J. Biochem. **132**, 23–27 (2002)

The Effect of β-Tubulin-Specific Antisense Oligonucleotide Encapsulated in Different Cationic Liposomes on the Supression of Intracellular *L. Donovani* Parasites *In Vitro*[1]

Dalia Dasgupta,[*] Samit Adhya,[†] and Mukul Kumar Basu[*,2]

Divisions of [*]Biomembrane and [†]Genetic Engineering, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Calcutta-700032, India

Received December 10, 2001; accepted March 26, 2002

An antisense oligonucleotide (20 mer) targeted to the parasite β-tubulin gene and encapsulated in cationic liposomes, was used to test its antileishmanial activity *in vitro*. Cationic liposomes containing dioleyl trimethyl ammonium propane (DOTAP) were found to have higher antileishmanial activity (88% at 4 μM oligonucleotide) compared to two other liposomes with stearyl amine (SA) and cetyl trimethyl ammonium bromide (CTAB) as cations. Dot-blot experiments were performed to analyse the expression of β-tubulin mRNA using β-tubulin–specific radiolabelled DNA as a probe. When compared with their respective controls, β-tubulin–specific gene expression was found to be diminished by treatment with a specific antisense oligonucleotide encapsulated in cationic liposomes (CTAB:DOPE) in a concentration-dependent manner. These experiments show that antisense oligonucleotides targeted to the β-tubulin gene of *Leishmania donovani* inhibit β-tubulin synthesis leading to the arrest of multiplication of intracellular parasites.

Key words: antisense oligonucleotides, β-tubulin, cationic liposomes, leishmania, RNA expression.

The clinical applications of antisense oligonucleotides are of particular interest due to their antiproliferative, antiviral, and antiparasitic properties (*1*). Antisense molecules are designed to modulate the expression of specific proteins by interfering with transcription, mRNA maturation and/or translation (*2*).

In case of leishmaniasis, the serious host toxicity of conventional drugs along with the evolving phenomenon of drug resistance has prompted the need for a "perfect" medication without side effects. Antisense oligonucleotides, with their exquisite specificity and high affinity for target sites and ease of design, promise to provide highly specific tools for overcoming many of the drawbacks of conventional chemotherapy (*1*). Selection of the target site is very important as antisense molecules are designed to modulate the expression of a specific gene. As reported, an attractive target site for the antisense inhibition of leishmania and other kinetoplastide protozoa is the 39-nucleotide miniexon sequence universally present at the 5′ end of all cytoplasmic mRNAs. Antisense oligonucleotides complementary to the miniexon sequence have been shown to inhibit *L. amazonensis* (*3*) and *Leishmania donovani* (*4*) growth in macrophages *in vitro*. Moreover, we have shown that encapsulation in cationic liposomes (CTAB: DPPE) targeted to the miniexon sequence has increased the leishmanicidal activity of the antisense oligonucleotide (*4*).

In this study, we selected the β-tubulin gene of *Leishmania* as the target site for antisense therapy. Tubulins are major cytoskeletal proteins in kinetoplastid protozoa, and play an important part in a number of cellular structures such as the cytoskeleton, pellicle, mitotic spindle, and flagellum. The microtubular system has been used as a potential target for drugs in curative and preventive chemotherapy of trypanosomiasis and leishmaniasis (*5, 6*). In this study, we investigated the effect of different cationic liposome–encapsulated anti–β-tubulin oligonucleotides on the suppression of intracelullar *L. donovani* growth and β-tubulin mRNA expression *in vitro*.

EXPERIMENTAL PROCEDURES

Materials—Dioleylphosphatidylethanolamine (DOPE), cetyltrimethyl ammonium bromide (CTAB), stearylamine (SA), dioleyl trimethyl ammonium propane (DOTAP), agarose, acrylamide, trizma, sodium dodecylsulfate, ammonium persulfate, chloroform, methanol, phenol, EDTA, β-mercaptoethanol, isoamyl alcohol, and isopropanol were purchased from Sigma Chemicals. Medium M199, RPMI 1640, heat inactivated fetal bovine serum, penicillin, streptomycin, and gentamycin were from Gibco Laboratories. Absolute ethanol was purchased from E-March. All other reagents were of analytical grade.

Oligonucleotides—Antisense oligonucleotide complementary to positions 1–20 of the *L. donovani* β-tubulin gene (5′CAGGAAACGATCACGCAT 3′) and the corresponding

[1] Financial support from the Department of Biotechnology, New Delhi, is gratefully acknowledged. Thanks are due to the Council of Scientific and Industrial Research, New Delhi, for providing financial assistance to Dr. M.K. Basu in the form of an ES scheme.

