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Original article

Synthesis of acridine-nuclear localization signal (NLS) conjugates and evaluation of their impact on lipoplex and polyplex-based transfection

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Abstract

We report on the synthesis of various acridine(Acr)-spacer-nuclear localization signal (NLS) peptide conjugates and explore whether their use as NLS-labeling agent of plasmidic DNA could improve gene nuclear import and expression into cells when mediated by synthetic DNA complexes. As the conditions of successful use of the NLS properties to enhance gene transfer are not clear, and with the aim of detecting and defining the requirements of NLS-enhanced transfection, we investigated gene delivery and expression into various cell lines with various DNA complexes (lipoplexes or polyplexes) that were formulated for various N/P ratios from various preformed Acr-spacer-NLS/DNA complexes (1:1, 5:1 and 10:1 molar ratio). For the in vitro transfection assays, the lipoplexes and polyplexes were formulated from the preformed Acr-spacer-NLS/DNA complexes and dioctadecylamidoglycylspermine (DOGS)/dioleylphosphatidylethanolamine (DOPE) 1:1 mol and branched polyethyleneimine (PEI) 25 kDa, respectively, which are very efficient in vitro gene transfer systems. We show by fluorescence experiments that part of the acridine-NLS-conjugates remains intercalated within the plasmid for most of the N/P lipoplexes and polyplexes investigated. We show that, as several other studies performed with NLS-conjugates that are not covalently linked to DNA, the expression of the transgene is in most cases not improved upon complexation of plasmidic DNA with NLS-intercalating conjugates prior to its formulation as lipoplexes or polyplexes.

Keywords: Gene transfer; DNA complex; Non-viral vector; Synthetic vector

1. Introduction

The development of gene transfer vectors allowing gene expression into cells constitute not only a most attractive therapeutical approach (gene therapy) and a major breakthrough in the biomedical field for the treatment of various inherited and acquired diseases but also a powerful tool to study gene and protein function and regulation. For example, the delivery of a therapeutical gene into cancer cells is indeed a promising technology for the development of various anticancer strategies (replacement of a deficient tumor suppressor gene to re-establish the balance between growth and apoptosis, inhibition of a dominant oncogene, stimulation and modification of immune effector cells to recognize and reject cancer cells, amplification of tumor cell immunogenicity, chemo-/radiosensitization of tumor cells, chemo-/radio-protection of normal cells, gene suicide, inhibition of tumor vasculature growth). Gene transfer systems based on lipoplexes or polyplexes have gained wide acceptance over the last decade as gene transfer vectors. At present, however, their usefulness and applications as therapeutical devices are limited by transient and low levels of gene expression observed in vivo. One of the limiting steps responsible for a low gene expression with these non-viral vectors resides in an inefficient intracellular trafficking of DNA from the cytoplasm to the nucleus [1,2]. This is particularly of concern with postmitotic and quiescent cells for which the nuclear membrane breakdown does not occur periodically. Nevertheless, they present several advantages as compared with adenoviral or retroviral vectors including low-cost and large-scale production, and safety.

To overcome the cytoplasmic degradation of the gene via an effective transport into the nucleus and to improve the efficiency of gene expression, the use of nuclear localization signal (NLS) peptides for non-viral gene transfer has been widely investigated ([3–17] and references therein). Except during

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mitosis, macromolecules such as proteins or nucleic acids cannot enter the nucleus through the nuclear pore. The intranuclear transfer of cellular and viral proteins, DNA, and/or RNA occurs by means of an energy-dependent mechanism which involves peptidic NLS sequences that bind to structures called nuclear pore complex (NPC) via transport receptors such as importins α/β [3].

To date, literature indicates that the NLS approach has potential for improving DNA nuclear delivery and expression with non-viral vectors. Most of the studies involved the well-characterized simian virus (SV)40 T large antigen NLS-sequence ^{126}PKKKRKV^{132}. The various approaches explored differ mainly in the method used for the attachment (covalent [4,8,6,10] or non-covalent [5,9,11–17]) of the NLS peptide(s) to DNA and in the use of linear DNA [4,5,9] or circular (plasmidic) DNA [6,8–17]. While many of these approaches have met with limited success, a significant (10–1000-fold) enhanced gene expression was obtained following ligation of a NLS-oligonucleotide conjugate to one or both ends of a linear DNA [4]. There is further a controversy concerning the optimum number of NLS per DNA to enhance its nuclear delivery [4–6,9]. Moreover, the neutralization of the positive charges of the lysine and arginine residues of many NLS peptides (which are critical for their interactions with transport receptors and importins) by the negatively charged DNA phosphates is likely expected to hinder cargo recognition and binding to NPC [8]. Very recently, a highly cationic NLS peptide based on the HTLV sequence was shown to be more effective for condensing plasmid DNA into discrete particles and for enhancing gene expression levels compared to polylysine controls [9].

As a part of our contribution into this field, we explored whether the use of a non-covalent attachment of a SV40 NLS peptide to plasmidic DNA via intercalating conjugates (see structures in Fig. 1) could improve gene nuclear import and expression mediated by lipoplexes and polyplexes. As DNA intercalating agent, we selected an acridine-derived moiety. Acridine (Acr) is a heteroaromatic polycyclic molecule, which inserts tightly, but reversibly, between two base pairs in a DNA duplex by a combination of hydrophobic, electrostatic, hydrogen bonding, and dipolar forces [18]. The framework of the Acr-spacer-NLS-conjugates consists of an Acr moiety bearing a spacer of variable length in an attempt to separate the NLS peptide from DNA and to overcome the problem of the electrostatic interactions between the cationic NLS and anionic DNA [8,9]. NLS attachment to this Acr-spacer was performed via a chemically stable thioether bond between the maleimide-labeled spacer unit and a cysteine residue at the carboxy terminal of the peptide. As the conditions of successful use of the NLS properties to enhance gene transfer are not clear from literature data [3–5] and with the aim of detecting and defining the requirements of NLS-enhanced transfection, we investigated gene delivery and expression into various cell lines (NIH-3T3, A549) with various DNA complexes (lipoplexes or polyplexes) that were formulated for various N/P ratios (0.8, 1.25, 2.5, 5 and 10) from various preformed Acr-spacer-NLS/DNA complexes (1:1, 5:1 and 10:1 molar ratio). For the in vitro transfection assays, the lipoplexes and polyplexes were formulated from the preformed Acr-spacer-NLS/DNA complexes and dioctadecylamidoglycylspermine (DOGS)/dioleylphosphatidylethanolamine (DOPE) (1:1 mol) and branched polyethyleneimine (PEI) 25 kDa which are very efficient in vitro gene transfer systems ([19–22] and references therein). Moreover, various amounts of DNA (0.1 and 0.5 μg per well) were tested.

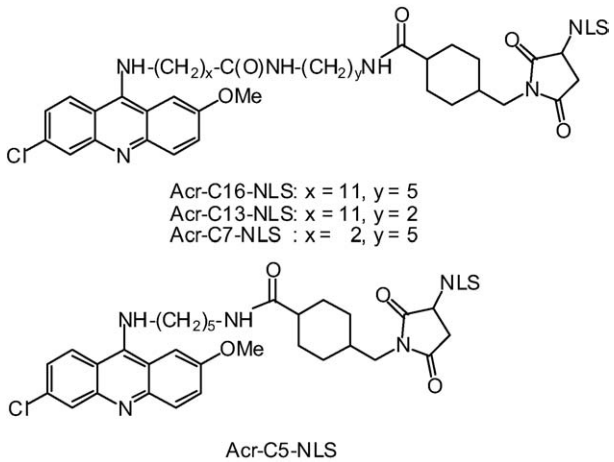
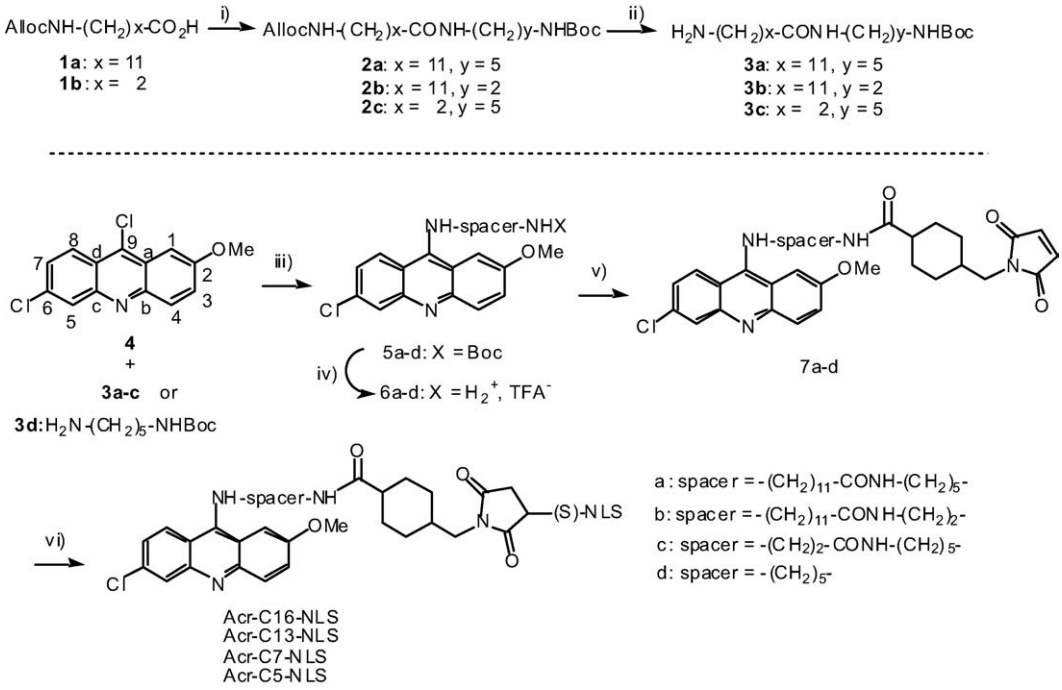


Fig. 1. Chemical structure and code name of the acridine-spacer-NLS-conjugates used in this study.

2. Chemistry

The synthetic approach to the targeted Acr-spacer-NLS-conjugates (Scheme 1) is based on the elaboration of the key acridine-spacer-maleimido synthons **7a–d** onto which the NLS peptide (via its cysteine-thiol function) was added. The various synthons **7a–d** were elaborated in three steps starting from the dichloro-acridine derivative **4** and the mono-protected α,ω-diamino spacers **3a–c** (which were obtained using conventional procedures) and commercially available **3d**, respectively. The first step consisted of nucleophilic displacement of one aromatic chlorine of **4** by the amine of the spacer units **3a–d** which was performed in phenol and in the presence of *N*-methyl morpholine (yields ranging from 70% to 80%). The next step was the Boc-deprotection of the terminal spacer amine function in **5a–d**, which was achieved, quantitatively in excess TFA. The target maleimide function in **7a–d** was then introduced by acylation of the resulting amine in derivatives **6a–d** with the heterobifunctional reagent *N*-succinimidyl-4-(maleimidomethyl)cyclohexane carboxylate (SMCC) with yields ranging from 80% to 90%).

