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J. Am. Chem. Soc., **2005**, 127 (3), 869-874• DOI: 10.1021/ja044006q • Publication Date (Web): 29 December 2004

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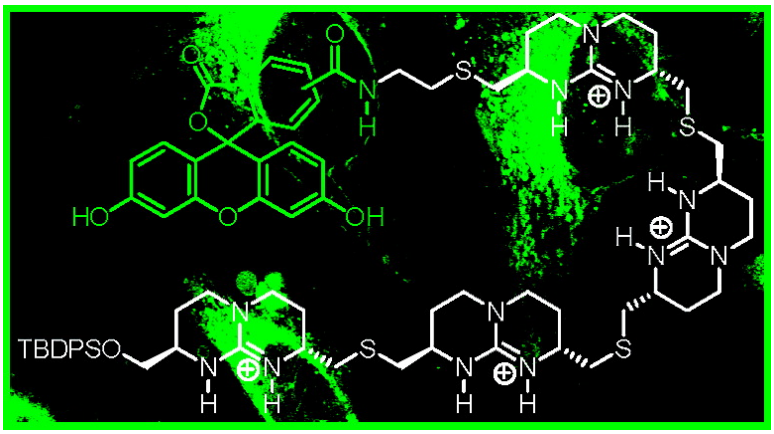

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Highly Efficient, Nonpeptidic Oligoguanidinium Vectors that Selectively Internalize into Mitochondria

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Abstract: Oligoguanidinium-based cell delivery systems have gained broad interest in the drug delivery field since one decade ago. Thus, arginine-containing peptides as Tat or Antp, oligoarginine peptides, and derived peptoids have been described as shuttles for delivering nonpermeant drugs inside cancer cells. Herein we report a new family of tetraguanidinium cell penetrating vectors efficiently internalized in human tumor cells. Their high internalization, studied by confocal microscopy and flow cytometry, as well as their specific accumulation in mitochondria makes these new vectors likely vehicles for the targeted delivery of anticancer drugs to mitochondria.

Introduction

In recent years, the previously unattainable goal of the cellular delivery of therapeutic moieties has been brought closer by the identification of proteins, peptides, and novel peptide derivatives able to transport nonpermeant molecules through the cell membrane.[1,2] Thus, the guanidinium-containing third helix of Antennapedia homeodomain (Antp) and Tat peptide are cell penetrating peptides (CPPs) that have received broad interest in the drug delivery field.[3,4] Since their discovery a wide number of studies have been focused on improving the permeabilization efficiency of CPPs and gaining a better understanding of the translocation process.[5,6] Efforts have been addressed recently to develop new cell-penetrating compounds other than canonical peptide structures, since peptides are prone to in vivo hydrolysis by peptidases and display complex pharmacokinetic properties. For instance, the Tat sequence has been shortened,[7] exchanged to linear or branched oligoarginine peptides,[8,9] or mutated into the all-D, inverso and retro forms, to improve its metabolic resistance and uptake.[8] Furthermore, Wender et al. have reported peptoids[10] and even polycarbamate, guanidinium-rich oligomers[11] based on their pioneering work with arginines.[12,13] Dendrimers,[14] β-oligoarginines,[15−17] and, more recently, γ-peptides are among other alternatives.[18] Most of these variants, however, still contain hydrolyzable bonds.

We report herein the cell-penetrating properties of well-defined, nonhydrolysable tetraguanidinium compounds **1** and **2** (Chart 1), consisting of highly preorganized chiral bicyclic guanidinium subunits linked together through short thioether spacers, as well as a study of their potential to specifically target cell organelles. In common with most CPPs and other cell penetrating vectors, these compounds contain an array of highly basic guanidine groups.[19] It was previously found that sym-

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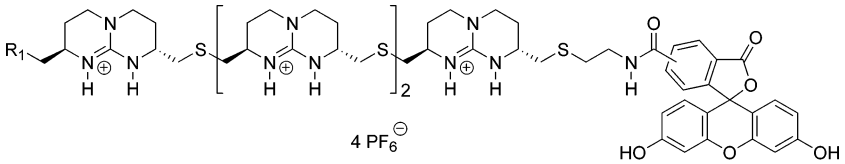
[⊥] Departament de Química Orgànica, Universitat de Barcelona.

