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International Journal of Pharmaceutics 311 (2006) 82–88
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Delivery of different length poly(L-lysine)-conjugated ODN to HepG2 cells using *N*-stearyllactobionamide-modified liposomes and their enhanced cellular biological effects

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Received 29 July 2005; received in revised form 22 November 2005; accepted 10 December 2005
Available online 19 January 2006

Abstract

Short (14–20-mer range) synthetic oligodeoxynucleotides (ODNs) allow specific modulation of cellular gene expression at various stages, thus providing a versatile tool for fundamental studies and a rational approach to anticancer chemotherapy. However, several problems, such as metabolic stability, efficient cell internalization of ODNs and their efficient entrapment into liposomes continue to markedly limit this approach. To improve the target specificity and biological activity of ODN, three different length of poly(L-lysine) (PLL) were conjugated to ODN and these conjugates were encapsulated in *N*-stearyllactobionamide *(N*-SLBA)-modified liposomes, *N*-SLBA is a ligand for the asialoglycoprotein receptor. Then, we investigated their effects on cell cycle and survivin protein levels of HepG2 cells. The results showed that the encapsulation efficiency was improved because the polycationic charges of PLL neutralized the polyanionic charges of ODN. Among them, PLL (M_W 2000 and 10,000)-conjugated ODN encapsulated in *N*-SLBA liposomes induced apoptosis of HepG2 cells and highly inhibited survivin gene expression.

Keywords: ODN; PLL; *N*-SLBA liposomes; Survivin protein expression

1. Introduction

ODNs have been shown to inhibit cellular and viral gene expression at the molecular level (Liang et al., 2005; Gaus et al., 2005). This technology offers a potential therapeutic action in various diseases including oncology. In order to be pharmacologically effective, the ODNs must exhibit: (1) sufficient binding to their target sequence; (2) sufficient specificity; (3) stability towards exo- and endo-nucleases; (4) and be able to cross cell membranes. Different strategies have been developed to overcome these problems (Weyermann et al., 2004; Dautzenberg et al., 2003; Jeong et al., 2003; Huang and Li, 1997).

To meet these above conditions, some derivatives including phosphorothioates (Takeshima et al., 2005), phosphoramidates (Michel et al., 2003), methylphosphonates (Katty and Michael, 2001), and mono, or dithiophosphates (Phillips et al., 1997) have been studied. Covalent attachment of peptide residues to synthetic ODNs is of great value not only for enhancing their resistance to nucleases, but also for the special functions produced by the peptide. For instance, a sensible increase in cell permeability can be easily achieved by conjugation with ODNs hydrophobic aminoacids or cationic residues (Leonetti et al., 1998; Pipkom et al., 2003), such as poly(L-lysine) (Zhou and Huang, 1994) or nuclear transport signals peptides (Torre et al., 1994).

Liposomes are widely used as carriers for a variety of drugs, including their recent application to gene delivery (Waelti and Gluck, 1998; Oliveira et al., 1997; Ma and Wei, 1996; Harashima and Kiwada, 1999). As carriers for the delivery of nucleic acids, liposomes offer a protective biocompatible and biodegradable delivery system that can enhance their cellular uptake (Zelphati and Szoka, 1996; Thierry et al., 1993). However, cell-specific drug delivery is sometimes urgently required for a variety of clinical purposes. In this text, we have investigated HepG2 cells (human hepatoblastoma cell line), hepatocytes exclusively expressed in large numbers of high-affinity cell-surface receptors (Noguchi et al., 2003) that can bind asialoglycoproteins and

Abbreviations: ODN, oligodeoxynucleotide; ODNs, oligodeoxynucleotides; PLL, poly(L-lysine); *N*-SLBA, *N*-stearyllactobionamide; PLL1, poly(L-lysine) (M_W 1000); PLL2, poly(L-lysine) (M_W 2000); PLL3, poly(L-lysine) (M_W 10,000)

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doi:10.1016/j.ijpharm.2005.12.014

subsequently internalize them to the cell interior. This mechanism would be an effective way to achieve hepatocyte targeting. It has been reported that liposomes modified by asialofetuin, an asialoglycoprotein (Kawakami et al., 2001), are taken up by asialoglycoprotein receptors. However, the introduction of asialoglycoproteins to liposomes is complicated, and so there are a number of problems associated with the carriers, such as reproducibility and immunogenicity (Kallinteri et al., 2001). In this sense, direct linkage of the carriers to sugar moieties would be promising. In our investigations, we have taken advantage of a novel galactosylated derivative, *N*-SLBA, to modify liposomes with galactose moieties for hepatocyte-selective gene delivery.