Condensation of the maleimides **7a–d** with the NLS peptide-thiol leading to the formation of a thioether bond was best performed by adding a DMF solution of **7a–d** to a solution of the NLS peptide in a 20 mM phosphate buffer at pH 7. Prior to use, the commercial NLS peptide was stirred overnight in a phosphate buffer 20 mM at pH 5–6 with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) [23] in order



Scheme 1. Synthetic route to the acridine-spacer-NLS-conjugates. i) H_2N–$(CH_2)_y$NHBoc (y = 2 or 5)/EDC/HOBt/NEt_3/DMF/25 °C (75–85% yield); ii) $Pd(PPh_3)_4/CH_2Cl_2/HNEt_2$ (35–70% yield); iii) NMM/phenol/100 °C (70–80% yield); iv) TFA/CH_2Cl_2 (quantitative); v) SMCC/DMF/NEt_3 (80–90% yield); vi) HCl 0.1 N in DMF, then NLS–(SH) (0.125 equiv.) in phosphate buffer pH 7 (NLS–(SH) = CGYGPKKKRKVGG).

to reduce the traces of $(NLS\text{-}S)_2$ disulfide. The condensation reaction was followed by analytical HPLC, and the Acr-spacer-NLS-conjugates were purified by semi-preparative HPLC. Their structure was confirmed by MALDI-TOF mass spectrometry and that of their key Acr-spacer-maleimido starting materials **7a–d** was attested by ESI-MS, and by ^1H and ^{13}C NMR.

3. Pharmacology

The observation that direct microinjection of DNA into the cell nucleus led to protein expression in over 50% of microinjected cells, whereas its injection into the cytoplasm led to protein expression in less than 0.01% of cells, indicates that inefficient DNA traffic from the cytoplasm to the nucleus is a major barrier restricting transgene expression [24].

The objectives of this study were to determine whether acridine-spacer-NLS (SV40) peptide conjugates used as NLS-labeling agent of DNA (via non-covalent intercalation of the acridine moiety into DNA) can improve gene nuclear import and expression of non-viral gene transfer systems (e.g. lipoplexes and polyplexes). Aiming at this goal, we explored the impact of the acridine-spacer-NLS-conjugates (Fig. 1) on the luciferase expression level in cells transfected with lipoplexes and polyplexes that were formulated from DNA (= luciferase reporter gene)/acridine-spacer-NLS complexes in comparison with non-labeled lipoplexes and polyplexes.

3.1. Lipoplex and polyplex formation and characterization

The lipoplexes and polyplexes (also used for the in vitro transfection assays described below) were formulated with the various Acr-spacer-NLS-conjugates and pTG11236 plasmid (pCMV-SV40-luciferase-SV40pA; 5739 bps), and for 1, 5 and 10 molecules of NLS-conjugates per plasmid. They were prepared in 5% glucose by preforming the acridine-NLS/DNA complexes which were then mixed with a liposomal dispersion of DOGS/DOPE 1:1 mol or a solution of branched PEI 25 kDa, respectively, and for N/P ratios of 5, 2.5, 1.25 and 0.8 for the lipoplexes, and 10, 5, 1.25 and 0.8 for the polyplexes (N = number of DOGS or PEI amine equivalents; P = number of DNA phosphate equivalents). The Acr-spacer-NLS: DNA complexes, and the lipoplexes and polyplexes formulated therefrom were analyzed by gel electrophoresis for attesting DNA complexation (results not shown). The lipoplexes and polyplexes were further investigated by dynamic light scattering for particle size measurements. For comparison, various control lipoplexes and polyplexes were also formulated and analyzed. These controls consisted of lipoplexes and polyplexes that were formulated from (i) DNA alone, (ii) dichloro-Acr (**4**)/DNA, (iii) Acr-spacer (**7a–d**)/DNA, or (iv) NLS peptide/DNA. Moreover, phenanthroline (Phen) and ethidium bromide (ETB) as DNA control intercalating agents were also tested for comparison.

3.2. In vitro transfection

To explore the ability of the Acr-spacer-NLS-conjugates to improve gene nuclear import and expression, we assayed

the transfection potential of all the DOGS/DOPE-based lipoplexes and PEI-based polyplexes that were formulated from Acr-spacer-NLS/DNA complexes described in the precedent section. To unambiguously highlight the impact of the Acr-spacer-NLS-conjugates on transfection, all the control formulations also described in the precedent section were tested as well. Moreover, these DNA complexes were assayed for low DNA amounts (i.e. 0.1 and 0.5 μg per well) and on two different cell lines (i.e. NIH-3T3 and lung epithelial A549 cells, from human pulmonary carcinoma) which possess the α-importins that bind to the NLS SV40 peptide sequence used. These assays were performed in the presence of 10% fetal calf serum for 48 h. The transfection efficiency of the lipoplexes and polyplexes was expressed in femtogram (fg) of luciferase per mg of proteins. Cells treated with naked DNA under equivalent conditions showed expression levels of about 10^{2-3} fg of luciferase per mg of proteins. The cell viability of the lipoplexes and polyplexes was also checked by determining the total protein amount per well of the transfected cells relative to that measured for untreated cells (for which the total protein amount per well is in a 30–60 μg per well range).

4. Results and discussion

4.1. Lipoplex and polyplex formation and characterization

The gel electrophoresis assays performed on the Acr-spacer-NLS/DNA (1:1, 5:1 and 10:1 mol) adducts and without ETB spreading showed the absence of a fluorescence trace, indicating that formation of these adducts cannot be ascertained using this technique (data not shown). However, when using ETB as intercalating agent, the ETB/DNA adducts were detectable but only for a ETB/DNA ratio of 10:1. When these ETB/DNA 10:1 adducts were complexed with DOGS/DOPE or PEI, fluorescently-labeled lipoplexes and polyplexes were detected but only for a N/P ratio of 0.8 and 1.25. For higher N/P ratios, a fluorescence trace was no more detected indicating that partial to full ETB decomplexation from DNA has occurred.

Analyses of the Acr-spacer-NLS-labeled and control lipoplexes and polyplexes by gel electrophoresis with ETB spreading (data not shown) indicated that fully complexed DNA (not accessible to ETB intercalation) was detected for N/P 2.5, 5 and 10, while "free" plasmid and partially complexed plasmid (accessible to ETB intercalation) was detected for N/P 1.25 and 0.8. Moreover, no electrophoretic migration differences between the Acr-spacer-NLS-labeled lipoplexes and polyplexes and their respective controls could be detected. These results show that complexation of DNA with such low amounts of Acr-spacer-NLS-conjugates, NLS, Acr, Phen or ETB per plasmid prior to complexation with DOGS/DOPE or PEI had no significant impact on DNA complexation nor on its electrophoretic migration.

The gel electrophoresis assays performed in the presence of excess sodium dodecylsulfate (SDS, data not shown) showed that the polyplexes displayed a greater stability than the lipoplexes with respect to SDS-induced dissociation. Indeed, for N/P > 1.25, the plasmid remained still partially complexed to PEI while fully-decomplexed plasmid was detected for any of the DOGS/DOPE lipoplexes investigated here.

To ascertain the presence of the intercalating conjugates within the plasmid in the lipoplexes and polyplexes, and more particularly for high N/P ratios, we used the fluorescence properties of the Acr-spacer-NLS-conjugates. These conjugates are fluorescent with a maximum of emission at 480–485 nm whether in the absence or presence of the DOGS/DOPE liposomes (Fig. 2A curve a), or of PEI (Fig. 2B curve a) but are no more fluorescent when intercalated into DNA (Fig. 2A, curve b). We found that upon adding the DOGS/DOPE lipo-

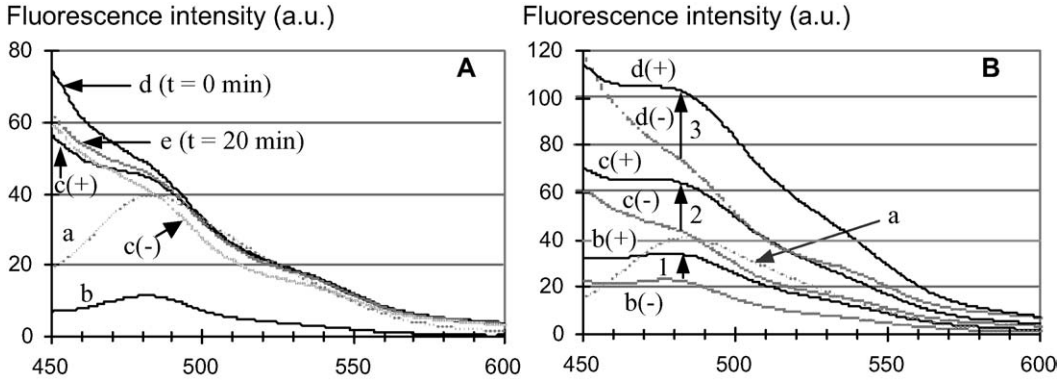


Fig. 2. (A): Fluorescence emission spectrum of (i) Acr-C13-NLS alone or in the presence of DOGS/DOPE liposomes (curve a), (ii) Acr-C13-NLS/DNA adduct or DNA alone (curve b), (iii) DOGS/DOPE/DNA N/P 5 lipoplexes (curve c(–)), (iv) DOGS/DOPE/[Acr-C13-NLS/DNA] N/P 5 lipoplexes, i.e. the lipids were added to the Acr-C13-NLS/DNA adduct (curve c(+)), and (iv) Acr-C13-NLS/[DOGS/DOPE/DNA] N/P 5 lipoplexes immediately after addition of Acr-C13-NLS to the lipoplexes (curve d, $t = 0$ min) and after 20 min of incubation (curve e, $t = 20$ min). (B): Fluorescence emission spectrum of (i) Acr-C13-NLS alone or in the presence of PEI (curve a), (ii) PEI/DNA N/P 1.25, 5 and 10 lipoplexes (curves b(–), c(–) and d(–), respectively), (iv) PEI/[Acr-C13-NLS/DNA] N/P 1.25, 5 and 10 polyplexes, i.e. PEI was added to the Acr-C13-NLS/DNA adduct (curves b(+), c(+) and d(+), respectively). The Acr-C13-NLS/DNA molar ratio is of 10:1. DNA is plasmid pTG11236. The concentration of Acr-C13-NLS is of 0.26 μM, that of DNA is of 15 μM phosphate. The emission spectra were recorded after excitation at 409 nm. For more details, see materials and methods section.

somes to the preformed Acr-spacer-NLS/DNA 10:1 complexes, the intercalating agent remained still complexed to DNA for a DOGS/DNA N/P ratio of 5. Indeed, the fluorescence emission curves recorded for the DOGS/DOPE/[Acr-spacer-NLS/DNA] and DOGS/DOPE/DNA lipoplexes were very close (Fig. 2A, curve c(+) and c(–), respectively). The full ejection of Acr-spacer-NLS from DNA upon complexation by DOGS/DOPE would have resulted in a more substantial fluorescence emission increase at 480 nm with respect to that recorded for the DOGS/DOPE/DNA lipoplexes. Moreover, we found that the addition of the Acr-spacer-NLS-conjugates to the preformed N/P 5 DOGS/DOPE/DNA lipoplexes (at a NLS/DNA molar ratio of 10:1) resulted in a decrease of the fluorescence signal with time (Fig. 2A, curve d for $t = 0$ vs. curve e for $t = 20$ min). This indicates that the intercalating agent penetrates progressively into lipid-complexed DNA which is still accessible at a N/P ratio of 5.

