsuperscript{19,22}

Structurally, the main difference between heparan sulfate and these other GAGs is the saccharide linkages, as their overall sulfation is similar.\textsuperscript{14} Does the difference linkages, having alternating $\alpha/\beta$ linkages versus pure $\beta$, enable flexibility of the glycan increasing interactions or assist in displaying the proper orientation of functionality?

2. Clathrin-mediated endocytosis has been suggested to be the primary mode of cell entry for guanidinoglycosides.\textsuperscript{22} Data suggest that endosomal trapping can be overcome, but the extent of this is still unknown. How these molecules escape endosomal vesicles is of interest as well. What effect does guanidinoglycoside valency have on this process?

**Outlook**

The study into guanidinoglycosides is far from over, and numerous questions still remain. Work is currently underway to investigate the upper capacity of transporter valency, as well as identifying the structural motifs of heparan sulfate necessary for promoting guanidinoglycoside binding/cellular uptake. One of the greatest avenues for growth of this project is in optimizing these two significant aspects of cellular uptake. Of equal prominence is the transition into mouse models to determine the therapeutic potential of this delivery system.
References


Appendix 1: Supporting Information for Chapter 2

Supporting Information

A1.1 Synthesis and characterization of bifunctional linkers

- Scheme A1.1 Synthesis of monomeric linker
- Scheme A1.2 Synthesis of dimeric linker

A1.2 Synthesis and characterization of guanidinoneomycin conjugates

- Scheme A1.3 Synthesis of 1 and 2

A1.3 Synthesis and characterization of guanidinoparomomycin conjugates

- Scheme A1.4 Synthesis of 3 and 4

A1.4 Synthesis and characterization of guanidinotobramycin conjugates

- Scheme A1.5 Synthesis of 5 and 6

A1.5 Schematic of Guanidinoneomycin-biotin-streptavidin-PE Cy5

- Figure A1.1 Streptavidin-PE Cy5
- Figure A1.2 The “monomeric” guanidinoneomycin-biotin-streptavidin-phycoerythrin Cy5 complex
- Figure A1.3 The “monomeric” guanidinoneomycin-biotin-streptavidin-phycoerythrin Cy5 complex

A1. Additional graphs for cellular uptake quantification

- Figure A1.4 Normalized cellular uptake at 200 nM by scaffold
- Figure A1.5 Normalized cellular uptake at 200 nM by cell line

A1.7 Example of crude histograms for competitive uptake experiment

- Figure A1.6 Example of crude histogram from the competitive uptake experiment involving monomeric guanidinoneomycin 1 and heparin.
Figure A1.7 Example of crude histogram from the competitive uptake experiment involving dimeric guanidinoneomycin 2 and heparin.

A1.8 References
A1.1 – Synthesis and characterization of bifunctional linkers (7, 8)

Scheme A1.1. Synthesis of monomeric linker. Synthesis of the Biotin-NHS ester and the sub-stoichiometric Staudinger reduction were preformed according to literature procedures.\[A1-3\]

Compound 14. Bis[2-(2-chloroethoxy)ethyl] ether (0.711 g, 3.08 mmol) was diluted into N,N-dimethylformamide (12 mL) and treated with an excess of sodium azide (1.01 g, 15.4 mmol) at 60°C for 4 h. The solvent was removed under reduced pressure and the resulting mixture was diluted into ether and filtered. The filtrate was again concentrated under reduced pressure, diluted into ether, and filtered. The final filtrate was concentrated under reduced pressure and used without further purification (0.645 g, 2.68 mmol, 87% yield). \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta\) 3.69–3.61 (m, 12H), 3.367 (t, \(J = 6.4\) Hz, 4H); ESI-MS calculated for C\(_8\)H\(_{16}\)N\(_6\)O\(_3\) [M+H]\(^+\) 245.14, [M+NH\(_4\)]\(^+\) 262.16, [M+Na]\(^+\) 267.12, [M+K]\(^+\) 283.09, found 244.84, 261.94, 266.97, 282.92, respectively.

Compound 15. 14 (0.381 g, 1.56 mmol) and triphenylphosphine (0.328 g, 1.25 mmol) were treated with diethyl ether (1.5 mL). To this, 1N HCl (1.5 mL) was added and the reaction was stirred vigorously for 12 h at RT. The reaction was diluted with ethyl ether (10 mL) and washed with 1N HCl. The
combined aqueous layers were then washed with ether and turned basic using NaOH pellets. The basic aqueous layer was washed with ether. The combined organic phases were concentrated under reduced pressure to yield the product as an oil (0.246 mg, 1.13 mmol, 72% yield). ¹H-NMR (CD₃CN, 400MHz): δ 3.66–3.56 (m, 12H), 3.51 (t, J = 5.2 Hz, 2H), 3.39 (t, J = 5.2 Hz, 2H), 2.83(s, 2H); ESI-MS calculated for C₈H₁₈N₄O₃ [M+H]+ 219.15, found 219.09.

**Compound 7.** N,N-dimethylformamide (400 μL), Biotin NHS (72 mg, 0.211 mmol), and N,N-diisopropylethylamine (33 mg, 0.25 mmol) were added to 15 (42 mg, 0.19 mmol) for 12 h at RT. The reaction was concentrated under reduced pressure and purified on silica gel using flash chromatography (2–5% methanol in CH₂Cl₂) to afford the product as an oil (84 mg, 0.189 mmol, 98% yield). ¹H-NMR (CDCl₃, 400 MHz): δ 6.71 (s, 1H), 6.42 (s, 1H), 4.50 (t, J = 3.6 Hz, 1H), 4.32 (t, J = 3.2 Hz, 1H), 3.68–3.61 (m, 12H), 3.56 (t, J = 5.2 Hz, 2H), 3.43 (t, J = 5.2 Hz, 2H), 3.40 (t, J = 5.2 Hz, 2H), 3.14 (q, J = 4.4 Hz, 1H), 2.90 (dd, J₁ = 5.2Hz, J₂ = 12.8 Hz, 1H), 2.23 (t, J = 7.2 Hz, 2H), 1.923–1.842 (m, 2H), 1.72–1.65 (m, 2H), 1.48–1.42 (m, 2H); ¹³C-NMR (DMSO, 100 MHz): δ 173.71, 164.03, 70.48, 70.45, 70.38, 70.25, 69.94, 69.85, 61.71, 59.87, 56.11, 50.67, 39.12, 35.78, 28.88, 28.72, 25.95; ESI-MS calculated for C₁₈H₃₂N₆O₅S [M+H]^+ 445.22, and [M+Na]^+ 467.21, found 445.18, and 467.21, respectively.
Compound 19. 18 (0.236 g, 0.466 mmol) was diluted into at 1:1 mixture of water and $N,N$-dimethylformamide (2 mL) and treated with sodium azide (0.242 g, 3.73 mmol) at 60°C for 4 h. The solvent was removed under reduced pressure and the resulting mixture was diluted into ether and filtered. The filtrate was again concentrated under reduced pressure, diluted into ether, and filtered. The final filtrate was concentrated under reduced pressure and used without further purification (0.194 g, 0.374 mmol, 80% yield). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 3.65–3.54 (m, 24H), 3.43–3.40 (m, 4H), 3.367 (t, $J = 6.4$ Hz, 4H), 1.48 (s, 9H); ESI-MS calculated for C$_{21}$H$_{41}$N$_7$O$_8$ [M+H]$^+$ 519.30, [M+Na]$^+$ 542.29, found 519.18, and 542.54, respectively.

