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Full Length Research Paper

Cholesteryl cytofectin-oligodeoxyribonucleotide lipoplexes: Protection against serum nuclease digestion and interaction with mammalian cells *in vitro*

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Submicron unilamellar cationic liposomes constructed from equimolar amounts of a cholesteryl cytofectin 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and dioleoylphosphatidylethanolamine (DOPE) were shown to bind a model antisense oligodeoxyribonucleotide (ODN) dT_{18} in a saturable manner to afford electropositive lipoplexes. Liposomes imparted to the ODN $[^3H]dT_{18}A_7$ full protection against nuclease catalyzed degradation in 10% foetal calf serum at 37°C for 3 h, while under the same conditions the naked ODN underwent approximately 50% degradation. Liposomes also promoted association of ODNs to Chinese hamster ovary (CHO) cells while at a liposome (+ve) : ODN (-ve) charge ratio of 1.3:1 lipoplexes were well tolerated by the human hepatoma cell line HepG2 at levels of ODN ordinarily employed in antisense ODN-mediated gene knockdown experiments. The findings of this study suggest that cationic liposomes based on the cationic cholesterol derivative Chol-T and the neutral co-lipid DOPE may be used in antisense ODN applications in mammalian cell lines grown in culture.

Key words: Cationic liposome, antisense oligodeoxyribonucleotide, CHO cells, HepG2 cells.

INTRODUCTION

The field of antisense therapeutics is based on the premise that oligonucleotides complementary to unique sequences in double stranded DNA, pre-messenger RNA or mature messenger RNA (mRNA) may hybridize to their cognate sequences and induce a gene knock-down effect. This has clear potential for the treatment of diseases with a genetic origin for which there are few or no conventional therapeutic alternatives. Mature messenger RNA is the preferred target for antisense oligodeoxynucleotides (ODNs), which on hybridization induce RNase H activity that hydrolyzes the RNA component of the heteroduplex or block the movement of ribosomes along the mRNA (Politz et al., 1995). The effect is a down regulation of the corresponding protein. The susceptibility of phosphodiester ODNs (PO-ODNs) to nuclease catalyzed degradation in the extracellular environment has however limited their application in the antisense approach to the modulation of specific gene expression for therapeutic purposes. Serum 3'-exonuclease, in particular, is responsible for most of the degradation experienced before entry into the cell is achieved (Shaw et al., 1991; Boado and Pardridge, 1992) while intracellular nucleases pose an added threat (Stein and Cheng, 1993). The degree of degradation by 3'-exonucleases

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Abbreviations: Chol-T, 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol; dT_{18}, octadecylthymidylate; $[^3H]dT_{18}A_7$, tritiated octadecylthymidylylheptosyldeoxyadenylate; **DOPE,** dioleoylphosphatidylethanolamine; **FBS,** foetal bovine serum; **HBS,** HEPES buffered saline; **HEPES,** 2-[4-(2-hydroxyethyl)-piperazinyl]-ethanesulfonic acid; **MEM,** minimum essential medium; ODN, oligodeoxyribonucleotide; **PO-ODN,** phosphordiester oligodeoxyribonucleotide; **PS-ODN,** phosphorothioate oligodeoxyribonucleotide, **PBS,** phosphate buffered saline.

may be reduced by introducing modifications to the 3'-terminal region, such as phosphorothioate caps (Hoke et al., 1991) and hairpin loops (Khan and Coulson, 1993: Tang et al., 1993). Although phosphorothioate oligodeoxynucleotides (PS-ODNs) offer substantial resistance to degradation by nucleases they display a lower binding affinity for the target RNA than their PO-ODN counterparts (Roth, 2005) and show a tendency to bind protein and other molecules in a sequence independent manner (Heinreich et al., 1997: Meunier et al., 2001). Strong and exclusive recognition of target sequences by antisense ODNs remains a primary requirement in the design of new antisense ODN transfection modalities and therefore PO-ODNs continue to be important candidates in conjunction with protective and target specific delivery systems (De Oliveira et al., 2000; Remaut et al., 2006; Remaut et al., 2007). We describe here an investigation into the interaction between model PO-ODNs with cationic liposomes composed of a cholesteryl cytofectin 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and the co-lipid dioleoyl phosphatidylethanolamine (DOPE) and the delivery of resultant lipoplexes to Chinese hamster ovary (CHO) cells known to be relatively impervious to naked PO-ODNs (Cumin et al., 1993). The stability of liposome-complexes PO-ODNs in foetal calf serum and their toxicity to the human hepatoma cell line HepG2 are also reported.

