siRNA-Lipoplex-Mediated Bcl-2 and Bcl-xL Gene Silencing Induces Apoptosis in MCF-7 Human Breast Carcinoma Cells

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Abstract: Bcl-2 family genes play a central role in cell apoptosis and cell proliferation, and are implicated in the pathology of many malignancies. We explored different ways of introducing siRNA duplexes into cells, comparing "naked" with lipoplex- and polyplex-based formulations in order to decrease the level of the Bcl-2 and Bcl-xL proteins. Our results show that siRNA binds efficiently to all cationic liposomes used. Upon binding, siRNA reduces the zeta potential of the particles, although in most cases they remain positively charged. 70% of MCF-7 cells took up fluorescently-labeled siRNA after 24 h. All siRNA sequences caused growth inhibition of cells, with variable efficiency in a dose-dependent manner. Significant decreases in Bcl-2 and Bcl-xL proteins were caused by two siRNA sequences. Both caused significant growth inhibition in concentrations as low as 100 nM. These two siRNAs caused the greatest increase in caspase-7 activity and DNA fragmentation level. Addition of $CaCl_2$ as a transfection enhancer resulted in marked increase of growth inhibition and Bcl-2 gene suppression by siRNA. Our lipoplexes containing siRNA showed equal or superior efficacy in comparison with commercial siRNA transfection kits. Efficiency of cell growth inhibition per RNA molecule using siRNA was found to be twenty fold higher than by a well-established Bcl-2 antisense oligonucleotide (ODN) molecule after optimization of ODN delivery to cells in culture. This study indicates the potential for efficient delivery of siRNA for treatment of various malignancies.

Keywords: Gene therapy, Bcl-2, Bcl-xL, siRNA, MCF-7, apoptosis, cationic lipids.

1. INTRODUCTION

Cancer is caused by multiple genomic aberrations that include oncogene activation, tumor suppressor inactivation, and suppression of apoptotic pathways. The regulation of apoptosis depends on the balance of pro- and anti-apoptotic proteins within the cell. In particular, the Bcl-2 homology (BH) family, encompassing both pro- and anti-apoptotic members, governs this important cell decision. Bcl-2 family genes play a central role in cell apoptosis and proliferation. Bcl-2 is an inhibitor of the mitochondrial apoptotic pathway and exerts its action by blocking the pro-apoptotic counterparts, including Bad and Bax, thereby preventing the release of cytochrome *c* and the activation of caspase 9. Overexpression of Bcl-2 is observed in several cancers, including hematologic malignancies, as well as a range of solid tumors, including nasopharyngeal, colorectal, prostate, and breast cancers [1, 2]. In several types of cancer, including breast cancer, it is known that there is increased expression of Bcl-2 and, to a lesser extent, of Bcl-xL [3-6]. However, paradoxically, Bcl-2-positive patients were reported to have a better prognosis and an overall better survival rate when compared with Bcl-2-negative patients [7, 8]. In mammary epithelial cells Bcl-2 expression is regulated by estrogen and correlates with estrogen receptor (ER) alpha expression [9, 10]. Thus, tumors expressing ER alpha usually express a high level of Bcl-2 and are also those that are less aggressive and less resistant to endocrine- and chemotherapy than ER-negative tumors. Therefore, cells expressing ER alpha are appropriate targets for Bcl-2 gene silencing in order to reduce the level of Bcl-2 and Bcl-xl proteins. Moreover, subsequent treatment with an estrogen antagonist such as tamoxifen may achieve a better effect after decreasing the expression of Bcl-2.

Bcl-2 is capable of inhibiting cancer-chemotherapy-induced apoptosis, and overexpression of Bcl-2 confers resistance to chemotherapy [2, 3]. One of the major limitations of cancer treatment is the marked ability of human tumors to develop and sustain resistance to chemotherapeutic drugs and radiation. The fact that defects in apoptosis can promote drug resistance downstream of the drug–target interaction strongly suggests a causal relationship between apoptosis and drug-induced cytotoxicity [11]. In this context, upregulation of the anti-apoptotic and downregulation of the pro-apoptotic Bcl-2 family members in tumors have been associated with their decreased susceptibility to chemo- and radio-

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therapy [2, 3]. Therefore, it has been proposed that functional blockade of either the anti-apoptotic Bcl-2 family members or overexpression of the pro-apoptotic ones could possibly change the balance of the apoptotic machinery in tumor cells and sensitize them to chemo- and radiotherapy.

It was demonstrated that chemotherapeutic drugs exert their effect in part by modulating the expression of several members of the Bcl-2 family in cancer cells. Doxorubicin causes a decrease in Bcl-2 expression and increase in Bax expression [12]. Schon and Schon have shown that imiquimod has proven clinical efficacy against basal cell carcinomas, actinic keratosis and cutaneous metastases of malignant melanoma. They found that, at least in part, this effect is mediated through interference with Bcl-2-dependent release of mitochondrial cytochrome *c* and subsequent activation of caspase-9 [13]. The therapeutic potential of modulation of Bcl-2 and Bcl-xL gene expression has been previously explored by using antisense oligonucleotides [14]. For example, it was shown that the Bcl-2 antisense oligonucleotide oblimersen has tumor suppressive activity and synergistic activity with chemotherapeutic agents [14, 15]. A different antisense oligonucleotide, which simultaneously downregulates Bcl-2 and Bcl-xL expression, induces apoptosis and inhibits growth of various tumor types *in vitro* and *in vivo* [16, 17].

Many research groups are now exploring the therapeutic potential of using Gene Silencing RNAi technology (GSRT), the process whereby a double-stranded RNA (dsRNA) induces the homology-dependent degradation of cognate mRNA and subsequent reduction of the intracellular concentration of a specific protein [18-21]. Good examples of such delivery systems are the complexes of nucleic acids with cationic lipids (lipoplexes) or with cationic polymers (polyplexes), which mediate transfection of DNA and of antisense oligonucleotides *in vitro* and *in vivo* [22-25].

In order to be efficacious, siRNA has to be delivered to the target cells; however, the chances for delivery of negatively charged siRNAs to cells in sufficient amounts are poor [26]. Therefore, convenient means should be developed for introducing siRNA into the desired cells *in vitro* and *in vivo* in order to achieve gene silencing. Cationic lipid-based lipoplexes and cationic polymer-based polyplexes are efficient delivery systems and are gaining increasing popularity. This is related to their availability, simplicity, reproducibility, and relatively low toxicity. Cationic lipids, which are the basis for lipoplexes, are amphiphiles possessing a positively-charged headgroup responsible for nucleic acid binding (complex formation) and for complex association to the cell, followed by cell uptake; in addition it has a linker, and a hydrophobic (nonpolar) part. More than 100 cationic lipids have been developed so far, most for *in vitro* use, and a few for *in vivo* use [26-30]. Many studies have shown that lipoplexes significantly improve the intracellular delivery of oligonucleotides [22, 23, 27-31].

Polyplexes are based on a variety of water-soluble cationic polymers chemically and physically very different from the cationic lipids [31]. Lipoplexes and polyplexes share similar properties of binding nucleic acids and introducing them to cells. So far, lipoplexes are more extensively used for delivery of nucleic acids than polyplexes.

In this work, we have taken advantage of the discovery of RNAi and gene silencing as an efficient way of reduction of gene expression. Therefore, we focused on screening for Bcl-2 siRNA delivery systems, especially cationic lipids, working out conditions for optimal *in vitro* transfection of synthetic siRNA duplexes using a number of our cationic lipid formulations and commercial cationic liposome transfection kits, as well as one polyplex system. We performed partial physicochemical characterization of the complexes and examined their effects on growth, apoptosis, and Bcl-2 and Bcl-xL protein expression, comparing different siRNA sequences. Special interest is given to silencing of the Bcl-2 family of anti-apoptotic proteins.