Compound 8. 19 (75 mg, 0.18 mmol) was diluted with CHCl$_3$ (6 mL) and treated with triisopropylsilane (200 µL), and trifluoroacetic acid (6 mL) for 15
min at RT. The reaction was diluted into toluene (30 mL) and concentrated under reduced pressure. The resulting oil was used without further purification, and was diluted with *N*,*N*-dimethylformamide (200 μL) and *N*,*N*-diisopropylethylamine (15 mg, 120 μmol) for 12 h at RT. The reaction was concentrated under reduced pressure and purified on silica gel using flash chromatography (2–5% methanol in CH₂Cl₂) to afford the product as an oil (51 mg, 0.079 mmol, 80% yield). ¹H-NMR (400 MHz, DMSO): δ 7.25 (s, 1H), 6.14 (d, *J* = 22.8 Hz, 1H), 5.61 (d, *J* = 31 Hz, 1H), 4.46 (d, *J* = 4.8 Hz, 1H), 4.29 (d, *J* = 4.8 Hz, 1H), 3.65–3.57 (m, 24H), 3.14 (t, *J* = 5.5 Hz, 1H), 2.88–2.85 (m, 1H), 2.74–2.71 (m, 1H), 2.28 (d, *J* = 6.4 Hz, 2H), 1.73–1.63 (m, 2H), 1.43–1.41 (m, 2H), 1.24–1.22 (m, 2H); ¹³C-NMR (CDCl₃ 100 MHz): δ 173.78, 164.03, 70.54, 70.24, 69.67, 69.49, 62.06, 60.38, 55.73, 50.89, 48.97, 46.39, 43.04, 40.75, 32.78, 29.89, 28.66, 28.53, 25.38; ESI-MS calculated for C₂₆H₄₇N₉O₈S [M+Na]⁺ 668.32, found 668.35.
A1.2 – Synthesis and characterization of guanidinoneomycin conjugates

Scheme A1.3 Synthesis of monomeric (1) and dimeric (2) guanidinoneomycin from neomycin. Precursors to compound 10 were prepared according to literature procedures.\(^{[32, 38]}\)

**Compound 11.** 10 (290 mg, 0.24 mmol) was dissolved in CH\(_2\)Cl\(_2\) (200 μL) and treated with hexynoic acid (40 mg, 0.35 mmol), \(N\)-(3-Dimethylaminopropyl)-\(N\')-ethylcarbodiimide hydrochloride (54 mg, 0.28 mmol), and \(N, N\)-diisopropylethylamine (67 mg, 0.52 mmol) for 12 h at RT. The reaction was diluted into ethyl acetate (15 mL) and washed with water (3×15 mL), brine (15 mL), and dried over sodium sulfate. The combined organic
layers were concentrated under reduced pressure and further purified on silica gel using flash chromatography (0–5% methanol in CH₂Cl₂) to afford the product as an off-white solid (260 mg, 0.20 mmol, 84% yield). ^1H-NMR (CD₃OD, 400 MHz): δ 8.20 (s, 1H), 5.49 (s, 1H), 5.11 (s, 1H), 4.33–4.31 (m, 1H), 4.10–4.07 (m, 1H), 3.98–3.89 (m, 4H), 3.79–3.69 (m, 3H), 3.60–3.15 (m, 16H), 2.87 (s, 1H), 2.79 (t, J = 8.0 Hz, 1H), 2.71 (s, 1H), 2.46 (t, J = 8.0 Hz, 2H), 2.35 (s, 2H), 2.27 (d, J = 5.5 Hz, 4H), 2.04 (s, 1H), 1.99–1.83 (m, 4H), 1.47 (s, 54H); ESI-MS calculated for C₅₉H₁₀₁N₇O₂₅ [M+H]^+ 1308.69, [M+Na]^+ 1330.67, found 1308.25, and 1330.52, respectively.

**Compound 12.** 11 (260 mg, 0.20 mmol) was dissolved in CH₂Cl₂ (4 mL), and treated with triisopropylsilane (200 μL) and trifluoroacetic acid (4 mL) for 15 min at RT. The reaction was diluted into toluene (30 mL) and concentrated under reduced pressure. The solid was then dissolved in water (10 mL) and washed with CH₂Cl₂ (3×15 mL). The aqueous phase was then reduced to a solid under reduced pressure. The crude product was dissolved in methanol (200 μL) and treated with a solution of N,N-di-tert-butoxycarbonyl-N''-triflylguanidine (2.70 g, 6.80 mmol) and triethylamine (220 mg, 2.2 mmol) in CH₂Cl₂ (2 mL) for 36 h at RT. The reaction was then diluted into CH₂Cl₂ (15 mL), washed with water (3×10 mL), brine (10 mL), and dried over sodium sulfate. The combined organic layer were concentrated under reduced pressure and further purified by flash chromatography (0–3% methanol in CH₂Cl₂) to afford the product as an off-white solid (290 mg, 0.14 mmol, 69%
yield over two steps). $^1$H-NMR (CD$_3$OD, 500 MHz): δ 7.65 (t, $J = 5.1$ Hz, 1H), 5.95 (d, $J = 4.4$ Hz, 1H), 5.09 (dd, $J_1 = 2.0$, $J_2 = 7.4$, 2H), 4.66–4.46 (m, 1H), 4.44 (s, 1H), 4.37 (dd, $J_1 = 1.5$ Hz, $J_2 = 4.9$ Hz, 1H), 4.32 (t, $J = 5.4$ Hz, 2H), 4.20 (m, 2H), 4.06–4.02 (m, 2H), 3.97–3.88 (m, 3H), 3.85–3.81 (m, 1H), 3.78–3.74 (m, 2H), 3.61–3.55 (m, 2H), 3.48–3.39 (m, 2H), 3.31 (t, $J = 9.3$ Hz, 1H), 2.58–2.43 (m, 3H), 2.34–2.29 (m, 4H), 1.91–1.84 (m, 2H), 1.70–1.49 (m, 108H), 1.35 (s, 2H); $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 175.50, 175.41, 164.64, 164.60, 164.34, 164.23, 164.17, 158.85, 158.00, 157.85, 157.52, 157.49, 154.73, 154.45, 154.24, 154.19, 153.27, 153.20, 151.33, 113.71, 99.19, 97.06, 89.19, 84.83, 84.75, 84.66, 84.63, 84.44, 84.08, 83.96, 80.72, 80.59, 80.54, 80.51, 80.32, 80.28, 77.02, 76.77, 75.39, 75.39, 74.28, 73.40, 73.31, 71.93, 71.12, 70.59, 68.15, 55.47, 53.01, 51.89, 50.19, 44.59, 44.43, 41.81, 36.24, 35.28, 30.73, 28.79, 28.73, 28.71, 28.67, 28.61, 28.58, 28.48, 28.43, 28.40, 28.24, 26.29, 23.70, 19.12; ESI-MS calculated for C$_{95}$H$_{161}$N$_{19}$O$_{37}$ [M+2H]$^{2+}$ 1081.08, [M+H+Na]$^{2+}$ 1092.07, [M+2Na]$^{2+}$ 1103.06, found 1081.19, 1092.10, and 1103.38, respectively.