MATERIALS AND METHODS

Materials

Dioleoylphosphatidylethanolamine (DOPE) was obtained from Sigma, (St Louis, MO, USA). dT_{18} was from the department of Virology, University of Natal Medical School, Durban, South Africa. Oligonucleotide markers (13 mer, 25 mer, 28 mer) were synthesized at the Comprehensive Cancer Centre, University of Southern California, Los Angeles, USA. Sephadex G-50 was purchased from Pharmacia Fine Chemicals, (Sweden). Mammalian cell lines CHO and HepG2 were supplied by Highveld Biologicals, South Africa. Minimum essential medium (MEM) was obtained from Gibco BRL (Inchinnan, Scotland). Terminal deoxyribonucleotidyl transferase was from Boehringer (Mannheim, Germany). Foetal bovine serum (FBS) was supplied by Delta Bioproducts, (Johannesburg, South Africa). Penicillin-Streptomycin mixtures and trypsin-EDTA were purchased from Whittaker, M.A. Bioproducts (Maryland, USA). Chol-T was prepared from Cholesteryl chloroformate as described elsewhere (Singh et al., 2001). All other reagents were of analytical grade and 18 mOhm water was used throughout.

3'-Terminal labeling of dT_{18}

A mixture (100 µl) of dT_{18} (2.7 nmole), [^{3}H] ATP (93 µCi, 3.4 Ci/m mole), potassium cacodylate (0.1 M), $MgCl_2$ (1 mM), Tris-HCl (25 mM, pH 6.9), mercaptoethanol (0.2 mM) and terminal deoxyribonucleotidyl transferase (50 U) was incubated at 37°C for 4 h. The reaction was terminated by the addition of EDTA (20 mM final concentration) whereupon the product was purified on a Sephadex G-50 column (90 x 0.6 cm, flow rate 13.2 ml/h). Equilibration and elution was with triethylammonium bicarbonate buffer (20 mM, pH 8.2). Fractions containing the product were pooled and evaporated to dryness *in vacuo*. The tritiated ODN was redissolved in water (200 µl) and stored at -20°C. Specific activity: 4.8 µCi/µg, corresponds to the 3'-addition of 7 A residues.

Liposome preparation

Unilamellar liposomes were prepared by depositing DOPE (2 µmole) and Chol-T (2 µmole) as a thin film on the inner wall of a test tube by evaporation in vacuo of a chloroform solution. This was followed by re-hydration in 20 mM 2-[4-(2-hydroxyethyl)-piperazinyl]-ethanesulfonic acid (HEPES), 150 mM NaCl (pH 7.5), (HEPES buffered saline, HBS) overnight at 4°C. Finally, sonication at 21°C for 5 minutes was effected in a Transonic bath type sonicator. Preparations were routinely stored at 4°C and remained stable after 4 months.

Effect of serum nucleases on liposome-complexed [^{3}H]$dT_{18}A_7$

[^{3}H]$dT_{18}A_7$ (0.17 µg, 4.77 µCi/µg) was incubated with liposomes (33 µg Chol-T, 63 nmole) in HBS (32 µl) for 20 min at 21°C. This was centrifuged at 4000 x g for 2 h at 4°C. The pellet was resuspended in HBS (40 µl) and to this was added MEM containing FBS (100 µl). Free [^{3}H]$dT_{18}A_7$ was similarly treated in control experiments. The mixtures were incubated at 37°C. At 0 and 180 min aliquots (30 µl) were removed and added to TE buffer (170 µl, 10 mM Tris-HCl, 1 mM EDTA pH 7.5). This was extracted successively with phenol, phenol:chloroform (1:1,v/v) and chloroform. The aqueous layer was concentrated to dryness in a Speedvac and the residue redissolved in HBS (200 µl). Samples were taken for analysis on denaturing (7 M urea) polyacrylamide (15%) gels in Tris-borate-EDTA buffer for 40 min at 20 mA. Individual lanes on the gel were cut into 3 mm segments which were digested with hydrogen peroxide solution (30%, 400 µl) for 24 h at 68°C. Radioactivity was measured by liquid scintillation.