2. MATERIALS AND METHODOLOGY

2.1. Lipids

Cholesterol was purchased from Sigma. The monocationic lipid 1,2-dioleyloxy-3-(trimethylamino) propane (DOTAP) and neutral lipid dioleoylphosphatidyl ethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL). DOTAP is a monocationic glycerolipid having a quaternary amine as a single pH-independent positive charge group (i.e., it is protonated at all pHs). Therefore, when interacting with nucleic acids, DOTAP N/P ratio (vide infra point 2.5) is equal to the N^+/P^- ratio. D-*erythro*-*N*-palmitoyl-sphingosyl-carbamoyl spermine (ceramide carbamoyl spermine (CCS)), a polycationic sphingolipid having 3 positive charges, composed of a mixture of D-*erythro*-*N*-palmitoyl-sphingosyl-1-carbamoyl spermine and D-*erythro*-*N*-palmitoyl-sphingosyl-3-carbamoyl spermine (at 4:1 mole ratio), was a gift of Dr. E. Rochlin of Bio-Lab Ltd., Jerusalem, Israel. CCS has one primary (pKa~10) and two secondary amines (pKa 8.9 and 8.0). As obvious from these pKa values, the degree of protonation is pH-dependent. In the case of liposomes and lipoplexes, these groups are located in the lipid/water interface and therefore degree of ionization is affected by the surface pH, which is high (pH~11) for the CCS-based liposomes when bulk (solution) pH is 7.4 [31]. Therefore, for the CCS-based liposomes the actual degree of protonation is not known, therefore, we used the N/P ratio (as used for polyplexes [25]) and not N^+/P^- ratio.

All lipids were at least 97% pure when analyzed by TLC. *tert*-Butanol was purchased from BDH, Poole, UK. Water was purified using WaterPro PS HPLC/Ultrafilter Hybrid model (Labconco, Kansas City, MO), providing sterile, pyrogen-free, highly pure water with low levels of total organic carbon and inorganic ions (18.2 megaohms), which is referred to here as "pure water". Linear polyethyleneimine (PEI) with molecular weight of 25,000 Da was obtained from Polysciences, Inc. (Warrington, PA).

2.2. siRNA Sequences

The E3 siRNA sequence was used by Cioca *et al*. to silence the Bcl-2 gene in the human myeloid leukemia cell line [32]. El and E4 siRNA sequences were previously employed to silence the Bcl-2 gene, and the E5 siRNA sequence was used to silence the Bcl-xL gene in the colorectal carcinoma cell line [29]. EJ2 and EJ5 sequences were chosen using standard rules for siRNA design [33]. The scrambled siRNA sequence has no homology whatsoever to any human gene, according to blast alignment.

Bcl-2 Sense Sequences

E1 5' G CUG CAC CUG ACG CCC UUC 3'

E3 5' GUA CAU CCA UUA UAA GCU G 3'

E4 5' GGG GCU ACG AGU GGG AUG C 3'

EJ2 5' GUG AGG UGU CAU GGA UUAA 3'

Bcl-xL Sense Sequences

E5 5' CAG GGA CAG CAU AUC AGA G 3'

EJ5 5' GGA GAC UAG AUU GCC UUUG 3'

Scrambled Sense Sequence

Scr 5' ACU CUA GCG GCA CCA UCG UGC C 3'

All siRNA molecules were purchased from Dharmacon (Lafayette, CO). The 18 mer G3139 Bcl-2 ODN antisense was obtained forum Genta (Lexington, MA).

2.3. Cellular Uptake of Fluorescently-Labeled siRNA

Fluorescein-labeled siRNA was used in order to determine the percent of cells that took up siRNA. Prior to the cell uptake experiments, the siRNA was labeled with 5' carboxy-fluorescein (FAM) using Silencer® siRNA Labeling Kit (Ambion, Austin, TX) according to the manufacturer's instructions. In this process, 5' carboxy-fluorescein (FAM) was conjugated to the siRNA sequences in a way that does not interfere with the silencing activity of the molecule. MCF-7 cells were incubated with 400 nM E1 siRNA, or 400 nM scrambled siRNA, or treated with lipid carrier only. Twenty four hours later, cells were fixed with paraformaldehyde solution (3% paraformaldehyde and 2% sucrose in PBS) and stained with 1.5 μg/mL DAPI (4, 6 diamidino-2-phenylindole) in mounting medium from Vector Laboratories, (Burlingame, CA). Cells were viewed under a fluorescent microscope, photographed and analyzed for percent of fluorescently labeled cells.

2.4. Characterization of Cationic Lipids and PEI

All cationic and helper lipids were analyzed for purity and quantified by TLC as described by Simberg *et al*. [34]. CCS and PEI were quantified based on elemental analysis. CCS was also quantified by its primary amino group using the TNBS assay [35]. In the case of CCS, both methods, nitrogen quantification and primary amines, were in good agreement.

2.5. Preparation and Characterization of siRNA Cationic Liposome Complexes (siRNA Lipoplexes) for Transfection Experiments

Preparation of lipoplexes: Lipoplexes were formed upon mixing of cationic liposomes and nucleic acids. The lipoplexes are defined by the specific cationic lipid used and by their specific +/– charge ratio. DOTAP is a monocationic lipid, while CCS has 3 positive charges per molecule. The mole ratio of 2:1 between the cationic and neutral lipid was used for the preparation of the lipoplexes. The phosphates (P^-) of the nucleic acids are the source of their negative charge. A 20 μM stock solution of siRNA, which is equivalent to 820 μM P^-, was diluted 10-fold in 5% dextrose (final concentration: 82 μM siRNA P^-). Stock solutions of lipids used for the lipoplex preparations were dissolved in *tert*-butanol and lyophilized. Lyophilized cationic lipids were hydrated with 5% dextrose in pure water to a final concentration of 164 μM, which results in formation of unsized heterolamellar vesicles (UHV) [36]. For downsizing, liposomes in 20 mM Hepes buffer, pH 7.4, were sonicated in an Elma transsonic 460/H bath sonicator for 10 s to obtain mostly large unilamellar vesicles (LUV). The siRNA solution was added dropwise until the desired charge ratios and concentrations were reached. The mixture was incubated for 10 min. In the experiments an siRNA P^- concentration of 41 μM and a cationic lipid concentration of 82 μM were used. A lipid N to siRNA P (N/P) ratio of 2 was used for the preparation of DOTAP-based lipoplexes and of 6, for CCS-based lipoplexes. Lipoplexes were formed at least 15 min before use. When precipitation occurred, the milky suspension was sonicated for a maximum of 2 min.

In the preparation of the lipoplexes containing $CaCl_2$, first, $CaCl_2$ at a concentration of 25 mM was added to the siRNA solution and incubated for 10 min, then siRNA solution was added dropwise to the LUV until the desired charge ratios and concentrations were reached.

In the preparation of the cationic polymer PEI complexes with siRNA, a stock solution of PEI (7.5 mM of nitrogen, which is 21% by elemental analysis) was prepared in Hepes buffer (20 mM, pH 7.4). Mixing of cationic polymers and nucleic acids resulted in formation of polyplexes. The polyplexes are defined by the ratio of positive charges expressed as number of nitrogen atoms (N) to number of nucleic acid phosphate (P) groups to give a N/P ratio. The siRNA was added to PEI at N/P ratio of 45 and incubated for 10 min.

Determination of zeta potential: Zeta potential was measured at 25 °C using a Zetasizer Nano-Z, Malvern Instruments Ltd, Malvern, UK. An aliquot of 100 μL of liposomes or lipoplexes was diluted in 600 μL of 20 mM Hepes (pH 7.4). The principle of zeta potential measurement and equation for calculation are described elsewhere [37].

Determination of level of siRNA binding to cationic liposomes by ethidium bromide (EtBr) intercalation exclusion assay: Liposomes made of 16.4 nmol of cationic lipid were mixed with 8.2 nmol of siRNA in order to achieve an siRNA P^- /cationic lipid mole ratio of 1/2. After 10 min of incubation at room temperature, 2.5 nmol of EtBr was added to each sample, followed by an additional 10 min of incubation [38]. EtBr fluorescence intensity was measured using an excitation wavelength of 260 nm and emission wavelength of 591 nm by LS50B luminescence spectrometer (Perkin Elmer, Norwalk, CT). The fluorescence intensity was expressed as the percentage of maximum fluorescence signal obtained for the same concentration (8.2 nmol) of free siRNA.