By contrast, partial to almost full ejection of Acr-spacer-NLS from DNA was observed upon addition of PEI to the preformed Acr-spacer-NLS/DNA adducts. As illustrated in Fig. 2B, the progressive increase of the PEI/DNA N/P ratio from 1.25 (curve b) to 5 (curve c) then to 10 (curve d), resulted in a progressive increase of the fluorescence emission at ~485 nm [Fmax(PEI/[Acr-spacer-NLS/DNA])–Fmax(PEI/DNA) for N/P 1.25 (arrow 1) < N/P 5 (arrow 2) < N/P 10 (arrow 3)], indicating the progressive release of Acr-spacer-NLS from DNA. This release was almost complete for N/P 10 (arrow 3 ≤ Fmax(Acr-spacer-NLS) at 485 nm (curve a).

Altogether, these fluorescence experiments indicate that part of the Acr-spacer-NLS-conjugates remains intercalated within the plasmid for any of the N/P lipoplexes and polyplexes investigated except for the N/P 10 polyplexes.

In terms of effect on lipoplex or polyplex mean size of the Acr-spacer-NLS-conjugates, NLS peptide, Acr, Phen or ETB, these additives had no significant impact. Indeed, lipoplexes and polyplexes with mean particle sizes in the 70–130 nm range for N/P ratios of 2.5, 5 and 10, and in the 100–200 nm range for N/P 0.8 and 1.25 were observed whether they were formulated or not with 1, 5 or 10 molecules of these additives per plasmid.

4.2. In vitro transfection

The various statistical analyses of the cell viability and transfection data obtained with the NIH and A549 cells are illustrated in Figs. 3 and 4, respectively. It is noticeable that the use of Acr-spacer-NLS-conjugates (as well as NLS, Acr, Phen or ETB) for the formulation of the lipoplexes and polyplexes had no detectable cytotoxic effects on the NIH-3T3 and A549 cell growth (Fig. 3) at the highest concentrations tested. Indeed, the 10:1 Acr-spacer-NLS/DNA, Acr/DNA, Acr-spacer/DNA, Phen/DNA, ETB/DNA, or NLS/DNA lipoplexes and polyplexes displayed a cell viability that is identical to that of their respective reference DOGS/DOPE lipoplexes and PEI polyplexes.

Importantly, the statistical analyses of the luciferase expression levels obtained with the different labeled and control formulations pointed out that the Acr-spacer-NLS-conjugates do not improve luciferase expression neither in NIH-3T3 cells (Fig. 4A, B) nor in A549 cells (Fig. 4C). Indeed, the detailed analyses which took into account (i) the spacer length of the Acr-spacer-NLS-conjugates, (ii) the N/P ratio (from 0.8 to 5) of the formulation, (iii) the Acr-spacer-NLS/DNA molar ratio (1, 5 or 10), and (iv) the DNA dose (0.5 or 0.1 μg per well), showed that the Acr-spacer-NLS-labeled lipoplexes display transfection efficiencies comparable to those of the control and DOGS/DOPE reference lipoplexes (Figs. 4A, C for a DNA dose of 0.5 μg per well; comparable tendencies were found for a dose DNA dose of 0.1 μg per well, data not shown). The presence of these conjugates was even found to decrease luciferase expression in the NIH-3T3 cells when their transfection is mediated with the labeled PEI-polyplexes (Fig. 4B). Moreover, neither the length of the spacer between the acridine intercalating and NLS units (Fig. 4A) nor the Acr-spacer-NLS/DNA ratio, even for low N/P ratios of the lipoplexes or polyplexes (see all cartoons throughout Fig. 4A–C), were found to have a significant impact on transfection.

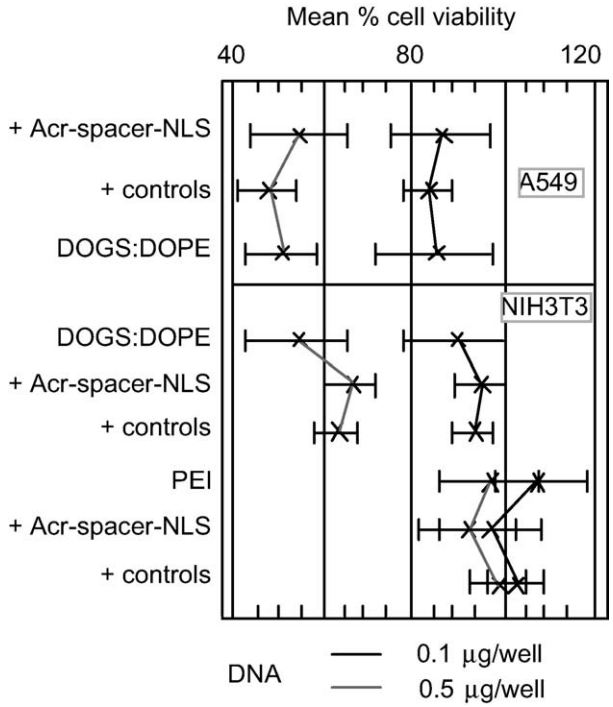


Fig. 3. Means and 95.0% Tukey HSD intervals of the percentage of cell viability for the DOGS/DOPE/[Acr-spacer-NLS/DNA] lipoplexes or PEI/[Acr-spacer-NLS/DNA] polyplexes in A549 and NIH-3T3 cells, irrespectively of (i) the spacer nature (C5, C7, C13 and C16), (ii) the Acr-spacer-NLS/DNA molar ratio (1:1, 5:1 and 10:1), and (iii) the N/P ratio (0.8, 1.25, 2.5 and 5 for the lipoplexes; 0.8, 1.25, 5 and 10 for the polyplexes) with respect to control/DOGS/DOPE or control/PEI formulations (control = DNA, Acr/DNA, Acr-spacer/DNA, Phen/DNA, ETB/DNA, NLS/DNA) and for a DNA dose of 0.1 or 0.5 μg per well. For more details concerning the statistical analysis, see materials and methods section.

5. Conclusion

It appears, in line with several other studies performed with NLS-conjugates not covalently linked to DNA [5,9,11–17],

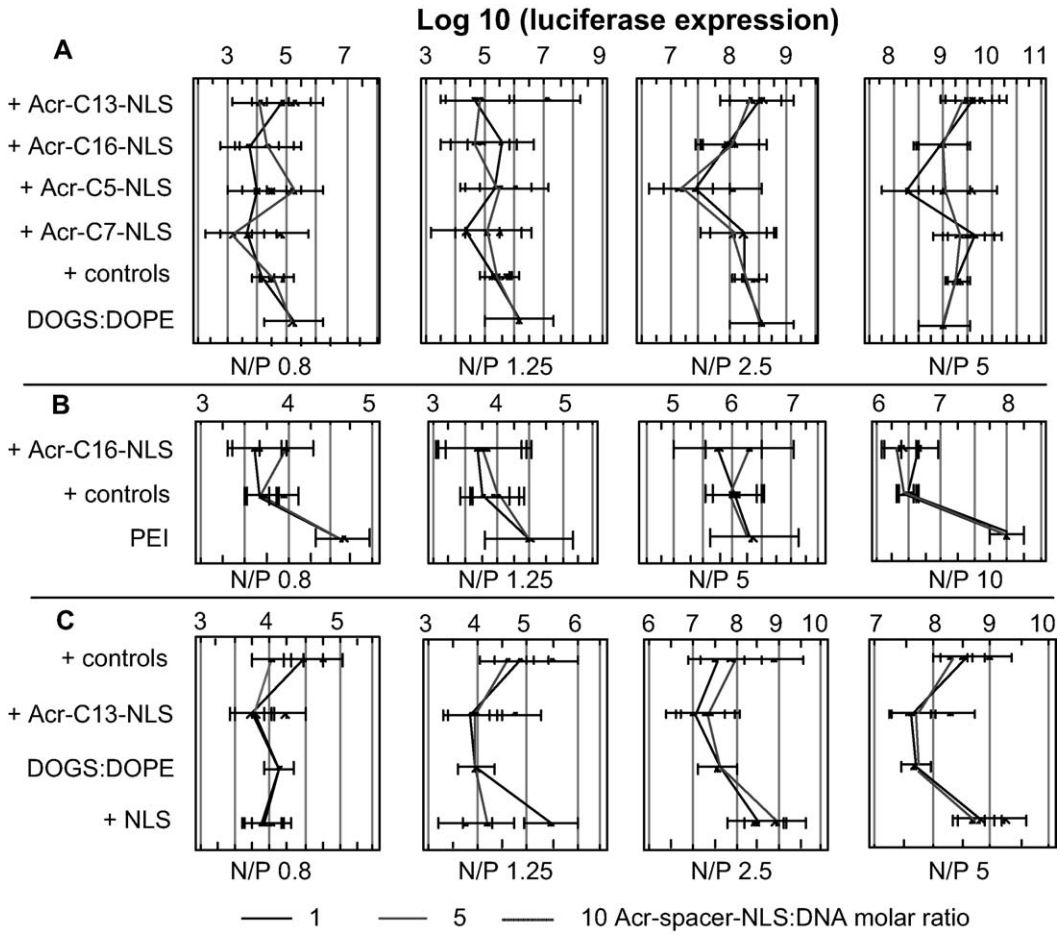


Fig. 4. Means and 95.0% Tukey HSD intervals of the logarithmic transformation of transfection levels (Log10(fg luciferase per mg protein)) for the DOGS/DOPE/[Acr-spacer-NLS/DNA] lipoplexes or PEI/[Acr-C16-NLS/DNA] polyplexes in NIH-3T3 (A and B, respectively) and A549 (C) cells, with respect to (i) the spacer length, i.e. C5, C7, C13 and C16 (A), (ii) the Acr-spacer-NLS/DNA molar ratio, i.e. 1:1, 5:1 and 10:1 (A–C), and (iii) the N/P ratio (0.8, 1.25, 2.5 and 5 for the lipoplexes (A, C); 0.8, 1.25, 5 and 10 for the polyplexes (B)) as compared to control/DOGS/DOPE or control/PEI formulations (control = DNA, Acr/DNA, Acr-spacer/DNA, Phen/DNA, ETB/DNA, NLS/DNA). The DNA dose was 0.5 μg per well. For more details concerning the statistical analyses, see materials and methods section.