Cell culture

Cells were routinely propagated in 25 cm^2 flasks at 37°C in MEM (5 ml) supplemented with 10% FBS, 20 mM HEPES (pH 7.5), penicillin (100 U/ml) and streptomycin (100 µg/ml). At confluence cells were split 1:3 - 1:5.

Delivery of [^{3}H]$dT_{18}A_7$ to CHO cells

Cells were seeded into 24 well plates (3.0 x 10^5 cells/well) and incubated in complete medium (0.5 ml) for 24 h. Cells then received [^{3}H]$dT_{18}A_7$ (0.05 µg, 6.1 pmole) free or pre-incubated for 20 min with increasing amounts of cationic liposomes (0 - 10 nmole Chol-T). After 24 h medium was removed and cells were washed with phosphate buffered saline (PBS, 150 mM sodium chloride, 2.7 mM potassium chloride, 1 mM potassium dihydrogen phosphate, 6 mM disodium hydrogen phosphate, pH 7.5) (3 x 0.5 ml). Thereafter, wells received Promega (USA) lysis buffer (5 mM Tris-phosphate pH 7.5, 0.4 mM 1,2-diaminocyclohexane-N,N,N',N'-tetra acetic acid, 0.4 mM dithiothreitol, 2% glycerol, 0.2% Triton X-100). Aliquots of cell lysates were taken for liquid scintillation.

Cell proliferation assays

HepG2 cells were seeded into 24 well plates (2.2 x 10^4 cells/well) and incubated in complete medium (0.5 ml) for 24 h to permit attachment. To cells was added dT_{18} in the range 0-5 µM. On a separate plate, cells received liposomes covering the range 0-120

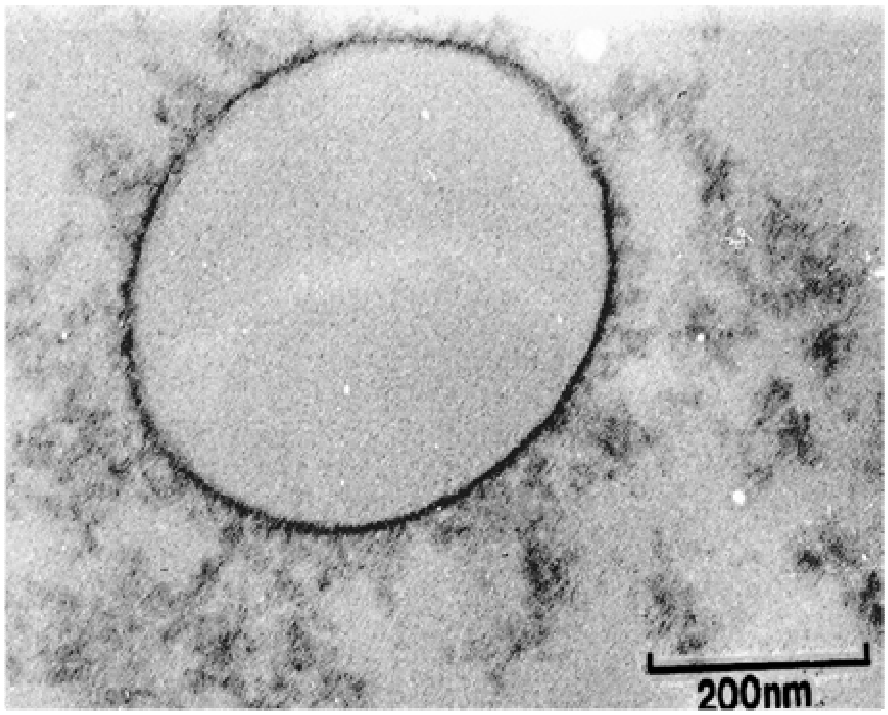

Figure 1. Transmission electron microcopy of cationic liposomes. A liposome suspension (50 µl) was mixed with 5% BSA (100 µl) and fixed with glutaraldehyde (25%). A section of the resultant gel was stained with OsO_4 and washed with cacodylate buffer (0.2 M). Dehydrated samples were fixed (Spurr's resin), sectioned and collected on C-200 copper grids and stained with uranyl acetate and lead citrate. Grids were viewed in a Philips 301 electron microscope at 60 kV.