(1) Lindgren, M.; Hallbrink, M.; Prochiantz, A.; Langel, U. *Trends Pharmacol. Sci.* **2000**, *21*, 99−103.
(2) Lundberg, P.; Langel, U. *J. Mol. Recognit.* **2003**, *16*, 227−233.
(3) Dunican, D. J.; Doherty, P. *Biopolymers* **2001**, *60*, 45−60.
(4) Silhol, M.; Tyagi, M.; Giacca, M.; Lebleu, B.; Vives, E. *Eur. J. Biochem.* **2002**, *269*, 494−501.
(5) Langel, U. *Cell-Penetrating Peptides: Processes and Applications*; CRC Press: Boca Raton, FL, 2002.
(6) Vives, E.; Richard, J. P.; Rispal, C.; Lebleu, B. *Curr. Protein Pept. Sci.* **2003**, *4*, 125−132.
(7) Vives, E. *J. Mol. Recognit.* **2003**, *16*, 265−271.
(8) Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836−5840.
(9) Futaki, S.; Nakase, I.; Suzuki, T.; Youjun, Z.; Sugiura, Y. *Biochemistry* **2002**, *41*, 7925−7930.
(10) 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.
(11) Wender, P. A.; Rothbard, J. B.; Jessop, T. C.; Kreider, E. L.; Wylie, B. L. *J. Am. Chem. Soc.* **2002**, *124*, 13382−13383.
(12) Rothbard, J. B.; Kreider, E.; VanDeusen, C. L.; Wright, L.; Wylie, B. L.; Wender, P. A. *J. Med. Chem.* **2002**, *45*, 3612−3618.
(13) Kirschberg, T. A.; VanDeusen, C. L.; Rothbard, J. B.; Yang, M.; Wender, P. A. *Org. Lett.* **2003**, *5*, 3459−3462.
(14) Crespo, L.; Sanclimens, G.; Montaner, B.; Perez-Tomas, R.; Royo, M.; Pons, M.; Albericio, F.; Giralt, E. *J. Am. Chem. Soc.* **2002**, *124*, 8876−8883.
(15) Rueping, M.; Mahajan, Y.; Sauer, M.; Seebach, D. *ChemBioChem* **2002**, *3*, 257−259.
(16) Umezawa, N.; Gelman, M. A.; Haigis, M. C.; Raines, R. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 368−369.
(17) Garcia-Echeverria, C.; Ruetz, S. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 247−251.
(18) Farrera-Singfreu, J.; Zaccaro, L.; Vidal, D.; Salvatella, X.; Giralt, E.; Pons, M.; Albericio, F.; Royo, M. *J. Am. Chem. Soc.* **2004**, *126*, 6048−6057.

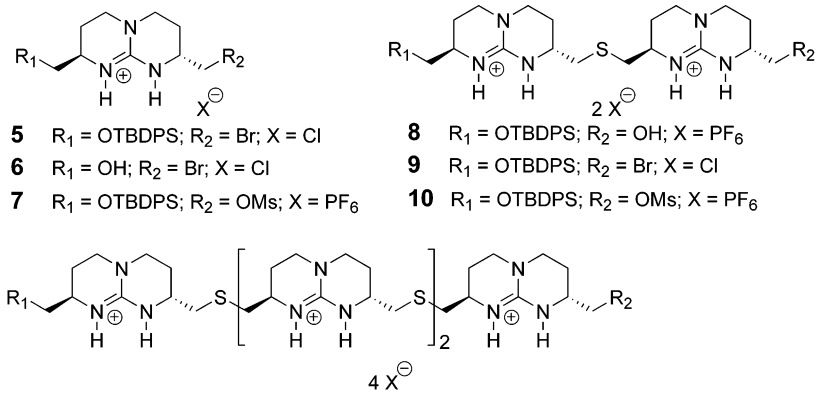
Chart 1. 5(6)-Carboxyfluorescein-Containing Tetraguanidinium Vectors **1** and **2**



1 R_1 = OTBDPS
2 R_1 = OH

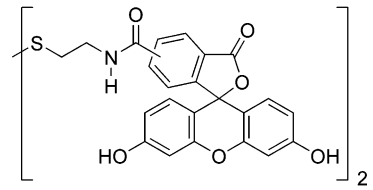
TBDPS = $SiPh_2{}^tBu$

Chart 2. Structure of Guanidinium Derivatives **3**−**12** and CF Disulfide **13**



5 R_1 = OTBDPS; R_2 = Br; X = Cl
6 R_1 = OH; R_2 = Br; X = Cl
7 R_1 = OTBDPS; R_2 = OMs; X = PF_6
8 R_1 = OTBDPS; R_2 = OH; X = PF_6
9 R_1 = OTBDPS; R_2 = Br; X = Cl
10 R_1 = OTBDPS; R_2 = OMs; X = PF_6

11 R_1 = OTBDPS; R_2 = OH; X = PF_6
12 R_1 = OTBDPS; R_2 = OMs; X = PF_6
3 R_1 = R_2 = OTBDPS; X = Cl
4 R_1 = R_2 = OH; X = Cl



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metrical analogues **3** and **4** (Chart 2) efficiently form stable complexes with negatively charged peptides or proteins.[20,21] The interesting properties of these guanidinium oligomers, as well as their similarity to Pro-rich CPPs[22] in terms of amphipathicity,[23] led us to study their internalization properties.

