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Cationic Nucleoside Lipids Based on a 3-Nitropyrrole Universal Base for siRNA Delivery

Claire Ceballos[†], **Carla A.H. Prata**[‡], **Suzanne Giorgio**[†], **Frédéric Garzino**[†], **Dominique Payet**[§], **Philippe Barthélémy**[#,ʄ], **Mark W. Grinstaff**[‡,*], and **Michel Camplo**[†,‡,*]

[†]Centre Interdisciplinaire de Nanosciences de Marseille CINaM, Upr-Cnrs 3118, Université Aix-Marseille II, Luminy, case 913, 13288 Marseille cedex 09, France.

[‡]Departments of Chemistry and Biomedical Engineering, Metcalf Center for Science and Engineering, Boston University, Boston MA 02215, USA.

[§]Centre d'Immunologie de Marseille Luminy, Parc scientifique de Luminy, 13288 Marseille, Cedex 9, France.

[#]Inserm, U869, Bordeaux, F-33076 France.

[ʄ]Université de Bordeaux, F-33076, Bordeaux, FranceInserm, U869, Université Victor Segalen Bordeaux 2, F-33076, Bordeaux, France.

Abstract

Cationic nucleoside lipids based on a 3-nitropyrrole universal base were prepared from D-ribose using a straightforward chemical synthesis. Several studies including DLS, TEM and ethidium bromide (EthBr) assay demonstrated that these amphiphilic molecules form supramolecular organizations of nanometer size in aqueous solutions and are able to bind nucleic acids. siRNA knockdown experiments were performed with these nucleolipids and we observed protein knockdown activity similar to the siPORT *NeoFX* positive control. No significant cytotoxicity was found.

Nucleolipids --amphiphiles possessing both nucleic acid recognition and lipophilic alkyl chains components-- are emerging as both tools for constructing supramolecular assemblies and for nucleic acid delivery.(1–13) The area of nucleic acid delivery is particularly interesting, since gene delivery provides a mechanism to introduce new genes in cells for basic *in vitro* research experiments as well as to replace or supplement a defective or missing gene in a patient as part of a clinical therapy.(14–20) Consequently, synthetic lipids, as well as other DNA carriers such as calcium phosphate particles, dendrimers, or surface-mediated assemblies(21), are highly sought-after. Minimally toxic to cells, these lipids also afford high DNA or RNA transfection activity. Recently, we have reported the synthesis and transfection activity of a series of cationic and glycosylated nucleolipids which are both nontoxic and active DNA transfection agents. (11) (13) In an ongoing effort to expand the scope and properties of this family of lipids we are 1) synthesizing analogs where the thymine nucleobase is replaced by the synthetic 3-nitropyrrole universal base, and 2) evaluating the potential of these nucleolipids for siRNA delivery. Herein, we report the synthesis, physico-chemical properties, and siRNA transfection efficiency of a universal-base nucleolipid, the tosylate salt of 1-(2', 3'-dioleyl-5'-trimethylammonium-(α,β)-D-ribofuranosyl)-3-nitropyrrole.

Contact : mgrin@bu.edu or camplo@univmed.fr.

The target nucleolipid, **7**, is shown in Figure 1, which possesses three key components: a quaternized amine at the 5' position, two oleic acid chains linked at the 2' and 3' positions, and a 3-nitropyrrole attached at the anomeric position. The cationic charge is responsible for electrostatic binding to the nucleic acids and the oleyl chains contribute to stabilization of the resulting nucleic acid-nucleolipid assembly through hydrophobic chain-chain interactions. The 3-nitropyrrole, which is perhaps the best characterized universal base analogue, was introduced to circumvent the restrictive hydrogen bonding pattern controlling typical purine-pyrimidine hydrogen motifs by incorporating the weakly hydrogen bonding nitro substituent.(22–24) It is known that the 3-nitropyrrole group can form complementary base-pairs with all four natural bases without significantly destabilizing neighboring base-pair interactions. As a result, it has been used for diverse applications including hybridization of DNA duplexes, oligonucleotide probes and primers, peptide nucleic acids, and sequencing by MALDI mass spectrometry. (25)