[2] To whom correspondence should be addressed: Tel: +91-33-473-6793, Fax: +91-33-473-5197, E-mail: biomembrane@iicb.res.in (O), mukulkbasu@yahoo.com (R)

sense oligonucleotide (5′ATGCGTGAGATCGTTTCGTG3′) were synthesized by Bangalore Genei (Pvt), Peenya, India. Crude oligonucleotides were purified by electrophoresis in denaturing 15% polyacrylamide gels. Oligonucleotides were ^{32}P-labeled using T4 polynucleotide kinase, and quantified by adsorption on DEAE paper, as described (*4*).

Preparation of Cationic Liposomes—Cationic liposomes using DOPE and different cations such as CTAB, SA, DOTAP in a molar ratio of 4:1 were prepared a using standard protocol (*4, 7*). The liposomal suspension was passed through Millipore filter paper for sterilization and the size of the vesicles was found to be ~200 nm in diameter.

Encapsulation of Labeled Oligonucleotide into Liposomes and Assessment of Encapsulation—The purified oligonucleotides were first labeled with [γ-^{32}P]ATP using a standard protocol (*4*). To the cationic liposomes prepared as described above, labeled oligonucleotides were added during swelling of the lipid film with PBS. The mixture was then sonicated and centrifuged at 100,000 ×*g*. Radioactivity present in the washed pellet was then measured. To calculate the percentage encapsulation, the liposomes were treated with pancreatic DNase I (50 units/ml) to remove the surface associated radioactivity. The total amount of encapsulated and surface-associated oligonucleotide was found to be 30% whereas 10% of oligonucleotide was encapsulated. The oligonucleotide to lipid molar ratio was calculated to be around 1:670.

Parasites—The pathogenic strain of *L. donovani* AG83 (MHOM/IN/1983/AG83) was used in this study. The preparation and maintenance of the amastigote and promastigote forms of the parasites were as described (*4*).

Macrophage Culture and Infection with Parasites—The TG-elicited peritoneal macrophages from swiss mice were isolated and plated on coverslips for 2 h at 37°C to obtain a monolayer of macrophages. The macrophage monolayer was challenged with *L. donovani* promastigotes at a ratio of (1:20) and incubated for 2 h at 37°C.

Treatment of Infected Macrophages with Oligonucleotides—To the infected macrophages, β-tubulin–specific free antisense oligonucleotides, complementary free sense oligonucleotides and different cationic liposome–encapsulated antisense oligonucleotides at concentrations of 1–4 μm were added and the mixtures were kept for 24 h at 37°C, and then in oligonucleotide–free medium for 18 h at 37°C in a CO_2 incubator. The cells were then either processed for the microscopic analysis of the fate of the intracellular parasites, or total RNA was isolated to analyse the β-tubulin RNA levels using a standard protocol. The viability of macrophages in presence of antisense oligonucleotides was examined by the trypan blue exclusion test..

RNA Isolation—Tolal RNA from infected macrophages and antisense oligonucleotide–treated infected macrophages was isolated using the general phenolchloroform method (*8*). To 100 μl of PCV (packed cell volume), 5 volumes of solution D (containing 4 M guanidium isothiocyanate, 50 mM Tris (pH 7.5), 10 mM EDTA, 2% sarcosyl) and 18 μl β-mercaptoethanol were added and mixed well. To this, 1 volume of 1 M NaAC (pH 4.0) and 5 volumes of phenol:H_2O were added, mixed well, and the mixtures were kept on ice for 10 min. To this, chloroform:isoamylalcohol (24:1 v/v) was added, and the mixtures were vortexed and kept on ice for 10 min. Then the mixtures were centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatants were collected. To the supernatants, equal volumes of isopropanol were added and the mixtures were kept at –20°C overnight. The next day, the solutions were centrifuged at 10 K for 15 min at 4°C. The pellets were washed with 70% alcohol, dried , dissolved in DEPC water and kept at –70°C until use. The total RNA from *L. donovani* promastigotes was prepared in the same way. The OD of 1 μl RNA was checked at 260 and 280 nm; its presence was confirmed by electrophoresis in 1% native agarose gels (data not shown).