**Compound 1.** 12 (25 mg, 12 μmol) and 7 (11 mg, 24 μmol) were dissolved in CH$_2$Cl$_2$ (800 μl) and treated with Cs$_2$CO$_3$ (400 μg, 1.2 μmol) and a 0.04 M solution of Cul in acetonitrile (40 μl) for 12 h at RT. The reaction was then diluted into ethyl acetate (10 mL) and washed with water (3×10 mL), brine (10 mL), and dried over sodium sulfate. The combined organic layers were concentrated under reduced pressure and used without further purification.
The crude product was dissolved in CH$_2$Cl$_2$ (2 mL) and treated with triisopropylsilane (20 μL, mmol) and trifluoroacetic acid (2 mL) for 1 h at RT. The reaction was diluted into toluene (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with CH$_2$Cl$_2$ (3×5 mL). The aqueous phase was reduced to a solid under reduced pressure and purified on a C-18 reverse phase HPLC column using a gradient of 5–20% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes (3mL/min). The compound eluted as a TFA salt at 16.6 min (13 mg, 11 μmol, 88% yield). $^1$H-NMR (D$_2$O, 500 MHz): δ 7.92 (s, 1H), 5.81 (d, $J$ = 3 Hz, 1H), 5.19 (d, $J$ = 2.4 Hz, 2H), 5.12 (d, $J$ = 2.4 Hz, 2H), 4.66(t, $J$ = 4.9 Hz, 3H), 4.48–4.45 (m, 1H), 4.42–4.40 (m, 1H), 4.21–4.19 (m, 2H), 4.07 (q, $J_1$ = 4.9 Hz, 1H), 4.02 (t, $J$ = 4.9 Hz, 2H), 3.86–3.81 (m, 3H), 3.78–3.76 (m, 1H), 3.73–3.64 (m, 12H), 3.61–3.51 (m, 4H), 3.43 (t, $J$ = 5.4 Hz, 2H), 3.39–3.34 (m, 1H), 3.25 (q, $J$ = 6.5 Hz, 3H), 3.03 (dd, $J_1$ = 5.2, $J_2$ = 13 Hz, 1H), 2.83 (s, 1H), 2.39 (t, $J$ = 7.5 Hz, 2H), 2.30 (t, $J$ = 7.3 Hz, 3H), 2.05–2.01 (m, 2H), 1.78–1.59 (m, 4H), 1.47–1.43 (m, 2H); $^{13}$C-NMR (D$_2$O, 125 MHz): δ 175.97, 175.37, 164.43, 162.06 ($J$ = 31.3 Hz), 156.79, 156.59, 156.44, 156.34, 156.24, 155.63, 146.52, 122.77, 115.58 ($J$ = 291 Hz), 97.51, 95.13, 84.15, 79.01, 77.85, 76.23, 73.64, 72.68, 71.77, 71.12, 70.05, 68.72, 68.57, 68.21, 67.96, 67.86, 65.80, 61.25, 59.38, 54.72, 54.51, 52.64, 51.07, 49.70, 49.05, 45.81, 40.85, 39.99, 38.78, 38.02, 34.59, 34.43, 31.17, 27.02, 26.86, 24.28, 23.27, 7.33; ESI-MS calculated for C$_{53}$H$_{99}$N$_{25}$O$_{18}$S [$M+2H$]$^2^+$ 703.0, [M+H]$^+$ 1403.72, [M+Na]$^+$ 1426.71, and found 703.31, 1404.81, 1426.79, respectively.
**Compound 2.** 12 (39 mg, 19 μmol) and 8 (5.4 mg, 8.4 μmol) were dissolved in CH$_2$Cl$_2$ (1.0 mL) and treated with Cs$_2$CO$_3$ (600 μg, 1.9 μmol), and a 0.04 M solution of CuI in acetonitrile (40 μl) for 12 h at RT. The reaction was then diluted into ethyl acetate (10 mL) and washed with water (3×10 mL), brine (10 mL), and dried over sodium sulfate. The combined organic layers were concentrated under reduced pressure. The crude product was dissolved in CH$_2$Cl$_2$ (2 mL) and treated with triisopropylsilane (20 μL) and trifluoroacetic acid (2 mL) for 1 h at RT. The reaction was diluted into toluene (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with CH$_2$Cl$_2$ (3×5 mL). The aqueous phase was then reduced to a solid under reduced pressure and purified on a C-18 reverse phase HPLC column using a gradient of 5–20% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes (3mL/min). The compound eluted at 18.7 min as a TFA salt (11 mg, 14 μmol, 76% yield). $^1$H-NMR (D$_2$O, 500 MHz, ): δ 7.79 (s, 2H), 5.68 (d, $J = 3$ Hz, 2H), 5.06 (d, $J = 2$ Hz, 2H), 4.99 (d, $J = 2$ Hz, 2H), 4.53 (t, $J = 4.6$ Hz, 6H), 4.32–4.32 (m, 1H), 4.30–4.29 (m, 1H), 4.25 (t, $J = 5.4$ Hz, 2H), 4.09–4.07 (m, 4H), 3.93 (q, $J = 5.4$ Hz, 2H), 3.89 (t, $J = 5.2$ Hz, 4H), 3.74–3.68 (m, 6H), 3.64–3.51 (m, 24H), 3.45–3.37 (m, 8H), 3.25–3.21 (m, 1H), 2.90 (dd, $J_1 = 4.9$ Hz, $J_2 = 13.2$ Hz, 1H), 2.67 (t, $J = 8.6$ Hz, 6H), 2.40 (t, $J = 7.6$ Hz, 2H), 2.26 (t, $J = 7.6$ Hz, 4H), 2.19–2.16 (m, 2H), 1.99 (s, 1H), 1.92–1.86 (m, 4H), 1.65–1.47 (m, 6H), 1.36–1.31 (m, 2H); $^{13}$C-NMR (D$_2$O, 125 MHz): δ 176.64, 176.15, 165.13, 162.86 ($J = 34.8$ Hz), 157.48, 157.26, 157.12, 157.01, 156.89, 156.30, 147.22, 123.46, 116.15 ($J = 289.8$ Hz),
110.28, 98.17, 95.85, 84.83, 79.53, 78.59, 76.93, 74.32, 73.29, 72.47, 71.83,
70.74, 69.90, 69.47, 69.39, 69.27, 69.23, 68.92, 68.63, 68.06, 67.66, 66.43,
61.93, 60.06, 55.39, 55.30, 51.75, 50.38, 49.70, 48.02, 44.92, 41.55, 41.49,
40.81, 39.58, 35.10, 32.23, 28.12, 27.32, 24.99, 24.73, 23.95; ESI-MS calculated for \( C_{96}H_{177}N_{47}O_{35}S \) \([M+3H]^3+\) 855.78, \([M+2H]^{2+}\) 1283.17, found 856.29 and 1283.62, respectively.
A1.3 – Synthesis and characterization of guanidinoparomomycin conjugates

Scheme A1.4. Synthesis of monomeric (3) and dimeric (4) guanidinoparomomycin from paromomycin. Precursors to compound 24 were prepared according to literature procedures.\textsuperscript{[41-43]}

