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Research paper

Cationic lipid–protamine–DNA (LPD) complexes for delivery of antisense c-myc oligonucleotides

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Abstract

In the present study, cationic lipid–peptide–DNA-complexes (LPDs) consisting of AH-Chol-liposomes and protamine–phosphodiester–oligonucleotide-particles (proticles) were introduced as carriers for antisense therapy. The LPDs were physically characterized, and a possible mechanism for adsorption of oligonucleotides (ODNs) was suggested. An increase in stability of ODNs against DNase I and serum nuclease digestion by these carriers was demonstrated. The hydrodynamic diameter increased after incubation with FCS which could be attributed to a protein coating of the particle surface. However, in cell culture medium lower particle sizes of the complexes occurred. In an antisense *c-myc* in vitro model, the effect of LPDs was tested using U937 cells. The C-MYC level was reduced after treatment of these antisense ODN carrier complexes. Furthermore, no changes in target mRNA concentration of the treated cells was found by reverse transcription and competitive multiplex-PCR.

Keywords: Protamine; Nanoparticles; Liposomes; Antisense oligonucleotides

1. Introduction

Over the past few years, major interest in antisense drugs has focused on the establishment of the mechanism and the relation to the biological activity. A wide range of chemical modifications of oligonucleotides with different properties were examined. A number of possible ways of action were identified such as RNase H cleavage [1], inhibition of translation, inhibition of splicing [2], and effects that resulted in loss of stability of RNA (for review, see [3,4]). Phosphodiester oligonucleotides (ODNs) were shown to serve as substrates when bound to target RNAs for RNase Hs [5]. These enzymes cleave the RNA in RNA/DNA duplexes. Also, unspecific mechanisms may exist that have an influence on, for example, the proliferation rate of cells. ODNs with four continuous guanosine residues have been shown to bind to special proteins like nucleolin [6]. On the other hand, Basu and Wickstrom have demonstrated that there is no formation of a higher order structure by antisense *c-myc* phosphorothioate ODNs containing tetraguanylate units at physiological conditions [7].

Despite demonstration of RNase H cleavage in lysate and purified enzyme assays a direct proof of RNase H activation by phosphodiester ODN/RNA duplexes in cells at low oligonucleotide concentrations is lacking. The major drawbacks of these antisense drugs as demonstrated in in vitro models are their low penetration rates through lipophilic cell membranes and their short half-life in biological fluids. In previous studies [8], the inhibition of *c-myc* induced proliferation of U937 cells with 1.6 μM ODNs was shown using positively charged protamine/phosphodiester ODN particles, which we called proticles. These particles are currently under investigation [9–11] and in addition to studies concerning cell proliferation the inhibitory effect of oligonucleotide loaded proticles also was investigated in antiviral assays [12]. In the present study, the antisense specific mechanism of action, i.e. a reduction of protein synthesis, was demonstrated on protein level using ternary

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complexes consisting of negatively charged proticles and cationic AH-Chol-liposomes [13] by Western blot analysis. The quantitative determination of *c-myc* mRNA content after the treatment by these cationic lipid–protamine–DNA complexes (LPDs) was performed by reverse transcription and competitive multiplex-PCR [14].

2. Materials and methods

2.1. Materials

The *c-myc* 20mer phosphodiester ODNs with an antisense (AS, 5′-AAG CTA ACG TTG AGG GGC AT-3′) or scrambled sequence (SC, 5′-TTG CAT TCG AAG TGG GGC TA-3′) [15] were obtained from ARK Scientific (Darmstadt, Germany). All ODNs were delivered as HPLC grade. AH-Chol was a gift from the Institute of Pharmaceutical Chemistry of the Johann Wolfgang Goethe-University (Frankfurt am Main, Germany). Protamine free base, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Deisenhofen, Germany), RNAzol™B from Wak-Chemie (Bad Homburg, Germany), and *c-myc* antibody (Clone 33) from Calbiochem (Bad Soden, Germany). All chemicals were of analytic grade and were used as delivered. The isotonic phosphate buffer solution, pH 7.4, (PBS) was prepared containing 137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate and 8.1 mM disodium hydrogen phosphate. The TBS-T buffer, pH 7.6, consisted of 20 mM Tris, 137 mM NaCl and 0.2% polysorbate 20.