Dot-Blot Analysis—For Dot-Blot analysis, the total RNA from infected macrophages, free and cationic liposome-encapsulated antisense oligonucleotide–treated infected macrophages, normal macrophages, and *L. donovani* parasites was spotted on a Hybond nylon membrane at a fixed concentration. The membrane was dried, UV crosslinked and kept at 4°C before hybridization.

Preparation of β-Tubulin Probe DNA—Plasmid PLDBT-1 containing the β-tubulin gene inserted in plasmid pUC 8 was isolated from *Escherichia coli* cells using a standard method (*9*). The coding region of the β-tubulin gene (540 bp) insert was recovered from the plasmid DNA (pUC8) with restriction enzymes *Hin*dIII and *Xho*I and isolated by electrophoresis followed by electroelution and purification.

Labeling of the DNA Probe—The isolated fragment was labeled with [α-^{32}P]dTTP by the random priming method (*9*).

Hybridization with Labeled β-Tubulin DNA Probe—The UV-crosslinked spots of RNA on the Hybond nylon membrane (dot-blot analysis) were prehybridized for 3–4 h at 42°C in 50% deionized formamide, 5× SSC, 5× Denhardt solution, 20 mM sodium phosphate, pH 7.0, 0.1% SDS, and 1 ml of heat chilled calf thymus DNA (2 mg/ml). Hybridization was performed in the same solution, additionally containing α-^{32}P–labeled denatured βT_1 DNA probe at 10^5–10^6 cpm/ml for 48 h at 42°C. Blots were washed at a stringency of 0.1× SCC, 0.1% SDS at room temperature as described (*9*) and autoradiographed.

RESULTS

The Effect of Different Cationic Liposome-Encapsulated β-Tubulin Targeted Antisense Oligonucleotides in Killing Intracellular Leishmania Parasites—The β-tubulin–specific antisense oligonucleotide sequence is shown in Fig. 1. We investigated the effect of the antisense oligonucleotide encapsulated in three different cationic liposomes using CTAB, SA, DOTAP as cations and DOPE as the lipid. The molar ratio used in this cationic liposome preparation is 4:1. The data in Table I show that the specific antisense oligonucleotides encapsulated in cationic liposomes are effective in killing intracellular leishmania parasites with a maximum effect at a concentration of 4 μM of about 73.3 ±

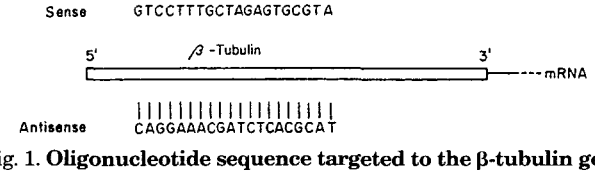

Fig. 1. **Oligonucleotide sequence targeted to the β-tubulin gene of *L. donovani*.** The sequences of the antisense and sense oligonucleotides used for targeting are shown as a box. The vertical lines show hybridization of the oligonucleotides and β-tubulin.

3, 80.0 ± 1, and 88.0 ± 2% for SA, CTAB, and DOTAP, respectively. Compared to this, the free antisense oligonucleotide at a concentration of 1 to 4 μm is effective in suppressing the parasite burden only about 22 to 28%. The corresponding free sense oligonucleotides at a concentration of 1–4 μM when used as controls, show insignificant parasite burden suppression (6.7 to 9.7%). Another control sense oligonucleotide (med RNA) with the sequence 5′CGCTATATAAGTATCAGTTT3′ (Bangalore Genei, India) in the concentration range of 1–4 μM, suppressed the intracellular parasite load only about 5–6%. Free antisense oligonucleotides had no effect on the viability of macrophages under these conditions (Fig. 2). From these results, it appears that there is a sequence–specific effect of antisense oligonucleotides, and that delivery through cationic liposomes increases their efficacy about 3.5 times. The choice of cations for the delivery of antisense oligonucleotides may also play a part as different cationic lipids showed different efficacies (Table I).

Measurement of Intracellular β-Tubulin Levels of L. donovani (Dot-Blot Analysis)—Total RNA from infected macrophages, antisense treated infected macrophages, *L. donovani* parasites and from normal macrophages was subjected to dot blot hybridization using a β-tubulin probe (Fig.