The alpha and beta anomers of 1'-(2',3'-dioleyl-5'-trimethylammonium–D-ribofuranosyl)-3-nitropyrrole, **7a** and **7b**, respectively, were synthesized from D-ribose as shown in Scheme 1. Briefly stated, 5-O-*tert*-butyldimethylsilyl-2, 3-O-isopropylidene-D-ribose, **2**, was prepared in two steps from D-ribose following a published procedure.(26) Next, the stereoselective chlorination of the protected sugar, **2**, with CCl_4 and hexamethylphosphorus triamide (HMPT) (27) was followed by coupling with the anion of 3-nitropyrrole formed from the commercially available 1-(triisopropylsilyl)pyrrole.(28) Deprotection of the resulting derivative **3** afforded the mixture of (α) **4a** (53%) and (β) **4b** (18%) anomers with the (α) form as the major isolated polar isomer. The relative stereochemistry of these compounds was assigned by NOESY and COSY experiments (data not shown). Compounds **4a** and **4b** were then separately tosylated with TsCl/TEA to give **5a** and **5b** (structures not shown), deprotected with TFA/H_2O, and coupled with oleic acid using DCC/DMAP to afford the nucleo-lipids **6a** and **6b**. Finally, **6a** and **6b** were reacted with trimethylamine for two days in a sealed tube to afford the nucleolipids **7a** and **7b**. The complete characterization data, including ^1H NMR, ^{13}C NMR, and elemental analysis, can be found in the SI. A differential scanning calorimetry (DSC) trace of the hydrated nucleolipid **7a** or **7b** shows no phase-transition temperature from −40 °C to 80 °C.

Next, to determine whether the universal base nucleolipids **7a** and **7b** bind nucleic acids, we performed a standard ethidium bromide fluorescence displacement assay with calf-thymus DNA as a model system.(29) DNA binding was observed for **7a** and **7b** as the fluorescence intensity decreased rapidly (see S.I. for curves and details). The nucleolipids formed a 6:1 and 2:1 complex for **7a** and **7b** with a binding constant of $\approx 10^{-7}$ M, $^{-1}$ similar in magnitude to DOTAP.(30) This universal base, 3-nitropyrrole, was expected to bind DNA or RNA based on the published data. Previous thermal denaturation studies with 1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrole showed that this base pairs with the four naturally occurring bases with little discrimination.(31,32) The nitro group of 3-nitropyrrole polarize the π–aromatic system enhancing the π–stacking interactions, and yet as a poor hydrogen bond participator it does not distinguish between the bases. Considering the minor differences between DNA and siRNA in terms of base composition and ability to hydrogen bond and from π-stacking arrangements, the 3-nitropyrrole base is likely to interact with the bases of siRNA as well as DNA.

As **7a** and **7b** are amphiphilic, the dispersion of these nucleolipids in water by sonication leads to vesicles in the presence and absence of DNA. Again DNA was used as a model system. In the absence of DNA, vesicle size, as measured by DLS, was 84.7 nm and 458.4 nm for compounds **7a** and **7b** respectively. For **7b** we observed a broad distribution of vesicle sizes. In the presence of DNA, and at different compound/DNA charge ratios, lipoplexes were formed of 132.4 nm (**7a**/DNA 2:1), 179.3 nm (**7a**/DNA 5:1), and 469.8 nm (ratio **7b**/DNA 2:1), 516.1 nm (ratio **7b**/DNA 5:1). Transmission electron microscopy (TEM) experiments were

performed on vesicle samples **7a** and **7b** using a Philips CM 10 (negative staining with ammonium molybdate 1% water, Cu/Pd carbon coated grids). Both anomers (α and β) of the nucleolipids formed small vesicles. Representative images of vesicles formed by **7a** and **7b** with diameters of roughly 80 nm and 220 nm respectively are shown in Figure 2. Similar experiments performed with vesicles formed from nucleolipid-DNA solutions were inconclusive as to the location of the DNA within the structure.