that the expression of the transgene is not improved upon complexation of plasmidic DNA with NLS-intercalating conjugates prior to its formulation as lipoplexes or polyplexes. These unsuccessful results might be due to (i) a too low concentration of NLS-labeled DNA (whether naked or partially complexed as lipoplexes and polyplexes) that has been released into the cytoplasm, (ii) dissociation of the NLS-conjugates from DNA inside the cytoplasm, (iii) too strong electrostatic interactions between the NLS signal and DNA, thus hampering the recognition of the NLS by the nuclear transport and import proteins, and/or (iii) to the globular plasmid used which owing to its size and topology does not permeate efficiently into the nucleus via the NPC. This later drawback could be circumvented by the use of linear double-stranded DNAs which have shown enhanced gene expression in the presence of NLS-PNA conjugates [9], or following ligation of an oligonucleotide-NLS conjugate [4] or incorporation of a modified nucleotide for chemical linkage of the NLS peptide [5].

6. Experimental protocols

Most of the reactions were performed in anhydrous solvents under dry and oxygen-free nitrogen. Anhydrous solvents were prepared by standard methods. The purification by column chromatography were carried out using silica gel 60 (70–230 mesh) and chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), acetone, methanol (MeOH), *N,N*-dimethylformamide (DMF) or mixtures thereof as indicated. Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volume. Advancing of the reaction was followed by thin layer chromatography (TLC) on silica plates F254 or by HPLC. The following developing systems were used: UV light, $KMnO_4$, H_2SO_4/EtOH, Dragendorff reagent, ninhydrin reagent. DOGS was synthesized in our laboratory, as described elsewhere [19]. DOPE was purchased from Sigma. The SV40 nuclear transport signal peptide analogue CGYGPKKKRKVGG was from Bachem. ^1H and ^{13}C spectra were recorded at 200 and 50.3 MHz, respec-

tively, on a Bruker AC-200. Chemical shifts were measured relative to $CHCl_3$ (δ 7.26 ppm) or CH_3OD (δ 3.31 ppm) for ^1H, relative to $CDCl_3$ (δ 77.1 ppm) for ^{13}C and expressed indirectly in relation to TMS. The atom numbering used for the description of the acridine derivative ^1H and ^{13}C spectra is shown in Scheme 1. The following abbreviations are used to describe the signal multiplicities: bs (broad signal), s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). Chemical shifts are expressed in parts per million (ppm) and listed as follows: shift in ppm (multiplicity, coupling, integration and attribution). Electrospray ionization mass spectrometry (ESI-MS) in positive mode was performed on a Finnigan MAT LCQ equipped with an atmospheric pressure ionization (API) source. Mass spectrometry of the final molecules were performed on a MALDI-TOF applied Biosystems Voyager DE-PRO. HPLC analyses were performed using either a Merck L-6200 pump system equipped with L-4000 UV detector (set at 280 nm), or on a Waters 600 pump equipped with a Waters 996 photodiode array detector (PDA, UV detector from 195 to 360 nm) and a column (250 × 4 mm) packed with Lichrospher 100 RP-18 (5 μm). A gradient with water (0.1% TFA) as solvent A and acetonitrile (0.1% TFA) as solvent B was used with a flow = 1 ml/min.

6.1. Chemistry

6.1.1. Synthesis of the BocNH-spacer-NH_2 spacers **3a–c**

*6.1.1.1. Synthesis of 12-*N*-(allyloxycarbonyl)aminododecanoic acid **1a**.* A solution of 0.59 ml (5.6 mmol) of allylchloroformate in 5 ml dioxane was added at 0 °C to a solution of 12-aminododecanoic acid (1.0 g, 4.6 mmol) in 20 ml THF and 20 ml NaOH (1 M). The resulting mixture was stirred during 1 h at 0 °C then at room temperature for 2 h. The solvents were evaporated in a Rotavap evaporation system. The residue was dissolved in a 5% citric acid solution. The resulting aqueous phase was extracted with $CHCl_3$. The organic phase was then washed with water, dried over Na_2SO_4 and evaporated, leading to 751 mg (2.51 mmol, 54%) of **1a** as a white powder. TLC ($CHCl_3$/MeOH: 9:1: v/v; ninhydrin, $KMnO_4$, H_2SO_4) Rf = 0.62. ^1H NMR ($CDCl_3$): δ 1.12–1.38 (m, 14H, $(C\underline{H}_2)_7(CH_2)_2CO_2H$); 1.38–1.71 (m, 4H, $NCH_2C\underline{H}_2$ and $C\underline{H}_2CH_2CO_2H$); 2.31 (t, ^3J = 7.4 Hz, 2H, $C\underline{H}_2CO_2H$); 3.04–3.23 (m, 2H, $NC\underline{H}_2$); 4.47–4.65 (m, 2H, $C\underline{H}_2O$); 4.72–4.94 (m, 1H, N<u>H</u>); 5.11–5.38 (m, 2H, $\underline{H}_2C$=CH); 5.78–6.10 (m, 1H, H2C=C<u>H</u>); 10.80–11.48 (bs, 1H, CO_2H). ^{13}C NMR ($CDCl_3$): δ 24.8 ($\underline{C}H_2CH_2CO_2H$); 26.8 ($N(CH_2)_2\underline{C}H_2$); 29.1, 29.3, 29.5, 30.0 ($NCH_2\underline{C}H_2CH_2(\underline{C}H_2)_6$); 34.2 ($\underline{C}H_2CO_2H$); 41.2 ($N\underline{C}H_2$); 65.5 ($\underline{C}H_2O$); 117.7 ($H_2\underline{C}$=CH); 133.2 ($H_2C$=<u>C</u>H); 156.4 <u>C</u>(O)NH); 179.6 ($\underline{C}O_2H$).

*6.1.1.2. Synthesis of 3-*N*-(allyloxycarbonyl)amino-propionic acid **1b**.* The procedure described for the synthesis of **1a** when applied to β-alanine (500 mg, 5.6 mmol) led to 865 mg (5.0 mmol, 52%) of **1b** as a colorless oil. TLC ($CHCl_3$/MeOH: 9:1: v/v; ninhydrin, $KMnO_4$) Rf = 0.54. ^1H NMR ($CDCl_3$): δ 2.55 (t, ^3J = 5.9 Hz, 2H, $C\underline{H}_2CO_2H$); 3.32–3.51 (m, 2H, $C\underline{H}_2N$); 4.42–4.67 (m, 2H, $C\underline{H}_2O$); 5.10–5.36 (m, 2H, $\underline{H}_2C$=CH); 5.38–5.57 (m, 1H, N<u>H</u>); 5.76–6.02 (m, 1H, H_2C=C<u>H</u>); 9.77–10.25 (bs, 1H, $CO_2\underline{H}$). ^{13}C NMR ($CDCl_3$): δ 34.3 ($\underline{C}H_2CO_2H$); 36.4 ($\underline{C}H_2N$); 65.8 (CH_2O); 117.9 ($H_2\underline{C}$=CH); 132.7 (H_2C=<u>C</u>H); 156.5 (<u>C</u>(O)NH); 177.2 ($\underline{C}O_2H$).

*6.1.1.3. Synthesis of [11-(5-*N*-(tert-butoxycarbonyl)aminopentylcarbamoyl)-undecyl]-carbamic acid allyl ester **2a**.* To a solution of 375 mg (1.25 mmol) of **1a** in 15 ml DMF and 0.52 ml (3.76 mmol) of triethylamine stirred at 0 °C, 203 mg (1.50 mmol) of 1-hydroxybenzotriazole (HOBt), 0.31 ml (1.50 mmol) of *N*-Boc-1,5-diaminopentane, and 288 mg (1.50 mmol) of *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) were added. After 30 min at 0 °C, the solution was stirred at room temperature during 1 day. The solvents were evaporated and the residue obtained was dissolved in $CHCl_3$. The organic phase was washed successively with a 5% citric acid solution, a 5% Na_2CO_3solution, and water, then dried over Na_2SO_4 and evaporated, leading to **2a** (521 mg; 1.08 mmol, 86%) as a white powder. TLC ($CHCl_3$/MeOH: 97:3: v/v; ninhydrin, $KMnO_4$) Rf = 0.45. ^1H NMR ($CDCl_3$): δ 1.11–1.69 (m, 33H, $(C\underline{H}_3)_3$, $(C\underline{H}_2)_3CH_2NHBoc$, $N–CH_2–(C\underline{H}_2)_9$); 2.11 (t, ^3J = 7.5 Hz, 2H, $C\underline{H}_2C(O)$); 2.98–3.28 (m, 6H, $NC\underline{H}_2$); 4.45–4.59 (m, 2H, $C\underline{H}_2O$); 4.59–4.75 and 4.75–4.97 (m, 2H, <u>H</u>N carbamic); 5.10–5.35 (m, 2H, $\underline{H}_2C$=CH); 5.67–6.01 (m, 2H, H_2C=C<u>H</u> and N<u>H</u> amide). ^{13}C NMR ($CDCl_3$): δ 24.0 ($\underline{C}H_2(CH_2)_2NHBoc$); 25.9 ($NH(CH_2)_9\underline{C}H_2$); 26.8 ($AllocNH(CH_2)_2\underline{C}H_2$); 28.5 ($C(\underline{C}H_3)_3$); 29.3, 29.4, 29.5, 29.5, 29.8, 30.0 ($NHCH_2\underline{C}H_2CH_2(\underline{C}H_2)_6$ and $\underline{C}H_2CH_2\underline{C}H_2CH_2NHBoc$); 36.9 ($NH(CH_2)_{10}\underline{C}H_2$); 39.3, 40.3 ($\underline{C}H_2(CH_2)_3CH_2NHBoc$); 41.1 ($AllocNH\underline{C}H_2$); 65.4 ($\underline{C}H_2O$); 79.1 ($\underline{C}(CH_3)_3$); 117.5 ($H_2\underline{C}$=CH); 133.1 ($H_2C$=<u>C</u>H); 156.2, 156.4 (<u>C</u>(O)Boc and <u>C</u>(O)Alloc); 173.3 (($CH_2\underline{C}(O)$).