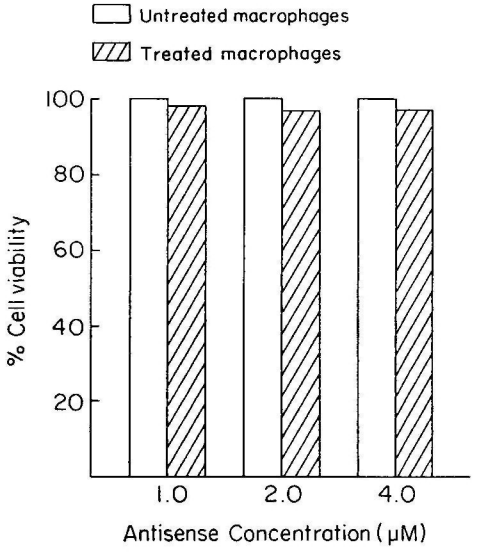

Fig. 2. **Trypan blue exclusion test to investigate the toxicity of antisense oligonucleotides.** Macrophages (both untreated heat killed and drug treated) were incubated *in vitro* with varying concentrations of antisense oligonucleotides for 1 h at 37°C. then 0.4% of trypan blue solution was added, incubatd for 10 min, and % viability was calculated.

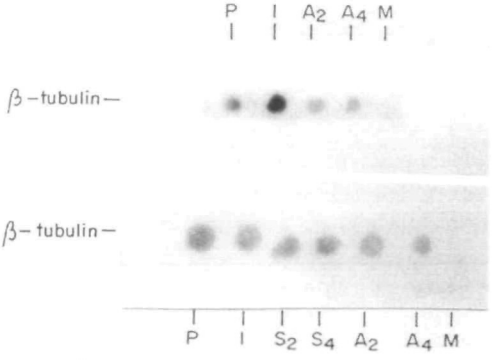

Fig. 3. **Autoradiogram of β-tubulin RNA levels (Dot-blot).** Total RNA from *L. donovani* parasites, normal macrophages, specific sense and liposomal (CTAB:DOPE) antisense oligonucleotide–treated infected macrophages were isolated and analyzed for β-tubulin levels using specific radiolabelled β-tubulin DNA as a probe. The results of two different experiments are shown in different panels. Upper panel: P, parasites; I, infected macrophages; A_2, liposomal antisense 2 μM; A_4, liposomal antisense 4 μM; M, Macropahges. Lower panel: P, I, A_2, A_4 and M represent the same samples as in the upper panel; S_2, sense oligonucleotde at 2 μM; S_4, sense oligonucleotide at 4 μM.

TABLE I. **Effect of β-tubulin–specific antisense oligonucleotides (20 mer) encapsulated in different cationic liposomes on the suppression of intracellular parasite (*L. donovani*) load.**

Treatment with β-tubulin–specific sense/antisense-oligonucleotides	Composition of cationic liposomes	% suppression
Free sense (MR), control (1 μM)	–	05.0 ± 1.5
Free sense (tubulin), control (1 μM)	–	06.7 ± 2.0
Free antisense (1 μM)	–	22.0 ± 2.0
Liposomal antisense (1 μM)	CTAB:DOPE	50.0 ± 2.0
Liposomal antisense (1 μM)	SA:DOPE	35.0 ± 3.0
Liposomal antisense (1 μM)	DOTAP:DOPE	55.0 ± 4.0
Free sense (MR), control (2 μM)	–	06.0 ± 1.7
Free sense (tubulin), control (2 μM)	–	08.2 ± 1.0
Free antisense (2 μM)	–	26.0 ± 3.0
Liposomal antisense (2 μM)	CTAB:DOPE	66.0 ± 3.0
Liposomal antisense (2 μM)	SA:DOPE	59.3 ± 4.0
Liposomal antisense (2 μM)	DOTAP:DOPE	69.3 ± 3.7
Free sense (MR), control (4 μM)	–	06.0 ± 2.0
Free sense (tubulin), control (4 μM)	–	09.7 ± 3.0
Free antisense (4 μM)	–	28.0 ± 2.0
Liposomal antisense (4 μM)	CTAB:DOPE	80.0 ± 1.0
Liposomal antisense (4 μM)	SA:DOPE	73.3 ± 4.0
Liposomal antisense (4 μM)	DOTAP:DOPE	88.0 ± 2.0

Infected macrophages were treated with β-tubulin–specific antisense oligonucleotides either in cationic liposomal or free form and with their complementary free sense oligonucleotides at a concentration of 1–4 μM for 24 h at 37°C in a CO_2 incubator. The cells were then again kept in oligonucleotide-free medium for 18 h under the same experimental conditions. The samples were then processed to analyze the fate of internalized parasites microscopically. Results are mean ± SD (n = 3). Control sense oligonucleotides [both specific to medRNA (MR) and tubulin] when used in cationic liposomal form (CTAB:DOPE) in the concentration range of 1–4 μM suppressed the intracellular parasite load by about 10–12% at most.

