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NOTE

Cellular uptake of modified aminoglycosides

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The uptake of modified amino- and guanidino-glycosides derived from kanamycin, tobramycin and neomycin in native and mutant CHO cells is examined using confocal microscopy and flow cytometry, illustrating the significance of multivalency for mammalian cell internalization of carriers that specifically interact with cell surface heparan sulfate proteoglycans.
The Journal of Antibiotics (2018) **71,** 142–145; doi:10.1038/ja.2017.131; published online 1 November 2017

Aminoglycoside antibiotics were among the first antibiotics discovered and used clinically.[1] Streptomycin, isolated from *Streptomyces griseus* was the first aminoglycoside discovered[2] and employed clinically to treat tuberculosis. Since then, various naturally occurring aminoglycosides such as kanamycin, neomycin (fradiomycin) and gentamycin have been discovered, and various semi-synthetic analogs have been introduced such as amikacin and dibekacin.[3,4] Aminoglycosides, although still clinically relevant, are not commonly employed due to their adverse effects and emergence of antibiotic resistance impeding their efficacy.[5,6] Nevertheless, although the prevalence of aminoglycoside antibiotics in the clinic may have decreased, the past half century of research has given way to alternate applications for this class of molecules.[7,8] One such application is as a scaffold for guanidinium-based molecular transporters, where they can serve as delivery vehicles for various biological entities into cells.[9,10]

Over the last two decades, our group has developed and investigated various driveatives of guanidinium-rich cellular delivery agents based on aminoglycosides, commonly referred to as guanidinoglycosides (Figure 1).[10–12] This subcategory of molecular transporters is synthesized by converting all ammonium groups on the aminoglycoside scaffolds into guanidinium groups.[13] Whereas the majority of cellular delivery agents and guanidinium-based molecular transporters are often used at micromolar concentrations, guanidinoglycosides have been shown to deliver large bioactive macromolecules into mammalian cells at nanomolar concentrations when both covalently or non-covalently bound to a cargo of interest.[12,14–18] The presence of heparan sulfate proteoglycans on the cell surface has been crucial to the observed cellular uptake.[12,14] Of significance is that multivalent systems have dominated these studies, with less information available for monomeric arrangements. As minimizing the number of molecular transporters used per cargo is desirable, we have sought to compare and contrast the uptake features of mono- and multi-valent amino- and guanidino-glycosides. In this communication, we report on cellular uptake studies with such highly charged carriers in their monovalent low MW (fluorescently tagged) form and multivalent arrangement (when bound to a high MW fluorescently labeled streptavidin via a biotin-linker) in both wild type (Chinese hamster ovarian, CHOK1) and heparan sulfate-deficient (pgsA-745) cells.[19] Our results illustrate intriguing carrier/uptake relationships that may impact the design of future transporters.

To investigate the effect of multivalency on cellular uptake, biotinylated and Cy3-conjugated amino- and guanidino-glycoside derivatives (Figure 1) were prepared according to previously reported synthetic procedures (full details to be published elsewhere). All compounds were tested in cell culture with wild type CHOK1 and with mutant Chinese hamster ovarian cells (pgsA-745) devoid of cell surface heparan sulfate. The internalization was quantified using flow cytometry (fluorescence-activated cell sorting (FACS)). Experimentally, wild-type CHOK1 and mutant pgsA-745 cells were seeded onto a 24-well plate at a density of 100 000 cells per well and grown to 80% confluency overnight. Biotinylated amino- and guanidino-glycosides were then bound to a Cy5-labeled streptavidin to form tetravalent biotin–streptavidin conjugates by incubating the compounds with streptavidin-Cy5 (5:1) in a MilliQ:PBS (1:1) solution protected from light for 20 min. Both the streptavidin conjugates and the Cy3 guanidinoglycosides were diluted to the desired concentrations in F-12 growth medium containing 10% FBS. The cells were washed with phosphate-buffered saline (PBS) and incubated with 300 μl of the fluorescent carrier solutions for 1 h at 37 °C in a 5% CO_2 atmosphere. The cells were then washed twice with 300 μl of PBS and detached with 60 μl of trypsin-EDTA for 10 min, followed by a dilution with 0.1% bovine serum albumin in PBS and analyzed by FACS (Figure 2).