Experiments have been performed in both fixed and living cells to compare the properties of the new vectors with the well-studied Antp and Tat. Interestingly, tetraguanidinium oligomers appear to specifically accumulate in mitochondria.

Materials and Methods

Synthesis of Vector 1. Tributylphosphine (10.3 μL, 0.039 mmol) was added to a solution of compound **13** (34 mg, 0.039 mmol) and Cs_2CO_3 (15.3 mg, 0.047 mmol) in a 1:1 mixture of MeOH/DMF (2 mL), and the mixture was stirred at room temperature for 40 min. Then a solution of **12** (66.2 mg, 0.039 mmol) in DMF (1.3 mL) was added, and the reaction was stirred for 1 h at room temperature after which more Cs_2CO_3 (30 mg, 0.09 mmol) was added. The solvent was evaporated, Et_2O was added, the mixture was sonicated, the precipitate was filtered off, and this process was repeated. The resulting combined solids were dissolved in acetone/MeOH, KPF_6 was added, and the mixture was sonicated. The precipitate was filtered off, and the filtrate (containing the product) was evaporated to dryness. The residue was dissolved in $CHCl_3$/MeOH 5%, where KPF_6 precipitates, the solid was filtered off, and the filtrate was concentrated to dryness. The resulting crude residue was purified by column chromatography on reverse-phase silica gel (H_2O/CH_3CN, 1:1 → 3:7), and the fractions containing product were evaporated. The residue was first precipitated in H_2O, and the solid was filtered off and further dissolved in CH_2Cl_2/MeOH 5%. The resulting solid was removed by filtration, and the solution was concentrated to dryness, affording **1** (40 mg, 50%) as tetrahexafluorophosphate salt. ^{1}H NMR (acetone-d_6, 500 MHz) δ 8.60 (br s, 1H), 8.42−7.82 (m, 3H), 7.80−7.70 (m, 6H), 7.65 (m, 1H), 7.58−7.47 (m, 8H), 6.85 (m, 1H), 6.77−6.67 (m, 3H), 3.85−3.70 (m, 8H), 3.70−3.50 (m, 18H), 3.10−2.90 (m, 10H), 2.85−2.70 (m, 8H), 2.35−2.20 (m, 8H), 2.05−1.90 (m, 8H), 1.10 (s, 9H); ^{13}C NMR (acetone-d_6, 125 MHz, DEPT) δ 153.8 (C), 152.3 (C), 136.7 (2 × CH), 134.2 (C), 134.1 (C), 131.3 (2 × CH), 130.7 (CH), 130.5 (CH), 129.2 (CH), 125.7 (CH), 113.8 (CH), 111.4 (C), 103.8 (CH), 67.2 (CH_2), 51.4 (CH), 51.2 (CH), 49.1 (CH), 46.4 (CH_2), 37.5 (CH_2), 27.6 (CH_3), 27.0 (CH_2), 23.8 (CH_2), 20.1 (C); MS (MALDI) *m*/*z* 1446.2 [M − 3HPF_6 − PF_6]$^+$, 1592.2 [M − 2HPF_6 − PF_6]$^+$.

Synthesis of Vector 2. A suspension of **1** (20 mg, 0.009 mmol) in a 2:1 mixture of CH_3CN/3 M HCl (3 mL) was stirred for 24 h at room temperature. The solvent was evaporated, Et_2O was added, the mixture was sonicated, and the solid was filtered off. To this solid H_2O and $KHCO_3$ were added (color changed), and the mixture was purified by column chromatography on reverse-phase silica gel (H_2O, then H_2O/CH_3CN, 7:3 → 65:35 → 6:4 → 1:1, all solvent mixtures containing 0.025 M KPF_6 and 0.1% TFA). The fractions containing product were

(19) Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000**, *56*, 318−325.
(20) Haack, T.; Peczuh, M. W.; Salvatella, X.; Sanchez-Quesada, J.; de Mendoza, J.; Hamilton, A. D.; Giralt, E. *J. Am. Chem. Soc.* **1999**, *121*, 11813−11820.
(21) Salvatella, X.; Haack, T.; Gairi, M.; de Mendoza, J.; Peczuh, M. W.; Hamilton, A. D.; Giralt, E. In *NATO ASI Series, Series C: Mathematical and Physical Sciences*; Pons, M., Ed.; Kluwer Academic Publishers: Dordrecht, 1999; pp 267−280.
(22) Fernandez-Carneado, J.; Kogan, M. J.; Castel, S.; Giralt, E. *Angew. Chem., Int. Ed.* **2004**, *43*, 1811−1814.
(23) Fernandez-Carneado, J.; Kogan, M. J.; Pujals, S.; Giralt, E. *Biopolymers* **2004**, *76*, 196−203.