The transfection of siRNA with **7a** and **7b** was next performed to evaluate the reduction in protein synthesis. Experiments were performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Ambion). Complete details can be found in the SI and the transfection experiments were performed in the presence of serum. Three distinct cell lines were used, including human liver (HepG2), mouse fibroblast (NIH 3T3), and Chinese Hamster Ovarian (CHO) cells. siRNA transfection results were accessed after 48h as a function of both anomer and cation /anion (N/P) mole ratio, and the experiments were performed in serum-containing media. In each experiment, controls using GAPDH siRNA and negative control siRNA were used at 30 mM. siPORT *NeoFX* was used as the positive control. As shown in Figure 3, siRNA knockdown was observed for both nucleolipids in the three different cell lines, with values comparable, not significantly significant, to the positive control - *NeoFX*. The transfection activity of **7a** was significant vs siRNA ($p < 0.05$) in NIH 3T3 and CHO cells with N/P ratios of 1, 5, 15, and 20 and 1 and 5, respectively. The transfection activity of **7b** was significant vs siRNA ($p < 0.05$) in CHO cells with N/P ratios of 15 and 20, respectively. The configuration of the anomeric position does not have a significant role on the transfection. In addition, none of the nucleolipids showed a significant cytotoxicity at this concentration using an MTS assay with CHO cells as the viability was similar to the negative control (data no shown).