**Compound 26.** 25 (65 mg, 0.054 mmol) was dissolved in CH$_2$Cl$_2$ (3 mL), and treated with triisopropylsilane (30 μL) and trifluoroacetic acid (3 mL) for 1 h at RT. The reaction was diluted into toluene (30 mL) and concentrated under reduced pressure. The solid was then dissolved in water (10 mL) and washed
with CH$_2$Cl$_2$ (3×15 mL). The aqueous phase was then reduced to a solid under reduced pressure. The crude product was dissolved in methanol (500 µL) and treated with a solution of N,N'-di-tertbutoxycarbonyl-N''-triflylguanidine (650 mg, 1.66 mmol) and triethylamine (160µL, 1.15 mmol) in CHCl$_3$ (2 mL) for 36 h at RT under argon. The reaction was then diluted into CH$_2$Cl$_2$ (15 mL), washed with water (3×10 mL), brine (10 mL), and dried over sodium sulfate. The combined organic layer were concentrated under reduced pressure and further purified by flash chromatography (2% methanol in CH$_2$Cl$_2$) to afford the product as an off–white solid (34 mg, 0.018 mmol, 39% yield). $^1$H-NMR (CD$_3$OD, 400 MHz): δ 5.64 (d, $J$ =3.8 Hz, 1H), 5.01 (br. s, 1H), 4.95 (d, $J$ =1.9 Hz, 1H), 4.27–4.33 (m, 1H), 4.28–4.30 (m, 1H), 4.13–4.17 (m, 2H), 4.10 (dd, $J$ =10.4, 3.8 Hz, 1H), 3.97–4.04 (m, 1H), 3.88–3.92 (m, 2H), 3.80–3.86 (m, 1H), 3.76–3.83 (m, 2H), 3.65–3.71 (m, 2H), 3.61–3.67 (m, 1H), 3.50–3.56 (m, 1H), 3.47–3.53 (m, 1H), 3.43–3.49 (m, 3H), 3.40–3.46 (m, 1H), 3.33–3.39 (m, 1H), 3.24–3.31 (m, 1H), 2.38 (t, $J$ = 7.7 Hz, 2H), 2.19–2.22 (m, 1H), 2.17–2.19 (m, 2H), 2.10–2.16 (m, 1H), 1.72–1.80 (m, 2H), 1.50–1.60 (m, 1H), 1.36–1.49 (m, 90H); $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 174.8, 162.7–163.3, 154.7–157.3, 110.5, 98.0, 96.0, 86.9, 82.8–83.4, 83.4, 79.9, 79.0–79.8, 79.0, 75.4, 74.7, 74.5, 72.9, 72.5, 72.4, 69.8, 69.6, 69.5, 66.5, 60.8, 54.2, 51.6, 50.4, 49.2, 42.8, 40.2, 34.8, 32.5, 27.2, 24.8, 17.6; ESI-MS calculated for C$_{84}$H$_{144}$N$_{16}$O$_{34}$ [M+2H]$^{2+}$ 960.50, found 960.41.
**Compound 3.** 26 (16 mg, 8.7 μmol) and 7 (30 mg, 16 μmol) were dissolved in CH₂Cl₂ (600 μl) and treated with Cs₂CO₃ (300 μg, 1 μmol) and a 0.04 M solution of Cul in acetonitrile (40 μl) for 12 h at RT. The solvents were removed under reduced pressure and the residue was redissolved in CH₂Cl₂ (2 mL) and treated with triisopropylsilane (20 μL, mmol) and trifluoroacetic acid (2 mL) for 1 h at RT. The reaction was diluted into toluene (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with CH₂Cl₂ (3×5 mL). The aqueous phase was reduced to a solid under reduced pressure and purified on a C-18 reverse phase HPLC column using a gradient of 10–50% acetonitrile (0.1% TFA) in water (0.1% TFA) over 30 minutes (3 mL/min). The compound eluted at 10.1 min (5 mg, 4 μmol, 56%). ¹H-NMR (D₂O, 500 MHz): δ 7.91 (s, 1H), 5.78 (d, J =2.6 Hz, 1H), 5.19 (d, J =1.6 Hz, 1H), 5.07 (d, J =0.9 Hz, 1H), 4.59–4.66 (m, 3H), 4.42 (dd, J =8.1, 4.5 Hz, 1H), 4.38 (dd, J =4.8, 1.8 Hz, 1H), 4.34 (dd, J =6.2, 5.1 Hz, 1H), 4.14–4.17 (m, 2H), 4.00–4.04 (m, 1H), 3.98 (t, J =5.0 Hz, 2H), 3.77–3.86 (m, 4H), 3.72–3.73 (m, 1H), 3.60–3.69 (m, 11H), 3.50–3.58 (m, 7H), 3.48 (dd, J =14.4, 4.6 Hz, 1H), 3.38–3.43 (m, 1H), 3.37–3.40 (m, 4H), 3.30–3.34 (m, 1H), 2.99 (dd, J =13.0, 4.9 Hz, 1H), 2.73–2.80 (m, 3H), 2.35 (t, J =7.2 Hz, 2H), 2.23–2.28 (m, 3H), 1.95–2.01 (m, 2H), 1.68–1.76 (m, 1H), 1.61–1.68 (m, 1H), 1.57–1.62 (m, 2H), 1.52–1.57 (m, 1H), 1.36–1.44 (m, 2H); ¹³C-NMR (D₂O, 125 MHz): δ 176.9, 176.3, 165.4, 156.4–157.5, 147.2, 124.1, 110.3, 98.0, 95.8, 85.1, 79.4, 78.7, 76.4, 74.5, 73.4, 72.9, 72.4, 72.3, 69.2, 68.9, 68.8–69.7, 66.8, 62.1, 60.5, 60.3, 55.8, 55.5, 53.6, 52.1, 50.6, 50.2, 41.9, 41.2, 39.8, 39.0, 35.5,
35.3, 32.1, 28.0, 27.7, 25.2, 25.1, 24.1; ESI-MS calculated for C\textsubscript{52}H\textsubscript{95}N\textsubscript{22}O\textsubscript{19}S \[M+H]\textsuperscript{+} 1363.69, found 1363.72.

**Compound 4.** 26 (20 mg, 10 μmol) and 8 (3.0 mg, 4.0 μmol) were dissolved in CH\textsubscript{2}Cl\textsubscript{2} (400 μL) and treated with Cs\textsubscript{2}CO\textsubscript{3} (300 μg, 1 μmol), and a 0.04 M solution of Cul in acetonitrile (50 μl) for 12 h at RT. The solvents were removed under reduced pressure and the residue was redissolved in CH\textsubscript{2}Cl\textsubscript{2} (1 mL) and treated with triisopropylsilane (10 μL) and trifluoroacetic acid (1 mL) for 1 h at RT. The reaction was diluted into toluene and (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with CH\textsubscript{2}Cl\textsubscript{2} (3×5 mL). The aqueous phase was then reduced to a solid under reduced pressure and purified on a C-18 reverse phase HPLC column using a gradient of 30–60% acetonitrile (0.1% TFA) in water (0.1% TFA) over 8 minutes (3 mL/min). The compound eluted at 4.5 min (17 mg, 6 μmol, 66% yield). \textsuperscript{1}H-NMR (D\textsubscript{2}O, 500 MHz): δ 7.83 (s, 2H), 5.74 (d, J =2.0 Hz, 2H), 5.15 (d, J =1.6 Hz, 2H), 5.03 (d, J =1.2 Hz, 2H), 4.57 (t, J =5.1 Hz, 4H), 4.52–4.58 (m, 1H), 4.36–4.41 (m, 1H), 4.34–4.36 (m, 2H), 4.29–4.34 (m, 2H), 4.11–4.13 (m, 4H), 3.95–4.00 (m, 2H), 3.93 (m, 2H), 3.73–3.83 (m, 8H), 3.68–3.69 (m, 2H), 3.50–3.65 (m, 30H), 3.48–3.52 (m, 14H), 3.43 (dd, J =14.3, 4.6 Hz, 2H), 3.36 (dd, J =14.0, 5.6 Hz, 2H), 3.25–3.31 (m, 1H), 2.94 (dd, J =13.1, 5.0 Hz, 1H), 2.70–2.76 (m, 5H), 2.43 (t, J =7.4 Hz, 2H), 2.30 (t, J =7.5 Hz, 4H), 2.18–2.22 (m, 2H), 1.90–1.97 (m, 4H), 1.65–1.73 (m, 1H), 1.61–1.69 (m, 2H), 1.53–1.61 (m, 2H), 1.471.56 (m, 1H),
1.31–1.43 (m, 2H); $^{13}$C-NMR (D$_2$O, 125 MHz): $\delta$ 176.7, 176.4, 164.4, 156.4–157.6, 147.3, 123.8, 110.1, 98.2, 95.9, 85.0, 79.5, 78.9, 76.2, 74.6, 73.5, 72.9, 72.7, 72.3, 69.2, 69.0, 68.2–70.4, 68.8, 66.7, 62.2, 60.4, 55.9, 55.3, 53.6, 52.2, 50.3, 50.1, 48.3, 45.4, 42.1, 41.3, 39.9, 35.4, 32.7, 32.1, 28.4, 28.0, 25.3, 24.9, 24.3; ESI-MS calculated for C$_{94}$H$_{174}$N$_{41}$O$_{36}$S $[M+3H]^{3+}$ 828.43, found 828.94.
A1.4 – Synthesis and characterization of guanidinotobramycin conjugates

Scheme A1.5. Synthesis of monomeric (5) and dimeric (6) guanidinotobramycin from tobramycin. Precursors to compound 28 were prepared according to literature procedures.[41-43]