Amongst the amino- and guanidino-glycoside streptavidin conjugates using derivatives **1e–3e** and **1f–3f**, respectively, the guanidinylated neomycin carrier (**3f**) conjugate was shown to enter cells with higher efficacy than the other guanidino- and amino-conjugates in CHO cells (Figure 2a). The internalization of the guanidinoglycoside conjugates was significantly higher compared with their aminoglycoside counterparts when bound to streptavidin-Cy5, demonstrating the

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Received 11 June 2017; revised 21 August 2017; accepted 10 September 2017; published online 1 November 2017

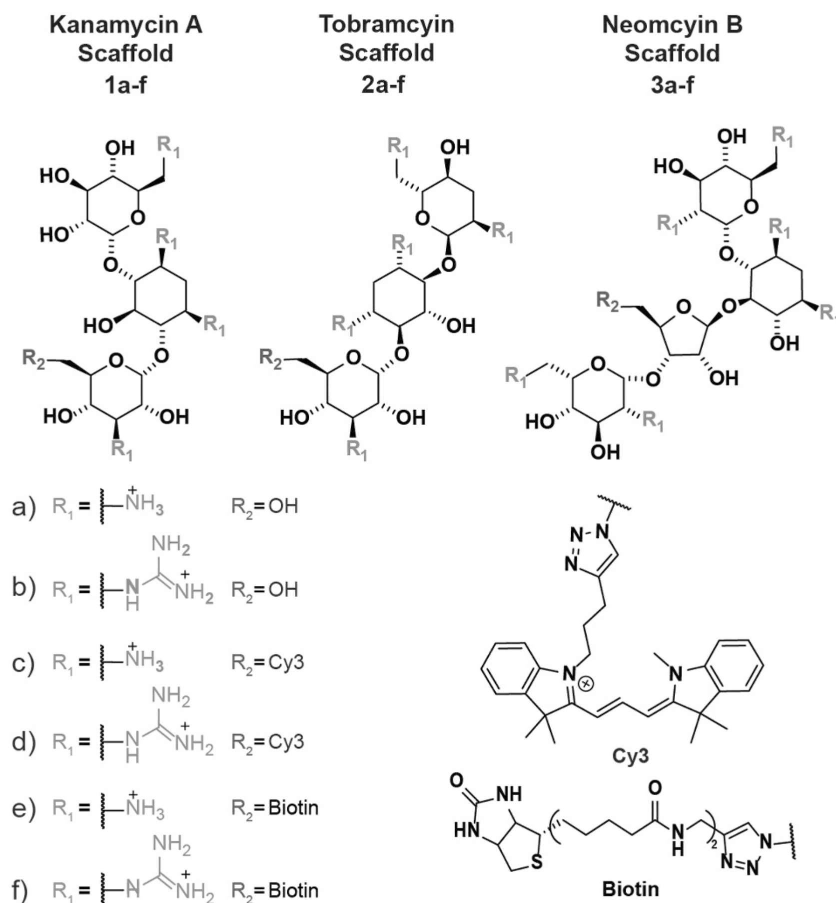

Figure 1 Structures of unmodified (**1**–**3a**,**b**), Cy3-conjugated (**1**–**3c**,**d**) and biontylated (**1**–**3e**,**f**) amino- and guanidino-glycoside derivatives. A full colour version of this figure is available at the *Journal of Antibiotics* journal online.

impact of the guanidinium group upon cellular delivery of macromolecules. This is consistent with previously published work performed solely on the GNeo-streptavidin conjugate.[12,18] The GNeo-streptavidin (**3f**) conjugate entered cells twice as effectively as the guandinotobramycin (**2f**) conjugate, highlighting the benefit of a higher number of guanidinium moieties on the transporter. Comparatively, the guanidinokanamycin (**1f**) conjugate showed minimal cellular uptake. The same behavior, albeit significantly reduced, was observed in mutant pgsA-745 cells, which do not express cell surface heparan sulfate.