In combination with the above-mentioned approach, in order to improve therapeutic applications of synthetic ODNs in an antisense strategy, our study investigated the efficiency of poly(L-lysine)-conjugated ODN (ISIS# 23665) encapsulated in targeted liposomes. An antisense ODN up to 18 nucleobases in length were designed to target a nucleic acid molecule encoding human survivin and which inhibits the expression of human survivin.

Firstly, ODN was conjugated with different lengths of poly(L-lysine) ε-amino groups through an *N*-morpholine ring. Then *N*-SLBA-modified liposomes were prepared to encapsulate ODN and its conjugates. Comparison of different molecular weight poly(L-lysine)-conjugated ODN encapsulated in *N*-SLAB liposomes was undertaken to investigate the efficacy of biological activity. Results showed that, when liposomes were incubated with cells at a drug concentration of 2 μM in the culture medium, poly(L-lysine) (M_W 2000 and 10,000)-conjugated ODN induced apoptosis of HepG2 cells and resulted in down-regulation of survivin expression.

2. Materials and methods

2.1. Materials

ISIS# 23665 (5′ cga tgg cac ggc gca ctt 3′) was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. Media and fetal calf serum for cell culture were obtained from (Gibco-BRL), poly(L-lysine) (M_W 1000, 2000 and 10,000), bacterial alkaline phosphatase (BAP), myokinase, spermine, pCp and sodium cyanoborohydride were purchased from Sigma. T4RNA ligase (RNase- and DNase-free) and creatine kinase were from MBI. *N*-SLBA was synthesized from lactobionic acid and stearylamine.

2.1.1. Cell culture

HepG2 cells were cultured in RPMI1640 (Gibco-BRL) containing 1 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2/95% air.

2.2. Methods

2.2.1. ODN covalent linkage to PLL

ODN was synthesized on a riboadenosine-derivatized support using a DNA synthesizer and purified by reversed phase chromatography. As described by Lemaitre and co-workers (Leonetti et al., 1998), covalent linkage of oligomers to PLL through an *N*-morpholine ring was achieved by periodic acid oxidation and borocyanohydride reduction of the 3′ end ribose. Each fraction obtained was assayed for ODN–poly(L-lysine) content using the Bradford method.

2.2.2. Preparation of N-SLBA liposomal ODN and N-SLBA liposomal PLL-conjugated ODN

Soy phospholipids, cholesterol and *N*-SLBA in a ration of 50:45:5% (mol/mol/mol) (Shi et al., 2004) were dissolved in ethanol in a round-bottom flask and dried in a rotary evaporator under reduced pressure at 50 °C to form a thin film on the flask wall. The film was hydrated overnight with saline solution containing 100 μM ODN (Sasaki et al., 2001), PLL1 (M_W 1000)-conjugated ODN, PLL2 (M_W 2000)-conjugated ODN and PLL3 (M_W 10,000)-conjugated ODN to give a lipid concentration of 20 mg/ml. MLV were downsized to form oligolamellar vesicles by extrusion at 50 °C in an Extruder device (Lipex Biomembranes, Canada) through polycarbonate membrane filters of variable pore size. Liposomes were extruded in three steps: three consecutive extrusions through a 0.8 μm pore diameter filter followed by three other consecutive extrusions through 0.4 μm membranes. The resulting lipid suspension was then extruded three times through a 0.2 μm filter. Similarly, liposomal PLL/ODN, liposomal PLL-conjugated ODN and *N*-SLBA liposomal PLL underwent the same procedures. After preparation, liposomes were divided into aliquots, a nitrogen stream was passed to displace the air, and finally the liposomes were stored at 4–7 °C in a refrigerator. These conditions avoid phospholipid oxidation and hydrolysis. Before use, they were warmed to 37 °C.