**Compound 30.** 29 (210 mg, 0.20 mmol) was dissolved in CH$_2$Cl$_2$ (5 mL), and treated with triisopropylsilane (50 μL) and trifluoroacetic acid (5 mL) for 1 h at RT. The reaction was diluted into toluene (30 mL) and concentrated under reduced pressure. The solid was then dissolved in water (10 mL) and washed with CH$_2$Cl$_2$ (3×15 mL). The aqueous phase was then reduced to a solid
under reduced pressure. The crude product was dissolved in methanol (2 mL) and treated with a solution of $N,N'$-di-tert-butoxycarbonyl-$N''$-triflylguanidine (1.88 g, 4.81 mmol) and triethylamine (644 μL, 4.620 mmol) in CHCl$_3$ (8 mL) for 36 h at RT under argon. The reaction was then diluted into CH$_2$Cl$_2$ (15 mL), washed with water (3×10 mL), brine (10 mL), and dried over sodium sulfate. The combined organic layer were concentrated under reduced pressure and further purified by flash chromatography (2% methanol in CH$_2$Cl$_2$) to afford the product as an off-white solid (300 mg, 180 mmol, 92% yield). $^1$H-NMR (CD$_3$OD, 500 MHz): δ 5.76 (d, $J$ =3.2 Hz, 1H), 5.01 (d, $J$ =3.3 Hz, 1H), 5.53 (t, $J$ =12.9 Hz, 1H), 4.39 (t, $J$ =12.5 Hz, 1H), 4.24–4.29 (m, 1H), 4.14 (td, $J$ =10.6, 2.6 Hz), 4.04–4.09 (m, 1H), 3.92 (td, $J$ =10.3, 2.6 Hz), 3.83 (td, $J$ =9.2, 2.8 Hz), 3.60–3.67 (m, 2H), 3.57–3.60 (m, 1H), 3.41–3.49 (m, 4H), 3.29–3.33 (m, 1H), 3.24 (td, $J$ =9.6, 2.6 Hz, 1H), 2.27–2.34 (m, 2H), 3.22–3.26 (m, 3H), 3.19–3.24 (m, 1H), 2.06–2.11 (m, 1H), 1.75–1.82 (m, 1H), 1.65–1.75 (m, 1H), 1.58–1.62 (m, 1H), 1.40–1.60 (m, 90H); $^{13}$C-NMR (CD$_3$OD, 125 MHz): δ 174.4, 162.7–163.4, 152.8–158.3, 99.3, 95.0, 82.6–83.4, 83.4, 82.7, 78.9–79.2, 77.3, 76.1, 71.8, 70.8, 70.6, 69.0, 65.2, 56.3, 50.0, 48.8, 48.6, 42.3, 40.3, 34.6, 34.2, 32.0, 26.8–27.0, 24.6, 17.4); ESI-MS calculated for C$_{79}$H$_{135}$N$_{16}$O$_{29}$ [M + H]$^+$ 1771.96, found 1772.38.

**Compound 5.** 30 (28 mg, 16 μmol) and 7 (7 mg, 24 μmol) were dissolved in CH$_2$Cl$_2$ (600 μl) and treated with Cs$_2$CO$_3$ (300 μg, 1 μmol) and a 0.04 M solution of Cul in acetonitrile (40 μl) for 12 h at RT. The solvents were
removed under reduced pressure and the crude product was dissolved in \( \text{CH}_2\text{Cl}_2 \) (4 mL) and treated with triisopropylsilane (40 \( \mu \)L, mmol) and trifluoroacetic acid (4 mL) for 1 h at RT. The reaction was diluted into toluene and (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with \( \text{CH}_2\text{Cl}_2 \) (3×5 mL). The aqueous phase was reduced to a solid under reduced pressure and purified on a C-18 reverse phase HPLC column using a gradient of 20–40% acetonitrile (0.1% TFA) in water (0.1% TFA) over 15 minutes (3 mL/min). The compound eluted at 9.0 min (22 mg, 14 \( \mu \)mol, 88% yield). \(^1\)H-NMR (\( \text{D}_2\text{O}, 500 \text{ MHz} \)): \( \delta \) 7.88 (s, 1H), 5.49 (d, \( J =3.5 \) Hz, 1H), 5.13 (d, \( J =3.0 \) Hz, 1H), 4.60–4.63 (m, 3H), 4.42 (dd, \( J =8.0, 4.6 \) Hz, 1H), 4.09–4.13 (m, 1H), 3.98 (t, \( J =5.5 \) Hz, 2H), 3.75–3.80 (m, 1H), 3.74–3.70 (m, 1H), 3.68–3.73 (m, 1H), 3.68–3.75 (m, 2H), 3.37–3.54 (m, 2H), 3.36–3.88 (m, 8H), 3.30–3.34 (m, 1H), 2.99 (dd, \( J =13.0, 5.0 \) Hz, 1H), 2.73–2.82 (m, 1H), 2.70–2.78 (m, 2H), 2.33 (t, \( J =7.6 \) Hz, 2H), 2.27–2.32 (m, 1H), 2.20 (t, \( J =7.3 \) Hz, 2H), 2.20–2.27 (m, 1H), 1.97 (p, \( J =7.5 \) Hz, 2H), 1.71–1.82 (m, 1H), 1.68–1.76 (m, 1H), 1.67–1.75 (m, 1H), 1.58–1.69 (m, 2H), 1.51–1.60 (m, 1H), 1.35–1.45 (m, 2H); ESI-MS calculated for \( \text{C}_{47}\text{H}_{87}\text{N}_{22}\text{O}_{14}\text{S} \) [M + H]\(^+\) 1215.65, found 1215.78.

**Compound 6.** 30 (30 mg, 17 \( \mu \)mol) and 8 (4.4 mg, 6.8 \( \mu \)mol) were dissolved in \( \text{CH}_2\text{Cl}_2 \) (400 \( \mu \)L) and treated with \( \text{Cs}_2\text{CO}_3 \) (1.7 \( \mu \)mol), and a 0.04 M solution of Cul (0.1 eq) in acetonitrile (85 \( \mu \)L) for 12 h at RT. The solvents were removed under reduced pressure and the crude product was dissolved in
CH$_2$Cl$_2$ (2 mL) and treated with triisopropylsilane (10 μL, mmol) and trifluoroacetic acid (2 mL) for 1 h at RT. The reaction was diluted into toluene and (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with CH$_2$Cl$_2$ (3×5 mL). The aqueous phase was then reduced to a solid under reduced pressure and purified on a C–18 reverse phase HPLC column using a gradient of 10–60% acetonitrile (0.1% TFA) in water (0.1% TFA) over 15 minutes (3 mL/min). The compound eluted at 8.7 min (6 mg, 7.5 μmol, 44% yield); $^1$H-NMR (D$_2$O, 500 MHz): δ 7.87 (s, 1H), 5.49 (d, J =3.6 Hz, 2H), 5.13 (d, J =2.8 Hz, 2H), 4.60–4.62 (m, 4H), 4.60 (t, J =5.0 Hz, 2H), 4.41 (dd, J =8.0, 4.4 Hz, 2H), 4.09–4.13 (m, 2H), 3.97 (t, J =4.6 Hz, 4H), 3.75–3.80 (m, 2H), 3.74–3.71 (m, 2H), 3.67–3.73 (m, 2H), 3.67–3.74 (m, 4H), 3.60–3.70 (m, 16H), 3.37–3.55 (m, 4H), 3.29–3.33 (m, 1H), 2.98 (dd, J =13.1, 5.1 Hz, 1H), 2.72–2.79 (m, 1H), 2.73–2.77 (m, 4H), 2.47 (t, J =7.6 Hz, 4H), 2.33 (t, J =7.3 Hz, 2H), 2.26–2.31 (m, 2H), 2.22–2.25 (m, 2H), 1.96 (p, J =7.3 Hz, 4H), 1.73–1.81 (m, 2H), 1.68–1.76 (m, 1H), 1.66–1.75 (m, 2H), 1.58–1.68 (m, 2H), 1.52–1.60 (m, 1H), 1.37–1.46 (m, 2H); $^{13}$C-NMR (D$_2$O, 125 MHz): δ 176.8, 176.6, 165.4, 156.0–158.5, 147.5, 123.8, 98.6, 96.2, 81.6, 80.4, 74.9, 72.0, 70.8, 69.1, 68.0–70.1, 62.1, 60.3, 57.0, 55.6, 51.3, 50.5, 49.9, 49.5, 48.8, 48.3, 45.4, 41.8, 39.5, 35.1, 33.0, 32.5, 31.8, 28.3, 28.0, 25.1, 24.9, 24.2; ESI-MS calculated for C$_{84}$H$_{153}$N$_{41}$O$_{26}$S [M+3H]$^{3+}$ 816.78 and found 817.27.
A1.5 Schematic of Guanidinoneomycin-biotin-streptavidin-PE Cy5

Figure A1.1. Streptavidin-phycoerythrin complex highlighting biotin binding sites.
Figure A1.2. The “monomeric” guanidinoneomycin-biotin-streptavidin-phycoerythrin Cy5 complex.
Figure A1.3. The “monomeric” guanidinoneomycin-biotin-streptavidin-phycoerythrin Cy5 complex
A1.6 – Additional graphs from cellular uptake studies

**Figure A1.4.** Normalized cellular uptake at 200 nM highlighting the enhanced uptake of the dimers (dashed) over the monomers (solid). Key: guanidineomycin (blue), guanidinoparomomycin (red), and guanidinotobramycin (green).