Size distribution measurements: Mass-weighted size distribution of the liposomes and complexes was determined at 25 ºC by dynamic light-scattering (DLS) using the ALV-NIBS/HPPS particle sizer ALV-Laser Vertriebsgesellschaft GmbH (Langen, Germany). This instrument allows measuring size without diluting the sample, even at the millimolar lipid concentration range, so that no dilution-induced changes occur during measurements.

2.6. Cell Cultures

MCF-7 cells were grown in DMEM medium supplemented with glutamine, antibiotics and 10% fetal calf serum in an incubator at 37ºC under 5% CO_2. All culture medium components were purchased from Biological Industries (Beit-HaEmek, Israel).

2.7. Transfection of MCF-7 Cells

For determination of siRNA transfection with commercial kits, transfection was performed in 24-well plates. Cells were seeded at 7.5×10^4 cells per well in a volume of 500 µL. The transfection was performed using siIMPORTER cationic lipid transfectants and the corresponding siRNA, following the manufacturer's instructions (Upstate Cell Signaling, Charlottesville, VA). All experiments were performed at 24, 48 and 72 h post transfection.

For determination of siRNA transfection with our lipid-based carriers, MCF-7 cells were plated into 96-well plates (4.0×10^3 cells/well) and incubated at 37ºC with the specified concentrations of siRNA complexed with 2, 4 and 8 µM cationic lipids in parallel and incubated for 48-72 h. A solution of 5% dextrose (without lipoplexes) was added to the control wells. For transfection of MCF-7 cells with PEI, 25, 50 and 100 nM siRNA was complexed with 0.25, 1.0 and 2.0 µM PEI in parallel and incubated for 48 h. Hepes buffer (without polyplex) was added to the control wells.

2.8. Determination of MCF-7 Cell Survival

Viable cells were counted using the trypan blue or the methylene blue (MB) staining assay [39]. For determination of cell survival by the MB assay, following 24 h of incubation in culture, different concentrations of drugs were added to each well. After fixed time intervals of drug exposure, the drug-treated cells, as well as control cells, were washed with pure water and fixed by adding 50 µL of 2.5% glutaraldehyde to each well for 15 min. The fixed cells were rinsed 10 times with pure water and once with borate buffer (0.1 M, pH 8.5), dried, and stained with MB (100 µL of 1% solution in 0.1 M borate buffer, pH 8.5) for 1 h at room temperature. Stained cells were rinsed thoroughly with pure water to remove any non-cell-bound dye, and then dried. The MB bound to the fixed cells was extracted by incubation at 37 ºC with 200 µL per well of 0.1 N HCl for 1 h, and the net optical density of the dye in each well was determined using an absorbance plate reader (Multiskan Bichromatic Labsystems, Finland) at 620 nm. For determination of viable cell counts by trypan blue staining, after fixed time intervals of drug exposure, the drug-treated cells, as well as control cells, were washed and the incubation continued in fresh medium until termination of the experiment. At the end of the experiment, cells were trypsinized, centrifuged, stained with trypan blue and counted using a hemocytometer.

2.9. Assessment of Apoptosis

Apoptosis assays were performed in 96-well plates. The caspase-7 activity was checked in MCF-7 cells by Apo-ONE Homogenous Caspase-3/7 assay (Promega, Madison, WI) according to manufacturer's instructions. This assay uses Rhodamine-110 conjugated to the amino acid sequence DEVD, a target sequence of caspases-3 and -7, which emits fluorescence upon cleavage. MCF-7 cells have a non-active truncated caspase-3 due to a deletion mutation of 47 bases in exon 3 of the caspase-3 gene [40]. Therefore, the activity measured in this test is that of caspase-7. Briefly, MCF-7 cells (4.0×10^3 cells/well) were treated for 24 h with various siRNA lipoplex formulations. The caspase-3/7 reagent (100 µL) was added to each well containing 100 µL of blank, control or treated cells. For detection of caspase-7 activity, cells were incubated with the kit components for 6 h, then fluorescence was measured at excitation wavelength 485 nm and emission wavelength 530 nm using the Synergy HT plate reader in fluorescence mode (Bio-Tek, Winooski, VT).

The level of DNA fragmentation was detected in treated MCF-7 cells after 48 h incubation "Cell Death Detection ELISA Plus" (Roche Applied Bioscience, Basel, Switzerland). The detection is quantitative and is based on the ELISA principle, using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific detection and quantitation of mono- and oligonucleosomes that are released into the cytoplasm of cells that die from apoptosis.

2.10. Determination of the Bcl-2 Protein by Bcl-2 ELISA Assay

Briefly, MCF-7 cells (1.0×10^6cells/well) were treated for 16 or 24 h with various siRNA lipoplexes. Cell lysates were prepared, and the level of Bcl-2 was determined in treated and untreated (control) cells. The Bcl-2 ELISA assay (Bender MedSystems Vienna, Austria) was used according to manufacturer's instructions. The absorbance at 450 nm was measured using the Multiscan Bichromatic.

2.11. Determination of the Level of Bcl-2 and Bcl-xL by Western Blotting

We used the procedure of Onuki *et al.* [41]. Briefly, 24 or 48 h post transfection, cells were washed with cold PBS, and then with lysis buffer containing sodium deoxycholate 0.5%, Tris-HCI 0.01M, NaC1 0.15M, NP_{40} 0.5% and Protease Inhibitor Cocktail (Sigma-Aldrich). A cell scraper was used to remove cells to Eppendorf tubes, which were put on ice for 30 min and then centrifuged at 14.000 rpm for 2 min. A sample was taken for determination of protein [42]. Laemmeli buffer was added at a volume equal to the cell lysate. This was heated to 100 ºC and loaded onto a 10% polyacrylamide gel. The membrane was blocked using SuperBlock blocking buffer (Pierce, Rockford, IL) for 1 h, followed by 1 h incubation with primary Bcl-2 or Bcl-xL antibody (1:1000, DakoCytomation, Glostrup, Denmark). After one hour of incubation with horseradish peroxidase, conjugated secondary antibody was added (1:10000, Jackson Immunoresearch, Minneapolis, MN). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used, and the membrane was exposed to X-ray film.

2.12. Determination of the Level of Bcl-2 and Bcl-xL by RT-PCR

Cells were seeded on a 6-well plate for 24 h. After 24 h, cells were transfected and incubated for another 48 h. RNA was extracted with the Mini Isolation RNA kit (Zymo Research Corporation, Orange, CA) according to manufacture's instructions. Equal amounts of RNA (500 ng) from each treatment were subjected to the two-step RT-PCR assay. cDNAs were made using the Reverse-iT 1^{st} strand synthesis

kit (Abgene, Epsom, UK). The PCR amplification was performed using the Ready mix reaction kit (dNTP, mM, buffer, Taq polymerase (Abgene) using specific primers.

RT-PCR Primers Sequences

Actin

5' CCTGGCACCCAGCACAAT 3'

5' GCCGATCCACACGGAGTACT 3'

Bcl-2

5'GTGAACATTTCGGTGACTTCC 3'

5' CCTTCACCATGTCCTTCTGA 3'

3. RESULTS

3.1. Bcl-2 siRNA Lipoplexes: Design and Physicochemical Characterization

The level of binding of siRNA to cationic liposomes was determined by the EtBr intercalation exclusion assay, as described in Materials and Methods. It was found that when E1 siRNA was complexed with several cationic unisized heterolamelar vesicles UHV (all lipid compositions, except that of DOTAP/Cholesterol), almost no EtBr fluorescence was observed (Table **1**), indicating that all siRNA is associated with the cationic liposomes. Similar results were obtained with other siRNA sequences that were tested.