concentrated to dryness, and the resulting residue was dissolved in CH_2Cl_2/acetone 5%, where KPF_6 precipitates. After filtration and evaporation of the filtrate, the product was precipitated in Et_2O, yielding **2** (13 mg, 74%) as tetrahexafluorophosphate salt. ^{1}H NMR (acetone-d_6, 500 MHz) δ 8.60−8.20 (m, 2H), 7.50−7.20 (m, 4H), 6.80−6.60 (m, 5H), 3.80−3.70 (m, 8H), 3.70−3.50 (m, 18H), 3.15−2.70 (m, 18H), 2.30−2.15 (m, 8H), 2.05−1.85 (m, 8H); MS (MALDI) *m*/*z* 1208.2 [M − $3HPF_6$ − PF_6]$^+$, 1354.1 [M − $2HPF_6$ − PF_6]$^+$.

Synthesis of CF-RKKRRQRRR-NH_2 (CF-Tat) and CF-RQIKIWFQNRRMKWKK-NH_2 (CF-Antp). The peptides were synthesized on Rink Amide resin and labeled at the N-terminus with 5(6)-carboxyfluorescein,[24] following the procedure described in the Supporting Information.

Cell Culture and Treatments with 1, 2, CF-Tat, and CF-Antp. HeLa cells were obtained from ATCC (Manasas, VA). They were maintained in DMEM (Dulbecco's Modified Eagle Medium) culture medium (1000 mg/L glucose; Biological Industries) containing 10% Fetal Calf Serum (FCS), 2 mM glutamine, 50 U/mL penicillin, and 0.05 g/mL streptomycin. For experiments, exponentially growing HeLa cells were detached from the culture flasks using a trypsin−0.25% EDTA solution, and the cell suspension was seeded at a concentration of 21.4 × 10^3 cells/cm^2 onto glass coverslips, 4-well Lab-TeckTM chambered coverglass, plastic dishes, or 96-well plates (Nalge Nunc International) depending on the experiment. Experiments were carried out 24 h later, when the confluence was ∼60−70%. CF compounds were dissolved in PBS (phosphate buffer saline) with 0.04% dimethyl sulfoxide and sterilized with 0.22 μm filters (Millex-GV, PVDF, Durapore, Millipore). Compounds **1** and **2**, labeled peptides, and 5(6)-carboxyfluorescein stock solutions were diluted in the cell culture medium. Nonadherent cells were washed away, and attached cells were incubated at 37 °C in 5% CO_2 in DMEM medium with a known concentration of guanidinium oligomers, CF peptides, or CF.

Confocal Laser Scanning Microscopy (CLSM). HeLa cells were incubated for 1 h at 37 °C in the presence of 5% CO_2 with CF (as negative control), CF-Tat and CF-Antp (as positive controls), and compound **1** at a concentration of 1 μM. Then cells were rinsed 3 times with PBS and fixed in a 3% paraformaldehyde solution in 0.1 M PBS containing 60 μM sacarose for 15 min. Cells were then washed with phosphate buffer for 5 min and mounted with Mowiol-Dabco mounting medium. CLSM was performed using an Olympus Fluoview 500 confocal microscope with a 60X/1.4 NA objective. CF fluorescence was excited with the 488 nm line of an argon laser, and its emission was detected over a range of 515−530 nm. As a control for the fixation step, similar experiments were performed in cells plated onto glass bottomed Lab-TekTM chambers for live cell imaging. After incubation for 1 h, cells were washed 4 times with PBS containing 1.1 mM $CaCl_2$ and 1.3 mM $MgCl_2$ and images were taken within the next 30 min. The microscope settings were identical for each compound and dose. In 4 °C experiments the culture medium was added with Hepes buffer at a final concentration of 25 mM. The addition of the CF compounds as well as the incubation for 1 h was carried out while controlling the temperature at 4 °C. For colocalization studies, MitoTracker Orange CM-H2 TM Ros or ERtracker blue-white DPX (Molecular Probes) was added at a final concentration of 500 nM or 700 nM, respectively, for the last 30 min of the incubation. The cells were washed and fixed as described previously. MitoTracker fluorescence was excited with the 543 nm line of an argon laser, and its emission was detected over a range of 560−600 nm. ERtracker fluorescence was observed in a DMRB fluorescence microscope equipped with a Apo 63 × 1.32 oil PH3 objective and a BP 340−380 filter (UV).