2.2. LPD preparation

Phosphodiester ODNs were adsorbed on protamine in 1:1 mass ratios by mixing an aliquot of 10 μg/ml ODNs to protamine solutions of 10 μg/ml protein in double-distilled water. Liposomes consisting of equimolar ratios of AH-Chol (cholest-5-en-3β-yl-6-aminohexyl ether) and phosphatidylcholin were prepared using a film method followed by a membrane extrusion homogenization step (200 nm, Liposo-Fast, Avestin, Germany) [13]. Pure oligonucleotide or proticle loading of liposomes was achieved by adsorption of the negatively charged ODNs or proticles to the cationic pre-formed liposomes at 10:1 (cationic lipid/ODN) or 1:1:1 (cationic lipid/protamine/ODN) mass ratios. All samples were incubated overnight and then sonicated for 1 min at 20 °C (operating frequency of 35 kHz in continuous mode at 230 W, Transsonic digital, Elma, Singen, Germany) [16].

2.3. Size determination

The hydrodynamic diameters were measured by photon correlation spectroscopy (PCS) using a BI-200 SM Goniometer Ver. 2.0. (Brookhaven Instruments Corp., Holtsville, NY) with a 30-mW He:Ne laser (Melles Griot, Cincinnati, CA) and a BI-2030-AT Digital Correlator (Brookhaven). The measurements were carried out in double-distilled water and additionally at physiological ionic strengths in FCS and cell culture medium at a scattering angle of 90° at room temperature. The influence of adsorption of serum compounds on particle size was measured with LPDs prepared in double-distilled water followed by a 10-fold dilution with FCS or RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin followed by 30 min of incubation at 37 °C and agitation at a frequency of 700 rpm using an Eppendorf Thermomixer 5437 (Eppendorf, Germany). The data from the BI-2030 AT correlator were processed using the NNLS mode of the Brookhaven software. Measurements were recorded ($n=3$) and mean values were given as effective diameters and standard deviations (SD) were calculated for statistical reasons.

2.4. Surface charge

The zeta potential of the particles, liposomes, and complexes of both was determined in double-distilled water (pH: 6.9) as well as after incubation in FCS (pH: 7.4) by measuring the electrophoretic mobility using a Model 501 Lazer Zee Meter™ (PenKem, Bedford Hills, NY). The final oligonucleotide concentration was 5 μg/ml. The measured zeta potential was corrected for a reference temperature of 20 °C.

2.5. Negative stain transmission electron microscopy

In order to investigate the mechanism of complex formation, transmission electron microscopy (TEM, Transmissionselektronenmikroskop EM 902, Zeiss, Germany) was performed. AH-Chol-liposomes, 1:1 proticles, and the complexes of both were adsorbed for 2 min on carbon-coated copper/paladium grids, then the supernatant was wicked off with filter paper and replaced with 2% aqueous uranyl acetate for 15 s. Then, the grids were washed with double-distilled water and dried.

2.6. Oligonucleotide loading

The oligonucleotide content of the proticle–AH-Chol-complexes was determined by an anion exchange HPLC assay, using a Dionex Nucleopac™ PA 100 4×250 column (Dionex, Idstein, Germany) and a Merck-Hitachi HPLC system (Merck, Darmstadt, Germany), as described previously [8]. The samples were centrifuged for 2 h at 100,000×*g* (Ultracentrifuge Optima L-80, Beckmann, Germany). The ODN concentration of the supernatant was determined by measuring the absorbance at 260 nm. The amount of bound ODN was calculated as the difference

between the amount of ODN added to the complex preparation and ODN content in the supernatant.

2.7. Stability against DNase I and serum nucleases

For the determination of the stabilization against endonuclease DNase I ODNs were adsorbed on AH-Chol-liposomes at 10:1 (catonic lipid/ODN) mass ratios. Further, LDPs were prepared as described before (see Section 2.2) and 1:1 proticles served as control. After overnight incubation and 1 min of ultrasonication the samples were exposed to 10 I.U. DNase I (Roche, Mannheim, Germany) for 30 min at 37 °C and agitation at 700 rpm (Thermomixer 5437, Eppendorf, Hamburg, Germany). The enzymatic digestion was terminated with 50 μl 1 M EGTA solution of each 120 μl sample. Then, the complexes were dissolved with 170 μl 4 M NaCl + 4% Triton X-100. After further overnight incubation, the content of intact ODN was measured by HPLC.

