J|A|C|S
COMMUNICATIONS

Published on Web 09/20/2003

Cellular Uptake of Aminoglycosides, Guanidinoglycosides, and Poly-arginine

Nathan W. Luedtke,† Peter Carmichael, and Yitzhak Tor*

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0358

Received May 7, 2003; E-mail: ytor@ucsd.edu

Charged molecules over 500 amu typically exhibit poor bioavailability.[1] This limits the delivery of many therapeutically active molecules to their intended targets. Polycationic molecules provide important exceptions to this generalization. Modification of bovine serum albumin (BSA) with ethylenediamine produces "cationionized BSA", a highly effective antigen carrier.[2] Despite its size (over 66 000 amu), cationized BSA efficiently enters cells via an unknown path involving adsorptive uptake.[3] More recently, a number of poly-arginine peptides,[4] peptoids,[5] and peptidomimetics[6] have been found to exhibit highly efficient uptake into a wide range of mammalian cell types. The conjugation of such poly-Arg peptides to large molecules can facilitate the transduction of peptide, protein, and nucleic acid conjugates into cells.[4a] The mechanism responsible for poly-Arg-mediated transport is still unclear, but may involve a receptor-mediated, nonendocytotic route.[4a,7] In this report, we present two guanidine-modified natural products that exhibit exceptional cellular membrane translocation efficiencies and share an uptake mechanism similar to that of poly-Arg peptides.

Aminoglycoside antibiotics are a therapeutically important family of polycations that selectively inhibit prokaryotic protein biosynthesis.[8] We recently reported that converting the amines on aminoglycosides into guanidine groups increases both the RNA affinity and the anti-HIV activities of the resulting compounds, termed "guanidinoglycosides".[9] To examine how the cellular uptake of these compounds is affected by guanidinylation, we have synthesized a series of BODIPY-tagged aminoglycosides and guanidinoglycosides based upon tobramycin and neomycin B (Figure 1). BODIPY is an excellent fluorescent probe for cellular uptake studies because its fluorescence is relatively insensitive to changes in the local environment. By using fluorescein as a reference ($\phi = 0.93$ at pH 9.0), we determined that the emission quantum efficiency (ϕ) of all five BODIPY conjugates **1**−**5** is equal to 1.0 at pH 7.5.

The uptake of BODIPY-containing glycosides by two different eukaryotic cell lines was studied using both fluorescence activated cell sorting (FACS) and fluorescence microscopy. Examples of FACS histograms are presented in Figure 2, and selected microscopy images are shown in Figure 3. In a typical experiment, cell cultures were treated with 0.5−5 μM of each compound for 0.5−1 h, washed twice with buffer, cleaved with trypsin, and quantified for fluorescence at 530 nm.[10] Both fluorescent aminoglycosides (**1** and **3**) display poor cellular uptakes (slightly above the autofluorescence of the cell itself) (Table 1).[11,12] Upon guanidinylation, the cellular uptake of tobramycin is enhanced by approximately 10-fold (relative to autofluorescence), and the enhancement for neomycin B is approximately 20-fold (Figure 2A,B and Table 1).[12,13] Because fluorescent poly-Arg peptides are known to exhibit better uptake than poly-Lys conjugates,[5,14,15] it appears that poly-guanidino compounds exhibit better transport properties relative to the corresponding polyamines, regardless of the molecular scaffold.[16]