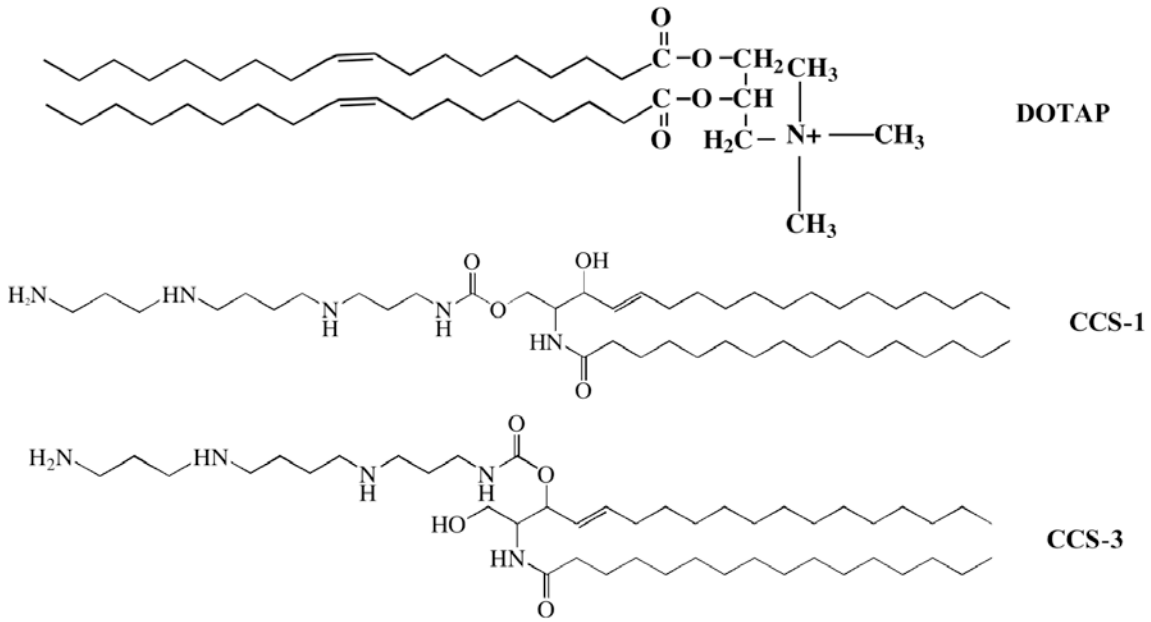
We also studied the zeta potential of cationic liposomes and lipolexes containing E1 siRNA. As expected, all DOTAP- and CCS-based liposomes showed positive zeta potential (Table **2**). The DOTAP-based liposomes had a higher zeta potential than those based on CCS, as expected from the difference between the quaternary amine of DOTAP and the three amines (one primary and two secondary) of the CCS (see Scheme **1** with structural formulas and [22]). Addition of siRNA to these UHV decreased their zeta potential. We found that decrease of the zeta potential of UHV was dependent on the cationic lipid/siRNA ratio and on the type of helper lipid. Namely, at the N/P ratio of 2 for DOTAP- and 6 for CCS-based lipoplexes, the decrease was more pronounced than in lipoplexes having the N/P ratio of 5 and 15 for DOTAP- and CCS-based lipoplexes, respectively (Table **2**). These results were also consistent with our results obtained from the EtBr binding assay, which showed that in DOTAP/Cholesterol lipoplexes at N/P ratio of 2, 20% of siRNA remained unbound (free) (Table **1**). In parallel, these lipoplexes show a reversal from positive to negative zeta potential (Table **2**). These results indicate that in almost all lipoplexes siRNA was efficiently bound to cationic lipids.

Table 1. Binding of E1 siRNA to Cationic Lipid-based Unsized Heterolamellar Vesicles (UHV).

Cationic Lipoplex Formulations	Free siRNA (%)
CCS/siRNA	0
DOTAP/siRNA	0
CCS/DOPE /siRNA	0
DOTAP/DOPE/siRNA	0
CCS/Chol/siRNA	0
DOTAP/Chol/siRNA	20

Note: N/P mole ratio of 2 or 6 was used for preparation of DOTAP- and CCS-based lipoplexes, respectively. The level of siRNA binding to cationic liposomes was measured by EtBr intercalation assay.

Size distribution analysis by DLS shows that DOTAP- and CCS-based UHV were in the size range of 100–500 nm, depending on their composition, with the size of DOTAP-based liposomes being about half of the CCS-based lipoplexes (Table **2**). Addition of siRNA to these cationic UHV (regardless of their composition) to form lipoplexes, resulted in an increase in particle size. The average size of DOTAP-based lipoplexes was ~2-fold that of the DOTAP-based UHV. On the other hand, addition of siRNA to



Scheme 1. Structural formulas of 1,2-dioleyloxy-3-(trimethylamino)-propane (DOTAP), D-*erythro*-*N*-palmitoyl-sphingosyl-1-carbamoyl spermine (CCS-1) and D-*erythro*-*N*-palmitoyl-sphingosyl-3-carbamoyl spermine (CCS-3).

Table 2. Physicochemical Characteristics of Different Liposomes and Lipoplexes

Formulations	$Lipid^+/siRNA^-$ charge ratio	Size (Range), nm	Zeta Potential (mV)
siRNA	siRNA only	-	–39±2.0
PEI	Polymer only	73.1±23.5	18.1±1.1
DOTAP	Lipid only	176±52	99±3.2
DOTAP/DOPE	Lipid only	105±23	82±1.0
DOTAP/Chol	Lipid only	206±28	67±2.6
CCS	Lipid only	301±76	62±1.2
CCS/DOPE	Lipid only	264±106	80±1.0
CCS/Chol	Lipid only	467±48	71±5
DOTAP/siRNA	2	356±54	59±1.0
DOTAP/DOPE/siRNA	2	226±36	60±2.0
DOTAP/Chol/siRNA	2	128±46	–65±6.0
CCS/siRNA	6	2883 (broad distribution)	40±2.0
CCS/DOPE/siRNA	6	3439 (broad distribution)	59±1.0
CCS/Chol/siRNA	6	2078 (broad distribution)	34±1.8
DOTAP/siRNA	5	338±12	61±5.6
DOTAP/DOPE/siRNA	5	296±38	63±5.5
DOTAP/Chol/siRNA	5	286±56	71±5.0
CCS/siRNA	15	3568 (broad distribution)	47±2.0
CCS/DOPE/siRNA	15	4236 (broad distribution)	61±2.5
CCS/Chol/siRNA	15	3698 (broad distribution)	71±3.5

Note: Mole ratio of cationic to neutral lipid of 2:1 was used for the preparation of liposomes and lipoplexes.

the CCS-based UHV caused a much larger increase in both mean particle size and in particle size heterogeneity (Table **2**).

3.2. Uptake of siRNA by MCF-7 Cells

MCF-7 cells were incubated with 400 nM E1 siRNA or scrambled siRNA. Cells incubated with cationic liposomes only were included in the control group. After 24 h, MCF-7 cells were viewed under a fluorescent microscope, photographed and the percent of cells that took up labeled lipoplexes was determined. Our results (Fig. **1**) indicate that 60-70% (±5%) of the MCF-7 cells took up the E1 siRNA (see green staining of the DAPI-stained nuclei). Moreover, MCF-7 cells treated with lipolexes containing scrambled siRNA, but not with cationic liposomes, revealed positive staining (see green-stained nuclei in Fig. **1**).