**Figure A1.5.** Normalized cellular uptake of compounds 1-6 200 nM highlighting the effect of the various cell lines on internalization of the monomeric (solid) and dimeric (dashed) constructs. Key: CHO-K1 wild type cells (black), pgsA (teal), pgsD (brown), pgsE (red), pgsF (green).
A1.7 – Example of crude histograms for competitive uptake experiment

**Figure A1.6.** Example of crude histogram for competition study involving monomeric guanidinoneomycin 1 and heparin. Data points 1-8 refer to the specific glycan concentrations tested, with 1 being the highest and 8 the lowest.

**Figure A1.7.** Example of crude histogram for competition study involving dimeric guanidinoneomycin 2 and heparin. Data points 1-8 refer to the specific glycan concentrations tested, with 1 being the highest and 8 the lowest.
A1.8 – References


Appendix 2: Supporting Information for Chapter 3

A2.1 Experimental information

A2.2 Synthesis and characterization of guanidinoneomycin conjugates

- **Scheme A2.1.** Synthesis of amino-guanidinoneomycin

- **Scheme A2.2.** Synthesis of guanidinoneomycin conjugates

A2.3 Synthesis and characterization of Arg₉ conjugates

- **Scheme A2.3.** Synthesis Arg₉ conjugates

A2.4 Figures for cellular uptake studies

- **Figure A2.1.** Cellular uptake after 20 minutes

- **Figure A2.2.** Time-lapsed cellular uptake of Cy5 labeled Arg₉ and guanidinoneomycin

- **Figure A2.3.** Inhibited uptake of Cy5-Arg₉ by TAMRA-Gneo

A2.5 Additional figures for FRET endosome assay

- **Table A2.1.** Sample of crude data

- **Figure A2.4.** Graph of crude data

- **Figure A2.5.** Calibration curve for CFP

- **Figure A2.6.** Graph of data in converted units

- **Figure A2.7.** Time-lapsed images of CFP-transfected HeLa cells treated with FlAsH-r₉
A2.1 – Experimental information

Materials: Unless otherwise specified, materials obtained from commercial suppliers were used without further purification. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. PBS (Dulbecco’s phosphate buffered saline), HBSS (Hanks’ balanced salt solution), and DMEM Media were purchased from Invitrogen. FACS buffer (Isotonic solution 0.85% w/v, phosphate buffered, pH 7.1–7.3) was purchased from BD Biosciences (San José, CA, USA). Trypsin/EDTA was purchased from VWR (Mediatech, Manassas, VA, USA). Corning Costar 3548 (Lowell, MA, USA) 48-well plates and NUNC (Rochester, NY, USA) 155411 8-well plates were used.

Instrumentation: NMR spectra were recorded on either a Varian Mercury 500 MHz spectrometer. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility by utilizing either an LCQDECA (Finnigan) ESI with a quadrupole ion trap or an MAT900XL (ThermoFinnigan) FAB double-focusing mass spectrometer. Reversed-phase HPLC (C18 column) purification and analysis were carried out on an Agilent 1200 series LC and 1100 series LC/MSD Trap XCT. Microscopy studies were performed using Zeiss Axiocam 200M and Slidebook 4.0 Software from Intelligent Imaging Innovations, Inc and Zeiss Observer.Z1 using the Zen 2009 Software from Carl Zeiss Microimaging. Flow-cytometry studies were performed on a
BD FACSCalibur, with excitation at 635 nm and emission monitored at 661/16 nm.

**Synthesis:** Detailed synthetic procedures and characterization data are located in the Supporting Information. Arg₉ (D-isomer) was synthesized on TGR resin by solid phase peptide synthesis.

**UV-Vis:** Concentrations for FlAsH and Cy5 labeled compound solutions were determined using Nano Drop 2000C and literature values for extinction coefficients.

**Flow Cytometry:** 75000 HeLa cells were counted by using a hemocytometer, transferred to 48-well plates, and incubated in DMEM (300 µL of media; with a 1% solution of penicillin/streptomycin and 10% FBS) overnight at 37°C. The cells were then washed with PBS and treated with a solution of the corresponding compound (75 µL; DMEM containing 10% FBS) and incubated. Following this, the cells were washed twice with PBS to remove any remaining extracellular compound. The cells were then detached with trypsin/EDTA (50 µL), diluted with medium (50 µL) and FACS buffer (50 µL), and analyzed by flow cytometry. Cellular uptake was quantified by the mean fluorescence intensity; the crude data were interpreted by using FlowJo v8.8.6 wherein the median value was determined and later plotted and further analyzed by using OriginPro 8.
**FRET Endosome Assay:** HeLa Cells were passaged and plated in 8-micro-well plates (Nunc, Lab-Tek Chambered Coverglass, Cat#155411) and incubated in medium (300 µL of Cellgro DMEM supplemented with 1% penicillin/streptomycin and 10% FBS) for 24 hours at 37°C. 25uL of a solution of Opti-MEM (190 µL), Fugene (6 µL) and cDNA of CFP4Cys (4 µL at 0.5 µg/µL) was added to each well and the cells were incubated for 48 hours in complete media. Prior to imaging, the cells were washed four times with HBSS and maintained in 400 µL of HBSS supplemented with 2 g/ml Glucose. Cell fluorescence was observed with a Zeiss Axiovert 200M with Zeiss Plan NeoFluor 40x NA 1.30 objective and Photometrics Cascade II 1024 Monochrome camera. CFP was imaged with excitation filter 420/20, dichroic 450, and emission filter 475/40, 1 second exposure. Cells were imaged for 90 minutes and maintained at 37°C.

**Calculations:** The data obtained from an experiment is in camera counts (CFP emission) with respect to time. This data is organized with respect to individual cells. For each specific concentration of transporter, an initial rate of change was determined using the change in signal over the change in time for a given window. Data was processed for experiments at several different transporter concentrations. These initial rates were plotted and analyzed. Using a calibration curve, data was converted from counts to concentration of CFP. This was done using the equation:

\[ Y \text{ (counts)} = 35503062.87 (\text{counts}/[\text{CFP}] \text{ µM}) \times [\text{CFP}] \text{ µM}. \]
Confocal Microscopy: HeLa Cells were passaged and plated in 8-micro-well plates (Nunc, Lab-Tek Chambered Coverglass, Cat#155411) and incubated in medium (300 µL of Cellgro DMEM supplemented with 1% penicillin/streptomycin and 10% FBS) for 24 hours at 37°C. Prior to imaging, the cells were washed four times with HBSS and maintained in 400 µL of HBSS supplemented with 2 g/ml Glucose. Cells were observed with Zeiss Observer Z.1 system using Zen 2009 LSM 7 Live Software (Carl Zeiss Microimaging, LLC, USA) with C-Apochromat 40x/1.20 W Korr UV-Vis-IR M27 objective. Cy5 was imaged with 635 nm laser line and long pass emission filter 650 nm. TAMRA was imaged with a 532 nm laser line and band pass emission filter 550–615.
A2.2 – Synthesis and characterization of guanidinoneomycin conjugates.