For the evaluation of ODN digestion in FCS or cell culture medium supplemented with 10% FCS, 30 μl sample was incubated with 120 μl medium for 24 h at 37 °C.

2.8. Cell cultures

U937 (human histiocytic lymphoma) cells were grown in RPMI 1640 medium (Sigma, Deisenhofen, Germany) supplemented with 10% FCS (Seromed, Berlin, Germany) and 1% penicillin/streptomycin (GIBCO, Eggenstein, Germany) in a humidified incubator containing 5% CO_2 at 37 °C.

2.9. MTT-assay

The toxicity of AH-Chol-liposomes, 1:1 proticles and their ternary complexes was determined with an MTT-assay. Approximately 100,000 cells were added to each well of a 96-well-plate (Nunc, Naperville, IL). The samples were formed in double-distilled water and then diluted 10-fold with cell culture medium. 100 μl samples were added to each well. After 4 or 24 h of incubation at 37 °C and 5% CO_2, 50 μl medium and 50 μl of a 0.25% (w/v) solution of MTT in PBS were added. After another 2 h of incubation the cells were centrifuged at 400 × *g* and 4 °C (Hettich Rotix A/P Zentrifuge, Hettich Zentrifugen, Tuttlingen, Germany) and then dissolved with a 25% (w/w) solution of sodium lauryl sulfate in 0.1 N NaOH. The number of living cells was calculated from the absorption at 550 nm (reference 630 nm) (ELISA-Reader, Dynatech MR 5000, Dynatech, Denkendorf, Germany) in comparison to a medium control.

2.10. Inhibition of C-MYC expression in U937 cells

Approximately 150,000 cells were added to each well of a 24-well-plate (Greiner GmbH, Frickenhausen, Germany) and were incubated for 12 or 24 h with 15 μg *c-myc* antisense or with 15 μg scrambled phosphodiester ODNs in form of AH-Chol/ODN, LPD complexes or 1:1 proticles. Proticles (protamine/ODN mass ratio) of about 2.5:1 served as control. Afterwards the cells were counted and centrifuged for 10 min at 14,000 rpm (Eppendorf-Zentrifuge 5417, Eppendorf, Hamburg, Germany) in 2 ml reaction tubes (Greiner GmbH). The supernatant was rejected and the cells were washed twice with ice-cold isotonic PBS. From this suspension approximately 200,000 cells/well were dissolved in 100 μl lysis buffer consisting of 62.5 mM Tris–HCl, 69.3 mM sodium lauryl sulfate, 1.086 M glycerol, 50.0 mM dithiothreitol and 1.5 mM bromophenol blue. The lysis was followed by SDS-PAGE [17] (Electrophoresis chamber, Whatman Biometra Power Pack P 25, Göttingen, Germany) and Western blot analysis [18] (Western-Blot Apparatur Bio-Rad Mini Trans Blot, Bio-Rad). The transfer-membrane (Transfer-Membran Millipore Immobilon™-P, Millipore GmbH, Eschborn, Germany) was blocked overnight at 4 °C with a 6% solution of skimmed milk powder in TBS-T. After a washing step, the membrane was incubated first with the *c-myc* antibody and then with a peroxidase-conjugate secondary anti-mouse antibody for 1 h, both times followed by washing steps. The detection was made with ECL Western blotting detection reagents using a chemiluminescence system.

2.11. Evaluation of the antisense effect by mRNA level determination

The quantitative determination of the antisense effect on *c-myc* mRNA level was determined by RT-competitive multiplex-PCR as described previously for CFTR [14,19]. Approximately, 200,000 cells were treated for 8, 16 or 24 h in a 24-well-plate (Greiner GmbH) with 20 μg *c-myc* antisense or scrambled ODNs bound to AH-Chol–proticle complexes. After incubation, the cells were centrifuged for 15 min at 500 × *g* (Eppendorf Zentrifuge 5417) and washed with ice-cold PBS. Then, RNA was isolated by RNAzol B™ treatment according to the manufacturer's instructions. After determination of the mRNA content (spectrophotometric measurement at 260 nm), RT-competitive multiplex-PCR was carried out. Three micrograms RNA were reverse transcribed into cDNA in 20 μl 1 × transcription buffer that additionally contained 1.5 mmol/L of each dNTP, 500 ng random hexamers, and 200 U Superscript II-reverse transcriptase (Life Technologies) according to the manufacturer's instructions. The PCR conditions were standardized using a master mixture containing 50 mmol/L KCl, 10 mmol/L Tris–HCl, 1.5 mmol/L $MgCl_2$, and 0.5 mmol/L dNTPs, with 50 pmol *c-myc* primer (Ex1, 5′- GGC ACT TTG CAC TGG ACC TT-3′; Ex2, 5′-GGT CAT AGT TCC TGT TGG TG-3′) [2], ARK Scientific, Germany), 5 pmol *GAPDH* primer (A, 5′-ACT TTC CAG GAG CGA GAT CC-3′ and B, 5′-ACC ACT GAC ACG TTG GCA GT-3′ from Life Technologies GmbH, Germany), and 0.5 μL of cDNA per 50 μL of total PCR volume. Aliquots of this