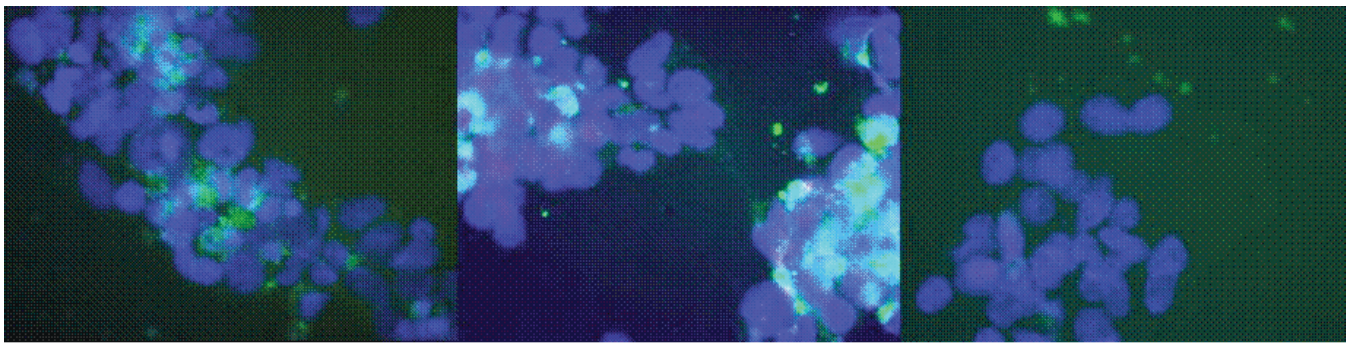


Fig. (1). Uptake of labeled E1 siRNA by MCF-7 cells. MCF-7 cells were transfected with E1 siRNA (400 nM), scrambled siRNA (400 nM) or treated with the tranfection agent only (mock). Prior to the transfection the siRNA was labeled with FAM using Silencer® siRNA labeling kit. 24 h after transfection, cells were fixed with paraformaldehyde solution (3% paraformaldehyde and 2% sucrose in PBS) and stained with DAPI (mounting medium containing 1.5 μg/mL of DAPI).

3.3. Growth Inhibitory Activity of Bcl-2 and Bcl-xL siRNA — Screening Studies for Optimal siRNA Sequences

To assess the growth inhibitory activity of siRNA duplexes, MCF-7 cells were plated at 25% confluence, transfected, and allowed to grow for 48 h. Microscope images of stained MCF-7 cells are presented in Figs. (**2A-J**). Inhibition of growth was clearly observed in cells transfected with either E1 Bcl-2 (Fig. **2E**, **F**) or E5 Bcl-xL (Fig. **2I**, **J**) siRNAs complexed with siIMPORTER cationic lipids. In contrast, no inhibition of growth was observed in untreated cells (Fig. 2A, G) or in cells that were treated with the transfection reagent only (mock) (Fig. **2B**, **H**).

Next we compared the efficacy of growth inhibition using the different siRNA sequences, E1, EJ2, E3 and E4, which target the Bcl-2 mRNA, and E5 and EJ5, which target the Bcl-xL mRNA. We also explored combining two siRNAs, the pair E1+E3 and the pair E1+E5. The appropriate duplexes were added to MCF-7 cells in complexes with siIMPORTER cationic lipids at increasing concentrations and compared to scrambled siRNA sequences complexed with cationic lipids. Then, cells were stained with trypan blue and counted by hemocytometer. The quantification of the growth inhibitory effect of the various siRNA duplexes is depicted in Fig. (**3**). We observed a decline in cell number as early as 24 h post transfection. At 250 nM both E1 and E5 cause a nearly 50% decrease (IC_{50}) in percentage cell survival (Fig. **3A**). The IC_{50} for EJ2, E3 and E4 sequences is over 1000 nM (Fig. **3A**). There is a significant difference (Wilcoxon, $p < 0.05$) between the growth inhibition achieved by the E1 and E5 siRNAs and the other sequences. Fig. (**3A**) shows that E1 siRNA, which is directed against Bcl-2, has a significantly higher growth inhibitory effect on cell survival than E5 and EJ5, which are directed against Bcl-xL. The combination of E1 and E3 resulted in antagonistic interaction and inhibited MCF-7 cell growth less than each duplex alone (Fig. **3B**). On the other hand, the combination of E1 and E5 was more effective than each one of the sequences; however, its effect was less than additive (Fig. **3B**). The transfection agent siIMPORTER caused minimal growth inhibition, less than 10%. Scrambled siRNA had no effect on growth of MCF-7 cells (Fig. **3A**). For the following experiments, we chose to work with the best siRNA sequence for each target. Therefore, we continued our studies with E1 Bcl-2 and E5 Bcl-xL siRNAs.

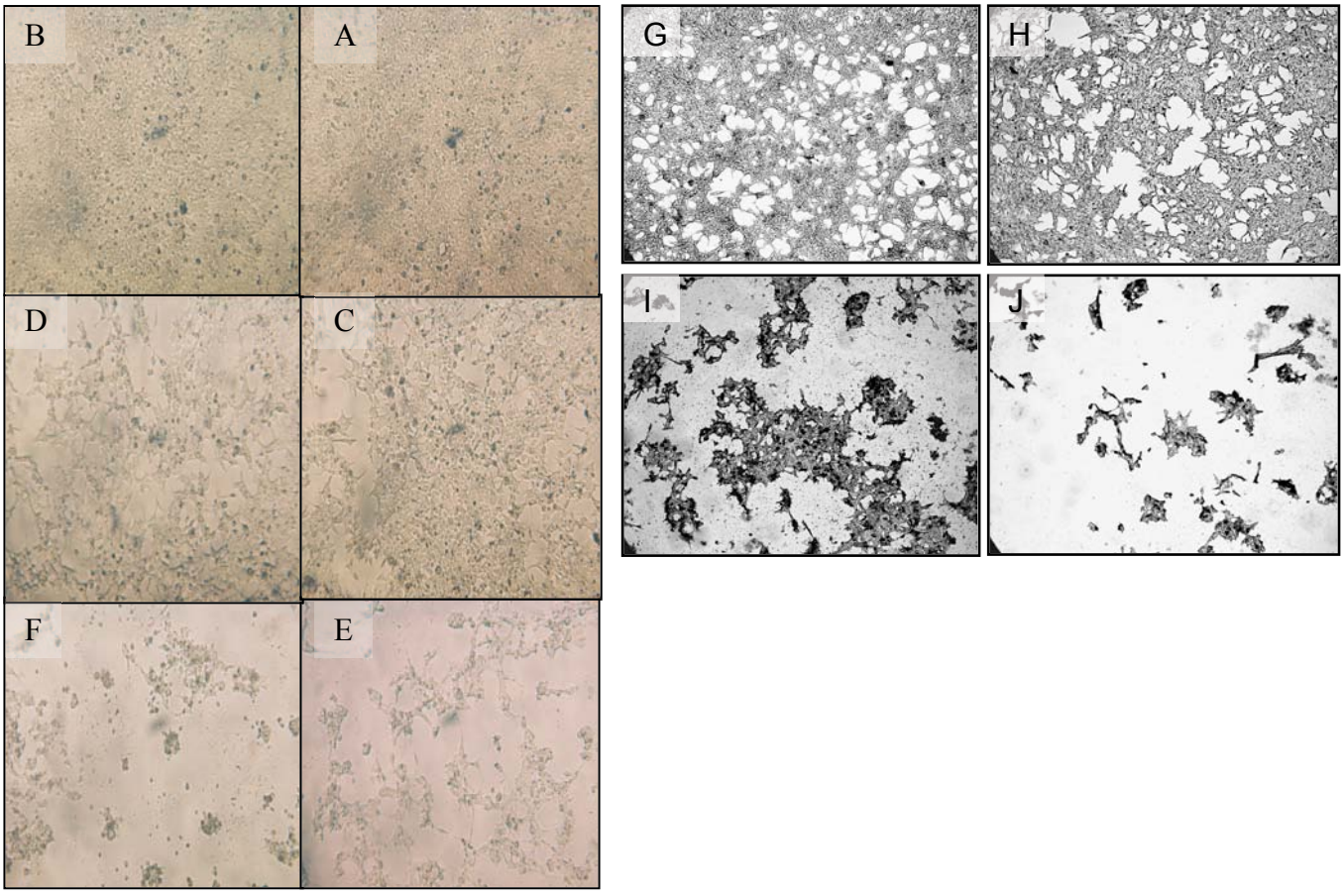


Fig. (2). In situ growth inhibitory effect of lipoplexes containing E1 Bcl-2 and E5 Bcl-xL siRNAs. MCF-7 cells were transfected with cationic lipid transfectant (siIMPORTER) containing E1 Bcl-2 and E5 Bcl-xL siRNAs. Images show MCF-7 cells treated for 48 h with E1 Bcl-2 siRNA (**A**-**F**) or with E5 Bcl-xL siRNA (**G**-**J**). **A** – Untreated cells. **B** – Cells treated with transfection reagent only (mock). **C** – 100 nM E1 treated cells. **D** – 250 nM E1 treated cells. **E** – 500 nM E1 treated cells. **F** – 1000 nM E1 treated cells. **G** – Untreated cells. **H** – Cells treated with transfection reagent only (mock). **I** – 500 nM E5 treated cells. **J** – 1000 nM E5 treated cells. The cells are photographed under an inverted microscope at × 40 magnification.