TABLE II. **Densitometric scanning analysis of β-tubulin-specific RNA bands.**

Oligonucleotides	Concentration	% inhibition of β-tubulin RNA levels
Sense	2μM	14 ± 2
	4μM	17 ± 3
Antisense	2μM	43 ± 1
	4μM	56 ± 4

Hybridization spots (Fig. 3) were quantified by scanning densitometry in a Biorad GS 710 Densitometer. Background values corresponding to uninfected macrophage control were subtracted. The hybridization intensity of untreated infected macrophage samples was taken as 100%. Mean and SD values ($p < 0.005$) for 3 independent experiments are shown.

3). There was a dose–dependent decrease in β-tubulin RNA levels in the case of antisense oligonucleotide encapsulated cationic liposome treatment (Table II). When compared with the untreated infected control, the cationic lipo-some mediated effect is greater (Fig. 3, upper panel). Sense oligonucleotide treatment does not produce any inhibitory effect at the β-tubulin RNA level (Fig. 3, lower panel). Therefore, the mechanism of killing of intracellular *L. donovani* parasites upon β-tubulin–targeted antisense oligonucleotide treatment is probably due to the inhibition of β-tubulin synthesis at the RNA level and thus the arrest of disease progression inside the host macrophages. The levels of control RNAs specific for host macrophages, *e.g.* actin mRNA and rRNA, were not altered by treatment with antisense oligonucleotides (data not shown). This observations is in keeping with the lack of toxicity of oligonucleotides shown towards the host macropages (Fig. 2).

DISCUSSION

Antisense oligonucleotides are of potential interest for clinical applications that would exploit their antiproliferative, antiviral or antiparasitic properties (*10*). Antisense oligonucleotides have also been reported to have little inhibitory effect against HIV I in culture (*11*). Reports have already appeared regarding the leishmaniacidal effect of cationic liposome encapsulated antisense oligonucleotides targeted to the miniexon sequence of *L. donovani in vitro* (*4*). Because such oligonucleotides are rapidly degraded in the medium (*12*), investigations are on going to overcome this problem. The two important approaches include (*13–15*): (i) the modification of oligonucleotides with increased nuclease resistance (*16*), which includes phosphorothioate oligonucleotides and oligonucleotides with the sugar moiety oriented in the α-configuration (*17*), (ii) the encapsulation of oligonucleotides in cationic liposomes or immunoliposomes (*18, 19*) to protect them against nuclease activity and to increase the efficiency of delivery into cells.

In the present study, β-tubulin targeted antisense oligonucleotides in different cationic liposomes have been found to inhibit *L. donovani* parasite survival inside macrophages when tested *in vitro*. Our results show a sequence-specific effect of β-tubulin targeted antisense oligonucleotides in killing *L. donovani* parasites. In comparison with free antisense oligonucleotides at concentrations of 1–4 μM, liposome encapsulated antisense oligonucleotides show greater leishmanicidal activity at the same concentration. The improved efficacy of liposomal antisense oligonucleotides may be due to their protection against nuclease activity and increased cellular uptake.

The mechanism of oligonucleotide release from liposomes has been studied previously and it has been seen that cationic liposome encapsulated antisense oligonucleotides are phagocytosed and fused with parasitophorous vacuoles, leading to increased intracellular antisense oligonucleotide concentrations in comparison with that delivered in the free form, in the vicinity of the parasites. The mode of entry of antisense oligonucleotides into the amastigote form of parasites is not clear. However, it has recently been reported that the entry probably takes place through the flageller pocket (*20, 21*).

There are two mechanisms that can account for the leishmanicidal effects induced by anti–β-tubulin oligonucleotides: (i) competition between the oligomers and the machinery in charge of reading the mRNA born information, thus blocking protein translation, and (ii) RNase H–mediated degradation of the target. Only indirect indications suggest that RNase H could be involved in the antisense effects in intact cells (*2*). Thus we designed our study to examine the β-tubulin-RNA levels in the case of untreated infected macrophages and antisense-treated infected macrophages. The Dot-blot experiment clearly shows a dose-dependent decrease in the β-tubulin RNA level in infected macrophages in the case of specific antisense treatment. Liposome encapsulated antisense oligonucleotides have been found to be more efficient in blocking β-tubulin RNA levels. As it has been previously reported that liposomal antisense oligonucleotides are taken up better by cells (*17*) *in vitro*, they show an enhanced effect in blocking β-tubulin RNA and also in suppressing the parasite burden. Thus we can conclude that β-tubulin targeted antisense oligonucleotides could be another specific antisense target in addition to the miniexon sequence to suppress the parasitic burden intracellularly.