2.2.3. Cell death features observed by staining with the DNA-specific dye Hoechst 33342

Nuclear morphological changes were detected by staining with Hoechst 33342 (O'Callaghan et al., 2001). HepG2 cells were seeded onto 12-well plate at a concentration of 1×10^4 cells/well, the medium was removed, and replaced with serum-free medium. After 6 h incubation with ODN, PLL or its conjugates incorporated in liposomes with or with *N*-ALBA at a drug concentration of 2 μM, the medium was replaced with complete medium. Cells were incubated for a further 72 h, then harvested and washed with PBS. The cells were fixed with 2% glutaraldehyde for 2 h and washed again with PBS. After resuspension in 10 μl PBS, the cells were stained with 1 mM Hoechst 33342 (10 μl in PBS) for 10 min. Cell suspensions were placed on glass slides and then using fluorescence microscopy the stained nuclei were counted as apoptotic cells containing condensed chromatin.

2.2.4. Cell cycle analysis

HepG2 cells (1×10^6) were cultured in 10% fetal medium and allowed to attach for 24 h at 37 °C, the medium was removed, and replaced with serum-free medium (Kočišová et al., 2005) containing 2 μM ODN, PLL or its conjugates incorporated in liposomes with or without *N*-SLBA. After 6 h at 37 °C, the

medium was replaced with complete medium. Cells were incubated for a further 72 h, then trypsinized and washed twice with cold PBS buffer. Then the cells were fixed with 2 ml ice-cold ethanol (70%, v/v in water) overnight at −2 °C. After centrifugation, the fixed cells were exposed to 500 μl 180 U/ml RNase for 30 min at 37 °C, then to propidium iodide (PI) staining solution (25 μg/ml PI, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer) for 30 min at 4 °C (Xia et al., 2002). The cell cycle distribution was analyzed using a FACScan flow cytometer and ModFit LT software.

2.2.5. Western blot analysis

As described above, after 72 h incubation approximately 1×10^6 cells were harvested and washed with phosphate-buffered saline (PBS) and lysed at 4×10^4 cells/μl in cell lysis buffer (20 mM HEPES, pH 7.4, 0.25% NP-40 containing protease inhibitor cocktail; Boehringer Mannheim, Indianapolis, IN) for 30 min on ice. Equal amounts of lysate (equivalent to 5×10^5 cells) were subjected to SDS-PAGE on 12% polyacrylamide gels (Tanaka et al., 2000). Then proteins were transferred to Hybond-P (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) membranes and reacted with monoclonal antibody against survivin for 2 h at room temperature. After washing, the membranes were probed with a horseradish peroxidase-conjugated secondary antibody and reacted with ECL reagent (Ambersham Pharmacia Biotech, Buckinghamshire, United Kingdom). An anti-β-actin blot was made in parallel as a loading control. Signals were detected by a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA) and quantified by Scion Image software (Scion, Frederick, MD). Results were expressed as survivin/β-actin ratios.

2.2.6. Data analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard error of the mean (S.E.M.) and were analyzed using one-way analysis of variance (ANOVA) for individual comparisons. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Conjugation of ODN and poly(L-lysine)

ODN was covalently linked to poly(L-lysine) via an *N*-morpholine (azahexopyranose) ring after periodate oxidation of their 3′-terminal ribose. However, it was necessary to add an oxidizable pCp, and this operation was performed using T4 RNA ligase (Morcos, 2001).

In our investigation, ODN was coupled to three different length of PLL (PLL1; PLL2; PLL3). Our study was performed to search for a suitable conjugate to be delivered in a functional form with HepG2 cells. In addition, a suitable length of poly(L-lysine) may help to improve hybridization with the target mRNA.

The synthetic ODN was covalently linked to PLL as described above in a yield of 23–32%.