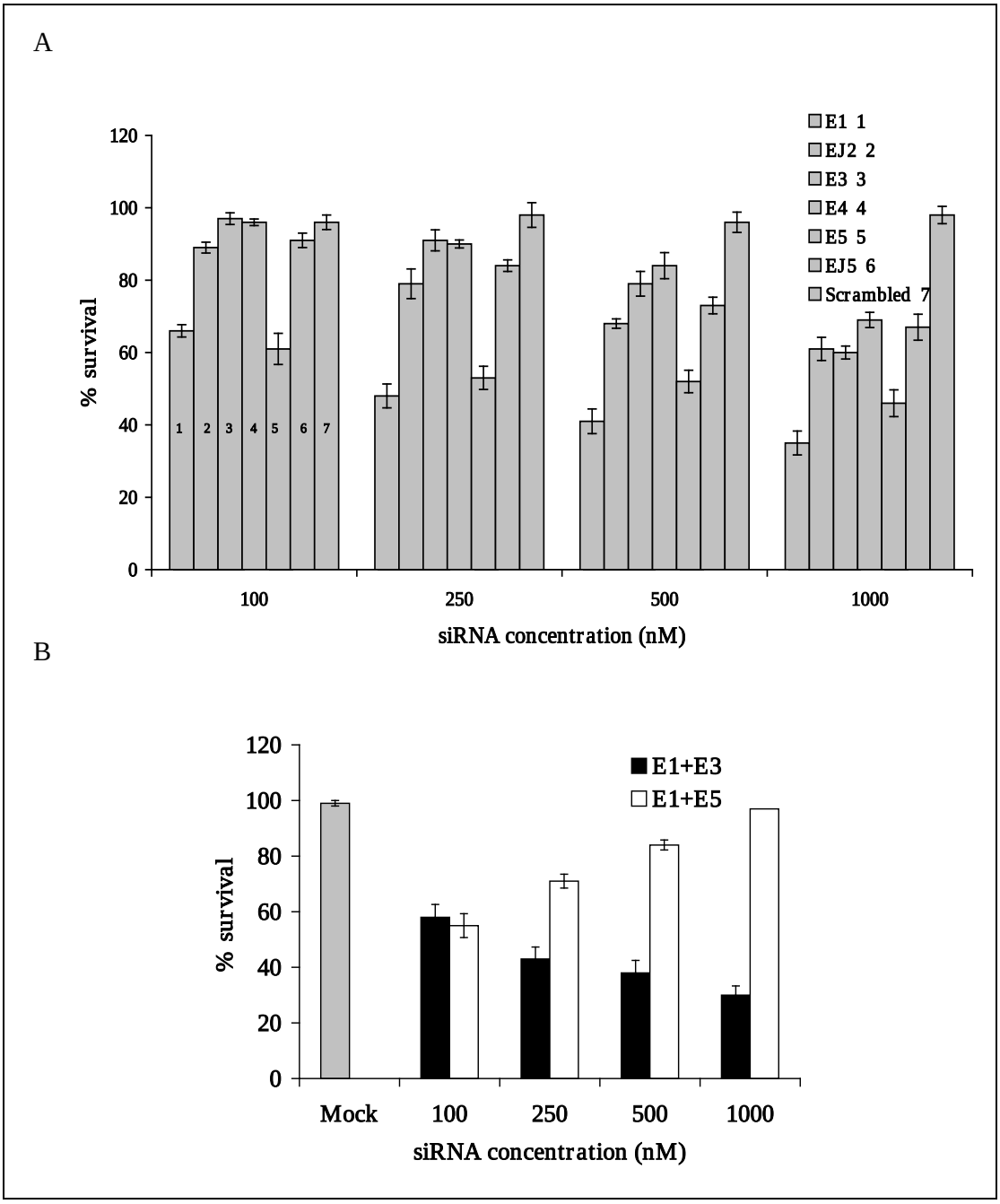


Fig. (3). Dose-dependent growth inhibitory effect of lipoplexes containing Bcl-2 and Bcl-xL siRNAs. MCF-7 cells were plated in 24-well plates (7.5×10^4 per well) and transfected in triplicate with siRNA duplexes using siIMPORTER transfectant 24 h after plating and grown for an additional 48 h. Growth-inhibitory effect of lipoplexes was compared to mock cells- cells treated with the tranfection reagent only, or cells transfected with scrambled siRNA. At the end of the transfection, cells were detached by trypsin and counted by hemocytometer using trypan blue to determine the number of viable cells. (**A**) E1, EJ2, E3, E4, E5, EJ5, Scrambled. (**B**) E1+E3 – black, E1+E5 – white, Mock – grey.

3.4. Growth Inhibitory Activity of Various Lipoplexes Containing E1 siRNA

E1 siRNA against Bcl-2 was the best Bcl-2 siRNA sequence. Therefore we checked the survival of MCF-7 cells transfected with E1 siRNA duplexes complexed with various cationic liposomes prepared by us. We found that lipoplexes containing E1 decrease the growth of MCF-7 cells in a time- and dose-dependent manner with a maximum inhibition at 72 h post transfection (Fig. **4**). When MCF-7 cells were transfected with 25, 50 or 100 nM E1 siRNA complexed with DOTAP/DOPE UHV, the growth of MCF-7 cells was 60, 31 or 11%, respectively, as compared to controls (Student *t* test, $p < 0.05$, Fig. **4**). In accord with the results in the previous section, growth inhibition was much smaller in MCF-7 cells treated with lipoplexes containing EJ2 siRNA than with E1 siRNA at identical concentrations. For instance, when MCF-7 cells were transfected with 100 nM EJ2 siRNA complexed with DOTAP/DOPE UHV, 81% of MCF-7 cells

