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Cationic liposomes as delivery systems for double-stranded PNA–DNA chimeras exhibiting decoy activity against NF-κB transcription factors

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Abstract

Peptide nucleic acids (PNAs) have been recently proposed as useful molecules in pharmacogenetic therapy, especially due to the fact that they show a very high stability with respect to DNA and RNA. However, PNAs are not efficient decoy molecules, are characterized by negligible cell internalization and low solubility and are not suitable to be delivered by liposomes. With respect to the biological activity of PNA-based molecules, PDP deserve great consideration, due to the fact that they exhibit high levels of solubility, and are expected to be resistant to proteinases and exonucleases. In this manuscript we determined whether double-stranded molecules based on PNA–DNA chimeras containing NF-κB binding sites, exhibit decoy activity against NF-κB transcription factors. In addition, we determined whether they can be complexed by cationic liposomes. The results obtained demonstrated that hybrids based on PNA–DNA chimeras are powerful decoy molecules against NF-κB p52 transcription factor. In addition, we found that cationic liposomes can be proposed for *in vitro* delivery to target cells of these decoy molecules. The results presented in this paper are thus of practical importance, since the simplicity and the versatility of the cationic liposome technology have made cationic liposomes useful nonviral gene delivery systems for human gene therapy.

Keywords: PNA; Decoy; Liposomes; Delivery

1. Introduction

PNAs [1] have been recently proposed as useful molecules in pharmacogenetic therapy, especially due to the fact that they show a very high stability with respect to DNA and RNA. PNAs are based on a pseudopeptide (polyamide) backbone constituted of *N*-(2-aminoethyl)glycine units [1–7]. Interestingly, PNAs exhibit (a) capacity to hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson–Crick double helices [1,3] and (b) resistance to DNases and proteinases [4,7]. Studies in cell free systems have demonstrated potent antisense (inhibition of translation) and antigene (inhibition of transcription) activity of PNAs [7–10].

However, it should be underlined that, unlike commonly used nucleic acids analogs, PNA oligomers are characterized by a negligible cell internalization [4–7] and, in some cases, by a low solubility [7]. In addition, PNAs are not suitable to be delivered by cationic liposomes, since, being neutral molecules, cannot form electrostatic interactions with the positively charged liposomes [5,7]. With respect to the biological activity of PNA-based molecules, PNA–DNA–PNA chimeras (PDP) deserve great consideration [11,12], due to the fact that they exhibit high levels of

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Abbreviations: PNA, peptide nucleic acid; PDP, PNA–DNA–PNA chimeras; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; HIV-1, human immunodeficiency virus, type-1; LTR, long terminal repeat.

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solubility, and are expected to be resistant to proteinases and exonucleases [7,11,12]. In addition, we have recently reported that double-stranded PDP chimeras are powerful decoy molecules [13]. We synthesized and analyzed a double-stranded PDP chimera mimicking the NF-κB binding sites of HIV-1 LTR [14,15]. These molecules were found to be an excellent decoy for p52 and p50 NF-κB transcription factors [13].

In this manuscript we determined whether PDP chimeras exhibiting decoy activity against NF-κB transcription factors can be delivered using cationic liposomes [16–18]. Cationic liposomes are of great interest in gene therapy based on DNA molecules and analogs, since they possess the great advantage to interact spontaneously with DNA, generating complexes that have an almost quantitative loading efficiency [16]. In other words, all DNA is complexed on the surface of the liposome, provided that enough positive charges are available. In this respect, it is assumed that a positive to negative charge ratio spanning from 2:1 to 4:1 represents the optimal ratio in order to obtain efficient transfection capacity [17,18]. In addition to those previously mentioned, liposomes possess other general advantages as delivery systems for gene medicine. In fact (a) they are able to protect nucleic acids from degradative processes [19], (b) they have a large loading capacity, accommodating also very large genes, potentially including chromosomes [20], and finally (c) liposomes can be targeted to specific cells or tissues by conjugation with appropriate vector molecules such as monoclonal antibodies [21].