3.2. Characteristics of PLL-conjugated ODN encapsulated in N-SLBA-modified liposomes

The current study was undertaken to investigate the influence of PLL length on the encapsulation efficiency. To do this, encapsulated ODN, PLL1-conjugated ODN, PLL-conjugated ODN and PLL3-conjugated ODN were determined by direct measurement using an LC-10 UV spectrometer (Dalian Johnsson Sepa-

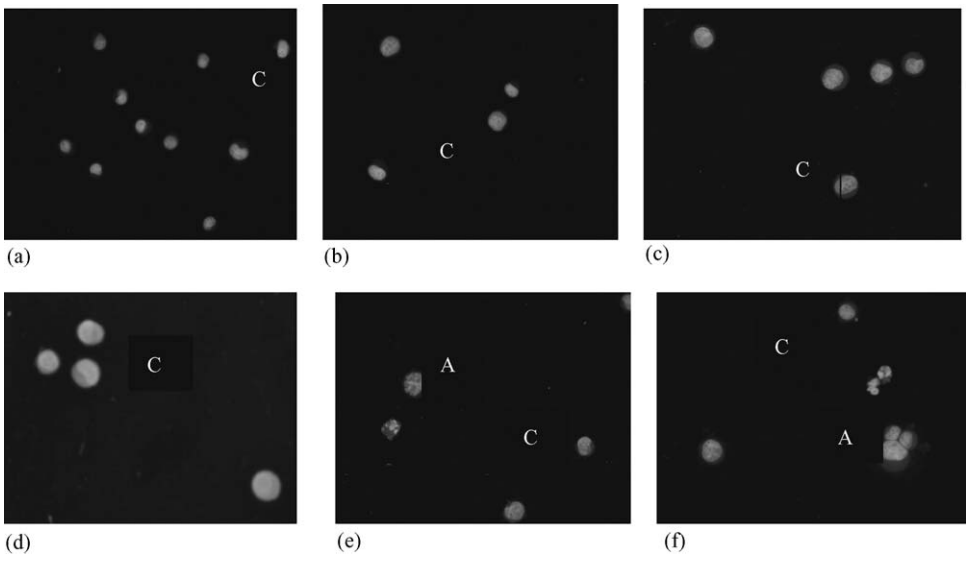

Fig. 1. Nuclear condensation and formation of apoptotic bodies by treatment with PLL-conjugated ODN encapsulated in *N*-SLBA liposomes. HepG2 cells treated with liposomal PLL1-conjugated ODN without *N*-SLBA (a), liposomal PLL2-conjugated ODN without *N*-SLBA (b), liposomal PLL3-conjugated ODN without *N*-SLBA (c), PLL1 conjugated-ODN encapsulated in *N*-SLBA liposomes (d), PLL2-conjugated ODN encapsulated in *N*-SLBA liposomes (e) and PLL3-conjugated ODN encapsulated in *N*-SLBA liposomes (f) were stained with Hoechst 33342 dye as described in Section 2. C represents a cell with "unique, typical" chromatin condensation; A, a cell with typical apoptotic chromatin condensation. Magnification 1000×. This is a representative result from three separate experiments.

ration Science and Technology Corporation in China). The 75 μl liposomal solution was loaded onto the Sephadex G-50 column, liposomes were separated from any free ODN or its conjugates and detected at a UV wavelength of −260 nm, while free ODN was collected and estimated by measuring the absorbance or peak area (at a wavelength of 260 nm) of the eluted fractions containing free ODN or its conjugates. The entrapment efficiency (%) was calculated as 100 × (administered ODN − unentrapped ODN)/administered ODN (Stuart et al., 2004). The final concentration of ODN and its derivatives encapsulated in the liposomes was approximately 8.0 ± 0.8, 12 ± 0.9, 18 ± 0.8, and 44 ± 1.2%. The obtained liposomes had diameters of approximately 200 nm and were useful for improving transfection efficiency. The leakage-rate of ODN from these liposomes was less than 6.5% after 10 h incubation at 37 °C.