µM with respect to the Chol-T component. On a third plate, cells received preformed lipoplexes at a positive: negative charge ratio of 1.3:1 up to 120 µM with respect to Chol-T. After 4 hours at 37°C the medium was changed and cells incubated for a further 48 h. Relative cell numbers were determined by the method of Schellekens and Stitz (1980). Briefly, medium was removed and cells washed with PBS (3 x 1 ml). Thereafter wells received 200 µl crystal violet solution (0.5% crystal violet, 0.8% NaCl, 5% formaldehyde and 50% ethanol). After 20 min, stain was removed and wells thoroughly washed with water and air dried for 24 h. Stain was extracted into 2 methoxyethanol (500 µl) overnight and absorbance of extracts measured at 550 nm.

RESULTS AND DISCUSSION

Liposome-ODN interactions

Liposomes were prepared from equimolar mixtures of the cholesteryl cytofectin Chol-T and the neutral co-lipid DOPE. This fusogenic phospholipid assists in the formation of liposomes with significantly increased transfection activity and reduced cytotoxicity (Sternberg et al., 1994). Liposomes were examined for size and lamellarity by transmission electron microscopy. Figure 1 clearly confirms the unilamellar nature of the cationic liposomes which were approximately 200 nm in diameter. In a centrifugation assay (Figure 2) it was established that liposome uptake of dT_{18} reached apparent saturation at a liposome (+ve): ODN (-ve) charge ratio of 3:1. This is consistent with a model in which approximately half of the cationic liposome positive charges are located on the inner leaf of the liposome membrane bilayer and therefore unlikely to interact with the negatively charged dT_{18} backbone. Results presented in Figure 3 indicate that liposome-bound $[^3H]dT_{18}A_7$ does not undergo measurable hydrolysis in the presence of 10% FBS at 37°C up to 3 h. Whereas $[^3H]dT_{18}A_7$ alone under the same conditions afforded significant amounts of radiolabelled ODNs corresponding to 18 mers and smaller with a 50% reduction in intact $[^3H]dT_{18}A_7$. This would support the notion that breakdown products resulted largely from 5'-exonuclease activity since the shortest possible radiolabelled oligonucleotide resulting from 3'-exonuclease digestion alone would be the 19 mer $[^3H]dT_{18}A_1$. Possible protection against 3'-exonuclease activity by duplex formation at the 3' end through intramolecular dT-dA base pairing (hairpin) may account for the apparent lack of 3'-exonuclease activity.

Lipoplex interactions with cells *in vitro*

Cationic liposomes moderately improved association of

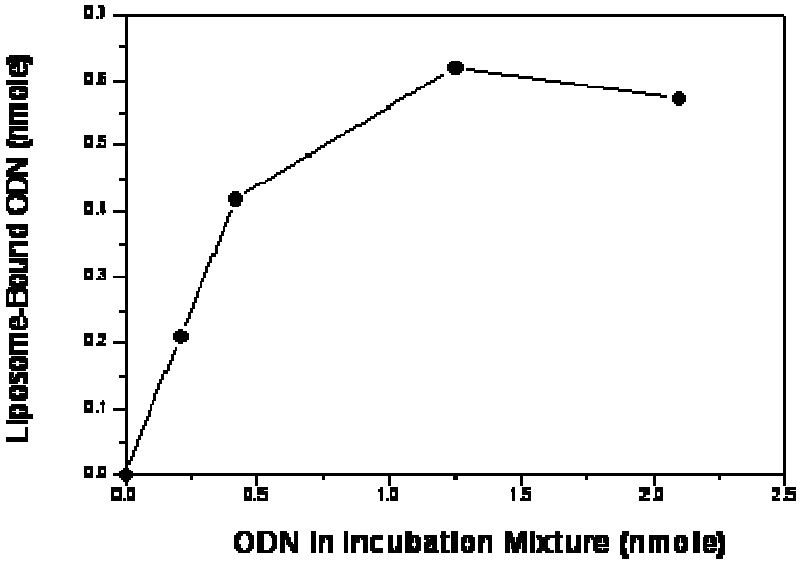

Figure 2. Liposome-ODN binding assay. Reaction mixtures (50 µl) contained cationic liposomes (17 µg Chol-T, 33 nmole) and increasing quantities of dT_{18} up to 12.3 µg (2.1 nmole) in HBS. After incubation at 21°C for 20 minutes mixtures were added to HBS (500 µl) and centrifuged at 4000 x g for 2 h at 4°C. ODN concentrations in supernatants were determined by measuring absorbance at 260 nm.