The present report describes (a) the characterization of the decoy activity of PDP/DNA, DNA/PDP and PDP/PDP hybrid molecules; (b) the preparation of positively charged liposomes using different cationic lipids, (c) the ability of liposomes to complex PNA–DNA chimeras, and (d) the short term toxicity of the cationic liposomes on *in vitro* cultured human cells.

2. Materials and methods

2.1. Synthetic oligonucleotides and production of NF-κB PNA–DNA chimeras

Fig. 1A shows the sequences of the produced PDP chimeras carrying binding sites for the transcription factors belonging to the NF-κB superfamily. NF-κB DNA/DNA was the reference decoy molecule, while PDP/DNA and PDP/PDP were the PNA-based decoy molecules analyzed in the present paper with respect to biological activity and complexation to liposomes.

The synthetic oligonucleotides used in this study were purchased from Sigma. PNA monomers for production of PNA–DNA chimeras were synthesized in Prof. Van Boom laboratories [12] (Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University); DNA monomers were obtained from Perseptive Biosystems. Methanol (Rathburn, HPLC grade) was stored over molecular sieves (3 Å) and used without other purification. All the other solvents (Biosolve DNA synthesis grade) were used as received. Automatized synthesis of the chimeras was performed on a Pharmacia Gene Assembler, using highly cross-linked polystyrene (loading 26–28 μmol/g) as the solid support on a 1 μmol scale, as reported elsewhere [13]. After the last elongation step, the oligomers were cleaved from the solid support and deprotected by treatment with 1.5 mL methanolic ammonia, at 50° for 16 hr. The samples were filtered and then purified by RP-HPLC on a LiChrosphere 100 RP-18 endcapped column (4 mm × 250 mm) on a Jasco HPLC system. Gradient elution was performed at 40° building up gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water, 1/1, v/v), with a flow rate of 1 mL/min. Chimera 1: HPLC purity 100%, $t_R = 18$ min (gradient 3–20% B in 25 min); chimera 2: HPLC purity 100%, $t_R = 16$ min (gradient 5–25% B in 25 min). HPLC-MS analysis was carried out on a Jasco LCMS system equipped with a LiChrosphere 100 RP-18 endcapped column (4 mm × 250 mm) using a gradient of acetonitrile in 10 mM ammonium acetate buffer with mass detection on a Perkin-Elmer Sciex API 165 equipped with an Electrospray Interface (ESI). Chimera 1: $t_R = 7$ min (gradient 5–20% acetonitrile in 29 min); ESI-MS: $[M + 4H]^{4+} = 1438.2$, $[M + 5H]^{5+} = 1150.5$, calculated for $C_{193}H_{245}N_{90}O_{95}P_{13}$ 5748.26. Chimera 2: $t_R = 8$ min (gradient 0–20% acetonitrile in 20 min); ESI-MS: $[M + 4H]^{4+} = 1443.6$, $[M + 5H]^{5+} = 1154.9$, calculated for $C_{194}H_{247}N_{86}O_{99}P_{13}$ 5770.26.

Sequences : Gly-ccg-5′-TGGAAAGTCCCCA-3′-gcg-Ac 1
Gly-cgc-5′-TGGGGACTTTCCA-3′-cgg-Ac 2

2.2. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay [13,14] was performed by using the double-stranded synthetic oligonucleotides mimicking the NF-κB binding sites (the nucleotide sequences have been reported in Fig. 1). The target molecules were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (MBI) in the case of DNA/DNA and DNA/PDP [14]. ^{32}P-labeled PDP/PDP molecules were labeled by nick-translation, using low concentration of DNase and [γ-^{32}P]dCTP [31]. Binding reactions were set up as described elsewhere [13] in a total volume of 25 μL containing buffer TF with 5% glycerol, 1 mM DTT, 10 ng of human NF-κB p52 protein (Promega Corporation) and 0.25 ng of ^{32}P-labeled oligonucleotides. After 30 min binding at room temperature, the samples were electrophoresed at constant voltage (200 V) under low ionic strength conditions (0.25 × TBE buffer = 22 mM Tris-borate, 0.4 mM EDTA) on 6% polyacrylamide gels. Gels