3.3. Staining with Hoechst 33342

Nuclear morphological changes were assessed by staining with Hoechst 33342 to identify apoptotic cells. As shown in Fig. 1, PLL2- and PLL3-conjugated ODN encapsulated in *N*-SLBA liposomes induced condensed chromatin fragments in HepG2 cells, typical apoptotic cells (designated as A) were detached from the monolayer and apoptotic bodies formed, and many smaller "dots" representing segregated condensed chromatin appeared. Interestingly, a unique and typical chromatin condensation (represented as C) was observed in HepG2 cells treated with control groups. However, liposomal ODN, *N*-SLBA liposomal ODN, liposomal PLL1/ODN, liposomal PLL2/ODN, liposomal PLL3/ODN, *N*-SLBA liposomal PLL1, *N*-SLBA liposomal PLL2, and *N*-SLBA liposomal PLL3 did not induce condensed chromatin fragments in HepG2 cells. At least 100 cells were observed in the random field in three independent experiments. It was suggested that in the HepG2 cells, PLL2-, and PLL3-conjugated ODN can increase the induced apoptosis by encapsulation in *N*-SLBA liposomes.

3.4. Anticancer effect of poly(L-lysine)-conjugated ODN encapsulated in N-SLBA liposomes

Conjugates were encapsulated in *N*-SLBA liposomes, and then incubated with HepG2 cells for 72 h in serum medium.

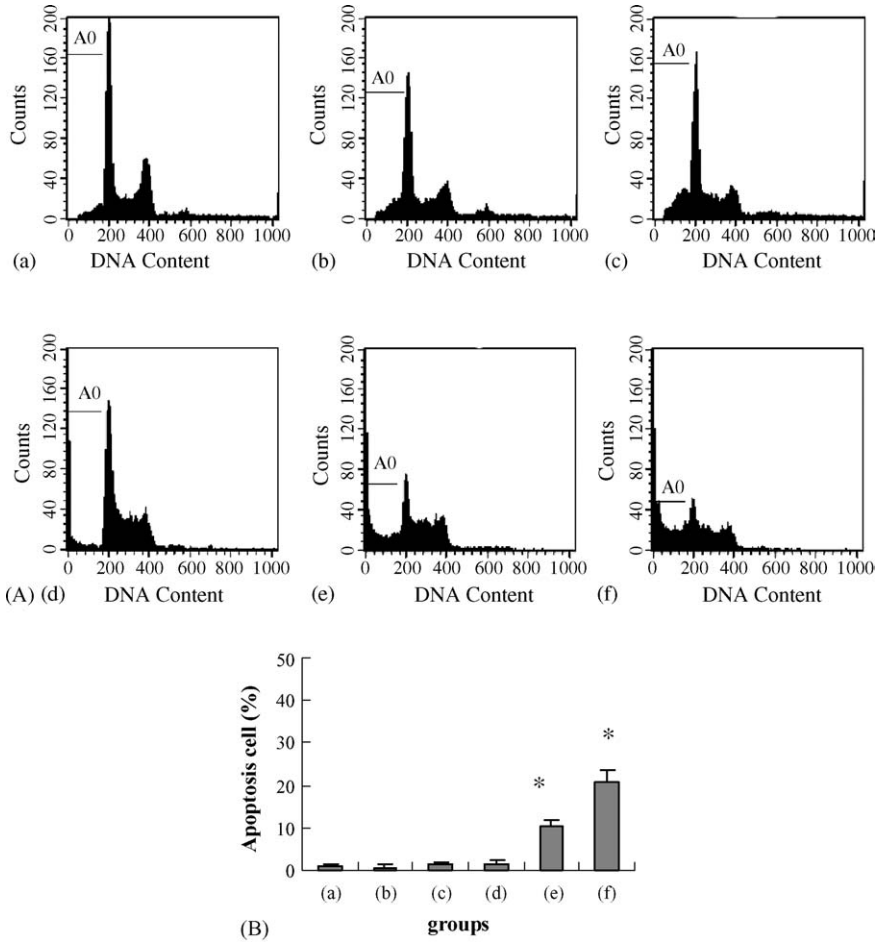

Fig. 2. Apoptosis assay of liposomal PLL1-conjugated ODN without *N*-SLBA (a), liposomal PLL2-conjugated ODN without *N*-SLBA (b), liposomal PLL3-conjugated ODN without *N*-SLBA (c), PLL1-conjugated ODN encapsulated in *N*-SLBA liposomes (d), PLL2-conjugated ODN encapsulated in *N*-SLBA liposomes (e) and PLL3-conjugated ODN encapsulated in *N*-SLBA liposomes (f) was performed, and the number of apoptotic cells was measured by flow cytometry as described in the text. The region designated as A_0 was defined as cells undergoing apoptosis-associated DNA degradation as shown in (A). Data represent the mean values of three replications with bars indicating S.E.M. as shown in (B): $^{*}p < 0.05$ compared with the control groups in which the cells were treated with PLL-conjugated ODN encapsulated in liposomes without *N*-SLBA.