Microplate Fluorimetry Assay. For each assay 21.4 × 10^3 cells/cm^2 were seeded and cultured for 24 h. After complete adhesion to the plate, the culture medium was exchanged. The cells were incubated for 1 h at 37 °C under 5% CO_2 with the fresh medium containing CF-labeled compounds or CF. Cells were washed 3 times with PBS. The measurement of emitted fluorescence was carried out after 30 min of incubation with lysis buffer (0.1% Triton X-100 in 50 mM Tris, pH 8.5) in a FL600 Microplate fluorescence reader (Bio-Tek). Fluorescence was measured at $\lambda_{excitation}$ = 485/20 nm and $\lambda_{emission}$ = 530/25 nm. Triplicates of each concentration were performed, and the fluorescence emitted for the blanks (only cells) was subtracted. The experiment was reproduced 3 times.

Flow Cytometry. For each assay 21.4 × 10^3 cells/cm^2 were seeded and cultured for 24 h in plastic dishes. After 24 h the culture medium was exchanged and the cells were incubated for 1 h at 37 °C under 5% CO_2 with fresh medium containing CF-labeled compounds or CF as a negative control. Cells were washed with PBS, treated with trypsin for 5 min at 37 °C, and collected in falcon tubes in cold PBS. After centrifugation at 1000 rpm for 4 min at 4 °C, the trypsin-containing solution was discarded and the cells were resuspended in 25 mM Hepes-buffered cell culture medium containing propidium iodide at a final concentration of 5 μg/mL. Fluorescence analysis was performed with a Coulter XL flow cytometer. Cells stained with propidium iodide were excluded from further analysis. A minimum of 10 000 events per sample were analyzed twice. Results shown are the average of two measures in the flow cytometer. Bars indicate standard deviations.

MTT Assay.[25,26] HeLa viability in the presence of **1**, **2**, CF-Tat, and CF-Antp was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see Supporting Information).

Results and Discussion

Synthesis. In an earlier work[27] we described the preparation of symmetric tetraguanidinium oligomers **3** (and **4**) (as their tetrachloride salts) from the bromo derivative **5**, which was transformed into an intermediate sulfide (by means of a thiouronium salt) that reacted with deprotected compound **6** to give a nonsymmetric dimer **8** (dichloride salt) in a 53% overall yield (Chart 2). Subsequent transformation of **8** into the symmetric tetramer **3** was achieved in two steps via the activated derivative **9**, in 66% yield.[27] However, attempts to expand iteratively this synthetic strategy to higher oligomers or to nonsymmetric tetramers (such as targets **1** and **2** endowed with the fluorescent label CF) were found inefficient and/or difficult to reproduce. A major improvement results from a few changes in the synthetic scheme, such as replacing bromide by mesylate as leaving group, use of hexafluorophosphate instead of chloride as counterion, or generating the nucleophilic sulfide via disulfide intermediates. Thus, readily available mesylate **7**[28] was first transformed into a thioester with potassium thioacetate, followed by treatment with methanesulfonic acid. Reduction of the resulting disulfide to the sulfide with tributylphosphine followed by in situ reaction with another equivalent of **7** afforded dimeric monoalcohol **8** (as dihexafluorophosphate salt) in a 75% overall yield. The alcohol was activated again as mesylate (94%), and the whole process was repeated to give the nonsymmetric tetramer **11** in a 77% overall yield.[29] Finally, CF-containing vectors **1** and **2** were prepared as follows: activation of the free OH in **11** by mesylation (compound **12**, 95% yield) and

(24) Fernandez-Carneado, J.; Giralt, E. *Tetrahedron Lett.* **2004**, *45*, 6079−6081.
(25) Holst-Hansen, C.; Brünner, N. In *Cell Biology: A laboratory Handbook*, 2nd ed.; Celis, J. E., Ed.; Academic Press: 1998; Vol. 1.
(26) Liu, Y.; Peterson, D. A.; Kimura, H.; Schubert, D. *J. Neurochem.* **1997**, *69*, 581−593.
(27) Sanchez-Quesada, J.; Seel, C.; Prados, P.; de Mendoza, J. *J. Am. Chem. Soc.* **1996**, *118*, 277−278.
(28) Breccia, P.; Van Gool, M.; Perez-Fernandez, R.; Martin-Santamaria, S.; Gago, F.; Prados, P.; de Mendoza, J. *J. Am. Chem. Soc.* **2003**, *125*, 8270−8284.
(29) This iterative strategy can be continued further to the corresponding octamer without substantial decrease in yield (manuscript in preparation).