In summary, we have synthesized and characterized a new universal base nucleolipid for siRNA delivery. The nucleolipids possess a cationic charge, a 3-nitropyrrole base, and two oleyl chains, and these lipids form vesicles in the presence and absence of nucleic acids. siRNA delivery and resulting protein knockdown was observed in three separate cell lines: hamster ovarian, mouse fibroblast, and human liver cells. This is the first report of such activity with a nucleolipid. Work is in progress to synthesize additional universal nucleolipids (including the nitroindole and nitroimidazole analogues for siRNA delivery), as well as to develop a structure-activity relationship. Nucleolipids are one of the newest types of synthetic lipid vectors being explored and, like others in the lipid family, offer the potential advantages of ease of synthesis, carriers of large and small nucleic acid payloads, and minimal toxicity.(11, 14–17,33–42). The synthesis and evaluation of new lipids that possess unique head groups, backbones, or hydrophobic chains for nucleic acid binding and/or self-assembly will facilitate the identification of optimal carriers for nucleic acids *in vitro* and *in vivo*; efforts in this area should be encouraged and promoted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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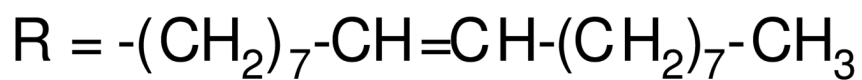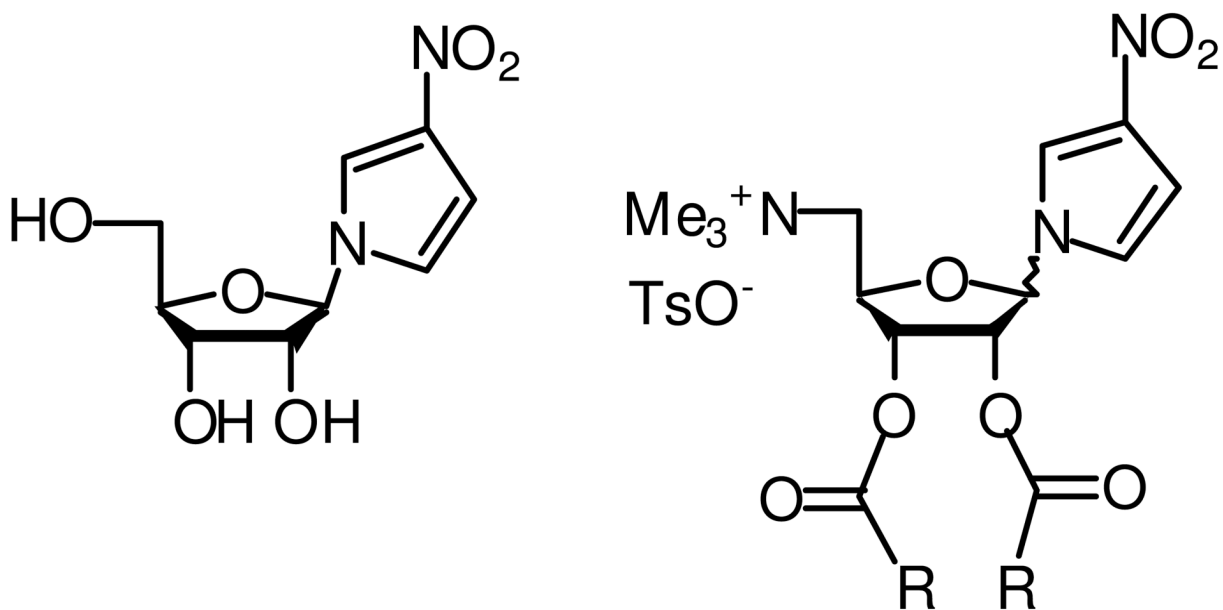
Figure 1.
Chemical structure of the universal nucleobase and nucleolipid (tosylate salt of 1'-(2',3'-dioleyl-5'-trimethylammonium-α β–D-ribofuranosyl)-3-nitropyrrole).