Apoptotic activity was detected with conjugates encapsulated in *N*-SLBA liposomes, and the drug concentration in the culture medium was 2 μM. Flow cytometric analysis of HepG2 cell distribution in the different cell cycle phases showed that exposure to PLL2- and PLL3-conjugated ODN incorporated in *N*-SLBA liposome induced the apoptosis of HepG2 cells at 72 h (as shown in Fig. 2). No significant effect was observed with liposomal PLL1-, PLL2- and PLL3-conjugated ODN, and *N*-SLBA liposomal PLL1-conjugated ODN unlike the antitumor activity of PLL2- and PLL3-modified ODN entrapped in *N*-SLBA liposomes. Similarly, Liposomal ODN, *N*-SLBA liposomal ODN, liposomal PLL1/ODN, liposomal PLL2/ODN, liposomal PLL3/ODN, *N*-SLBA liposomal PLL1, *N*-SLBA liposomal PLL2, and *N*-SLBA liposomal PLL3 did not induce the apoptosis of HepG2 cells (data not shown). This demonstrated the anticancer activity action of the 3′ end sequence conjugated with poly(L-lysine) and the importance of the length of PLL.

3.5. Targeted liposome-induced inhibition of survivin protein expression

Like other antiapoptotic proteins, such as Bcl-2 and Mac-1, survivin expression is likely to be differentially regulated during differentiation. We therefore examined the effect of liposome-induced HepG2 cell survivin expression. HepG2 cells were treated with ODN or conjugates (2 μM) encapsulated into liposomes with *N*-SLBA or without *N*-SLBA for up to 72 h, and survivin protein levels were determined (Table 1). These were significantly reduced in the PLL2- and PLL3-modified ODN incorporated in *N*-SLBA liposome-treated HepG2 cells studied (Fig. 3). Survivin levels were reduced by approximately 80 and 95% compared with the levels in control cells. No significant effect was observed with liposomal PLL1-, liposomal PLL2- and liposomal PLL3-conjugated ODN, and *N*-SLBA liposomal PLL1-conjugated ODN. The same results were observed with liposomal ODN, *N*-SLBA liposomal ODN, liposomal PLL1/ODN, liposomal PLL2/ODN, liposomal PLL3/ODN, *N*-SLBA liposomal PLL1, *N*-SLBA liposomal PLL2, and *N*-SLBA liposomal PLL3 (data not shown). Concomitant with survivin downregulation, PLL2- and PLL3-conjugated ODN incorporated into *N*-SLBA-modified liposome-treated HepG2 cells exhibited apoptosis.

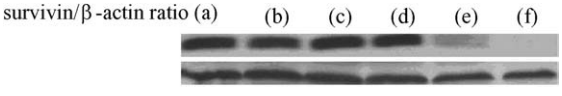

Fig. 3. Western blot analysis of survivin expression in HepG2 cells. This shows survivin protein expression in cells treated with liposomal PLL1-conjugated ODN without *N*-SLBA (a), liposomal PLL2-conjugated ODN without *N*-SLBA (b), liposomal PLL3-conjugated ODN without *N*-SLBA (c), PLL1-conjugated ODN entrapped in *N*-SLBA liposomes (d), PLL2-conjugated ODN entrapped in *N*-SLBA liposomes (e) and PLL3-conjugated ODN encapsulated in *N*-SLBA liposomes (f). All experiments in this study were performed at least in triplicate.