master mix were added to serial dilutions (1:3) of a mixture of the competitors *C-MYC* IS (6×10^4 molecules/μL) and *GAPDH* IS (6×10^5 molecules/μL, gift from the Institute of Internal Medicine, Division of Pulmonary Medicine, Johann Wolfgang Goethe-University hospital, Frankfurt, Germany). The PCR products were separated by a 2% agarose gel electrophoresis, visualized with ethidium bromide and documented with a Docu-Gel IV System (MWG Biotech, Ebersberg, Germany). The ratio of target to competitor was determined for each dilution of the competitors and compared to the constant ratio of GAPDH and its competitor. Consequently, the number of molecules of competitor corresponding to a 1:1 ratios is equivalent to the number of molecules of input target cDNA.

3. Results

3.1. Characterization of AH-Chol–proticle-complexes

ODNs were bound to protamine at 1:1 mass ratios by combining aliquots of 10 μg/ml oligonucleotides with solutions of protamine of the same concentration in double-distilled water. The mixing resulted in spontaneous particle formation with sizes ranging between 90 and 100 nm [8,9]. These negatively charged proticles were adsorbed on AH-Chol-liposomes with hydrodynamic diameters of about 250 nm [13]. The cationic lipid–proticle-complexes had mean diameters of about 320 nm (see Table 1) During sonication the structure of the liposome-particle-complexes was reorganized and a certain part of the proticles were transferred into the interior of the liposomes resulting in particles similar to cationic lipid–peptide–DNA-complexes (LPDs) obtained with plasmid-DNA [20] (Fig. 1). There was no change in mean size after sonication. With AH-Chol/ODN liposomes, no change in hydrodynamic diameters occurred during complexation compared to the unloaded 250 nm AH-Chol-liposomes.

The structural alterations of the proticle–liposome complexes led to an important change in surface charge: 1:1 proticles had a zeta potential of −32 mV, AH-Chol liposomes one of +40 mV. After the adsorption of proticles on the surface of the liposomes, the complexes became negatively charged (−17 mV). Finally, after ultrasonication the structural changes led to the migration of a part of the proticles and, consequently, the negative charge into the liposomes causing almost complete neutralization of the whole complexes, i.e. a zeta potential of about −2 mV. Changes also were observed with AH-Chol/ODN complexes. After incubation of AH-Chol-liposomes with ODNs in 10:1 mass ratios, the complexes were negatively charged (−23 mV). Ultrasonication induces a shift of the neutralized cationic lipids into the interior of the liposomes and yielded positive zeta potentials of +22 mV.

Table 1
Particle diameter determined by PCS

Preparation	Particle diameter (nm), mean(±SD)		
	Water	FCS	RPMI 1640
1:1 Proticles	95 (±8)	145 (±10)	82 (±8)
AH-Chol	250 (±15)	271 (±21)	201 (±20)
AH-Chol/ODN	253 (±21)	275 (±12)	198 (±22)
LDP (after sonication)	320 (±18)	n.d.	n.d.

$n=3$; mean value = effective diameter ± SD; n.d., not detected.

The content of ODN loading was analyzed after ultracentrifugation in the supernatant. In the 1:1 proticle preparation, 40% ODN remained unbound. This result could be improved by complexation with the AH-Chol-liposomes and 97% (SD = 2.7%, $n=3$) of the added ODNs were bound. After ultrasonication, the content of intact ODNs was slightly reduced (93%, SD = 0.4%, $n=3$). This was caused by degradation of ODNs during ultrasonication [16]. With AH-Chol-liposomes, the total binding of ODNs was achieved at a 10:1 mass ratio (AH-Chol/ODN) [13].