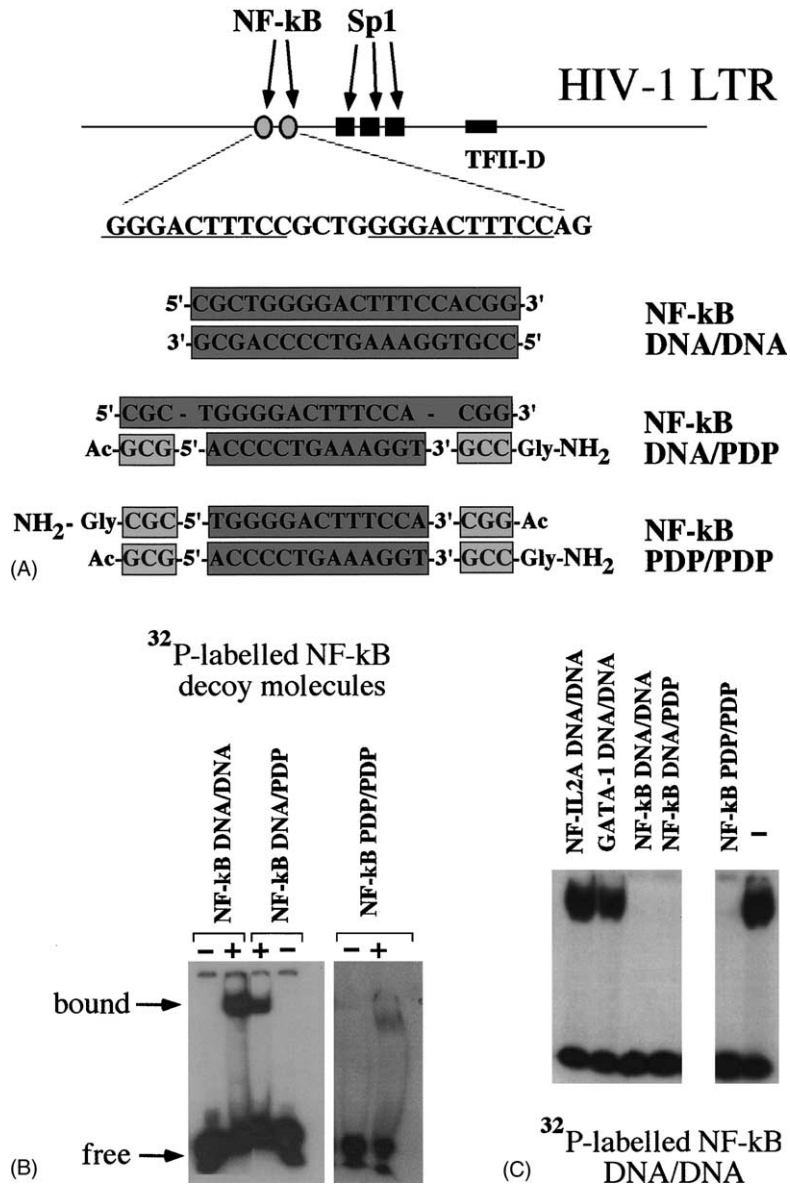

Fig. 1. (A) Structure of the HIV-1 LTR, location of NF-κB and Sp1 binding sites, sequences of the ODNs and PDP chimeras used. (B) Binding of purified NF-κB p52 to ^{32}P-labeled HIV-1 NF-κB DNA/DNA, DNA/PDP, and PDP/PDP target molecules. ^{32}P-labeled molecules were incubated in the absence (−) or in the presence (+) of 10 ng of NF-κB factor for 20 min in binding buffer [14]. (C) Effects of NF-κB, NF-IL2A, GATA-1 DNA/DNA hybrids, NF-κB DNA/PDP hybrids and PDP/PDP on the interaction between purified NF-κB p52 and ^{32}P-labeled HIV-1 NF-κB DNA/DNA target molecules. Ten nanograms of NF-κB factor was incubated for 20 min in binding buffer in the absence (−) or in the presence of 100 ng of the decoy molecules, as indicated. After this incubation period, a further 20 min incubation step was performed in the presence of ^{32}P-labeled HIV-1 NF-κB DNA/DNA target molecule.