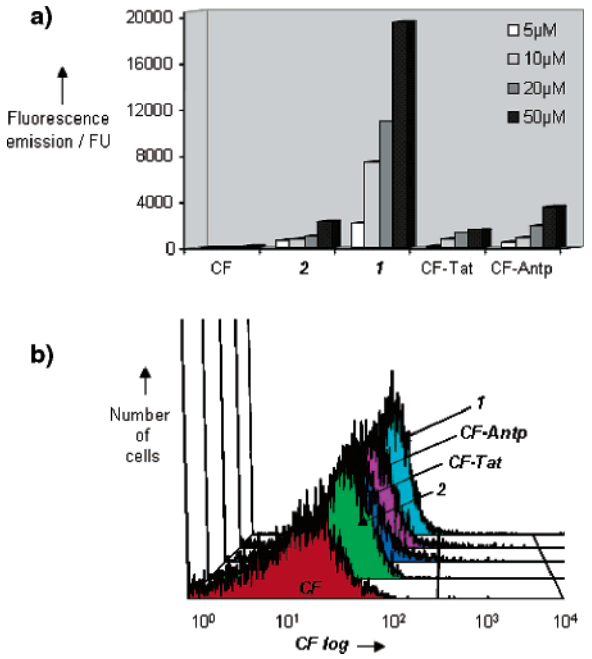

Figure 1. (a) Fluorescence measured in a microplate fluorescence reader after incubation of HeLa cells for 1 h at 37 °C with the tetraguanidinium oligomers **1** and **2**, CF-Tat and CF-Antp (as positive controls), and CF (as negative control) at concentrations ranging from 5 μM to 50 μM. (b) Fluorescence obtained by flow cytometry after incubating HeLa cells with **1**, **2**, CF-Tat, CF-Antp, or CF at 1 μM for 1 h at 37 °C.

subsequent reaction (50% yield) with the CF disulfide derivative **13** (obtained from CF and cystamine dihydrochloride in a 50% yield). For further details see the Supporting Information.

Internalization and Cytotoxicity Studies. Preliminary experiments were performed by incubating HeLa cells with oligomers **1** and **2** on a 96-well microplate. The 16-amino acid sequence from the third helix of the Antennapedia homeodomain (Antp), RQIKIWFQNRRMKWKK-NH_2, and the 9-amino acid peptide corresponding to the 49−57 shortened Tat peptide (Tat), RKKRRQRRR-NH_2, both labeled at the N-terminal position with CF, were used as positive controls, whereas CF itself was used as a negative one. All compounds were internalized in a dose-dependent manner at concentrations varying from 5 to 50 μM (Figure 1a). Interestingly, **1** was internalized at 5 μM at the same rate as **2** or controls CF-Tat and CF-Antp at much higher concentrations (50 μM). Since the only difference between **1** and **2** is the *tert*-butyldiphenylsilane group (TBDPS) positioned at one end of the tetraguanidinium chain, the increased uptake by a factor of 4 highlights the importance of the presence of a hydrophobic moiety well apart from the guanidinium groups.

Preliminary cytotoxicity studies (viability assays at concentrations up to 10 μM with MTT) showed that the best internalized compound **1**, incubated for 24 h with HeLa cells, reduced cell viability to 28% at 10 μM, but no cytotoxicity was evidenced at 5 μM (Figure 2), so the internalization process was studied again at much lower concentrations, ranging from 0.1 to 1 μM (Figure 3a).[30] In good agreement with the preliminary data obtained using a microplate fluorimeter, flow cytometry revealed that **1** was better internalized than CF-Antp, while **2** and CF-Tat were both internalized to a similar degree (Figure 1b).

(30) The technique was also changed to avoid nondesired and noncontrolled attachment of the compounds to the plastic of the fluorimeter plates.

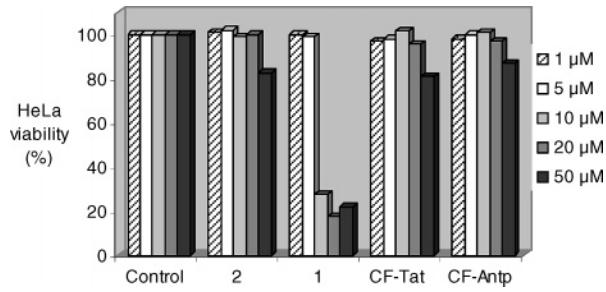

Figure 2. Cytotoxicity of **1**, **2**, CF-Tat, and CF-Antp. HeLa viability was quantified by MTT staining after 24 h of incubation.