Scheme A2.1. Synthesis of amino-guanidinoneomycin. a) Boc₂O, NEt₃, DMF, H₂O (72%); b) TIBS-Cl, pyridine (75%); NH₃, MeOH, 80°C (91%); d) hex-5-ynoic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 4-dimethylaminopyridine, N,N-diisopropylethylamine, CH₂Cl₂ (84%); e) TFA, triisopropylsilane (TIPS), CH₂Cl₂ (94 %); f) (BocNH)₂(C=NTf), NEt₃, MeOH, H₂O (73%); g) 2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine, CuI, Cs₂CO₃, CH₂Cl₂, CH₃CN (75%); h) TFA, TIPS, CH₂Cl₂ (87%).

Precursors synthesized according to literature procedures.

Amino Gneo. 12 (12 mg, 5.5 μmol) and 11-azido-3,6,9-trioxanodecan-1-amine (2.4 mg, 11 μmol) were dissolved in CH₂Cl₂ (200 μl) and treated with Cs₂CO₃ (400 μg, 1.2 μmol) and a 0.04 M solution of CuI in acetonitrile (25 μl) for 36 h at RT. The reaction was then diluted into ethyl acetate (5 mL) and washed with water (3×5 mL), brine (5 mL), and dried over sodium sulfate. The combined organic layers were concentrated under reduced pressure and used without further purification. The crude product was dissolved in CH₂Cl₂ (2 mL) and treated with triisopropylsilane (20 μL, mmol) and trifluoroacetic acid (2 mL)
for 1 h at RT. The reaction was diluted into toluene (5 mL) and concentrated under reduced pressure. The solid was then dissolved in water (5 mL) and washed with CH$_2$Cl$_2$ (3×5 mL). The aqueous phase was reduced to a solid under reduced pressure and purified on a C-18 reverse phase HPLC column using a gradient of 5–17.5% acetonitrile (0.1% TFA) in water (0.1% TFA) over 15 minutes (3mL/min). The compound eluted as a TFA salt at 11.9 min (8.5 mg, 4.3 μmol, 78% yield). $^1$H-NMR (500 MHz, D$_2$O): δ 7.72 (s, 1H), 5.63 (m, 1H), 5.01 (m, 1H), 4.94 (m, 1H), 4.74 (m, 1H), 4.70 (m, 12), 4.62 (m, 12H), 4.58 (m, 1H), 4.47 (t, J=5, 2H), 4.25–4.23 (m, 1H), 4.2 (t, J=5.5, 1H), 4.03–4.01 (m, 2H), 3.89 (q, J=5.5, 1H), 3.84 (t, J=5, 2H), 3.68 (s, 4H), 3.67 (t, J=2, 3H), 3.66 (m, 4H), 3.64 (m, 10H), 3.56 (m, 6H), 3.55 (m, 2H), 3.52–3.50 (m, 10H), 3.38 (t, J=5, 18H), 3.34–3.33 (m, 4H), 3.14 (t, J=5, 6H), 3.09 (t, J=4.8, 10H), 2.63 (s, 9H), 2.21 (t, J=7.7, 2H), 1.83 (t, J=7.7, 2H), 1.55 (q, J=12, 2H); $^{13}$C-NMR (125 MHz, DMSO): δ 176.29, 162.9 (q, J=37), 157.60, 157.36, 157.24, 157.13, 157.00, 156.42, 123.46, 116.30 (q, J=298), 98.27, 85.92, 84.93, 76.97, 74.43, 69.56, 69.49, 69.35, 69.33, 69.12, 69.11, 66.27, 65.10, 55.48, 53.41, 51.87, 50.05, 48.26, 39.03, 32.49, 25.10, 24.03; ESI-MS calculated for C$_{43}$H$_{83}$N$_{23}$O$_{16}$ [M+H]$^+$ 1177.64, [M+H+Na]$^{+2}$ 600.62, [M+2H]$^{+2}$ 589.83, found 1178.63, 601.01, and 590.25.
Scheme A2.2. Synthesis of guanidinoneomycin conjugates. a) Cy5-NHS, 4-dimethylaminopyridine, N,N-diisopropylethylamine, DMF; b) FlAsH-NHS, 4-dimethylaminopyridine, N,N-diisopropylethylamine, DMF; c) TAMRA-NHS, 4-dimethylaminopyridine, N,N-diisopropylethylamine, DMF.

**Cy5-Gneo.** Cy5-NHS, 4-dimethylaminopyridine, and N,N-diisopropylethylamine were added to amino-gneo in DMF for 12 h at RT. The crude product was purified on a C-18 reverse phase HPLC column using a gradient of 10–90% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes (3mL/min). The compound eluted as a TFA salt at 9.3 min. HR-ESI-FT-MS calculated for C\textsubscript{76}H\textsubscript{124}N\textsubscript{25}O\textsubscript{23}S\textsubscript{2} [M+3H]\textsuperscript{+3} 606.2909 and found 606.2902.

**Flash Gneo.** Flash-NHS, 4-dimethylaminopyridine, and N,N-diisopropylethylamine were added to X in DMF for 12 h at RT. The crude
product was purified on a C-18 reverse phase HPLC column using a gradient of 10–90% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes (3mL/min). The compound eluted as a TFA salt at 10.3 min. HR-ESI-TOFMS calculated for C_{68}H_{102}As_{2}N_{23}O_{22}S_{4}[M+3H]^{+3} 623.4956 and found 623.4952.

**TAMRA Gneo.** TAMRA-NHS, 4-dimethylaminopyridine, and N,N-diisopropylethylamine were added to X in DMF for 12 h at RT. The crude product was purified on a C-18 reverse phase HPLC column using a gradient of 25–34% acetonitrile (0.1% TFA) in water (0.1% TFA) over 12 minutes (3mL/min). The compound eluted as a TFA salt at 10.8 min. HR-ESI-FT-MS calculated for C_{72}H_{111}N_{25}O_{20}[M+3H]^{+3} 549.62333 and found 549.62125.
A2.3 – Synthesis and characterization of Arg₉ conjugates.

Scheme A2.3. Synthesis Arg₉ conjugates. Cy5-NHS/FlAsH-NHS, 4-dimethylaminopyridine, N,N-diisopropylethylamine, DMF.

**Cy5-Arg₉.** Cy5-NHS, 4-dimethylaminopyridine, and N,N-diisopropylethylamine were added to r₉ in DMF for 12 h at RT. The crude product was purified on a C-18 reverse phase HPLC column using a gradient of 10–90% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes (3mL/min). The compound eluted as a TFA salt at 6.2 min. HR-ESI-FT-MS calculated for C₁₀₁H₁₇₈N₄₂O₁₉S₂ [M+4H]⁺⁺⁺ 586.8418 and found 586.8416.

**Flash-Arg₉.** Flash-NHS, 4-dimethylaminopyridine, and N,N-diisopropylethylamine were added to X in DMF for 12 h at RT. The crude product was purified on a C-18 reverse phase HPLC column using a gradient of 10–90% acetonitrile (0.1% TFA) in water (0.1% TFA) over 20 minutes
(3mL/min). The compound eluted as a TFA salt at 9.7 min. HR-ESI-TOFMS calculated for C$_{93}$H$_{155}$As$_2$N$_{46}$O$_{23}$S$_2$ [M+3H]$^+$ 799.3247 and found 799.3235.
**A2.4 – Figures for cellular uptake studies.**

Note that in some cases, the symbol size may be larger than the error bars for a given data point.