were dried and subjected to standard autoradiographic procedures [13]. In competition experiments, the competitor molecules carrying HIV-1 NF-κB binding sites (DNA/DNA, PDP/PDP, and DNA/PDP) were preincubated for 20 min with purified NF-κB p52 protein before the addition of labeled target DNA. Forward nucleotide sequences of NF-IL2A and GATA-1 competitor ODNs were 5′-TAA TAT GTA AAA ACA TT-3′ and 5′-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3′, respectively. Double-stranded ODNs were produced after annealing of the forward oligonucleotides with the complementary ODNs.

2.3. Liposome preparation

Egg phosphatidyl choline was purchased from Lipid Products (Surrey). Tetralysine cationic lipids, tetralysine-cholesterol (Lys4-Chol) and tetralysine-palmitate (Lys4-Palm), were a generous gift of Prof. M Marastoni (Department of Pharmaceutical Sciences, University of Ferrara). Positively charged liposomes were produced by a protocol based on reverse phase evaporation followed by extrusion of the liposome suspension through polycarbonate filters with homogeneous pore size. Liposomes were subjected to one extrusion cycle through two stacked

400 nm pore size filters followed by three extrusion cycles through two stacked 200 nm pore size membranes, in order to obtain unilamellar liposomes with an homogeneous size distribution. Different cationic detergents were alternatively used for the production of the liposomes, namely Lys4-Chol and Lys4-Palm [2]. The resulting liposomal formulations were named as follows: lipo-Lys4-Chol and lipo-Lys4-Palm. The complexation of PNA–DNA chimeras to liposomes was performed just before the analysis, simply mixing the target decoy molecules to "preformed" cationic vesicles, employing increasing liposome concentrations, including those which were known to cause a full complexation of target DNA/DNA hybrids [22].

2.4. Morphological and physicochemical analysis of cationic liposomes

The morphological and dimensional analysis of the produced liposomes was performed by freeze-fracture electron microscopy technique and photo correlation spectroscopy, using a Zetasizer 3000 photo correlation spectroscopy (Malvern) [22,23], equipped with 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for injections. Measurements were made at 25° at an angle of 90°. Data were interpreted using the Contin software. The freeze-fracture electron microphotographies confirmed that the extruded liposomal suspension was mainly constituted by unilamellar vesicles (see Fig. 2A). Photo correlation spectroscopy studies demonstrated that the extruded vesicles present a narrow size distribution with an average diameter reflecting the pore size of the employed membrane. Table 1 and Fig. 2B report the hydrodynamic mean diameter and the cumulative size distribution of the two cationic liposomal formulations.

2.5. Complexation of NF-κB DNA/DNA, DNA/PDP and PDP/PDP hybrids to liposomal formulations

Different amounts of Lys4-Palm (lipo-lys4-palm) or Lys4-Chol (lipo-lys4-chol) liposomes were incubated in the presence of either ^{32}P-labeled DNA/DNA or DNA–PDP hybrid molecules or cold PDP/PDP, and formed complexes were electrophoresed through an agarose gel and exposed to autoradiographic procedure (DNA/DNA or DNA/PDP) or stained with ethidium bromide (PDP/PDP molecules).