*6.1.1.4. Synthesis of [11-(2-*N*-(tert-butoxycarbonyl)aminoethylcarbamoyl)-undecyl]-carbamic acid allyl ester **2b**.* The procedure described for the synthesis of **2a** when applied to **1a** (375 mg, 1.25 mmol) and *N*-Boc-1,2-diaminoethane (0.24 ml, 1.50 mmol) afforded, after purification by liquid–liquid extraction ($CHCl_3$–H_2O), 415 mg (0.94 mmol, 75%) of **2b** as a white powder. TLC ($CHCl_3$/MeOH: 97:3: v/v; ninhydrin, $KMnO_4$) Rf = 0.48. ^1H NMR ($CDCl_3$): δ 1.04–1.70 (m, 27H, $NHCH_2(C\underline{H}_2)_9$ and $(C\underline{H}_3)_3$); 2.13 (t, ^3J = 7.6 Hz, 2H, $C\underline{H}_2C(O)$); 3.04–3.39 (m, 6H, $C(O)NHC\underline{H}_2$); 4.46–4.62 (m, 2H, $C\underline{H}_2O$); 4.71–4.94 (m, 1H, <u>H</u>N carbamic); 5.03–5.35 (m, 3H, $\underline{H}_2C$=CH and N<u>H</u> carbamic); 5.76–6.01 (m, 1H,H_2C=C<u>H</u>); 6.31–6.48 (m, 1H, N<u>H</u> amide). ^{13}C NMR ($CDCl_3$): δ 25.8 ($NH(CH_2)_9\underline{C}H_2$); 26.8 ($NH(CH_2)_2\underline{C}H_2$); 28.5 ($C(\underline{C}H_3)_3$); 29.3, 29.3, 29.5, 29.5 ($NH(CH_2)_3(\underline{C}H_2)_6$); 30.0 ($AllocNHCH_2\underline{C}H_2$); 36.8 ($NH(CH_2)_{10}\underline{C}H_2$); 40.4, 40.7 ($HN(\underline{C}H_2)_2NH$); 41.1 ($AllocNH\underline{C}H_2$); 65.5 ($\underline{C}H_2O$); 79.6 ($\underline{C}(CH_3)_3$); 117.6 ($H_2\underline{C}$=CH); 133.1 ($H_2C$=<u>C</u>H); 156.4, 157.0 (<u>C</u>(O)Boc and <u>C</u>(O)Alloc); 174.1 ($CH_2\underline{C}(O)$).

*6.1.1.5. Synthesis of [5-(3-N-(allyloxycarbonyl)aminopropionylamino)-pentyl]-carbamic acid tert-butyl ester **2c***. The procedure described for the synthesis of **2a** when applied to 400 mg (2.31 mmol) of **1b** and 0.58 ml (2.77 mmol) of *N*-Boc-1,5-diaminopentane, afforded 719 mg (2.01 mmol, 87%) of **2c** as a white powder. TLC ($CHCl_3$/MeOH: 9:1: v/v; ninhydrin, $KMnO_4$) Rf = 0.46 . ^{1}H NMR ($CDCl_3$): δ 1.18–1.56 (m, 15H, $(CH_3)_3$ and $(CH_2)_3CH_2NH$); 2.36 (t, ^{3}J = 6.0 Hz, 2H, $CH_2C(O)$); 2.96–3.12 (m, 2H, AllocNHCH_2); 3.12–3.27 (m, 2H, CH_2NHBoc); 3.34–3.49 (m, 2H, $CH_2C(O)NHCH_2$), 4.44–4.54 (m, 2H, H_2CO); 4.69–4.83 (m, 1H, NH carbamic); 5.09–5.32 (m, 2H, H_2C=CH); 5.58-5.73 (m, 1H, NH carbamic); 5.73–5.96 (m, 1H, H_2C=CH); 6.16–6.35 (m, 1H, NH amide). ^{13}C NMR ($CDCl_3$): δ 28.5 ($C(CH_3)_3$); 29.1, 29.6 ($(CH_2)_2CH_2C(O)$); 36.0 (AllocNHCH_2CH_2); 37.2 ($CH_2C(O)NH$); 39.2 (C(O)NHCH_2); 40.3 (AllocNHCH_2); 65.5 (CH_2NHBoc); 79.2 ($C(CH_3)_3$); 117.5 (H_2C=CH); 132.9 (H_2C=CH); 156.3, 156.5 (OC(O)NH); 171.4 (C(O)NH).

*6.1.1.6. Synthesis of [5-(12-*N*-(aminododecanoylamino)-pentyl]-carbamic acid tert-butyl ester, **3a** (Alloc-deprotection procedure)*. To a solution of 521 mg (1.08 mmol) of **2a** in 20 ml CH_2Cl_2 and 1.7 ml (16.2 mmol) of diethylamine, 125 mg (0.11 mmol) of tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) were added. The mixture was stirred during 2 h at room temperature. After evaporation of the solvents, the residue was dissolved in $CHCl_3$ and washed with water. The organic phase was dried over Na_2SO_4 and evaporated. Chromatography of the residue over a silica gel column (40 g, $CHCl_3$/MeOH: from 95:5 to 80:20: v/v) led to an orange powder. Steric exclusion chromatography over Sephadex LH-20 gel column (7 g, MeOH) led to 172 mg (0.43 mmol, 40%) of **3a** as a white powder. TLC ($CHCl_3$/MeOH: 9:1: v/v; ninhydrin, $KMnO_4$) Rf = 0.46. ^{1}H NMR ($CDCl_3/CD_3OD$, 50:50): δ 0.90–1.65 (m, 33H, $(CH_2)_3CH_2NHBoc$, $H_2NCH_2(CH_2)_9$); 1.90 (t, ^{3}J = 7.5 Hz, 2H, $CH_2C(O)$); 2.40 (t, ^{3}J = 7.1 Hz, 2H, H_2NCH_2); 2.78 (t,^{3}J = 6.7 Hz, 2H, C(O)NHCH_2); 2.92 (t, ^{3}J = 6.8 Hz, 2H, CH_2NHBoc). ^{13}C NMR ($CDCl_3/CD_3OD$, 50:50): δ 23.7 ($CH_2(CH_2)_2$NHBoc); 25.7 ($H_2N(CH_2)_9CH_2$); 26.6 ($H_2N(CH_2)_2CH_2$); 28.0 ($C(CH_3)_3$); 28.6, 29.0, 29.1, 29.1, 29.2, 29.3 ($H_2N(CH_2)_3(CH_2)_6$ and $CH_2CH_2CH_2CH_2$NHBoc); 32.3 ($H_2NCH_2CH_2$); 36.1 ($H_2N(CH_2)_{10}CH_2$); 38.9, 39.9 ($CH_2(CH_2)_3CH_2$NHBoc); 41.2 (H_2NCH_2); 78.9 ($C(CH_3)_3$); 156.7 (C(O)Boc); 174.6 (C(O)NH).

*6.1.1.7. Synthesis of [2-(12-amino-dodecanoylamino)-ethyl]-carbamic acid tert-butyl ester **3b***. The Alloc-deprotection procedure when applied to 415 mg (0.94 mmol) of **2b** afforded, after chromatography over a silica gel column (30 g, $CHCl_3$/MeOH: from 95:5 to 80:20: v/v) and steric exclusion chromatography over Sephadex LH-20 gel column (7 g, MeOH), 235 mg (0.66 mmol, 70%) of **3b** as a white powder. TLC ($CHCl_3$/MeOH: 9:1: v/v; ninhydrin, $KMnO_4$) Rf = 0.50. ^{1}H NMR ($CDCl_3/CD_3OD$): δ 0.97–1.53 (m, 27H, $H_2NCH_2(CH_2)_9$ and $(CH_3)_3$); 1.96 (t, ^{3}J = 7.5 Hz, 2H, $CH_2C(O)$); 2.45 (t, ^{3}J = 6.9 Hz, 2H, H_2NCH_2); 2.89–3.13 (m, 4H, $HN(CH_2)_2NH$). ^{13}C NMR ($CDCl_3/CD_3OD$): δ 25.6 ($H_2N(CH_2)_9CH_2$); 26.6 ($H_2N(CH_2)_2CH_2$); 28.0 ($C(CH_3)_3$); 29.0, 29.1, 29.2, 29.2, 29.3 ($H_2N(CH_2)_3(CH_2)_6$); 32.5 ($H_2NCH_2CH_2$); 36.2 ($H_2N(CH_2)_{10}CH_2$); 39.5, 39.7 ($HN(CH_2)_2NH$); 41.3 (H_2NCH_2); 79.3 ($C(CH_3)_3$); 157.1 (C(O)Boc); 175.0 (C(O)NH).

*6.1.1.8. Synthesis of [5-(3-amino-propionylamino)-pentyl]-carbamic acid tert-butyl ester **3c***. The Alloc-deprotection procedure when applied to 719 mg (2.01 mmol) of **2c** afforded, after chromatography over a silica gel column (90 g, $CHCl_3$/MeOH: from 95:5 to 80:20: v/v) and steric exclusion chromatography over Sephadex LH-20 gel column (7 g, MeOH), 198 mg (0.72 mmol, 36%) of **3c** as a white powder. TLC ($CHCl_3$/MeOH: 95:5: v/v; ninhydrin, $KMnO_4$) Rf = 0.63. ^{1}H NMR ($CDCl_3$): δ 1.00–1.59 (m, 17H, $(CH_2)_3CH_2NH$, $C(CH_3)_3$, NH_2); 2.14 (t, ^{3}J = 5.9 Hz, 2H, $CH_2C(O)$); 2.64–3.15 (m, 6H, NCH_2); 5.00–5.14 (m, 1H, NH carbamic); 7.33–7.49 (m, 1H, NH amide). ^{13}C NMR ($CDCl_3$): δ 23.8 ($HN(CH_2)_2CH_2$); 28.2, 29.0 ($HN–CH_2–CH_2–CH_2–CH_2$); 29.4 ($C(CH_3)_3$); 38.2 (H_2NCH_2); 38.5 ($H_2NCH_2CH_2$); 38.8 (C(O)NHCH_2); 40.1 (CH_2NHBoc); 78.6 ($C(CH_3)_3$); 156.0 (C(O)Boc); 172.3 (C(O)NH).