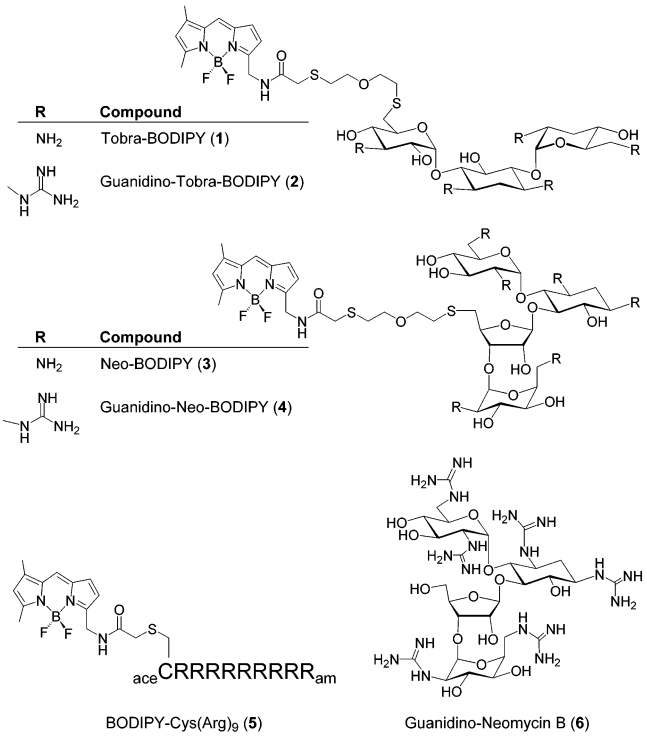

Figure 1. Aminoglycosides and guanidinoglycosides evaluated for cellular uptake. See Supporting Information for the synthesis and characterization of **1**−**5**. Compound **6** has already been reported.[9b]

As compared to a common poly-Arg transduction peptide,[4a,5,7,14,15] the guanidinoglycosides show the same, or even better, cellular uptake efficiencies. Guanidino-tobra-BODIPY (**2**) has four fewer guanidinium groups than BODIPY-Cys(Arg)$_9$ (**5**), but shows approximately the same transport efficiency (Table 1). Importantly, guanidino-neo-BODIPY (**4**) consistently has a better cellular uptake as compared to the poly-Arg peptide BODIPY-Cys(Arg)$_9$ (**5**) (Figure 2C, Table 1). This suggests that the semirigid preorganization of the guanidinium groups on the glycoside core may better facilitate translocation across the cell membrane.[17] In contrast to the results obtained for a family of poly-Arg peptoids,[5] the amphipathic properties provided by the flexible methylene chains of poly-(Arg)$_9$ residues do not appear essential for membrane transport of guanidinoglycosides. To address the possibility that guanidino-neomycin B enters cells through a different mechanism than poly-Arg, a competition experiment was conducted between BODIPY-Cys(Arg)$_9$ (**5**) and the unlabeled guanidino-neomycin B (**6**). FACS analysis shows that guanidino-neomycin B (**6**) effectively inhibits the transport of BODIPY-Cys(Arg)$_9$ into cells (Figure 2D and Table 1), suggesting a common pathway responsible for the uptake of both compounds.

Microscopy experiments have been conducted using both fluorescein-labeled and BODIPY-labeled guanidinoglycosides. The relative intensities of individual cells, following treatment with either

† Present address: Department of Chemistry and Biochemistry, Yale University, New Haven, CT 06520-8107.

10.1021/ja0360135 CCC: $25.00

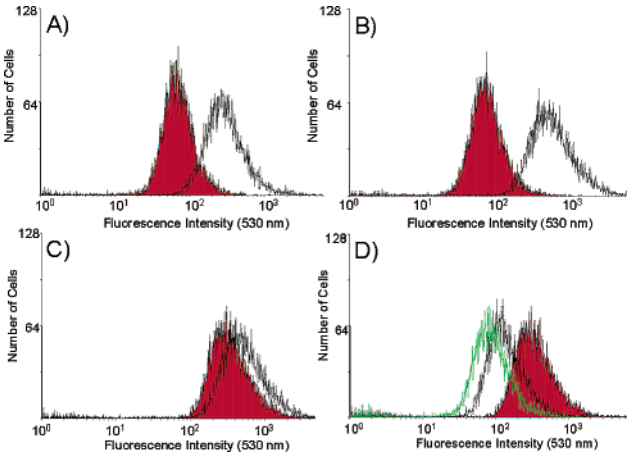

Figure 2. FACS histograms showing the fluorescence intensity versus cell count for 10 000 individual $10T^1/_2$ cells following a 1 h incubation with 0.5 μM of the following: (A) tobra-BODIPY (red) and guanidino-tobra-BODIPY (white); (B) neo-BODIPY (red) and guanidino-neo-BODIPY (white); (C) BODIPY-Cys(Arg)$_9$ (red) or guanidino-neo-BODIPY (white); (D) uptake of BODIPY-Cys(Arg)$_9$ inhibited by guanidino-neomycin B (**6**), at 0 μM (red), 10 μM (black), and 200 μM (green).