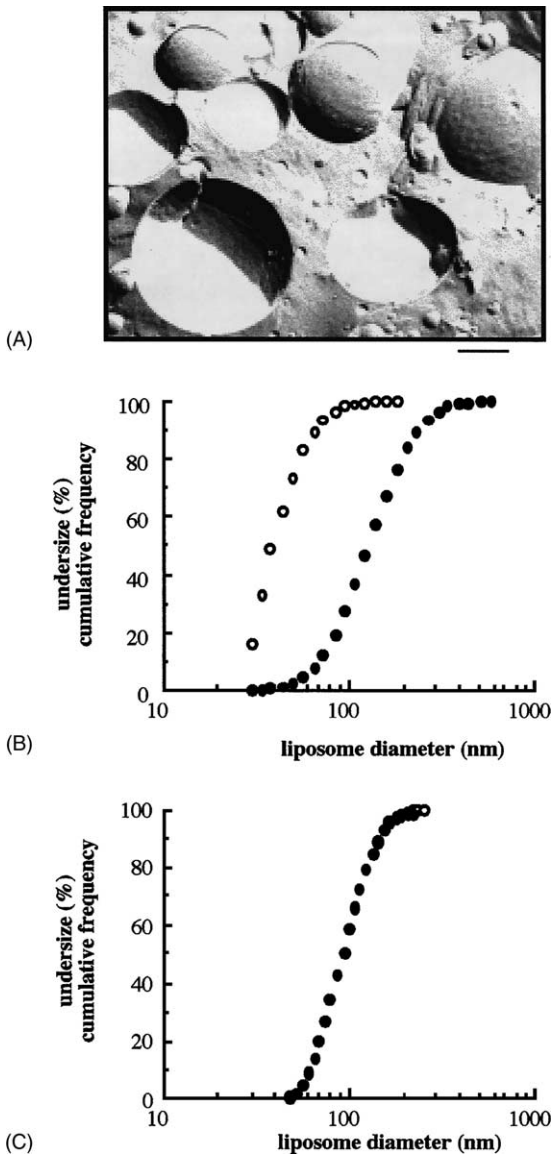

Fig. 2. (A) Freeze-fracture electron microscopy of liposomes containing DNA–PNA chimeras. Cumulative frequency distribution plot (undersize, by number) of (B) lipo-Lys4-Chol, and (C) lipo-Lys4-Palm.

2.6. Resistance of decoy molecules

The resistance of ^{32}P-labeled DNA/DNA and DNA/PDP target molecules was evaluated as follows: the decoy molecules were incubated without or with different concentrations (2–25 μg/reaction) of Lys4-Palm or Lys4-Chol for 30 min at room temperature and then serum (fetal calf

Table 1
Particle size and zeta potential of cationic liposomes

Liposome formulation	Mean diameter ± SD before complexation (polydispersity) (nm)	Mean diameter ± SD after complexation (polydispersity) (nm)	Zeta potential before complexation (mV)
Lys4-Chol (1:1, mol/mol)	110.2 ± (0.23)	135.6 (0.25)	+63.1
Lys4-Palm (1:1, mol/mol)	130.7 ± (0.10)	138.4 (0.16)	+66.0

Data were obtained by using a photon correlation spectrophotometer-zeta meter equipped with a helium neon laser.

serum, Eurobio; 30 g/L protein concentration) was added (3 μL/reaction). After overnight incubation, the reactions were phenol extracted, ethanol-precipitated, electrophoresed through a polyacrylamide gel and autoradiography was performed. Disappearence of the decoy molecule was considered as an evidence of degradation by the employed enzymes [13].

3. Results and discussion

3.1. Synthesis and characterization of the decoy activity of PNA–DNA chimeras mimicking NF-κB binding sites

Before complexation to liposomes, DNA/DNA, DNA/PDP, and PDP/PDP hybrids were characterized by gel