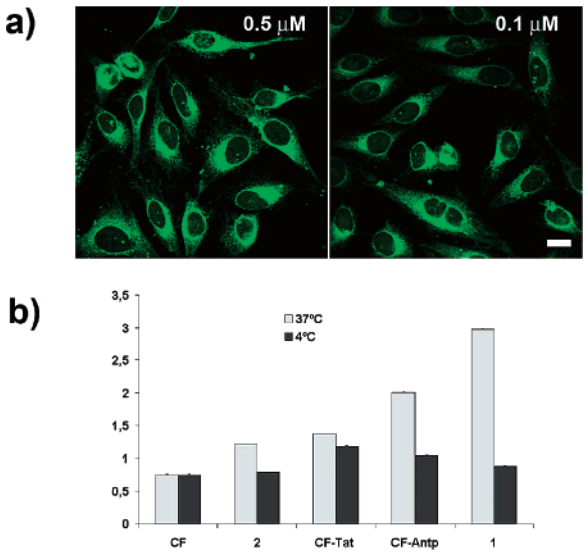

Figure 3. (a) CLSM images of HeLa cells incubated for 1 h with 0.5 μM (left) or 0.1 μM (right) **1** at 37 °C (Scale bar = 10 μm). (b) Fluorescence measured by flow cytometry after incubating HeLa cells with **1**, **2**, CF-Tat and CF-Antp (1 μM) at 37 °C or 4 °C.

The internalization of **1** in HeLa cells was also studied by confocal laser scanning microscopy (CLSM). After incubation of HeLa cells with **1** for 1 h at 37 °C, the cells were washed with HBBS-Hepes and directly observed by CLSM. Tetraguanidinium **1** showed a higher accumulation in HeLa cells than either CF-Tat or CF-Antp. Remarkably, **1** was only found in the cytoplasm, whereas CF-Tat and CF-Antp also accumulated in the nucleolus (Figure 4).

To gain further insight into the pathway of guanidinium oligomer translocation,[31−33] flow cytometry experiments with **1** were also performed at 4 °C. Cellular uptake proved to be temperature dependent, as uptake of **1** at 4 °C was substantially reduced, accounting for an active energy-dependent transport (Figure 3b).

Furthermore, kinetics of the internalization process showed that a significant accumulation of **1** was observed inside the cells after only 5 min, although the internalization increased progressively and did not reach saturation after 2 h of incubation (Figure 5a). This result is in agreement with a nonsaturable, passive diffusion internalization mechanism. Thus, both paths could be involved. A more detailed observation by CLSM of

(31) Suzuki, T.; Futaki, S.; Niwa, M.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2002**, *277*, 2437−2443.
(32) Thoren, P. E. G.; Persson, D.; Isakson, P.; Goksor, M.; Onfelt, A.; Norden, B. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 100−107.
(33) Rothbard, J. B.; Jessop, T. C.; Lewis, R. S.; Murray, B. A.; Wender, P. A. *J. Am. Chem. Soc.* **2004**, 126, 9506−9507.

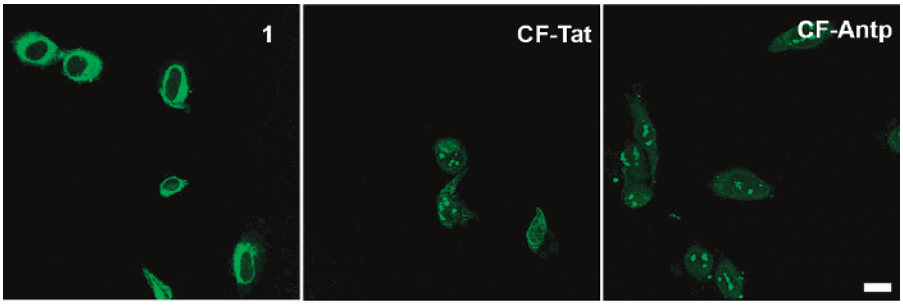

Figure 4. CLSM observation in vivo of HeLa cells after incubating them for 1 h with **1**, CF-Tat, or CF-Antp at 1 μM concentration and 37 °C (Scale bar = 10 μm).