3.2. Protection of oligonucleotides against DNase I digestion

The protective effect of binding of ODNs into proticle–AH-Chol-complexes against nucleases was determined by exposing 1:1 proticle, AH-Chol/ODN- and proticle–AH-Chol-complexes to relatively high concentrations of DNase I for 30 min. Unbound ODN was almost completely digested, only 1.7% (SD = 0.5, $n=3$) intact ODN was found. In 1:1 proticle samples, 20.4% (SD = 9.4%, $n=3$) of the ODN was digested, and in AH-Chol/ODN complexes 4.4% (SD = 2.7%, $n=3$). Proticle–AH-Chol-complexes completely protected ODNs against DNase I digestion.

3.3. Stability in cell culture medium

Incubation of 1:1 proticles with cell culture medium resulted in a decrease in particle diameter. This probably was caused by a difference in hydration layer at physiological ionic strength. In contrast, after incubation with FCS, the hydrodynamic diameter of 1:1 proticles increased compared to double-distilled water. This might be the result of the higher protein content in this medium. AH-Chol/ODN-complexes showed an increase of about 20 nm in mean diameter in FCS and a significant decrease in cell culture medium.

In most previous in vitro antisense experiments, treatment of the cells with carrier-ODN complexes was performed in serum-free or heat inactivated medium to avoid degradation of ODNs by nucleases. In our experiments, serum containing medium nearly completely digested unbound ODNs within 24 h (Fig. 2). After this time, only 3.5% intact ODNs were detected by HPLC, whereas in a control experiment with unbound ODNs, in which the enzymatic digestion was stopped immediately,

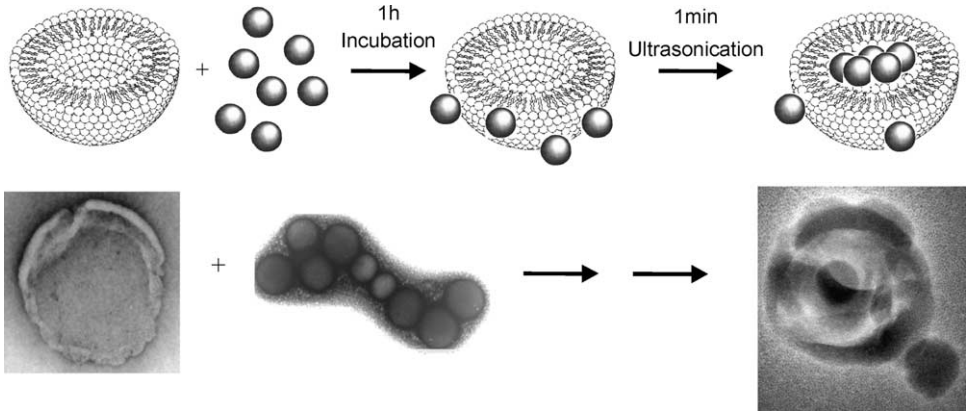

Fig. 1. Postulated mechanism of LPD formation. Pure AH-Chol-liposomes, 1:1 proticles and the product after incubation and sonication of AH-Chol-liposomes with 1:1 proticles were visualized by transmission electron microscopy.

no degradation occurred. A 1:1 proticle preparation yielded a significant stabilization (13.9% undigested ODNs), followed by AH-Chol/ODN-complexes (37% intact ODNs). The highest stabilization was achieved with ODNs bound into proticle–AH-Chol-complexes, 43% of the ODNs remained intact after 24 h.

3.4. MTT-assay

The effects of free AH-Chol-liposomes, proticle–AH-Chol-complexes and negatively charged proticles on the cellular metabolism were determined in U937 cells using a MTT-assay after 4 or 24 h of incubation. Free AH-Chol-liposomes exhibited the strongest toxicity in this assay by reducing the cell viability to 66% ($n=8$, SD = 16.8%) after 4 h and to 47% ($n=8$, SD = 4.9%) compared to a medium control after 24 h of incubation. The complexation of AH-Chol-liposomes with proticles decreased the toxicity, and increased the number of living cells up to 88% ($n=8$, SD = 10.7%) after 4 h and 71% ($n=8$, SD = 13.2%) after 24 h. Proticles alone were least toxic. After 4 h of incubation 80% ($n=8$, SD = 16.2%), and after 24 h 96% ($n=8$, SD = 7.3%) cell viability was found.