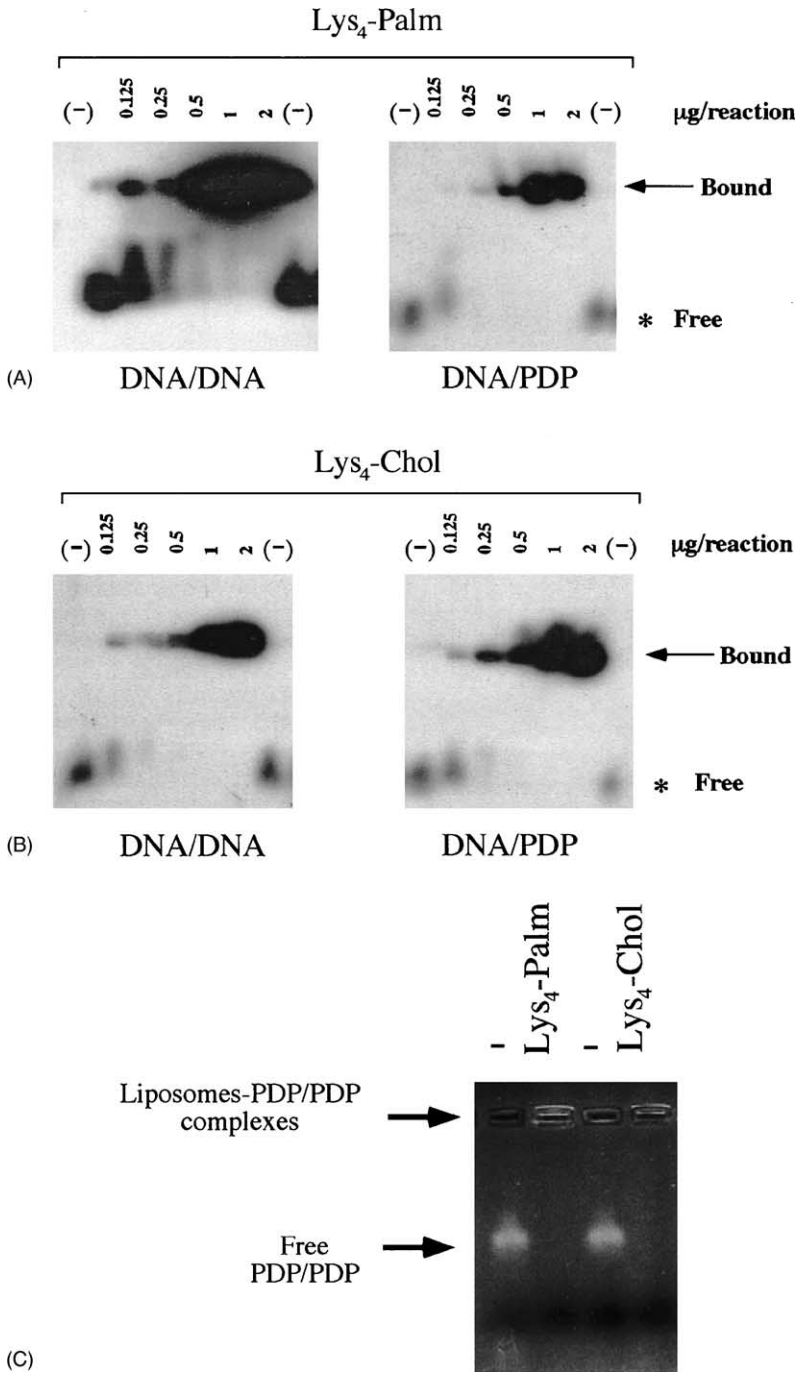

Fig. 3. Comparison of complexation efficiencies of NF-κB DNA–DNA and NF-κB DNA–PDP hybrids to the different liposomal formulations. The indicated amounts of liposomes were incubated in the presence of ^{32}P-labeled DNA–DNA or DNA–PDP hybrid molecules, and formed complexes were electrophoresized through an agarose gel and exposed to autoradiographic procedure. Complexation of DNA–DNA or DNA–PDP with liposomes were performed using the multivalent cationic lipid (A) Lys4-Palm (lipo-lys4-palm) or (B) Lys4-Chol (lipo-lys4-chol). (C) Complexation of PDP/PDP molecules to lipo-lys4-chol (Lys4-Chol) and lipo-lys4-palm (Lys4-Palm). In this case detection was performed on unlabeled PDP/PDP hybrids by ethidium-bromide staining of molecules after agarose-gel electrophoresis [37].

electrophoretic analysis in order to demonstrate complete annealing (data not shown). Biological activity was determined by electrophoretic mobility shift assay. Direct binding to p52 NF-κB factor was analyzed (Fig. 1B), as well as the ability to inhibit the interaction between NF-κB and ^{32}P-labeled target DNA (Fig. 1C). The results obtained show that both ^{32}P-labeled DNA/PDP and PDP/PDP chimeras bind to NF-κB p52 protein (Fig. 1B). Accordingly, both DNA/PDP and PDP/PDP chimeras recognizing NF-κB binding sites inhibit the binding of NF-κB p52 to ^{32}P-labeled target DNA (Fig. 1C, right side of the panel). As expected, control experiments demonstrate that NF-κB DNA/DNA molecules, but not NF-IL2A DNA/DNA and GATA-1 DNA/DNA decoys, are able to suppress molecular interactions between NF-κB p52 and target DNA (Fig. 1C, left side of the panel).