Table 1
Inhibition of human survivin mRNA levels by *oligodeoxynucleotides*

ISIS#	Region	Target site	Sequence	Inhibition (%)
23652	5′ UTR	1	gcgattcaaatctggcgg	0
23653	5′ UTR	19	cctctgccaacgggtccc	4
23654	5′ UTR	75	tgagaaagggctgccagg	46
23655	5′ UTR	103	ttcttgaatgtagagatg	0
23656	5′ UTR	128	ggcgcagccctccaagaa	38
23657	Coding	194	c aagtctggctcgttctc	0
23658	Coding	226	tccagctccttgaagcag	32
23659	Coding	249	ggtcgtcatctggctccc	36
23660	Coding	306	gcttcttgacagaaagga	35
23661	Coding	323	ggttaattcttcaaactg	0
23662	Coding	363	tcttggctctttctctgt	34
23663	Coding	393	tcttattgttggtttcct	0
23664	Coding	417	tcgcagtttcctcaaatt	37
23665	**Coding**	**438**	**cgatggcacggcgcactt**	**72**
23666	Coding	511	cctggaagtggtgcagcc	16
23667	Coding	542	acaggaaggctggtggca	70
23668	Coding	587	tttgaaaatgttgatctc	8
23669	Coding	604	acagttqaaacatctaat	0
23670	Coding	625	ctttcaagacaaaacagg	0
23671	Coding	650	acaggcagaagcacctct	0
23672	Coding	682	aagcagccactgttacca	64
23673	Coding	700	aaagagagagagagagag	18
23674	Coding	758	tccctcacttctcacctg	29
23675	3′ UTR	777	agggacactgccttcttc	43
23676	3′ UTR	808	ccacgcgaacaaagctgt	62
23677	3′ UTR	825	actgtggaaggctctgcc	0
23678	3′ UTR	867	aggactgtgacagcctca	62
23679	3′ UTR	901	tcagattcaacaggcacc	0
23680	3′ UTR	1016	attctctcatcacacaca	26
23681	3′ UTR	1054	tgttgttaaacagtagag	0
23682	3′ UTR	1099	tgtgctattctgtgaatt	20
23683	3′ UTR	1137	gacttagaatggctttgt	37
23684	3′ UTR	1178	ctgtctcctcatccacct	41
23685	3′ UTR	1216	aaaaggagtatctgccag	39
23686	3′ UTR	1276	gaggagcggccagcatgt	47
23687	3′ UTR	1373	ggctgacagacacacggc	41
23688	3′ UTR	1405	ccgtgtggagaacgtgac	22
23689	3′ UTR	1479	tacgccagacttcagccc	1
23690	3′ UTR	1514	atgacagggaggagggcg	0
23691	3′ UTR	1571	gccgagatgacctccaga	66

4. Discussion

ODNs represent an interesting tool for selective inhibition of gene expression, but their efficient entrapment into liposomes has proved difficult to achieve (Weyermann et al., 2004). At the same time, the biological effectiveness of ODNs is hindered by rapid degradation and poor cellular uptake (Wu-Pong et al., 1997). As a step towards overcoming this, small (18-mer) synthetic ODNs have been coupled at their 3′ end to ε-amino groups of lysine residues of PLL. The positive charges of PLL neutralized the negative charges of ODN, then PLL-conjugated ODN can be effectively incorporated into liposomes.

We have also developed novel galactosylated liposomes consisting of Gal-C_{18}, soy phospholipids and cholesterol (Chol) for hepatocyte-directed drug delivery. When applying *N*-SLBA liposomes to PLL-conjugated ODN delivery, we assessed the effect of different length of PLL-modified ODN on liposome encapsulation efficiency. *N*-SLAB is a ligand for the asialogly-

coprotein receptor (Pacuszka and Fishman, 1991). The results showed that the entrapment ratios of ODN and its derivatives are different. The contents of PLL2- and PLL3-modified ODN encapsulated in *N*-SLBA liposomes increased significantly, and the leakage rate was low. This demonstrated that the conjugates are associated with the liposomes during the incubation period.

The reason for the high-entrapment efficiency may be that, firstly, lipid hydroxy groups interacted with amino groups in PLL. Secondly, positive charges neutralized negative charges in ODN to some extent, then reduced liposomal membrane repulsion.