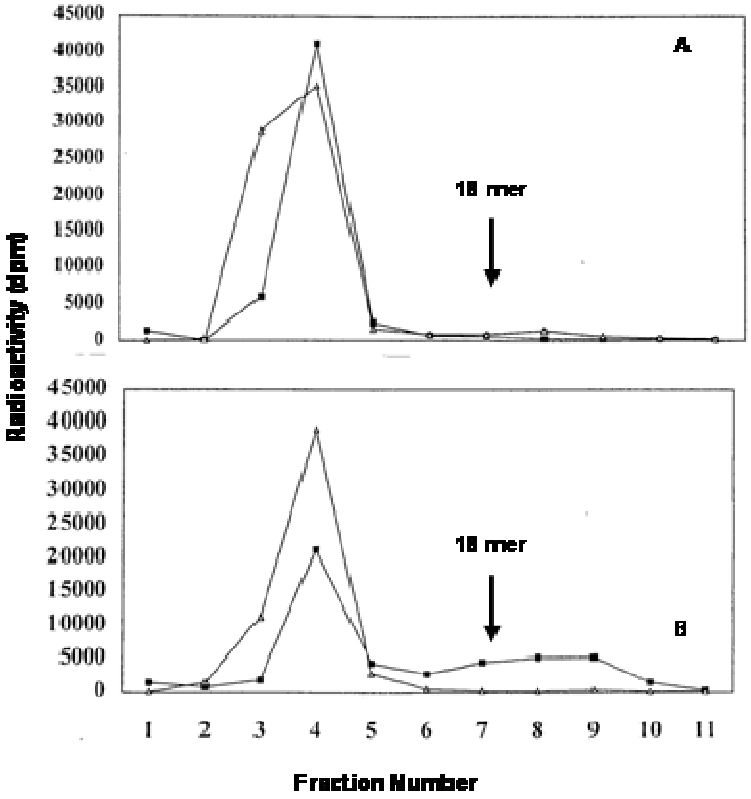

Figure 3. Effect of serum nucleases on free and liposome-complexed [^{3}H]$dT_{18}A_7$. Lipoplexes (Δ) and free [^{3}H]$dT_{18}A_7$ (■) were treated with 10% FBS. At 0 (A) and 180 (B) min aliquots were taken and ODNs extracted and separated by denaturing polyacrylamide gel electrophoresis.