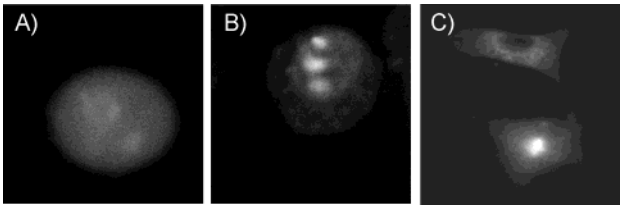

Figure 3. (A and B) Cross-sectional images of two individual HeLa cells in solution following a 30 min treatment with 5 μM of guanidino-neo-BODIPY and cleavage by trypsin (see Supporting Information for full cross-sectioning of each cell).[10] (C) Two neighboring $10T^1/_2$ cells growing on a culture plate following a 1 h exposure to 1 μM of **4**. See Supporting Information for uptake experiments using fluorescein-containing molecules.

Table 1. Mean Fluorescence Intensities of Treated Cells[a]

compound	$10T^1/_2$[b]	HeLa[c]
none (autofluorescence)	~40[d]	830
tobra-BODIPY (**1**)	60	1000
guanidino-tobra-BODIPY (**2**)	240	2100
neo-BODIPY (**3**)	60	~1400[e]
guanidino-neo-BODIPY (**4**)	430	7900
BODIPY-Cys(Arg)$_9$ (**5**)	280	2000
BODIPY-Cys(Arg)$_9$ (**5**) + 10 μM (**6**)	110	n.d.[f]
BODIPY-Cys(Arg)$_9$ (**5**) + 50 μM (**6**)	90	n.d.
BODIPY-Cys(Arg)$_9$ (**5**) + 200 μM (**6**)	70	n.d.

[a] The FACS data are not directly comparable between cell types, as a higher instrumental gain (about 10-fold) was used for the HeLa experiments. [b] Average intensity of 10 000 individual cells treated with 0.5 μM of each compound for 1 h. [c] Average intensity of 2000 individual cells treated with 1 μM of each compound for 0.5 h. Under these conditions, a "free" BODIPY dye molecule Tris-BODIPY shows poor uptake into HeLa cells (similar to Tobra-BODIPY).[10,12] [d] Estimate based upon data set collected at a higher instrumental gain. [e] Estimate based upon data set collected at a lower instrumental gain. [f] n.d. = not determined.

fluorescent aminoglycosides or guanidinoglycosides, are consistent with the trends from FACS experiments.[10] Optical cross-sectioning using scanning confocal fluorescence microscopy indicates that guanidinoglycosides are found inside of living cells (Figure 3).[10,18] Interestingly, two distinct types of cellular localization of guanidino-neo-BODIPY are observed (Figure 3). Approximately one-half of the cells exhibit a highly diffuse, cytoplasmic, and nuclear distribution (Figure 3A), while the other one-half exhibit more localized nucleolar staining, similar to that reported for poly-Arg peptides (Figure 3B).[4a,7] We have observed similar results with fluorescein-labeled conjugates,[10] as well as $10T^1/_2$ cells (Figure 3C). Taken together, this suggests that the relative uptake efficiencies and cellular localization of these compounds are not highly dependent on the cell type or dye molecule used.[19]

In summary, we have found that, unlike aminoglycosides, guanidinoglycosides exhibit highly efficient uptake by eukaryotic cell cultures via a mechanism similar to that of a poly-arginine peptide.[20] Guanidine-containing modified natural products, including guanidino-neomycin, may facilitate the efficient cellular transport of pharmacologically important cargo molecules.[21]

Acknowledgment. We thank Dr. Roger Tsien for his comments, and W. Coyt Jackson for technical assistance with the FACS experiments. Financial support was provided by the NIH (AI 47673) and the DOE (DE-FG03-01ER63276).