3.5. Inhibition of C-MYC expression in U937 cells

The sequence-specific antisense effect of unmodified ODNs as proticle, AH-Chol/ODN, and proticle–AH-Chol preparations was analyzed by Western blot. The changes in C-MYC concentration after 12- and 24-h incubation time are shown in Fig. 3. With proticle–AH-Chol complexes, no reduction of the target protein concentration was shown after 12 h of incubation. However, after 24 h, the C-MYC levels were significantly reduced with the antisense preparations. An unspecific reduction also was observed with the scrambled proticle–AH-Chol-complexes. With AH-Chol/ODN-liposomes no change in C-MYC level occurred. In order to analyze the influence of the cationic lipid further experiments were carried out with pure 1:1 and 2.5:1 proticles (protamine/ODN mass ratio). In our previous study, positively charged 2.5:1 proticle preparations already showed a sequence specific inhibition of the cell proliferation [8] and, hence, we used this preparation as positive control in the present study. In agreement with these results, a reduction of C-MYC protein again was observed with the 2.5:1 proticles now and a less pronounced reduction was measured with the 1:1 proticles. Particles with scrambled oligonucleotides showed no effect. Consequently, an unspecific effect of the scrambled phosphodiester ODNs as well as the protamine can be excluded. Hence, the reduction of the cellular C-MYC concentration caused by the proticle–cationic lipid-complexes must be due to the AH-Chol lipid. This unspecific reduction of C-MYC might be due to the influence on cell viability which has been observed by the MTT-test.

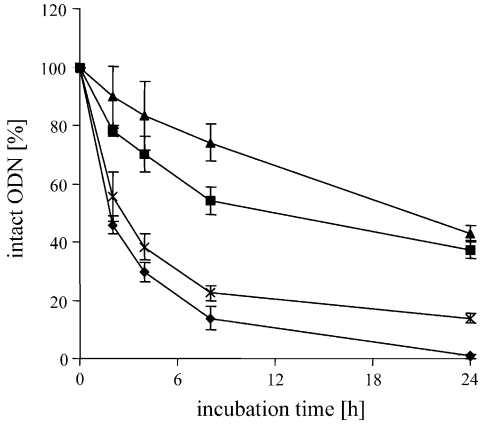

Fig. 2. Degradation of ODNs by serum nucleases. Free (—◆—) and complexed ODNs (—■— AH-Chol/ODN, —▲— LPDs, —×— 1:1 proticles) were incubated for 2, 4, 8 and 24 h with serum containing cell culture medium. The ratio of intact ODNs was quantified by HPLC analysis ($n=3$).

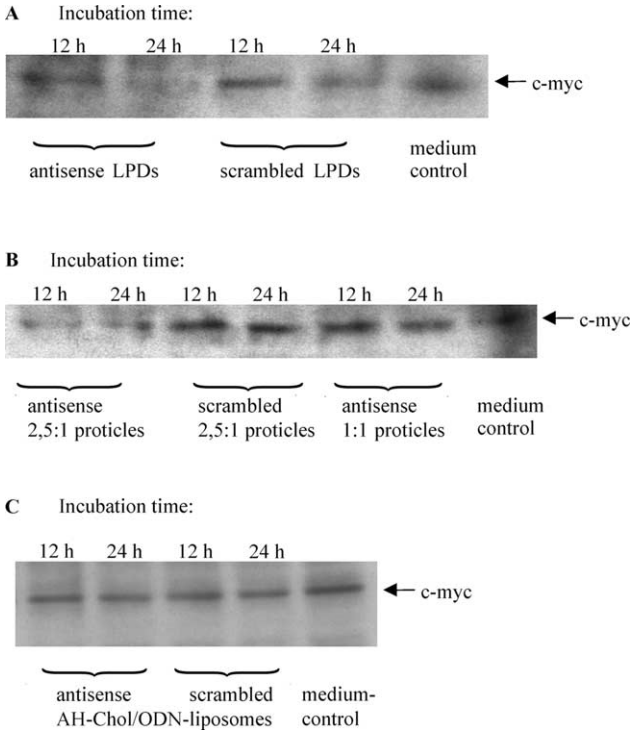

Fig. 3. Antisense effects visualized by determination of the C-MYC expression. Western blots of proteins captured by the lysates of U937 cells which had been treated with antisense and scrambled (A) LPD, (B) proticle, and (C) AH-Chol/ODN-liposome preparations for 12 and 24 h.