3.2. Complexation of double-stranded DNA–PNA chimera to liposomes

Fig. 3A and B demonstrates that Lys4-Chol and Lys4-Palm are able to complexate to DNA/PDP hybrids. This is demonstrated by the formation, when high concentrations of liposomes were used (1–2 μg/reaction), of complexes (identified as bound material) unable to migrate into the gels. It should be noted that complexation of NF-κB DNA/PDP to Lys4-Chol is very similar to the complexation to this liposomal formulation of NF-κB DNA/DNA hybrids (Fig. 3B). By contrast, complexation of DNA/PDP hybrids to Lys4-Palm exhibits a rather lower efficiency (Fig. 3A). Both Lys4-Chol and Lys4-Palm are also able to complexate to PDP/PDP hybrids (Fig. 3C). In this case, due to difficulty to obtain ^{32}P-labeled PDP/PDP chimeras, unlabeled PDP/PDP molecules were used and the gels were stained with ethidium bromide.

In order to verify whether the complexation observed leads to protective effects, the experiment described in Fig. 4 was performed. When ^{32}P-labeled DNA/PDP chimeras are incubated in serum overnight, a fast, time, and concentration dependent decrease of radioactivity is observed, due to the activity of a variety of enzymes, including 5′-phosphatases, that remove the 5′-end ^{32}P-phosphate group (compare lines (−), no serum, to lines (−) plus serum). Control assays based on gel electrophoresis and ethidium bromide staining of DNA/PDP chimeras demonstrated that nearly 100% of the DNA/PDP molecules maintain the same molecular weight after incubation in the presence of serum (data not shown). When the DNA/PDP chimeras are complexated with increasing amounts of Lys4-Chol and Lys4-Palm before incubation with serum, they are protected from the 5′-phosphatase activity present in serum. Results similar to those shown in Fig. 4 were obtained by repeating the experiments with commercially available 5′-phosphatase (data not shown).

3.3. Cytotoxicity of cationic liposomes

The effect of the liposomal formulations on *in vitro* cell growth was determined by a colorimetric assay, performed using, as a model system, the human leukemia K562 cell line. In order to perform a comparison with commercial

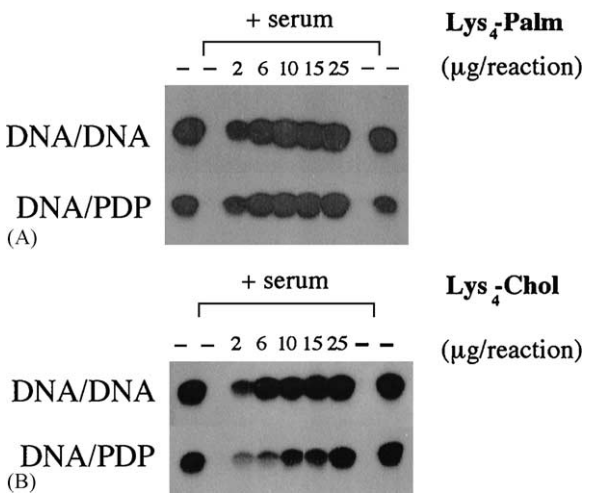

Fig. 4. (A) and (B) represents protective effects of the multivalent cationic lipid Lys4-Palm (lipo-lys4-palm) or Lys4-Chol (lipo-lys4-chol) on ^{32}P-labeled HIV-1 NF-κB DNA/DNA and DNA/PDP target molecules. DNA/DNA and DNA/PDP target molecules were incubated without (−) or with the indicated concentrations (2–25 μg/reaction) Lys4-Palm or Lys4-Chol for 30 min at room temperature and then serum (fetal calf serum, Eurobio) was added (3 μL/reaction). After overnight incubation, the reactions were phenol extracted, ethanol-precipitated, electrophoresed, and autoradiography was performed.