*6.1.2. Synthesis of the acridine-maleimide conjugates **7a–d***

*6.1.2.1. Synthesis of 4-(2,5-dioxo-2,5-dihydro-pyrrol-1-ylmethyl)-cyclohexanecarboxylic acid {5-[12-(6-chloro-2-methoxy-acridin-9-ylamino)-dodecanoylamino]-pentyl}-amide (**7a**)*. **Synthesis of 5a (General condensation procedure A)**. **3a** (172 mg, 0.43 mmol) was added to a solution of 2-methoxy-6,9-dichloroacridine **4** (60 mg, 0.21 mmol) in phenol (2.5 g, 26.6 mmol) and 4-methylmorpholine (NMM) (0.07 ml, 0.65 mmol) heated at 100 °C. The mixture was stirred at 100 °C during 2 h, then phenol was distilled under reduce pressure. Chromatography over a silica gel column (15 g, CH_2Cl_2/acetone: from 100:0 to 70:30: v/v), of the brown oily residue led to 113 mg (0.18 mmol, 82%) of **5a** as a dark yellow oil. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.49. ^{1}H NMR ($CDCl_3$): δ 1.03–1.80 (m, 33H, $(CH_3)_3$, $(CH_2)_3CH_2$NHBoc, AcrNHC$H_2(CH_2)_9$); 2.09 (t, ^{3}J = 7.6 Hz, 2H, $CH_2C(O)$); 2.96–3.10 (m, 2H, CH_2NHBoc); 3.10–3.25 (m, 2H, C(O)NHCH_2); 3.63 (t, ^{3}J = 7.1 Hz, 2H, AcrNHCH_2); 3.84–3.94 (bs, 3H, OCH_3); 4.63–4.82 (m, 1H, NHBoc); 5.87–6.03 (m, 1H, NH amide); 7.14–7.39 (m and 1dd, ^{3}J = 9.4 Hz, ^{3}J = 2.5 Hz, 3H, $AcrH_1$, $AcrH_3$, $AcrH_7$); 7.84–8.01 (m, 3H, $AcrH_4$, $AcrH_5$, $AcrH_8$). ^{13}C NMR ($CDCl_3$): δ 24.0 ($CH_2(CH_2)_2$NHBoc); 25.8 (AcrNH$(CH_2)_9CH_2$); 26.9 (AcrNH$(CH_2)_2CH_2$); 28.5 ($C(CH_3)_3$); 29.3, 29.4, 29.4, 29.9 (AcrNH$(CH_2)_3(CH_2)_6$, $CH_2CH_2CH_2CH_2$NHBoc); 31.7 (AcrNHCH_2CH_2); 36.8 (AcrNH$(CH_2)_{10}$–CH_2); 39.3, 40.3 ($CH_2(CH_2)_3CH_2$NHBoc); 50.6 (AcrNHCH_2); 55.5 (OCH_3); 79.1 ($C(CH_3)_3$); 99.4 ($AcrC_1$); 115.5, 117.7 ($AcrC_a$, $AcrC_d$); 124.2, 124.4, 124.5, 127.8, 131.0 ($AcrC_3$, $AcrC_4$, $AcrC_5$, $AcrC_7$, $AcrC_8$); 134.8, 146.4, 148.2 ($AcrC_6$, $AcrC_b$, $AcrC_c$);

150.1, 155.9 (AcrC$_2$, AcrC$_9$); 156.2 (C(O)Boc); 173.3 (C(O)NH).

Synthesis of 6a (Boc-deprotection procedure). The Boc-deprotection was quantitatively achieved by dissolving 113 mg of **5a** (0.18 mmol) in CH_2Cl_2 with a large excess of TFA during 1 h at room temperature. The excess TFA was removed by co-evaporation with cyclohexan leading to **6a** (162 mg, 0.18 mmol). ^{1}H NMR (CD_3OD): δ 1.09–1.80 (m, 22H, (CH_2)$_3$$CH_2$$NH_3^+$, AcrNHCH$_2CH_2$($CH_2$)$_8$); 1.83–2.05 (m, 2H, AcrNHCH$_2$$CH_2$); 2.16 (t, ^{3}J = 7.5 Hz, 2H, CH_2C(O)); 2.92 (t, ^{3}J = 7.5 Hz, 2H, $CH_2NH_3^+$); 3.18 (t, ^{3}J = 6.8 Hz, 2H, C(O)NHCH_2); 3.89–3.99 (bs, 3H, OCH_3); 4.05 (t, ^{3}J = 7.5 Hz, 2H, AcrNHCH_2); 7.39 (dd, ^{3}J = 9.3 Hz, ^{3}J = 2.0 Hz, 1H, AcrH$_1$); 7.38 and 7.52 (2dd, ^{3}J = 9.3 Hz, ^{3}J = 2.0 Hz, 2H, AcrH$_3$, AcrH$_7$); 7.60–7.73 (m, 3H, AcrH$_5$, AcrH$_4$, AcrH$_1$); 8.32 (d, ^{3}J = 9.5 Hz, 1H, AcrH$_8$). ^{13}C NMR (CD_3OD): δ 24.7 (CH_2(CH_2)$_2$$NH_3^+$); 27.1 (AcrNH(CH$_2$)$_9$$CH_2$); 27.8 (AcrNH(CH$_2$)$_2$$CH_2$); 28.1, 29.9, 30.2, 30.3, 30.4, 30.5 (AcrNH(CH$_2$)$_3$(CH_2)$_6$ and $CH_2CH_2CH_2CH_2$NHBoc); 30.7 (AcrNHCH$_2$$CH_2$); 37.1 (AcrNH(CH$_2$)$_{10}$$CH_2$); 39.9, 40.5 ($CH_2$($CH_2$)$_3$$CH_2NH_3^+$); 50.4 (AcrNHC$H_2$); 56.6 (OC$H_3$); 103.8 (AcrC$_1$); 111.2, 115.3 (AcrC$_a$, AcrC$_d$); 118.5, 121.4, 125.0, 128.6, 129.1 (AcrC$_3$, AcrC$_4$, AcrC$_5$H, AcrC$_7$, AcrC$_8$); 135.7, 141.3, 141.7 (AcrC$_6$, AcrC$_b$, AcrC$_c$); 157.6, 158.1 (AcrC$_2$, AcrC$_9$); 162.4 (q, CF_3CO_2H); 173.3 (C(O)–NH).

Synthesis of 7a (general condensation procedure B). To a solution of **6a** (185 mg, 0.21 mmol) and 0.12 ml NEt_3 (0.84 mmol) in 10 ml DMF, 77 mg (0.23 mmol) of 4-(*N*-maleimidomethyl)cyclohexane-carboxylic acid *N*-hydroxysuccinimide ester (SMCC) were added. The resulting mixture was stirred overnight at room temperature, then the solvents were evaporated under reduced pressure. Chromatography of the residue over a silica gel column (15 g, CH_2Cl_2/acetone: from 100:0 to 70:30: v/v) led to 130 mg (0.17 mmol, 81%) of **7a**. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.48. ^{1}H NMR ($CDCl_3$): δ 0.71–2.32 (m, 36H, (CH_2)$_3$$CH_2$NHSMCC, AcrNHCH$_2$($CH_2$)$_{10}$, and cyclohexyl); 2.99–3.42 (m, 6H, C(O)NHCH_2 and CHCH_2N); 3.64–3.90 (m, 5H, AcrNHCH_2 and OCH_3); 6.03–6.34 (m, 2H, NH amide); 6.62–6.73 (bs, 2H, CH=CH); 6.92–7.12 (m, 2H, AcrH$_7$, AcrH$_3$); 7.34–7.52 (m, 2H, AcrH$_1$, AcrH$_8$); 7.56–7.68 (m, 1H, AcrH$_5$); 7.94 (d, ^{3}J = 9.5 Hz, 1H, AcrH$_4$). ^{13}C NMR ($CDCl_3$): δ 23.8 (CH_2(CH_2)$_2$NH–C(O)); 25.9 (AcrNH(CH$_2$)$_9$$CH_2$); 26.9 (AcrNH(CH$_2$)$_2$$CH_2$); 28.9, 29.0, 29.1, 29.3, 29.4, 29.8, 30.1 (AcrNHCH$_2$$CH_2$$CH_2$($CH_2$)$_6$, $CH_2CH_2CH_2CH_2$NHC(O) and CH_2(cyclohexyl)); 36.4 (CHCH_2N); 36.8 (AcrNH(CH$_2$)$_{10}$$CH_2$); 39.0, 39.1 ($CH_2$($CH_2$)$_3$$CH_2$NH); 43.7 ($CH_2$NC(O)); 45.1 (C(O)CH); 49.0 (AcrNHCH_2); 55.8 (OCH_3); 102.5 (AcrC$_1$); 109.8, 113.9 (AcrC$_d$, AcrC$_a$); 118.2, 120.0, 120.5, 123.6, 126.7, 127.1 (AcrC$_3$, AcrC$_4$, AcrC$_5$, AcrC$_6$, AcrC$_7$, AcrC$_8$); 134.1 (CH=CH); 134.5, 140.0 (AcrC$_b$, AcrC$_c$); 154.9, 156.0 (AcrC$_9$, AcrC$_2$); 171.1 (C(O)–CH=CH–C(O)); 173.8 (C(O)NH(CH$_2$)$_5$); 176.1 (C(O)NHCH). MS (ESI+): m/z = 760.7 in agreement with the calculated mass for $[M]^+ = C_{43}H_{58}ClN_5O_5$.

6.1.2.2. Synthesis of 4-(2,5-dioxo-2,5-dihydro-pyrrol-1-ylmethyl)-cyclohexanecarboxylic acid {2-[12-(6-chloro-2-methoxy-acridin-9-ylamino)-dodecanoylamino]-ethyl}-amide 7b. **Synthesis of 5b**. The above described condensation procedure A when applied to **3b** (235 mg, 0.66 mmol) and **4** (91 mg, 0.33 mmol) afforded, after chromatography over a silica gel column (20 g, CH_2Cl_2/acetone: from 100:0 to 70:30: v/v), 150 mg (0.25 mmol, 76%) of **5b** as a dark yellow powder. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.50. ^{1}H NMR ($CDCl_3$): δ 1.00–1.83 (m, 27H, AcrNHCH$_2$(CH_2)$_9$ and (CH_3)$_3$); 2.10 (t, ^{3}J = 7.6 Hz, 2H, CH_2C(O)); 3.12–3.40 (m, 4H, HN(CH_2)$_2$NH); 3.63 (t, ^{3}J = 7.1 Hz, 2H, AcrNHCH_2); 3.81–3.97 (bs, 3H, OCH_3); signals for AcrH as for **5a**. ^{13}C NMR ($CDCl_3$): δ 25.7 (AcrNH(CH$_2$)$_9$$CH_2$); 26.9 (AcrNH(CH$_2$)$_2$$CH_2$); 28.4 (C($CH_3$)$_3$); 29.3, 29.4, 29.4 (AcrNH(CH$_2$)$_3$(CH_2)$_6$); 31.7 (AcrNHCH$_2$$CH_2$); 36.7 (AcrNH(CH$_2$)$_{10}$$CH_2$); 40.4 ($CH_2$NHBoc); 40.7 ($CH_2CH_2$NHBoc); 50.6 (AcrNHC$H_2$); 55.6 (OC$H_3$); 79.6 (C($CH_3$)$_3$); signals for AcrC and C(O) as for **5a**.