![Graph showing cellular uptake after 20 minutes. Cy5 labeled Arg9 (■) and guanidinoneomycin (■).](image)

**Figure A2.1.** Cellular uptake after 20 minutes. Cy5 labeled Arg₉ (■) and guanidinoneomycin (■).
Figure A2.2. Time-lapsed cellular uptake of Cy5 labeled Arg₉ (■) and guanidinoneomycin (■) at 200 μM. Note, that while the quantified internalization of the two transporters differs (Gneo is 2/3 that of Arg₉), the rate at which they enter cells is similar (4.6 and 2.9 counts/min respectively).

Figure A2.3. Inhibited uptake of Cy5-Arg₉ by TAMRA-Gneo. Cy5-Arg₉ (■) and Cy5-Arg₉ + TAMRA-guanidinoneomycin (■) after 120 minutes.
A2.5 – Additional figures for FRET endosome assay.

Table A2.1. Sample of crude data. Each column represents an individual cell. This data is for exposure to 5 μM of the transporter. The data obtained from the experiment is camera counts (CFP emission) with respect to time. The transporters were added at the third time interval, 8.56 minutes which is highlighted in bold. For each cell, an initial rate of change was determined using the change in signal over the change in time for a given window. These values were averaged together and statistically analyzed.

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| df/dt     | 2.23E+01   | 2.23E+01   | 2.23E+01   | 2.23E+01   | 2.23E+01   | 2.23E+01   | 2.23E+01   | 2.23E+01   | 2.23E+01   |
| df/dt     | 4.29E+03   | 8.07E+03   | 4.08E+03   | 1.20E+04   | 1.09E+04   | 1.83E+04   | 2.27E+04   | 6.91E+03   | 2.47E+03   |
| Fmax      | 2.38E+06   | 4.65E+06   | 3.55E+06   | 7.21E+06   | 3.81E+06   | 7.09E+06   | 1.92E+06   | 1.66E+06   | 2.25E+06   |

| Initial Rate | 9.957.10 | 6.806.40 | 2.268.80 |
Figure A2.4. Graph of crude data. Data was processed for experiments at several different transporter concentrations. The initial rates were plotted and analyzed.
Figure A2.5. Calibration curve for CFP. Using known concentration of CFP, a calibration curve was generated and curve fit. This enables a quantified correlation between the measured CFP emission counts and the concentration of CFP.

\[ y = 35503002.87x \]

\[ R^2 = 1.00 \]
Figure A2.6. Graph of data in converted units. Using the calibration curve, data was converted from counts to concentration of CFP. This was done using the equation: \( Y \text{ (counts)} = 35503062.87 \times X \text{ (CFP concentration)} \).
Figure A2.7. Heat map of CFP-4Cys Fluorescence in HeLa cells exposed to 1 µM r$_5$-FlAsH response over 60 min; recovery with 2,3-dimercaptopropanol (BAL).
Appendix 3: Supporting Information for Chapter 4

A3.1 Synthesis and characterization of coumarin-labeled tobramycin
  
  – Scheme A3.1. Synthesis of coumarin-labeled tobramycin (6)

A3.2 Synthesis and characterization of coumarin-labeled kanamycin A
  
  – Scheme A3.2. Synthesis of coumarin-labeled kanamycin A (7)
A3.1 – Synthesis and characterization of coumarin-labeled tobramycin

Scheme A3.1. Synthesis of coumarin-labeled tobramycin. (a) 7-(diethylamino)coumarin-3-carboxylic acid, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide, 4-dimethylaminopyridine, iPr₂EtN, Cl₂CH₂, 78%. (b) Trifluoroacetic acid, triisopropylsilane, Cl₂CH₂, 86%.

Boc5-protected coumarin-labeled tobramycin (10). Anhydrous dichloromethane (300 μL), and 7-(diethylamino)coumarin-3-carboxylic acid (12.4 mg, 0.0473 mmol) were added to 9 (38.1 mg, 0.0394 mmol). To this, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (11.3 mg, 0.0473 mmol), N,N-diisopropylethylamine (15 μL, 0.087 mmol), and 4-(dimethylamino)pyridine (6 mg, 0.04728 mmol) were added. The reaction was stirred for 18 h. The solvent was removed under reduced pressure and the resulting solid was dissolved in ethyl acetate and washed with water and brine. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (3% methanol in dichloromethane). Product: yellow powder. (37.2 mg, 0.0307 mmol, 78% yield). 1H-NMR (400 MHz, D2O): 8.63 (s, 1H), 7.56 (d, J = 9 Hz, 1H), 6.83 (dd, J1 = 9 Hz, J2 = 2.5 Hz, 1H), 6.57 (s, 1H), 5.16 (s, 1H), 5.03
(s, 1H), 4.59 (s, 1H), 4.23 – 4.19 (m, 1H), 3.75 – 3.69 (m, 2H), 3.61 – 3.58 (m, 2H), 3.53 (q, $J_1 = 7$ Hz, $J_2 = 5$ Hz, 4H), 3.44 – 3.36 (m, 6H), 3.20 (t, $J = 10.5$ Hz, 1H), 1.45 – 1.38 (m, 45H), 1.24 (t, $J = 7.5$ Hz, 4H); $^{13}$C-NMR (125 MHz, DMSO): 162.65, 161.47, 157.41, 155.01, 152.37, 132.10, 131.70, 129.75, 110.35, 108.85, 107.71, 104.56, 97.87, 95.68, 77.89, 77.19, 69.76, 44.29, 35.16, 29.07, 28.73, 28.19, 26.53, 21.95, 13.77, 12.32; ESI-MS calculated for C57H91N7O21 [M+H]$^+$ 1210.63 and [M+Na]$^+$ 1232.62, found 1210.43 and 1232.65.
A3.2 – Synthesis and characterization of coumarin-labeled kanamycin A

Scheme A3.2. Synthesis of coumarin-labeled kanamycin A. Reagents: (a) 7-(diethylamino)coumarin-3-carboxylic acid, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide, 4-dimethylanomopyridine, iPr₂EtN, Cl₂CH₂, 87%. (b) Trifluoroacetic acid, triisopropylsilane, Cl₂CH₂, 72%.

Boc4-protected coumarin-labeled-kanamycin A (12). Anhydrous N,N-dimethylformamide (300 μL), and 7-(diethylamino)coumarin-3-carboxylic acid (20 mg, 76.6 μMol) were added to the 11 (45.2 mg, 51.1 μMol). To this, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (11.8 mg, 61.3 μMol), N,N-diisopropylethylamine (22 μL, 127.7 μMol), and 4-(dimethylamino)pyridine (1.9 mg, 15.3 μMol) were added. The reaction was stirred for 18 h. The solvent was removed under reduced pressure and the resulting solid was dissolved in ethyl acetate and washed with water and brine. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The product was isolated by flash chromatography (5-10% methanol in dichloromethane). Product: green oil (50.11 mg, 44.46 μMol, 87% yield). ¹H-NMR (400 MHz, CD₃OD): δ 8.61 (s, 1H), 7.54 (d, J=12, 1H), 7.31-7.24 (m, 1H), 6.82 (dd, J=4, 10, 1H), 6.56 (d, J=4, 1H), 5.14 (s, 1H),
4.96 (s, 1H), 4.72 (s, 1H), 4.51-4.43 (m, 1H), 3.85-3.81 (m, 1H), 3.63-3.30 (m, 19H), 3.19-3.14 (m, 4H), 3.08 (t, J=10, 1H), 2.06-1.97 (m, 2H), 1.44 (s, 36H), 1.23-1.17 (m, 10H); $^{13}$C-NMR (125 MHz, DMSO): δ 174.25, 162.64, 161.80, 161.75, 157.25, 156.17, 154.89, 152.49, 147.79, 131.67, 129.64, 110.20, 107.64, 95.79, 77.85, 77, 71, 77.27, 44.30, 35.13, 31.29, 29.09, 29.03, 28.99, 28.83, 28.69, 28.58, 28.22, 28.16, 27.33, 26.56, 25.09, 22.08, 13.89, 12.30; ESI-MS calculated for C$_{52}$H$_{82}$N$_{6}$O$_{21}$ [M+Na]$^{+}$ 1149.33, found 1149.54 and 1059.40.