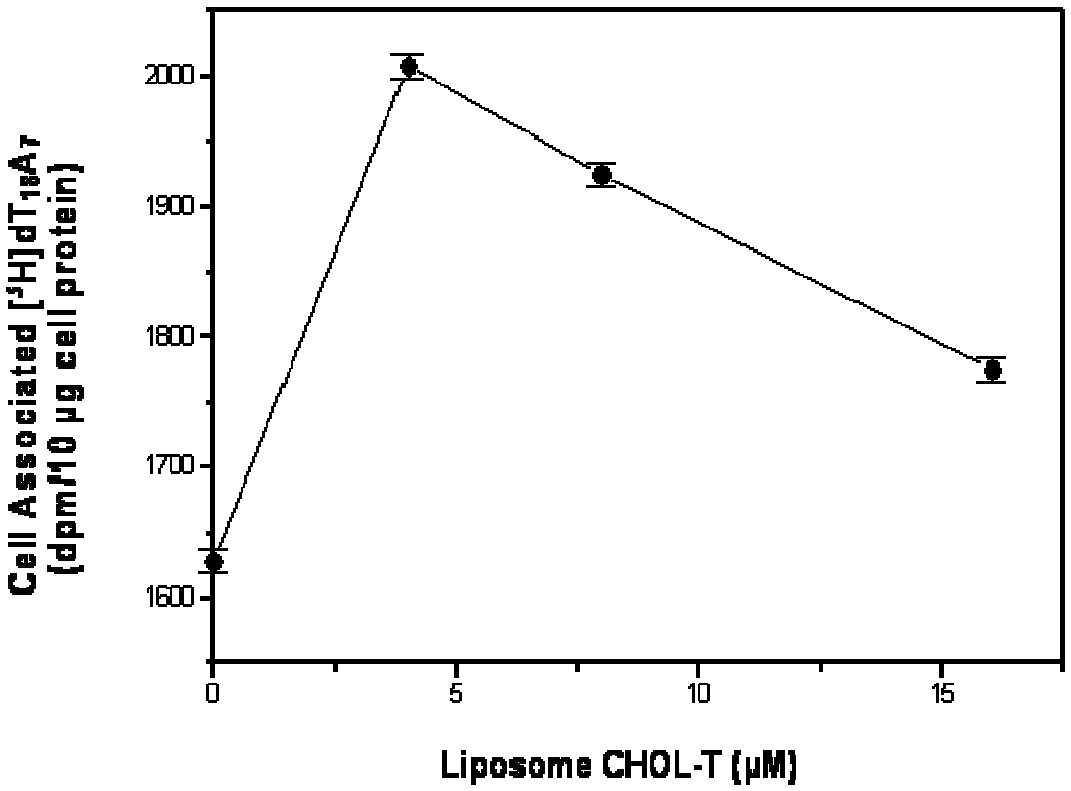

Figure 4. CHO cell-associated [^{3}H]$dT_{18}A_7$. Cells in 24 well plates were incubated with [^{3}H]ODN (12.2 nM) and varying amounts of cationic liposomes up to 16 µM for 24 h at 37°C. Experiments were conducted in triplicate and results are presented as means ± S.D.

[^{3}H]$dT_{18}A_7$ with CHO cells and showed a maximal effect at a Chol-T concentration of 5 µM (Figure 4). At higher liposome concentrations up to 20 µM the enhancement of ODN cell association was less pronounced. The ODN level in the incubation medium was set at 0.18 µM. It is noteworthy that in an earlier study it has been reported that at low ODN concentrations a cationic liposome preparation based on the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP) fails to enhance uptake of ODN by mammalian cells in culture (Capaccioli et al., 1993). Antisense ODNs which act stoichiometrically are typically effective against target mRNA molecules in cells at levels up to 25 µM in the medium (Wang et al., 2001). This is a higher quantity of nucleic acid than is commonly delivered in gene transfer experiments where the delivered transgene is usually an integral component of a plasmid vector. Liposome facilitation of this delivery may be accompanied by unwanted toxicity derived from the liposomes and/or lipoplexes. In a previous study with Chol-T liposomes and the plasmids pSV2CAT and pRSVCAT, the HepG2 cell line was shown to tolerate liposomes well at a 36 µM Chol-T level (Kisoon et al., 2002). However, since the liposome-assisted delivery of ODNs requires higher levels of liposomes it was decided to compare the toxicity of dT_{18} and Chol-T liposomes alone with that of lipoplexes up to 5 µM dT_{18} and 120 µM Chol-T at a constant positive: negative charge ratio of 1.3:1 in HepG2 cells. The ODN dT_{18} was only weakly cytotoxic (10% growth inhibition at 5 µM, Figure 5A) in agreement with findings of a previous study carried out in the human hepatoma cell line SMMC-7721 with dT_{15} (Wang et al., 2001). Although liposomes alone exhibited marked toxicity at 96 µM Chol-T and higher (50% inhibition and greater, Figure 5B) this effect was reduced by prior complexation with dT_{18} (30% growth inhibition at 96 µM Chol-T, Figure 5C).

Conclusion

It has been shown that cationic liposomes based on the cholesteryl cytofectin Chol-T exhibit saturable uptake of a model antisense ODN and lend it protection against serum nuclease catalyzed degradation. Moreover these cationic liposomes facilitate association of ODNs to the mammalian cell line CHO even under demanding conditions of low ODN concentration while ODN lipoplexes are well tolerated by the human hepatoma cell line HepG2. Thus it appears that Chol-T liposomes which have been used successfully in gene transfer applications may also be suitable for the development of delivery protocols in antisense ODN applications.