The monomeric amino- and guanidino-glycosides showed lower levels of entry into mammalian cells compared to their multivalent counterparts (Figure 2b, note different scales). Although the GNeo-biotin conjugate depicted high fluorescence intensities, the only monomeric derivative that approached this fluorescence intensity was guanidinotobramycin-Cy3 (**2d**) albeit at 10 μM, nearly three orders of magnitude higher than the concentration used in multivalent system (25 nM). It is important to note, however, that the quantum yield of Cy3 is significantly lower than that of Cy5, and that such a large difference in concentration could potentially originate from this disparity. Among the fluorescently tagged monomeric amino- and guanidino-glycosides, the guanidinoglycosides entered cells better than their parent aminoglycosides, similar to the multivalent system, indicating that the guanidinium group is important for these monomeric transporters as well. Cells treated with guanidinotobramycin-Cy3 (**2d**), exhibited nearly six-fold higher fluorescence intensity compared with guanidinoneomycin-Cy3 (**3d**) and guanidinokanamycin-Cy3 (**1d**), which is intriguing as it suggests lack of correlation between the number of charges on the molecular transporter and cellular uptake. This might appear to be contradictory to the obvious trend in the multivalent system, where increasing the number of positive charges augmented cellular uptake, hinting at distinct cell entry pathways. Indeed, the same fluorescence intensities are observed when pgsA-745 cells are treated with the monomeric derivatives, indicating that uptake of the guanidino-Cy3 derivatives (**1d**, **2d** and **3d**) is not exclusively dependent on cell surface heparan sulfate, as is well established for the multivalent derivatives. Other monomeric derivatives containing a different fluorescent probe were analyzed previously,[20] yet those compounds exhibited different uptake behavior than the Cy3 derivatives, which demonstrates that the uptake of fluorescently tagged monomeric guanidinoglycosides could also be dependent on the label (that is, cargo) of interest as well as the molecular transporter. This is not entirely unthinkable, as under such circumstances the fluorescent tag is almost as large as the carrier itself.

The results discussed above provide insight into the reduced cellular delivery efficacy of the monomeric carriers, yet fail to explain why compound **2d** enters cells more efficiently than its other monomeric counterparts, including compound **3d**. We speculate that compound **2d**, having a total of six positive charges (five on the guanidinotobramycin scaffold and one on the dye), may possess an effective combination of functional groups and positive charges in the optimal orientation to take advantage of multiple cell entry pathways (e.g., endocytosis, micropinocytosis). Preliminary confocal microscopy experiments in CHO cells, fixed after treatments with the fluorescently tagged carriers, indeed show broad cytoplasmic distribution for **2d**,