3.6. Evaluation of the antisense effect by mRNA level determination

The *c-myc* mRNA levels of U937 cells were quantified using RT-competitive multiplex-PCR. *C-myc* and *GAPDH* were co amplified with specific internal standards. *GAPDH* in constant concentrations served as controls. The PCR products were separated by gel electrophoresis and visualized with ethidium bromide. The ratio between *c-myc* and its internal standard was compared to the constant ratio of *GAPDH/GAPDH* IS. Over the measured time, 8, 16 and 24 h of incubation, no changes in relative cellular *c-myc* mRNA levels were found (Fig. 4). For this reason, we conclude that the antisense action with our phosphodiester 20mer *c-myc* antisense ODNs is mediated by a posttranscriptional inhibition due to sterical blockade of the translation initiation codon.

4. Discussion

After introduction of cationic lipids [21], these carriers became the most used non-viral penetration enhancers for antisense and gene delivery. Nevertheless, cationic lipid based gene and oligonucleotide transfer remains relatively inefficient compared to viral vectors such as adenovirus.

In 1996, the group of Huang, Gao et al. [20] introduced a new technique to potentiate the transfection efficacy of cationic lipid/DNA complexes. Before binding on the cationic carrier, the DNA was condensed with a polycationic peptide such as poly(L-lysine) or protamine sulfate. The ternary complexes were supposed to mimic viral carriers containing condensed DNA, which is surrounded by a lipid bilayer. The pre-condensation of DNA resulted in a reduction of the particle size and in an increased stability against nucleases. Gao et al. [20] named these particles LPDs (cationic lipid–peptide–DNA-complexes).

In our paper, a similar approach was employed for antisense oligonucleotides. In addition to previous studies,

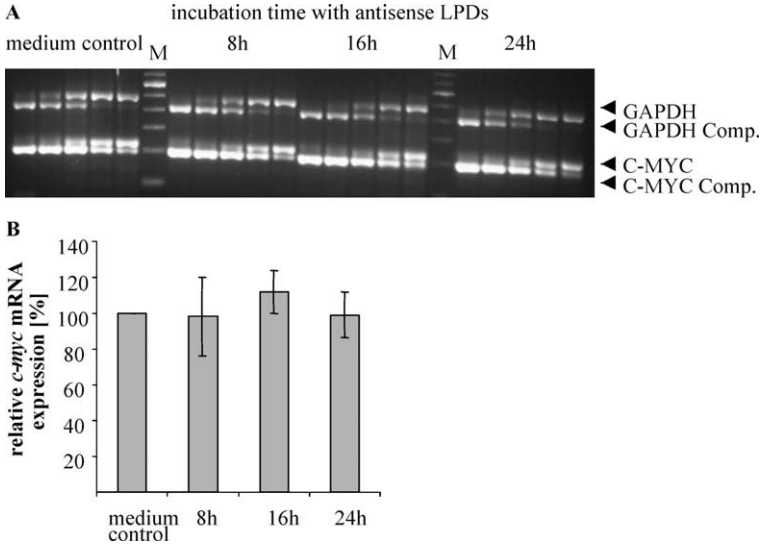

Fig. 4. Effect of *c-myc* antisense LPDs on target mRNA expression. U937 cells were incubated with antisense LPDs for 8, 16 and 24 h. (A) Agarose gel electrophoresis was performed with ethidium bromide-stained marker (M) and *GAPDH* and C-MYC products of a RT-competitive multiplex PCR. Each sample was examined five times with serial dilution (1:3) of cDNAs of the internal standards. (B) *C-myc* mRNA levels were quantified by densitometry and normalized to GAPDH expression as described. Results ($n=3$ for each group) are represented as percentage of control at time 0 (means $\pm$ SD).

where we have introduced the formation of proticles that result from an aggregation between protamine and oligonucleotides [8], the present study describes the possibility to encapsulate these proticles into liposomes. Because of the similarity of the present particles to those of Gao et al., we also call them LPDs in the following. In concordance to the results reported by this group, the complexation of our AH-Chol-liposomes with 1:1 proticles also caused an increased stability of ODNs against nucleases. However, there was no formation of fingerprint-like colloidal particles [22] or particles which were surrounded by a lipid bilayer [20]. Both 1:1 proticles and AH-Chol-liposomes kept their physical characteristics. An increase of the effective diameter was observed after incubation with FCS, which could be explained by an adsorption of serum proteins onto the particle surface. In addition, as published before [8] by our group, the measurements in cell culture medium resulted in a decrease of the particle diameters due to a lower protein content and the influence of salts on the hydrodynamic layer of the particle surface.