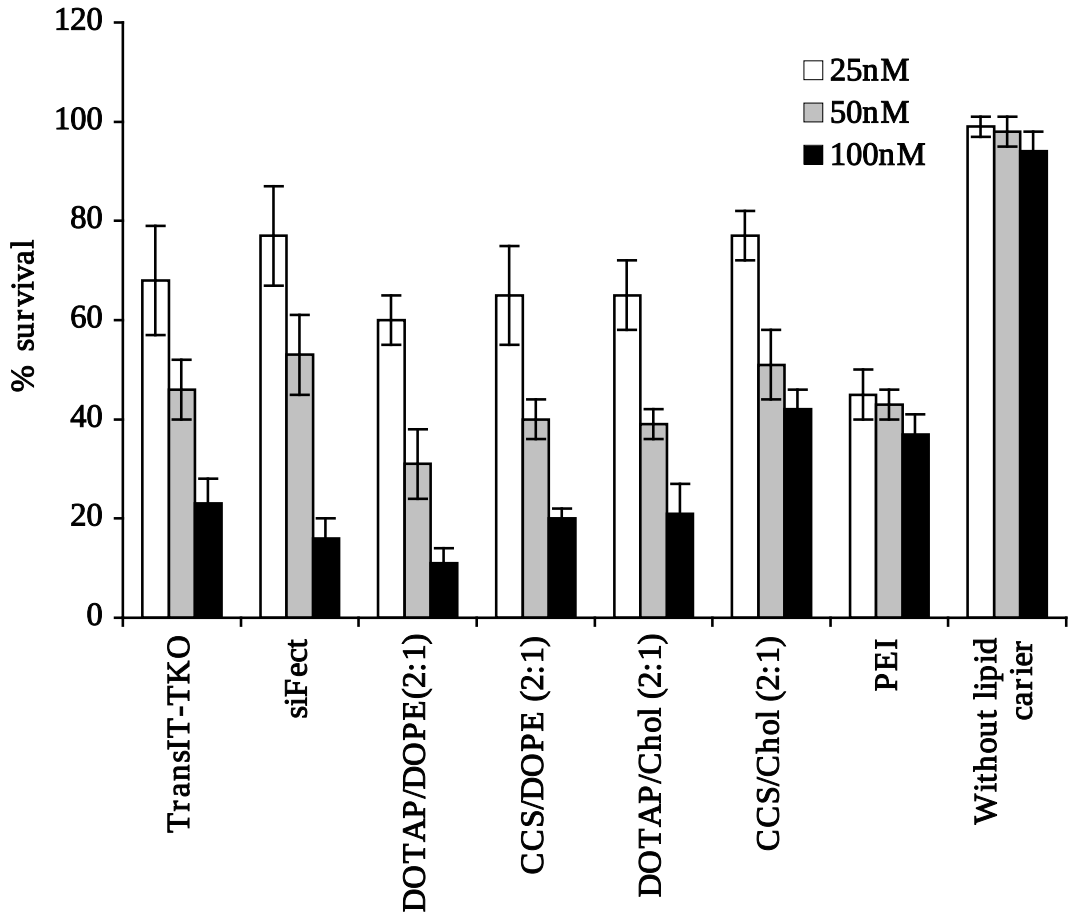


Fig. (4). Effect of lipoplexes having different lipid compositions on MCF-7 cell survival. For transfection experiments 4.0×10^3 cells were incubated with 25 nM (white columns), 50 nM (grey columns) and 100 nM (black columns) E1 siRNA complexed with 2, 4 and 8 mM lipids, respectively, and grown for 72 h. In the case of transfection with cationic polymer PEI, MCF-7 cells were treated with 25 nM, 50 nM and 100 nM E1 siRNA complexed with 0.25, 1 and 2 mM PEI, respectively. The commercially available carriers TransIT-TKO and siFect were compared to our lipid carriers. Cell survival was determined using methylene blue staining.

survived compared with only 11% when lipoplexes containing E1 siRNA were used. Regarding the other cationic lipoplexes, almost all showed comparable growth inhibition. 80-95% survival of cells treated with transfection reagent only (in all cases) was observed, indicating their low cytotoxicity (data not shown). In all cases, no growth inhibition was observed using naked duplexes (without lipid carrier). These studies confirm that siRNAs require an efficient carrier to achieve productive transfection.

We studied the effect of calcium(II) ions on siRNA-lipoplex-mediated transfection. We showed that Ca^{2+} ions also enhance the efficacy of siRNA lipoplexes, as addition of $CaCl_2$ to siRNA lipoplexes further improved their growth inhibitory effect (Fig. **5**).

3.5. Comparing Bcl-2 3139 Antisense Oligonucleotide Lipoplexes with Bcl-2 E1 siRNA Lipoplexes

Antisense Bcl-2 oligonucleotides such as G3139 were studied extensively [31]. Here we compared the ODN Bcl-2 lipoplexes previously optimized by us [43] with the Bcl-2 E1 siRNA lipoplexes used in this study. The growth inhibitory effect of E1 siRNA lipoplexes requires only 5% of the nucleic acid used to achieve similar inhibition by the Bcl-2 3139 ODN lipoplexes (Fig. **6**). Others found that lower concentrations of siRNA are needed to achieve levels of knockdown comparable to antisense reagents [44]. Moreover, in our previous work we have been able to knockdown Bcl-2 expression in MCF-7 cells with antisense DNA technology [40]. However, to achieve knockout of Bcl-2 with antisense, 50 μM of DNA was required, while in the case of the siRNA, only 400-1000 nM concentration was required in order to achieve similar effect.

3.6. Determination of Bcl-2 and Bcl-xL Expression in MCF-7 Cells Treated by the Appropriate siRNA

In order to confirm that growth inhibitory effect was related to the level of Bcl-2 or Bcl-xl proteins, we evaluated the expression of these proteins by ELISA assay and Western blot analysis.

Fig. (**7**) depicts the results of ELISA assay of Bcl-2 expression in MCF-7 cells that were transfected with cationic lipid lipoplexes containing E1 siRNA for 16 h. Suppression of Bcl-2 expression was observed only in cells transfected with lipoplexes containing siRNA, but not in cells transfected with cationic liposomes or with free siRNA. Addition of $CaCl_2$ (25 mM) to the lipoplexes improved the efficacy and reduced further the level of Bcl-2 by 35-40%.

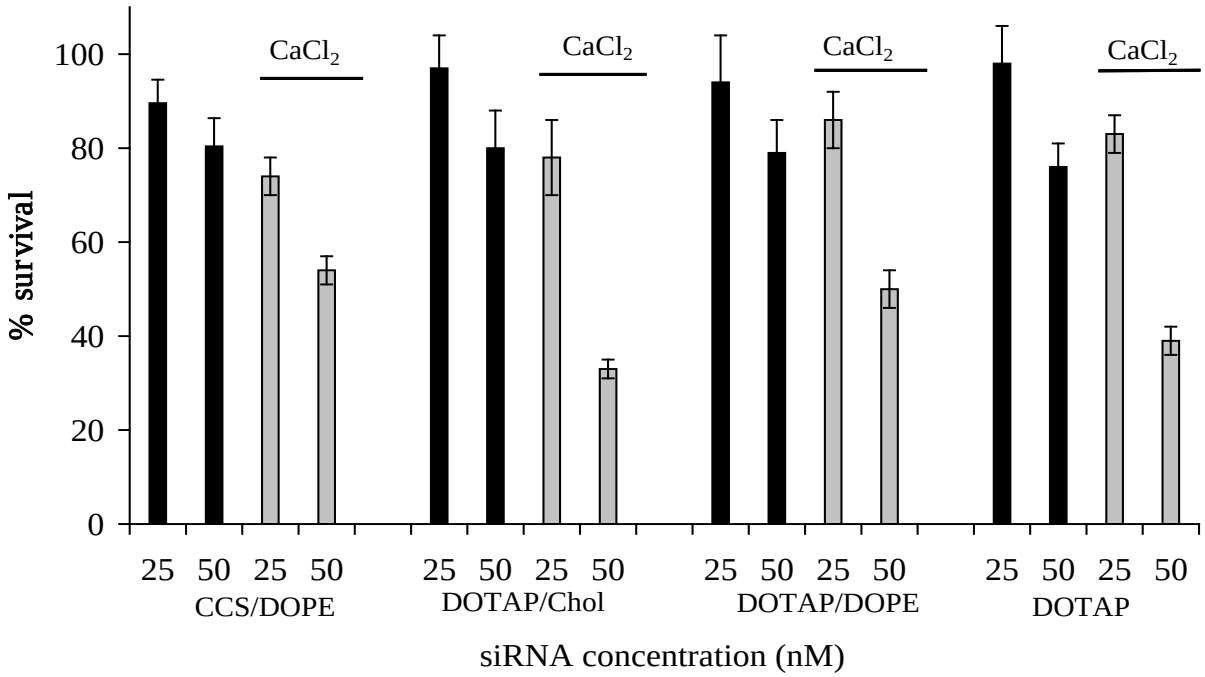


Fig. (5). Effect of $CaCl_2$ as transfection enhancer of MCF-7 cell growth. Inhibition level of MCF-7 cell growth was quantified by methylene blue staining assay. Briefly, MCF-7 cells were plated in 96-well plates (4.0×10^3 cells per well) and allowed to grow for 24 h. Then cells were incubated at 37ºC for 24 h with either 25 or 50 nM E1 siRNA complexed with either 2.0 or 4.0 μM lipids. 25 mM $CaCl_2$ was used. Error bars represent SD of two independent experiments.