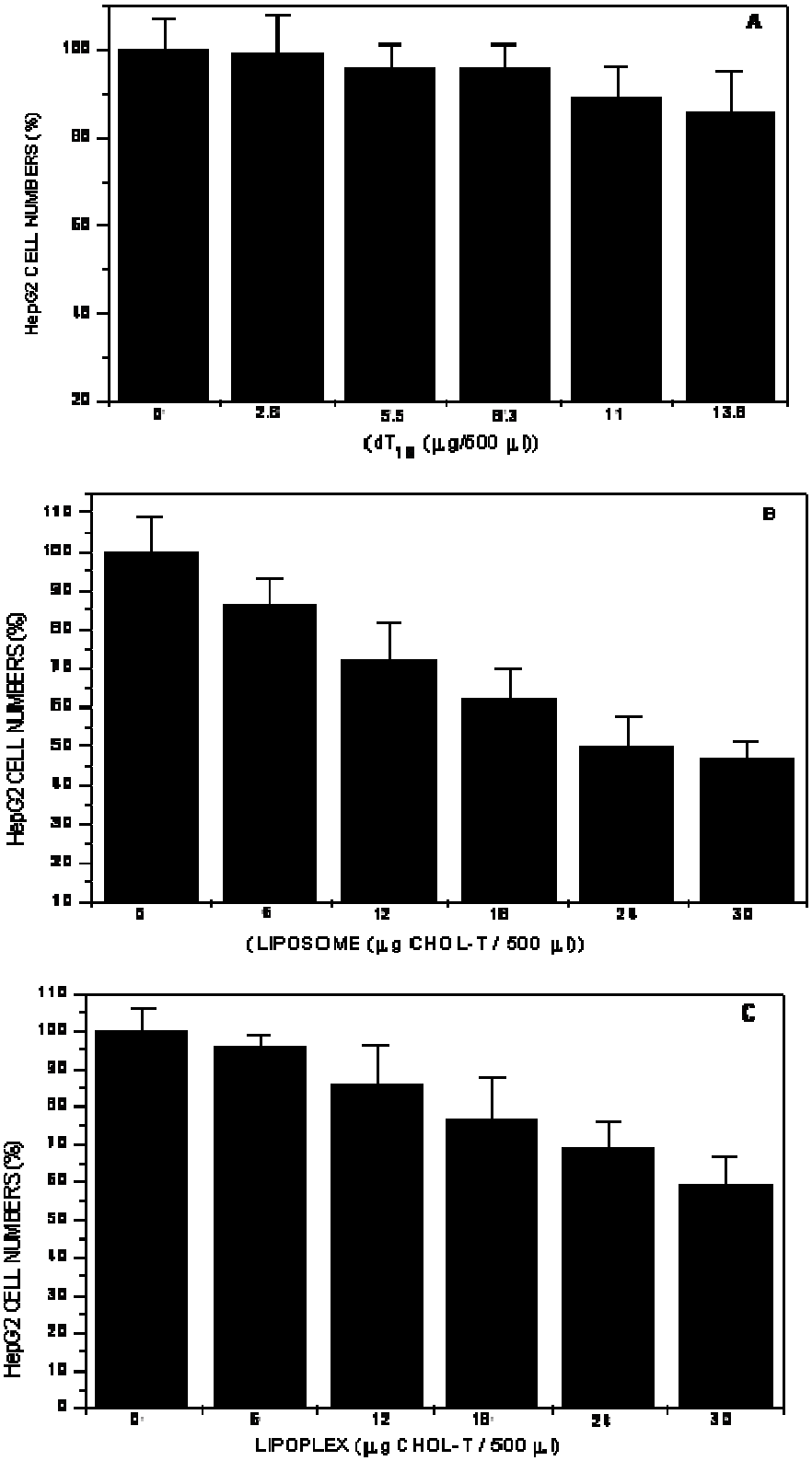

Figure 5. HepG2 cell growth inhibition assays. Cells in multiwell plates were exposed to dT_{18} up to 5 µM in the absence (A) or presence (C) of cationic liposomes up to 120 µM Chol-T at a liposome (+ve): ODN (-ve) charge ratio of 1.3:1. Growth inhibition by liposomes alone is also shown (B). Experiments were conducted in triplicate and results are presented as means ± S.D.

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