Summarizing the proposed mechanism, in an initial step, the negatively charged proticles were adsorbed to the surface of the cationic liposomes followed by ultrasonication which caused a reorganization of the lipid bilayer (Fig. 1). Parts of the proticles were surrounded by neutralized lipids and were encapsulated into the liposomes. Finally, after reaching an equilibration proticles were present in the interior as well as at the surface of the liposomes. Proticle encapsulation was confirmed by electron microscopy and led to an increase in the hydrodynamic diameters of the LPDs determined by PCS.

The complexation of ODNs with protamine also resulted in an increased loading and partial encapsulation of ODNs in AH-Chol-liposomes in comparison to pure AH-Chol/ODN preparations. It is remarkable that approximately 40% ODN used for the 1:1 proticle preparation was found to be in the supernatant after ultracentrifugation, however, about only 20% was susceptible for DNase I digestion which led us to the conclusion that a certain amount of ODN could be bound to soluble protamine complexes and the conditions applied for the 1:1 proticle preparation are not sufficient for a complete ODN precipitation.

Furthermore, the toxic effects of the AH-Chol lipid was significantly reduced by binding of proticles to the cationic liposomes. These effects confirmed earlier results reported by Fischer et al. [23], who showed that the neutralization of cationic lipids by oligonucleotides also reduced the toxicity. Most likely, the toxicity of such cationic lipids is a result of a charge neutralization of the cell membrane which does not occur in the presence of negatively charged ODNs. Thus, the toxicity of the preparation containing protamine is lower than that of samples without the cationic peptide.

Proticles of about 1:1 have been shown to increase the cellular uptake of ODNs [24] and to accumulate in the nucleus. This was caused by amino acid sequences resembling that of a nuclear localization signal [25]. However, it seems to be not necessary for all ODN applications to transport the ODNs into the nucleus since specific ODN effects are expected to take place into the cytoplasma. In one of our recent studies, LPDs consisting of AH-Chol-liposomes together with 1:1 proticles showed a similar cellular uptake than the pure 1:1 proticles (data not shown). Nuclear targeting was also seen with LPDs consisting of cationic protamine–plasmid-DNA-complexes and negatively charged liposomes [26]. The combination of AH-Chol lipid with the 1:1 proticles resulted in a reduction of C-MYC within 24 h of incubation, whereas the pure 1:1 proticle preparation showed no effect. After this time also, a sequence-unspecific effect was recognized with scrambled ODN preparations. This reduction of the C-MYC level could be due to the cationic lipid AH-Chol, because experiments with other proticle preparations without the cationic lipid resulted only in sequence-specific antisense effects.

Initially we expected RNase H cleavage to be the major antisense mechanism because of the nuclear targeting of protamine. However, there was no decrease in target mRNA concentration over the measured time in comparison to an internal standard. No cleavage of *c-myc* mRNA occurred although a reduced content of target protein was observed at the same incubation time. Consequently, it has to be concluded that the ODNs used in this study were targeted to the translation initiation region of complementary mRNA. Similar results were obtained by Rosolen et al. [27]. The RNase H content of U937 cells did not influence the antisense-induced C-MYC protein reduction and growth inhibition. Our present study also indicates that the antisense mechanism with phosphodiester ODNs delivered by LPDs is inhibition of mRNA translation by sterical blockade of the translation initiation codon.

5. Conclusion

The combination of proticles with cationic lipids can improve the efficacy of liposomal preparations in terms of ODN loading and toxicity. The ODN delivery of the LPDs was similar to pure 1:1 proticle formulations. Additionally, an improvement of sequence specific antisense effects was demonstrated with the 1:1 proticle–AH-Chol-complexes in comparison to pure negatively charged 1:1 proticles and AH-Chol/ODN liposomes. An effect of Ah-Chol lipids alone on the protein expression can not be excluded since the cationic lipid turned out to be cytotoxic.

The antisense mechanism with phosphodiester ODNs is based on the inhibition of mRNA translation by sterical blockade of the translation initiation codon and is independent of RNase H activity.

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