Synthesis of 6b. The Boc-deprotection procedure when applied to 150 mg of **5b** (0.25 mmol) led quantitatively to **6b** (213 mg; 0.25 mmol): ^{1}H NMR (CD_3OD): δ 1.04–1.71 (m, 16H, AcrNHCH$_2$CH$_2$(CH_2)$_8$); 1.80–2.04 (m, 2H, AcrNHCH$_2$$CH_2$); 2.21 (t, ^{3}J = 7.6 Hz, 2H, CH_2C(O)); 3.05 (t, ^{3}J = 5.9 Hz, 2H, $CH_2NH_3^+$); 3.45 (t, ^{3}J = 6.0 Hz, 2H, C(O)NHCH_2); 3.82–4.09 (m, 5H, OCH_3 and AcrNHCH_2); signals for AcrH as for **6a**. ^{13}C NMR (CD_3OD): δ 26.7 (AcrNH(CH$_2$)$_9$$CH_2$); 27.8 (AcrNH(CH$_2$)$_2$$CH_2$); 30.2, 30.3, 30.4, 30.5 (AcrNH(CH$_2$)$_3$(CH_2)$_6$); 30.7 (AcrNHCH$_2$$CH_2$); 36.9 (AcrNH(CH$_2$)$_{10}$$CH_2$); 38.1 ($CH_2NH_3^+$); 40.7 ($CH_2CH_2NH_3^+$); 50.3 (AcrNHC$H_2$); 56.6 (OC$H_3$); signals for AcrC and C(O) as for **6a**.

Synthesis of 7b. The condensation procedure B when applied to **6b** (135 mg, 0.27 mmol) led to 163 mg (0.23 mmol, 84%) of **7b**. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.44. ^{1}H NMR ($CDCl_3$): δ 0.70–2.27 (m, 30H, AcrNHCH$_2$(CH_2)$_{10}$ and cyclohexyl); 3.15–3.51 (m, 6H, HN(CH_2)$_2$NH and CHCH_2N); 3.66–4.00 (m, 4H, AcrNHCH_2 and OCH_3); 6.48–6.82 (m, 3H, CH=CH and NH amide); signals for AcrH and H(maleimido) as for **7a**. ^{13}C NMR ($CDCl_3$): δ 25.8 (AcrNH(CH$_2$)$_9$$CH_2$); 26.8 (AcrNH(CH$_2$)$_2$$CH_2$); 28.8, 29.1, 29.2, 29.2, 29.3, 29.8, 30.2 (AcrNHCH$_2$$CH_2$$CH_2$($CH_2$)$_6$ and CH_2(cyclohexyl)); 36.4 (CHCH_2N); 36.6 (AcrNH(CH$_2$)$_{10}$-CH_2); 39.9, 40.2 (HN(CH_2)$_2$NH); 43.7 (CH_2NC(O)); 45.0 (C(O)CH); 49.2 (AcrNHCH_2); 55.8 (OCH_3); signals for AcrC, C(O) and C(maleimido) as for **7a**. MS (ESI+): m/z = 718.7 in agreement with the calculated mass for $[M]^+ = C_{40}H_{52}ClN_5O_5$.

6.1.2.3. Synthesis of 4-(2,5-dioxo-2,5-dihydro-pyrrol-1-ylmethyl)-cyclohexanecarboxylic acid {5-[3-(6-chloro-2-methoxy-acridin-9-ylamino)-propionylamino]-pentyl}-amide 7c. **Synthesis of 5c**. The above described condensation procedure A when applied to **3c** (198 mg, 0.66 mmol) and **4** (101 mg, 0.36 mmol) afforded, after chromatography over a silica gel column (30 g, CH_2Cl_2/acetone: from 100:0 to 70:30:

v/v), 148 mg (0.29 mmol, 79%) of **5c** as a dark yellow powder. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.62. ^{1}H NMR ($CDCl_3$): δ 1.17–1.59 (m, 15H, $(CH_2)_3CH_2NHBoc$, $C(CH_3)_3$); 2.47 (t, ^{3}J = 5.6 Hz, 2H, $CH_2C(O)$); 2.98–3.15 (m, 2H, CH_2NHBoc); 3.18–3.33 (m, 2H, CH_2–C(O)NHCH_2); 3.84–4.02 (m, 5H, OCH_3, AcrNHCH_2); 4.56–4.73 and 6.23–6.39 (m, 2H, AcrN**H**, N**H**Boc); 7.17–7.28 (m, 2H, AcrH_1 and AcrH_3); 7.30–7.39 (m, 2H, AcrH_7, N**H** amide); 7.88 (d, ^{3}J = 10.2 Hz, 1H, AcrH_8); 7.96 (d, ^{3}J = 1.8 Hz, 1H, AcrH_4); 8.04 (d, ^{3}J = 9.2 Hz, 1H, AcrH_5). ^{13}C NMR ($CDCl_3$): δ 24.3 ($CH_2(CH_2)_2$NHBoc); 28.8 ($C(CH_3)_3$); 29.3, 30.2 ($CH_2CH_2CH_2CH_2CH_2$); 36.1 ($CH_2C(O)$); 39.8, 40.2 ($CH_2(CH_2)_3CH_2$NHBoc); 47.0 (AcrNHCH_2); 56.1 (OCH_3); 79.6 ($C(CH_3)_3$); signals for AcrC and C(O) as for **6a**.

Synthesis of 6c. The Boc-deprotection procedure applied to 148 mg of **5c** (0.29 mmol) led quantitatively to **6c** (218 mg, 0.29 mmol). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 1.01–1.55 (m, 8H, $CH_2C(O)$ and $(CH_2)_3CH_2NH_3^+$); 2.56–2.79 (m, 4H, $CH_2(CH_2)_3CH_2NH_3^+$); 3.01 (t, ^{3}J = 6.3 Hz, 2H, AcrNHCH_2); 3.82 (s, 3H, OCH_3); signals for AcrH as for **6a**. ^{13}C NMR ($CDCl_3$/CD_3OD): δ 23.2 ($CH_2(CH_2)_2NH_3^+$); 26.5, 28.0 ($CH_2CH_2CH_2CH_2NH_3^+$); 34.4 ($CH_2C(O)$); 38.7 ($CH_2NH_3^+$); 39.1 (C(O)NHCH_2); 46.2 (AcrNHCH_2); 55.7 (OCH_3); signals for AcrC and C(O) as for **6a**.

Synthesis of 7c. The condensation procedure B when applied to **6c** (204 mg, 0.27 mmol) led to 133 mg (0.21 mmol, 78%) of **7c**. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.47. RMN ^{1}H ($CDCl_3$/CD_3OD): δ 0.71–2.03 (m, 16H, $(CH_2)_3CH_2$NH and cyclohexyl); 2.67 (t, ^{3}J = 5.9 Hz, 2H, $CH_2C(O)$); 2.88–3.08 (m, 4H, C(O)NHCH_2); 3.25 (d, ^{2}J = 7.1 Hz, 2H, $CH_2N(C(O))_2$); 3.84 (s, 3H, OCH_3); 4.18 (t, ^{3}J = 5.9 Hz, 2H, AcrNHCH_2); 6.61 (s, 2H, C**H**=C**H**); 6.72–6.95 (m, 1H, N**H** amide); 7.17–7.25 (m, 1H, AcrH_3); 7.30–7.40 (m, 1H, AcrH_7); 7.49–7.68 (m, 3H, AcrH_1, AcrH_5 and AcrH_8); 8.15 (d, ^{3}J = 9.3 Hz, 1H, AcrH_4). ^{13}C NMR ($CDCl_3$/CD_3OD): δ 23.8 ($CH_2(CH_2)_2$NHCOCH); 28.4, 28.6, 29.6, 34.6 (CH_2(cyclohexyl), CH_2CH_2NH); 36.2 ($CHCH_2$N); 38.7, 39.1 (C(O)NHCH_2); 43.5 ($CH_2N(C(O))_2$); 44.8 (C(O)CH); 46.3 (AcrNHCH_2); 55.7 (OCH_3); signals for AcrC, C(O) and C(maleimido) as for **7a**. MS (ESI+): m/z = 634.6 in agreement with the calculated mass for $[M]^+ = C_{34}H_{40}ClN_5O_5$.

*6.1.2.4. Synthesis of 4-(2,5-dioxo-2,5-dihydro-pyrrol-1-ylmethyl)-cyclohexanecarboxylic acid [5-(6-chloro-2-methoxy-acridin-9-ylamino)-pentyl]-amide **7d**.* **Synthesis of 5d**. The condensation procedure A described above when applied to 0.22 ml (1.08 mmol) of *N*-Boc-1,5-diaminopentane and 150 mg (0.54 mmol) of **4** afforded, after chromatography over a silica gel column (30 g, CH_2Cl_2/acetone: from 100:0 to 70:30: v/v), 170 mg (0.38 mmol, 71%) of **5d** as a dark yellow powder. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.46. ^{1}H NMR ($CDCl_3$): δ 1.14–1.57 (m, 13H, AcrNH$(CH_2)_2(CH_2)_2$, $C(CH_3)_3$); 1.57–1.83 (m, 2H, AcrNHCH$_2$CH_2); 2.90–3.20 (m, 2H, CH_2NHBoc); 3.44–3.70 (m, 2H, AcrNHCH_2); 3.86 (s, 3H, OCH_3); 4.63–4.90 and 4.90–5.16 (m, 2H, N**H**); signals for AcrH as for **5a**. ^{13}C NMR ($CDCl_3$): δ 24.1 (NH–$(CH_2)_2$–CH_2); 28.5 (CH_3(Boc)); 29.9, 31.2 (NHCH$_2$CH_2CH$_2$CH_2); 40.3 (CH_2NHBoc); 50.4 (AcrNHCH_2); 55.6 (OCH_3); 79.2 (C(Boc)); 156.2 (C(O)Boc); signals for AcrC as for **5a**.