REFERENCES

1. Agrawal, S. and Zhao, Q (1998). Antisense-therapeutics. *Curr. Opin. Chem. Biol.* **2**, 519–528
2. Wagner, R.W. (1994) Gene inhibition using antisense oligodeoxy-neucleotides. *Nature* **372**, 333–335
3. Ramazeilleus, C., Mishra, R.K., Moreau, S., Pascolo, E., and Toulme, J.J. (1994) Antisense phosphorothioate oligonucleotides selective killing of the intracellular parasite leishmania amazoneusis. *Proc. Natl. Acad. Sci. USA* **91**, 7859–7863
4. Chakraborty, R., Dasgupta, D., Adhya, S., and Basu, M.K. (1999) Cationic liposome encapsulated antisense oligonucleotides mediates efficient killing of intracellular leishmania. *Biochem J.* **340**, 393–396
5. Seebeck, T. and Gehr, P.C. (1983) Trypanocidal effect of neuroleptic phenothiazines in *Trypanosoma. Bruicei. Mol. Biochem. Parasitol.* **9**, 197–208
6. Raviarola, H.W., Fernandez, A.R., Enders, J.E., Freters, R., Gea, S., Suligny, M., Palma, J.A., and Paglini-oliva, P. (1999) Thioridazine treatment modifies the evolution of *Trypanosoma cruzi* infection in mice. *Ann. Trop. Med. Parasitol.* **93**, 695–702
7. Pinnaduwage, P., Schmitt, L., and Huang, L. (1989) Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells. *Biochim. Biophys. Acta* **985**, 33–37
8. Maniatis, T., Fritsch, E., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor
9. Adhya, S., Das, S., and Bhaumik, M. (1990) Transcription and processing of β-tubulin mRNA is *L. donovani* promastigotes. *J. Biosci.* **15**, 249–259

10. Crooke, S.T. (1992) Therapeutic applications of oligonucleotides. *Annu. Rev. Pharmacol. Toxicol.* **32**, 329–376
11. Chaduwic, D.R. and Lever, A.M. (2000) Antisense RNA sequences targeting the 5′ leader packaging signal regions of human immunodeficiency virus type-1 inhibits viral replication of post transcriptional stages of the life cycle. *Gene Ther.* **7**, 1362–1368
12. Wickstrom, E. (1986) Oligodeoxynucleotide stability in subcellular extracts and culture media. *J. Biochem. Biophys. Methods* **13**, 97–102
13. Shaw, J.P., Kent, K., Bird, J., Fishblack, J., and Froehler, B. (1991) Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res.* **19**, 747–750
14. Akhtar, S., Kole, R., and Juliano, R.L. (1991) Stability of antisense DNA oligodeoxynucleotides analogs in cellular extracts and sera. *Life Sci.* **49**, 1793–1801
15. Lasic, D.D. and Papahadjopoulos, D. (1995) Liposomes revisited *Science* **267**, 1275–1276
16. Felgner, J.H., Kumar, R., Sridhar, C.N., Whecler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M., and Felgner, P.L. (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* **269**, 2550–2561
17. Cruiel, D.T., Agarwal, S., Wagner, E., and Cotton, M. (1991). Adenovirus enhancement of transferring-mediated gene delivery. *Proc. Natl. Acad. Sci. USA* **88**, 8850–8854
18. Jaasketaimen, I., Peltola, S., Honkakoski, P., Monkkoneu, J., and Urtti, A. (2000) A lipid carrier with a membrane active component and a small complex size are required for efficient cellular delivery of antisense-phosphorothioate oligonucleotide. *Eur. J. Pharm. Sci.* **10**, 187–193
19. Leonetti, J.P., Machy, P., Deglos, G., Lebleu, B., and Lesserman, L. (1990) Antibody targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication. *Proc. Natl. Acad. Sci. USA* **87**, 2448–2451
20. Zelphati, O. and Szoka, F.C. (1996) Mechanism of oligonucleotides releasae from cationic liposomes. *Proc. Natl. Acad. Sci. USA* **93**, 11493–11498
21. Ren, T., Song, Y.K., Zhang, G., and Liu, D. (2000) Structural basis of DOTMA for its intravenous transfection activity in mice. *Gene. Ther.* **7**, 764–768