Through the PLL-conjugated ODN interaction with electrostatic forces and the PLL-conjugated ODN with liposomal bilayers, it appears that in this study the PLL–ODN conjugates are stably associated with galactosylated liposomes and achieve an improved entrapment ratio and offer effective hepatic targeting. Analysis by size exclusion chromatography indicated that the entrapment efficiency differs from one derivative to another, and this may be induced by different polycationic charge intensities and interaction between PLL–ODN conjugates and liposomal bilayers. So the presence of the PLL moiety in the conjugates is essential, as the unconjugated ODN does not interact with *N*-SLBA liposomes.

Numerous studies (Fattal et al., 2004; Hughes et al., 2001) have indicated that ODNs enter cells by endocytosis and accumulate in the endosomal–lysosomal compartment. ODN is released from this compartment, and subsequently exerts antisense activity. By associating ODN and its conjugates with target liposomes, the influx of ODN via endocytosis is probably increased. On increasing the entrapment ratio of PLL-modified ODN, the increased influx leads to a higher ODN concentration in the endosomal–lysosomal compartment, which is assumed to result in a proportionally higher transfer to the cytosol and higher antisense activity. This galactosylated liposome increased uptake of survivin-specific antisense oligodeoxynucleotide by the HepG2 cells is expected to down-regulate survivin expression in these cells. A low expression of survivin in HepG2 cells will lead to cell apoptosis.

To address this issue, we next assessed the effect of ODN encapsulated in *N*-SLBA liposomes, liposomal PLL/ODN, liposomal PLL-conjugated ODN, *N*-SLBA liposomal PLL and *N*-SLBA liposomal PLL-conjugated ODN on apoptosis of HepG2 cells and survivin expression. Morphological features showed that PLL2- and PLL3-conjugated ODN increased the apoptosis induced through encapsulation in *N*-SLBA liposomes, with the formation of many smaller “dots” representing segregated condensed chromatin as shown in Fig. 1. As shown by the data in Fig. 2, the apoptosis assay employing PI staining followed by FACS analysis clearly showed a similar apoptotic effect of PLL2- and PLL3-modified ODN incorporated into *N*-SLBA liposomes in HepG2 cells. Using the optimal combination of PLL2- or PLL3-modified ODN and target liposomes, we were able to achieve more than 11 and 20% apoptosis avoiding the toxicity of cationic liposomes.

Survivin has been shown to play important roles in cell proliferation and survival (Ambrosini et al., 1997; Zaffaroni et al., 2005). Most of the supporting data comes from the reports that overexpression of survivin protects cells from death and down-regulation of survivin expression results in defects and apoptosis. Our studies demonstrated that PLL (M_W 2000 and 10,000)-modified ODN encapsulated in *N*-SLBA liposomes had a significant effect on HepG2 cells. Western blot analysis of survivin expression in HepG2 cells showed that the control samples were positive (Fig. 3.), PLL2- and PLL3-conjugated ODN encapsulated in *N*-SLBA liposomes induced down-regulation of survivin expression to different degrees by more than 0.75 and 0.95, compared with levels in cells treated with liposomal PLL/ODN, liposomal PLL-conjugated ODN and *N*-SLBA liposomal PLL (some data not shown). The reported overexpression of survivin in HepG2 cells and our finding that its down-regulation induces cell apoptosis suggests that survivin is a good target for HCC therapy (Mooon and Tarnawski, 2003).

The results obtained demonstrate that we successfully introduced ODN–PLL conjugates into HepG2 cells using target liposome technology. In addition, it is necessary to improve ODN biological activity using modified PLLs (M_W 2000 and 10,000), PLL covalent binding to ODN can avoid nuclease digestion. The galactosylated liposomes and PLL modification increased uptake of ODN by HepG2 cells may be used to improve antisense-based therapeutic intervention during cancer therapy, or may be of help in the development of novel strategies for the treatment of HCC by targeting antiapoptotic survivin.

Acknowledgements

This work was supported by the National Natural Science Foundation of China. We thank Prof. Yongkui Jing and his students for their valuable help, and also acknowledge Dr. David Jack for correcting this paper.

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