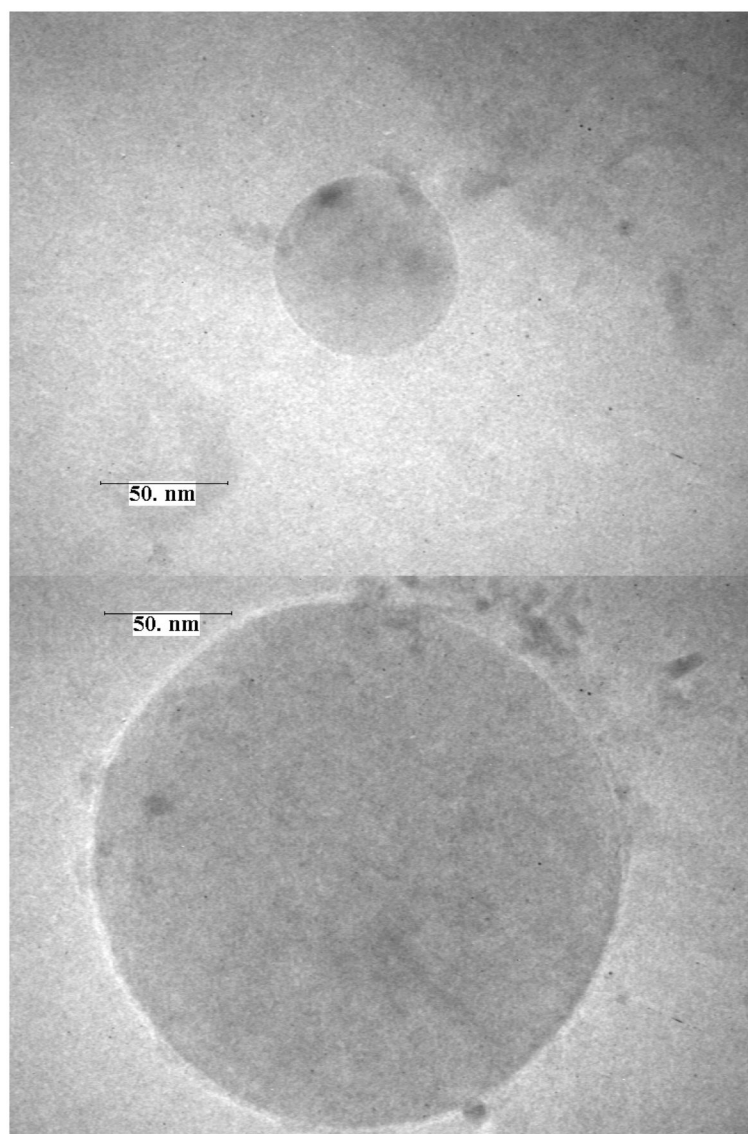
Figure 2.
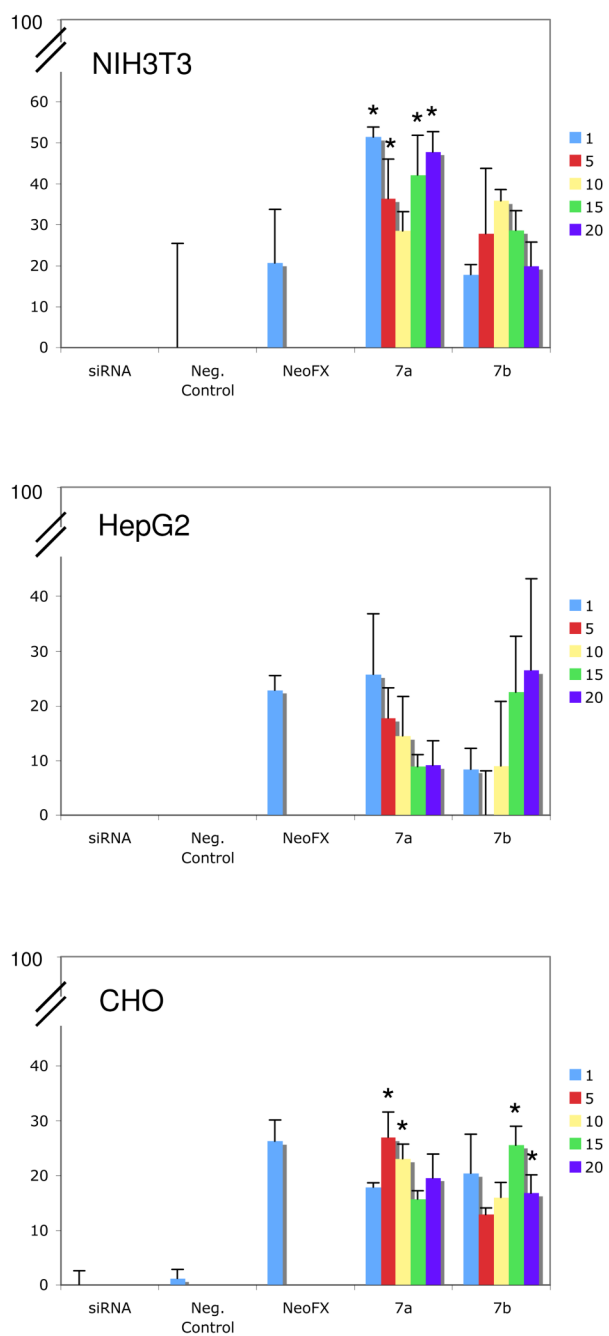TEM image of single isolated vesicles obtained with 7a (top) and 7b (bottom).
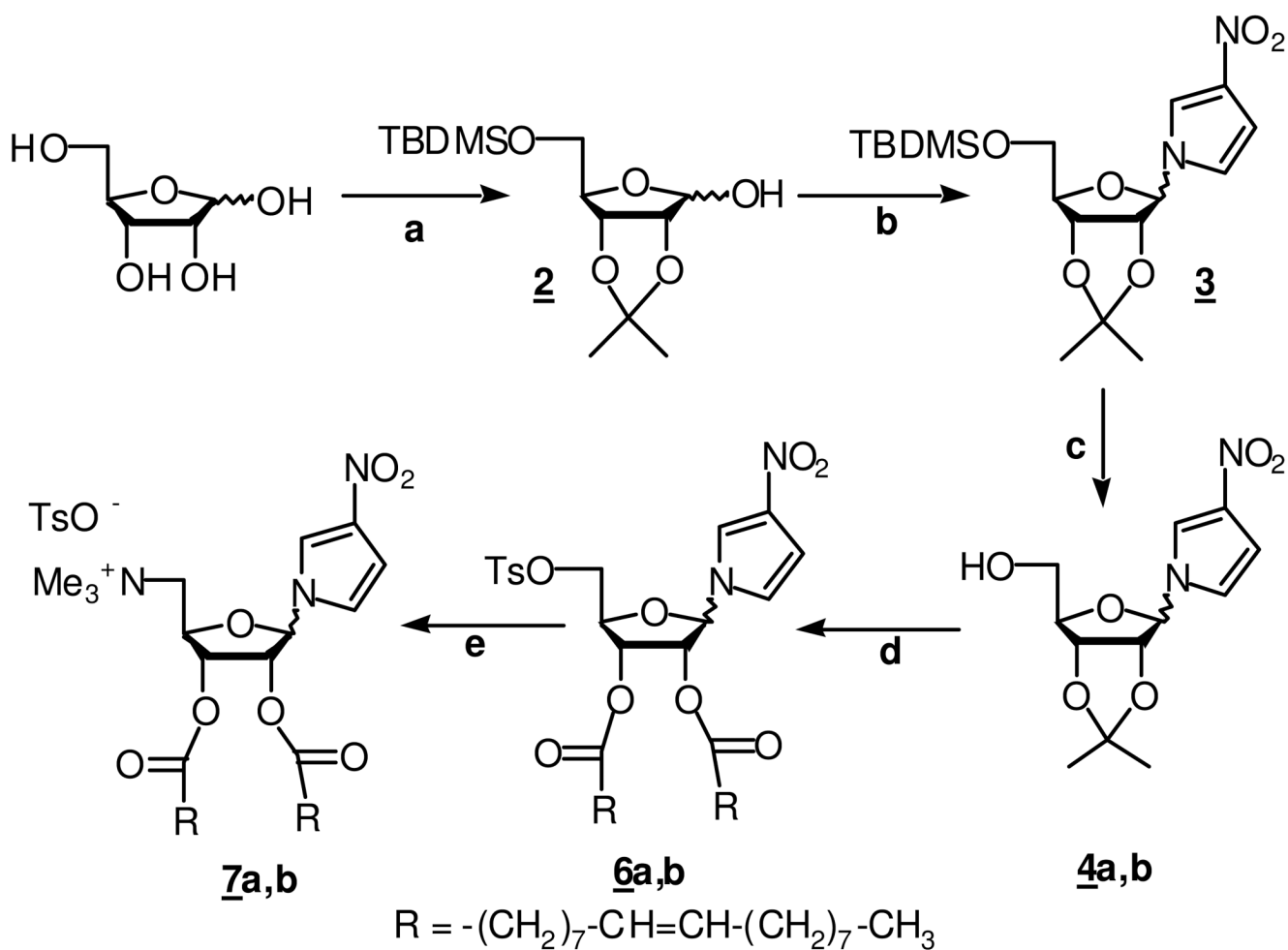
Figure 3.
siRNA transfection results after 48h in HepG2, NIH 3T3, and CHO cells as a function of
nucleolipid (**7a** and **7b**) and N/P mole ratio. N = 3; * p< 0.05 vs siRNA control.

Scheme 1.
Reaction conditions: (a) (i) acetone, CuSO$_4$, 94% yield, (ii) TBDMSCl, DMAP, TEA 35% yield; (b) (i) CCl$_4$, HMPT, (ii) 3-nitropyrrole, NaH, CH$_3$CN, 40% yield; (c) TBAF dry THF, 71% yield, α/β3:1; (d) (i) TsCl, TEA, DCM **5a** (94% yield), **5b** (56% yield)) (ii) TFA/H$_2$O, quant. (iii) Oleic acid, DCC/DMAP, **6a** (77% yield), **6b** (87% yield); (e) NMe$_3$, CH$_3$CN 60° C, 40 hrs, **7a** and **7b** quant. yields.