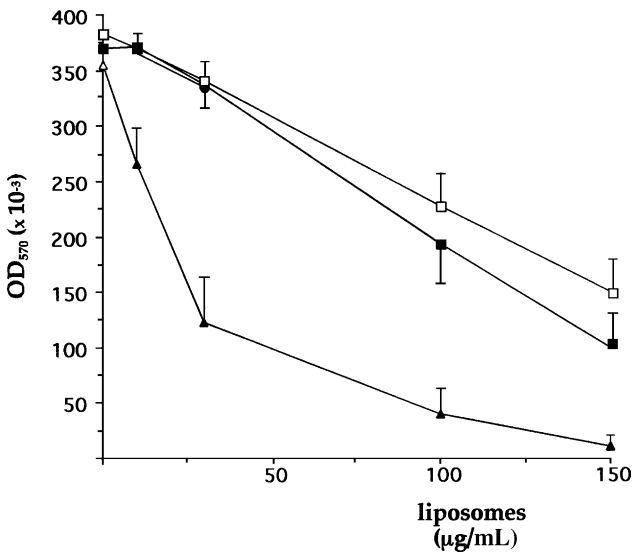

Fig. 5. Dose-dependent inhibition of K562 cell proliferation in the presence of cationic liposomes. Cells were seeded in complete cell culture medium and grown for 72 hr in the presence or in the absence of increasing amount (0–10–30–100–150 μg/mL) of liposomes (□, Lys4-Palm; ■, Lys4-Chol; ▲, lipofectin). After this time, cells number was determined by a colorimetric procedure as described elsewhere [22]. Absorbance measured at 570 nm, proportional to the cells number and expressed in OD ($\times 10^{-3}$) ($OD_{570\ nm} \times 10^{-3}$) was plotted as function of liposome concentration. Values represent the average ± SD of three independent experiments.

cationic liposomes formulations, lipofectin was also included in the experiment. The results of the experiment are shown in Fig. 5. As clearly appreciable, low toxicity (in term of antiproliferative effects) was shown by both lipo-Lys4-Palm and lipo-Lys4-Chol. It should be underlined that, on the contrary, lipofectin displayed a higher antiproliferative activity. When cellfectin and lipofectamine were used, an antiproliferative activity similar to lipofectin was obtained (data not shown).

4. Conclusions

In spite of a number of possible advantages over commonly used nucleic acids, PNA present two major drawbacks: (a) they are not taken up by the cells [3,5,7] and (b) being neutral molecules, they are not suitable for an efficient delivery with nonviral cationic formulations (liposomes, dendrimers, polymers) [7].

The goal of the present report was to determine whether cationic liposomes could efficiently bind PNA–DNA chimeras exhibiting decoy activity and deliver them to target cells.

PNA–DNA chimeras represent an excellent candidate for gene therapy with the decoy approach [24–28], since they are resistant to exonucleases [7] and active in inhibiting transcription factors/DNA interactions [7].

The results presented in this paper demonstrate that PDP/PDP, PDP/DNA, and DNA/PDP hybrids carrying NF-κB binding sites are powerful decoy molecules against NF-κB p52 transcription factor (Fig. 1). In addition, the major conclusion of our results is that cationic liposomes can be proposed for *in vitro* delivery to target cells of decoy molecules based on PNA–DNA chimeras.

With respect to the decoy activity, it should be noted that this issue is of growing interest [24–30] and many recent papers reported the modulation of gene expression using decoy molecules against a variety of transcription factors, including ICP4 [24], E2F [27], CRE-binding proteins [25,27], Sp1 [26], NF-κB [29,30].

The results presented in this paper are thus of practical importance, since the simplicity and the versatility of the cationic liposome technology have made cationic liposomes useful nonviral gene delivery systems for human gene therapy [16–19]. In this respect, further studies are in progress in order to assess the *ex vivo* effectiveness of liposomes for PNA–DNA chimeras administration.

Acknowledgments

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