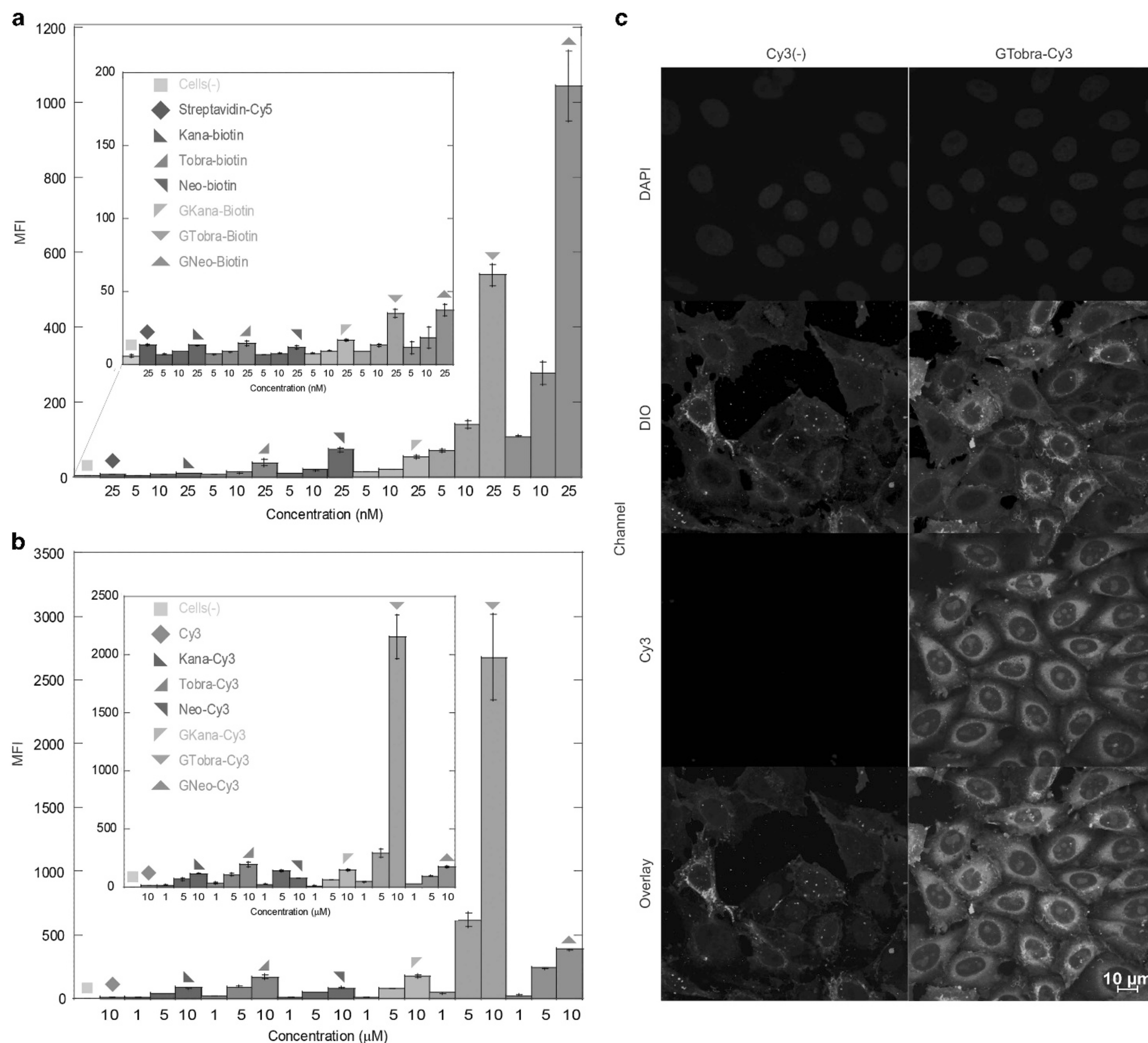

Figure 2 Mean fluorescence intensity of amino- and guanidino-glycoside derivatives bound to Cy5-labeled streptavidin in (**a**) wild-type chinese hamster ovarian (CHOK1) cells and mutant pgsA-745 cells (inset). (**b**) Mean fluorescence intensity of Cy3-labeled amino- and guanidino-glycoside derivatives in CHOK1 cells and pgsA-745 cells (inset). All experiments were performed in triplicate. (**c**) Confocal microscopy images of CHOK1 cells treated with Cy3 (left) and guanidinotobramycin-Cy3 **2d** (right). Please see online article for full color images. A full colour version of this figure is available at the *Journal of Antibiotics* journal online.

clearly distinct from previous observations for multimeric guanidinoglycoside-based transporters (Figure 2c).[14] Further experiments are required, however, to cement alternative localization and cellular distribution mechanisms for guanidinylated tobramycin derivatives.

In summary, we have demonstrated the significance of multivalency in cellular uptake of aminoglycoside-based guanidinium-rich cellular delivery agents that exclusively utilize heparan sulfate as a cell entry pathway. Furthermore, when selectivity drops and this internalization route into mammalian cells is no longer exclusively exploited, other factors appear to become significant, including the number of positive charges present as well as the cargo of interest. These observations may have universal significance as structural features and fluorescent labels may be having a role in the cellular delivery of other cell penetrating agents.

DEDICATION

In honor of professor Hamao Umezawa and his discoveries that made the world a better place.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the National Science Foundation for support (via grant number CHE-1303554) as well as the Chemistry and Biochemistry MS and NMR Facilities, and Dr Kristina Hamill.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)