Synthesis of 6d. The Boc-deprotection procedure applied to 170 mg of **5d** (0.38 mmol) led to **6d** (263 mg, 0.38 mmol). ^{1}H NMR (CD_3OD): δ 1.43–1.67 (m, 2H, NH$(CH_2)_2$CH_2); 1.67–1.87 (m, 2H, AcrNHCH$_2$CH_2); 1.87–2.13 (m, 2H, $CH_2CH_2NH_3^+$); 2.84 (t, ^{3}J = 7.3 Hz, 2H, AcrNHCH_2); 3.86 (s, 3H, OCH_3); 4.03 (t, ^{3}J = 7.1 Hz, 2H, $CH_2NH_3^+$); 7.20–7.63 (m, 5H, AcrH_1, AcrH_3, AcrH_5, AcrH_7 and AcrH_8); 8.19 (d, ^{3}J = 9.3 Hz, 1H, AcrH_4). ^{13}C NMR (CD_3OD): δ 24.8 (NH$(CH_2)_2$CH_2); 28.2 and 30.2 (NHCH$_2$CH_2CH$_2$CH_2); 40.5 ($CH_2NH_3^+$); 49.9 (AcrNHCH_2); 56.6 (OCH_3); signals for AcrC as for **6a**.

Synthesis of 7d. The condensation procedure B when applied to **6d** (204 mg, 0.27 mmol) led to 133 mg (0.21 mmol, 88%) of **7d**. TLC (CH_2Cl_2/MeOH: 9:1: v/v; ninhydrin, UV) Rf = 0.52. ^{1}H NMR ($CDCl_3$): δ 0.73–2.07 (m, 16H, NHCH$_2$(CH_2)$_3$, CH_2(cyclohexyl) and C**H**CH_2); 3.00–3.39 (m, 4H, CH_2NHC(O) and $CH_2N(C(O))_2$); 3.49–3.73 (m, 2H, AcrNHCH_2); 3.87 (s, 3H, OCH_3); 5.76–5.94 (m, 1H, N**H**); 6.63 (s, 2H, C**H**=C**H**); 7.04–7.40 (m, 4H, AcrH_1, AcrH_3, AcrH_7 and N**H**); 7.76–8.03 (m, 3H, AcrH_4, AcrH_5 and AcrH_8). ^{13}C NMR ($CDCl_3$): δ 24.2 (NH$(CH_2)_2$CH_2); 28.9 (C(O)CHCH_2); 29.5 (CH_2CH_2NHC(O)); 29.8 (NCH$_2$CHCH_2); 31.1 (AcrNHCH$_2$CH_2); 36.3 ($CHCH_2$N); 38.9 (C(O)C**H**); 43.7 (CH_2NHC(O)); 45.2 ($CH_2N(C(O))_2$); 50.4 (AcrNHCH_2); 55.6 (OCH_3); signals for AcrC and C(maleimido) as for **7a**. MS (ESI+): m/z = 563.5 in agreement with the calculated mass for $[M]^+ = C_{31}H_{35}ClN_4O_4$.

6.1.3. Synthesis of the acridine-spacer-NLS-conjugates Acr-spacer-NLS

To a solution of the NLS SV40 peptide (585 μg, 0.21 μmol; *M* = 1377 g/mol as checked by MALDI-TOF MS) in 64.1 μl phosphate buffer (pH 7, 20 mM), 3 μl of HCl 0.1 N and 4 μl of a 0.53 mM solution of TCEP were added. The mixture was stirred overnight at room temperature. Then, 22.5 μl of DMF and 6.7 μl of a solution containing 1.70 μmol of **7a–d** in DMF were added. The reaction was monitored by HPLC. After completion of the reaction (about 15 min at room temperature), the solvents were evaporated. The resulting **Acr-spacer-NLS** compounds were purified by reverse-phase semi-preparative HPLC (A/B 90:10 to 0:100 over 35 min).

6.1.3.1. Acr-C16-NLS. t_R = 30.43 min, MS (MALDI-TOF): m/z = 2138.3 in agreement with the calculated mass for $[M]^+ = C_{103}H_{162}ClN_{25}O_{20}S$.

6.1.3.2. Acr-C13-NLS. t_R = 28.58 min, MS (MALDI-TOF): m/z = 2096.2 in agreement with the calculated mass for $[M]^+ = C_{100}H_{156}ClN_{25}O_{20}S$.

6.1.3.3. Acr-C7-NLS. t_R = 22.44 min, MS (MALDI-TOF): m/z = 2012.2 in agreement with the calculated mass for $[M]^+ = C_{94}H_{144}ClN_{25}O_{20}S$.

6.1.3.4. Acr-C5-NLS. t_R = 24.55 min, MS (MALDI-TOF): m/z = 1941.1 in agreement with the calculated mass for $[M]^+ = C_{91}H_{139}Cl_{18}N_{24}O_{19}S$.

6.2. Preparation of the DNA complexes

The plasmid pTG11236 (pCMV-SV40-luciferase-SV40pA) used for the preparation of the DNA complexes and for transfection assays was produced by Transgène (Strasbourg, France). pTG11236 is a plasmid of 5739 bp. The quantities of compounds used were calculated according to the desired DNA concentration of 0.1 mg/ml in the lipoplex or polyplex dispersion, the N/P ratio, the molar weight and the number of amino groups in the cationic lipid (DOGS) or cationic polymer (PEI). The N/P ratio of 5, for example, corresponds to the molar amount of DOGS or PEI necessary to have a ratio of five amino group nitrogens (for 1 mol of DOGS or PEI) per one phosphate in the DNA (330 Da mean MW for one nucleotide unit), as described elsewhere [19–22].

First of all, DOGS/DOPE 1:1 mol liposomal solutions in a 5% glucose were prepared at a DOGS concentration of 1 mg/ml. Thus, for the preparation of N/P 5 DOGS/DOPE/(Acr-NLS/DNA) complexes, 100 μl of DOGS solution (10 mg/ml in EtOH) and 58.91 μl of DOPE solutions (10 mg/ml in $CHCl_3$) were transferred to a borosilicate glass tube (16 × 100 mm). The solvent was evaporated in a Rotavap evaporation system (45 °C, 30 pm, 0.2 bar, 40 tr/min). One milliliter of a 5% glucose solution was added to the film obtained. The preparation was vortexed for 12 h, then sonicated for 5 min to yield a liposomal preparation. The lipoplex dispersions were then prepared by mixing 1.96 μl of 1.29 mg/ml DNA solution with (i) 10 μl of the Acr-NLS conjugate (the concentration of this solution depends on the desired DNA/Acr-NLS mol:mol ratio (1:1, 1:5 or 1:10), then (ii) with the liposomal solution (11.96 μl for N/P 5) and glucose 5% to reach the final volume of 25 μl and a DNA concentration of 0.1 mg/ml. This preparation was vortexed for 10 s and used within 1 h for the particle size measurements and the in vitro transfection experiments. As for PEI, the polymer solution was prepared at a concentration of 0.431 mg/ml in glucose 5%. The polyplexes were prepared according to the same procedure than for the preparation of the lipoplexes.

6.3. Measurement of the size of the DNA complexes

The sample was diluted with glucose 5% in the measurement tube and homogenized and the average sizes were measured by photon correlation spectroscopy using a Coulter N4Plus particle size analyzer, as described elsewhere [25]. The formulations and analyses were reproduced twice.

6.4. Agarose gel electrophoresis

Each sample was analyzed and plasmid integrity in each sample was confirmed by electrophoresis after decomplexing the lipoplexes and polyplexes with sodium dodecyl sulfate, following the procedures described elsewhere [19].

6.5. Fluorescence assays

The labeled lipoplexes and polyplexes were prepared as described above from a liposomal DOGS/DOPE dispersion or PEI solution in 5% glucose, respectively, which was added to preformed Acr-spacer-NLS/DNA complexes in 5% glucose to reach a final volume of 1.5 ml, a Acr-spacer-NLS concentration of 0.26 μM, a DNA concentration of 15 μM phosphate. These preparations were then immediately placed in thermoregulated cuvettes at 25.0 ± 0.5 °C. Then, the emission fluorescence spectrum was recorded using a Perkin–Elmer spectrofluorometer LS 50B after excitation at 409 nm. Alternatively, the Acr-spacer-NLS-conjugates in 5% glucose were added to DOGS/DOPE liposomes, PEI, preformed lipoplexes or polyplexes in the thermoregulated fluorescence cuvettes. The quantities of compounds used were calculated according to the final DNA concentration, the N/P ratio (2.5, 5 and 10), and the Acr-spacer-NLS/DNA molar ratio (1, 5 and 10).

6.6. In vitro transfection of NIH-3T3 and A549 cells

NIH-3T3 (mouse fibroblasts) and A549 cells (epithelial cells derived from human pulmonary carcinoma) were grown 24 h before the in vitro transfection experiments in Dulbecco-modified Eagle culture medium (DMEM, Gibco-BRL) containing 10% foetal calf serum (FCS, Sigma), in 96-well plates (2×10^4 cells per well), in a wet (37 °C) and 5% CO_2/95% air atmosphere. Five microliters of the lipoplex or polyplex preparation were diluted to 100 μl in DMEM. The cell culture medium was removed and replaced with this 100 μl lipoplex (or polyplex solution which corresponds to a DNA concentration of 0.5 μg and a lipid concentration of 18.94 μM for DOGS (N/P 5) and 0.26 μM for PEI (N/P 5) in each well. After 4 and 24 h, 50 and 100 μl DMEM supplemented with 30% and 10% FCS, respectively, were added. Forty-eight hours after transfection, the culture medium was discarded and the cells were washed twice with 100 μl phosphate-buffered saline (PBS) and then lysed with 50 μl lysis buffer (Promega, Charbonnières, France). The lysates were frozen at –40 °C awaiting analysis of luciferase activity. This measurement was done for 10 s on 10 μl lysis mixture in a LB96P luminometer (Berthold, Evry, France) in dynamic mode, using the 'luciferase' determination system (Promega) in 96 well plates. The total protein concentration per well was determined by the BCA test (Pierce, Montluçon, France). For cells grown in the absence of lipoplexes or polyplexes, a well contained approximately 30–50 μg protein. Luciferase activity was calculated as femtograms (fg) of luciferase per milligram (mg) of protein. The percentage of cell viability of the lipoplexes or polyplexes was calculated as the ratio of the total amount of protein per well of the transfected cells relative to that measured for untreated cells ×100%.

6.7. Statistical analysis

Statistical tests were performed with STATGRAPHICS Plus5.0 software. Analysis of variance (ANOVA) was run on the logarithmic transformation of transfection levels (Log10(fg luciferase per mg protein)) and on the cell viability to fit normal distributions of the data.

Five factors, i.e. nature of the Acr-spacer-NLS-conjugates (length of the spacer) or of the intercalating agent (Acr, Phen, BET), Acr-spacer-NLS/DNA ratios (1, 5, and 10), N/P ratios (0.8, 1.25, 2.5, 5 and 10), and amount of DNA (0.1 and 0.5 μg per well) were analyzed as source of the variation of logarithmic transformation of the transfection levels and of cell viability percentages using a multiple comparison procedure. The Tukey's honestly significant difference (HSD) method was used to discriminate among the means of cell viability percentages and the logarithmic transformation of luciferase expression levels.

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