Supporting Information Available: Synthesis and characterization of compounds **1**−**5**, conditions for the cellular uptake studies, and microscopy images of $10T^1/_2$ cells treated with aminoglycoside- and guanidinoglycoside-fluorescein conjugates (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Lipinski, C. A.; Lombardo, F.; Dominsy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 4−25.
(2) Muckerheide, A.; Apple, R. J.; Pesce, A. J.; Michael, J. G. *J. Immunol.* **1987**, *138*, 833−837.
(3) Apple, R. J.; Domen, P. L.; Muckerheide, A.; Michael J. G. *J. Immunol.* **1988**, *140*, 3290−3295.
(4) For a review, see: (a) Futaki, S. *Int. J. Pharm.* **2002**, *245*, 1−7. For β-peptides, see: (b) Umezawa, N.; Gelman, M. A.; Haigis, M. C.; Raines, R. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, *124*, 360−369.
(5) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003−13008.
(6) Litovchick, A.; Lapidot, A.; Eisenstein, M.; Kalinkovich, A.; Borkow, G. *Biochemistry* **2001**, *40*, 15612−15623.
(7) Suzuki, T.; Futaki, S.; Niwa, M.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2002**, *277*, 2437−2443.
(8) (a) Davies, J.; Davis, B. D. *J. Biol. Chem.* **1968**, *243*, 3312−3316. (b) Moazed, D.; Noller, H. F. *Nature* **1987**, *327*, 389−394.
(9) (a) Luedtke, N. W.; Baker, T. J.; Goodman, M.; Tor, Y. *J. Am. Chem. Soc.* **2000**, *122*, 12035−12036. (b) Baker, T. J.; Luedtke, N. W.; Tor, Y.; Goodman, M. *J. Org. Chem.* **2000**, *65*, 9054−9058.
(10) See Supporting Information for experimental details.
(11) Preliminary experiments indicate that for prokaryotic cell cultures (*E. coli*), compounds **1**−**4** all show highly efficient cellular uptake.
(12) Similar results are observed for fluorescein-labeled conjugates.[10]
(13) A recent report describes the incorporation of aminoglycoside-cholesterol conjugates into liposomal phospholipid complexes capable of delivering DNA into cells. Guanidinylation of a kanamycin A-cholesterol conjugate actually decreases its efficiency as a delivery agent. See: Belmont, P.; Aissaoui, A.; Hauchecorne, M.; Oudrhiri, N.; Petit, L.; Vigneron, J. P.; Lehn, J. M.; Lehn, P. *J. Gene Med.* **2002**, *4*, 517−526. Our preliminary results indicate that a minimum of five guanidinium groups is needed to stimulate efficient cellular uptake (kanamycin A has four amines).
(14) Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000**, *56*, 318−325.
(15) Uemura, S.; Rothbard, J. B.; Matsushita, H.; Tsao, P. S.; Fathman, C. G.; Cooke, J. P. *Circ. J.* **2002**, *66*, 1155−1160.
(16) Contrary to experiments conducted with peptide conjugates of small organic fluorophores,[5,14,15] a recent paper reports that poly-Lys protein conjugates mediate transduction at the same or even higher levels than poly-Arg. See: Mai, J. C.; Hongmei, S.; Watkins, S. C.; Cheng, T.; Robbins, P. D. *J. Biol. Chem.* **2002**, *277*, 30208−30218.
(17) For peptides, secondary structure formation can increase transport efficiencies. See: Ho, A.; Schwarze, S. R.; Mermelstein, S. J.; Waksman, G.; Dowdy, S. F. *Cancer Res.* **2001**, *61*, 474−477.
(18) Treatment of guanidinoglycoside-containing cells with propidium iodide (a common viability test) indicates that the cells used for these experiments have intact membranes.
(19) Microscopy experiments suggest that trypsin-mediated cleavage prior to FACS analysis does not affect the cellular localization, or the relative uptake efficiencies of these compounds (Figure 3 and Figure S1).[10]
(20) Previous studies have shown that peptidomimetics based upon neomycin-arginine conjugates also show efficient cellular uptake.[6] This type of modification doubles the total number of basic groups and results in compounds possessing an equal number of amide, amine, and guanidine functionalities. The results presented here represent a direct comparison of purely amino- versus guanidino-containing glycosides.
(21) For potential commercial applications, see: Bonetta, L. *The Scientist* **2002**, *16*, 38−40.

JA0360135