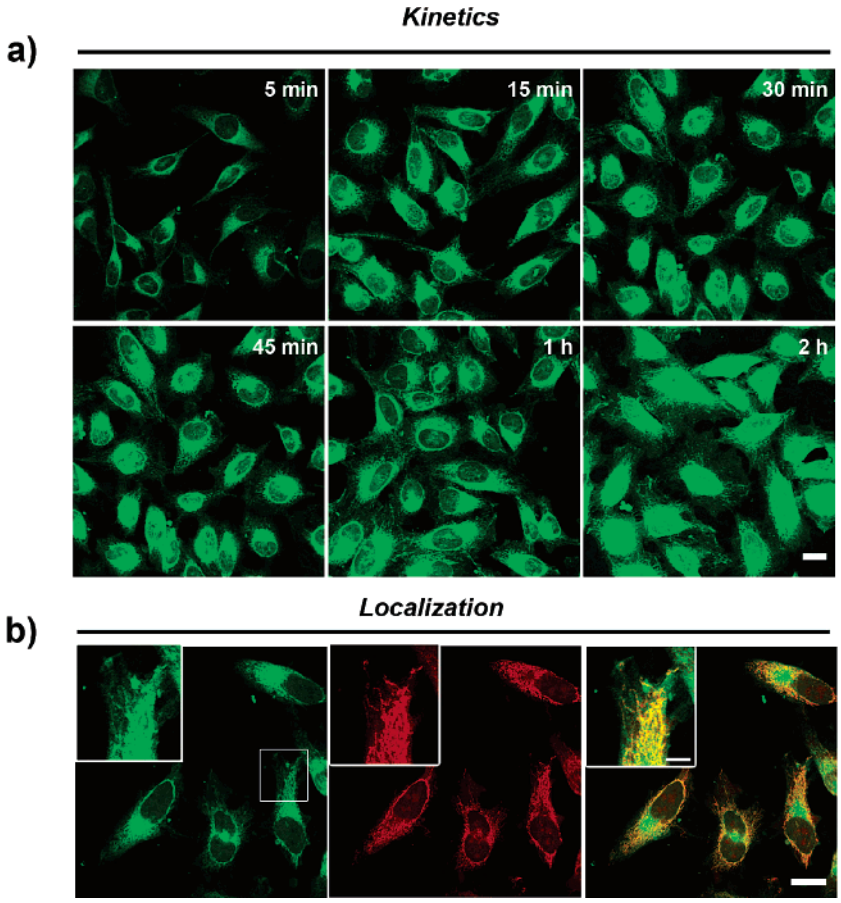

Figure 5. (a) Kinetic assay: images obtained by CLSM after incubation of HeLa cells with 0.5 μM **1** at 37 °C. (b) Distribution of **1** and colocalization with the mitochondrial marker MitoTracker Orange. HeLa cells were treated with 1 μM **1** (green) for 1 h and 500 nM MitoTracker (red) for 30 min (Scale bar = 10 μm). The combined image shows costained regions in yellow (Inset scale bar = 2 μm).

the subcellular localization of **1** revealed accumulation in certain subcellular organelles, that pointed to mitochondrial or endoplasmic reticulum localization, difficult to identify by simple observation (Figure 3a). A series of experiments incubating 1 μM **1** with 500 nM MitoTracker Orange, a dye that accumulates in active mitochondria, showed colocalization of both compounds in HeLa cells at 37 °C, indicating that **1** was retained (at least partially) in the mitochondria (Figure 5b). Experiments to rule out whether accumulation occurred in the endoplasmic reticulum revealed no colocalization in this organelle.

Conclusion

We have reported a new family of powerful nonpeptidic cell-penetrating tetraguanidinium oligomers. These compounds show more efficient translocation through HeLa membranes than Antp or Tat at very low concentrations and, interestingly, accumulate in mitochondria.[34] The potential use of this new type of internalizing vectors in the delivery of therapeutic agents into cells is currently under investigation. Their specific accumulation in mitochondria could be exploited for the delivery of antioxidants in cancer therapies.[35,36]

Acknowledgment. This article is dedicated to Professor José Elguero on the occasion of his 70th birthday. Financial support from the European Union (QLK2-CT-2001-01451), MCYT-FEDER (Bio2002-2301), CICYT (BQU2002-03536), Gener-

(34) As recently reported, Tat and Antp do not cross the mitochondrial membrane: Ross, M. F.; Filipovska, A.; Smith, R. A. J.; Gait, M. J.; Murphy, M. P. *Biochem. J.* **2004**, *383*, 457−468.
(35) Murphy, M. P.; Smith, R. A. J. *Adv. Drug Delivery Rev.* **2000**, *41*, 235−250.
(36) Smith, R. A. J.; Porteous, C. M.; Gane, A. M.; Murphy, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5407−5412.

alitat de Catalunya (CeRBa and SGR), Universidad Autónoma de Madrid (V. M.), and ICIQ Foundation is gratefully acknowledged.

Supporting Information Available: Synthesis and characterization of compounds **8**−**13**, synthetic procedure for the preparation of labeled peptides CF-Tat and CF-Antp, and description of the MTT assay. This material is available free of charge via the Internet at http://pubs.acs.org.

JA044006Q