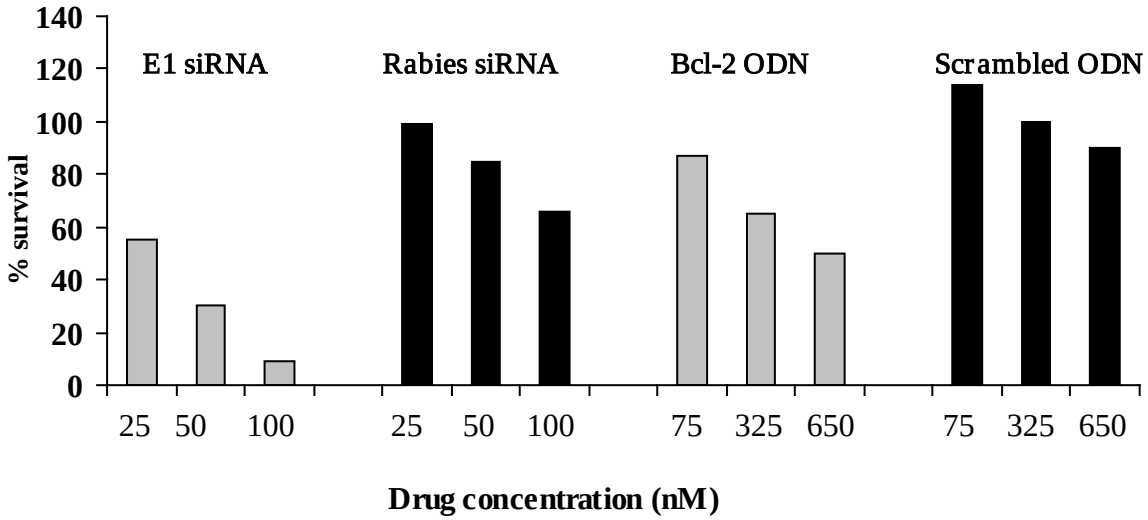


Fig. (6). Survival of MCF-7 cells treated with Bcl-2 E1siRNA or G 3139 Bcl-2 antisense ODN. Survival of MCF-7 cells treated with CCS/DOPE (2:1) lipoplexes containing Bcl-2 E1siRNA or G 3139 Bcl-2 antisense ODN for 72 h was evaluated by methylene blue staining assay. Controls included rabies siRNA and scrambled ODN.

In agreement with the ELISA assay, RT-PCR analysis (shown in Fig. **8C**), indicated that Bcl-2 mRNA was dramatically reduced in cells treated with 400-1000 nM E1 Bcl-2 siRNA. The effect was specific, as scrambled siRNA, like transfection agent, did not decrease Bcl-2 mRNA level. In addition, in cells treated with Bcl-xL E5 siRNA, a dramatic reduction of Bcl-xL mRNA was observed. As in the case with Bcl-2, the effect was specific. In addition, all three siRNA (Bcl-2, Bcl-xL and scrambled) did not decrease the level of the actin mRNA. Western blot analysis indicated that the protein level of Bcl-2 and Bcl-xL are significantly reduced as early as 24 h after siRNA lipoplex addition. The specificity of the siRNA effect can be demonstrated by the fact that Bcl-xL expression was not affected by siRNA directed towards Bcl-2 and vice versa. None of these siRNAs affected levels of Bax, p53 or actin expression (Fig. **8A**, **B**). The scrambled siRNA sequence did not cause any change in the tested protein levels as well. In summary, our results demonstrated that the growth inhibitory effect of Bcl-2 and Bcl-xL lipoplexes was caused by specific reduction in the level of these two proteins, each of them by its specific siRNA sequence.

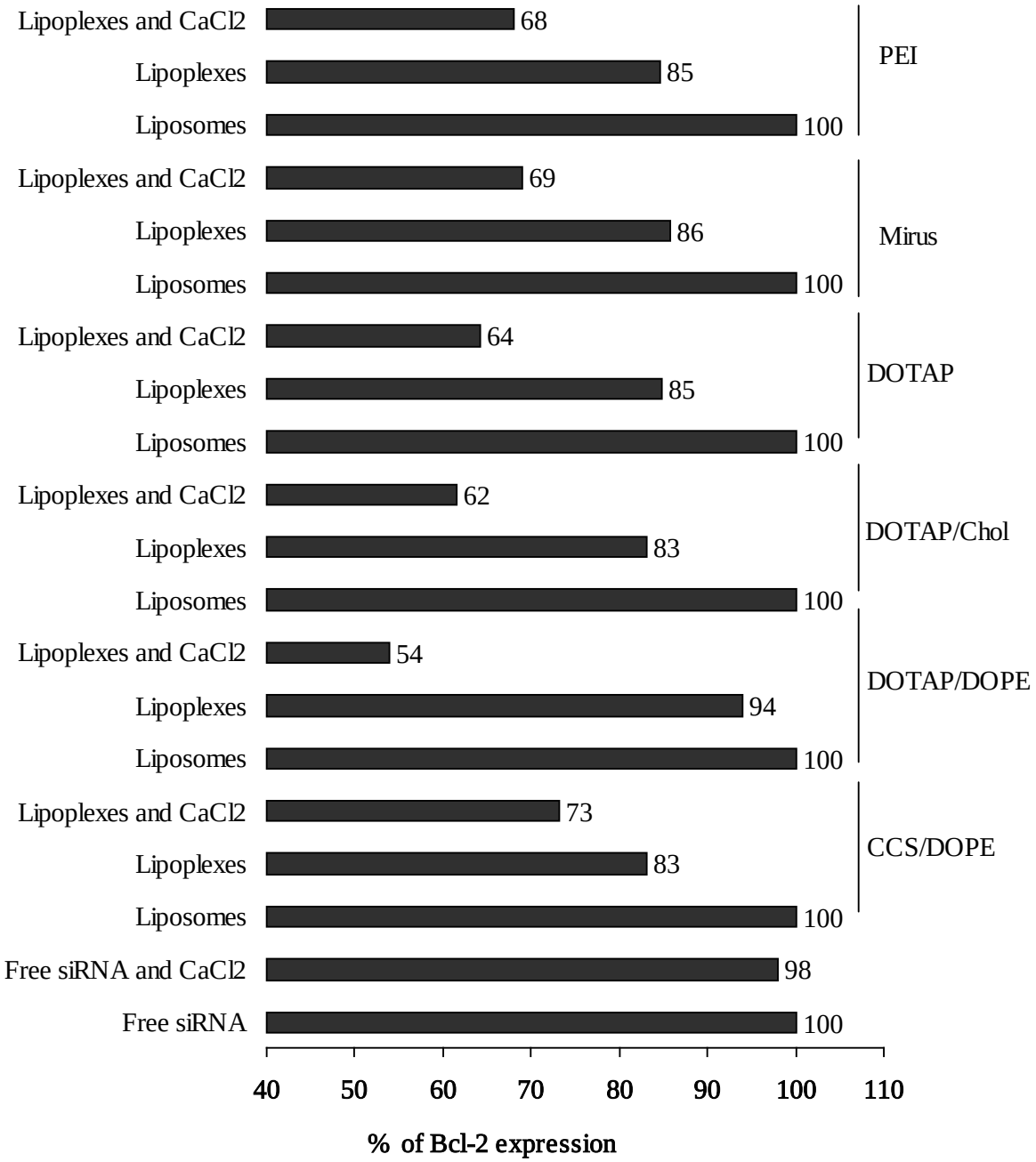


Fig. (7). Determination of the level of Bcl-2 protein expression in MCF-7 cells transfected with cationic lipid lipoplexes or liposomes. The level of Bcl-2 was determined in MCF-7 cells by Bcl-2 ELISA assay. Briefly, MCF-7 cells (10^6cells/well) were treated for 16 h with 400 nM of various lipid/E1 siRNA formulations. Cell lysates were prepared and the level of Bcl-2 was checked in treated (**lipoplexes)** as compared to mock-transfected (**liposomes**) cells or cells transfected with free siRNA.

3.7. Evaluation of Apoptosis

To confirm that the growth inhibitory effect of Bcl-2 and Bcl-xL lipoplexes was due to induction of apoptosis by siRNA duplexes, MCF-7 cells transfected with E1, E5 or E1+E5 siRNA were compared to mock-transfected cells. The transfected cells were assessed 48 h later for level of caspase-7 activity and level of DNA fragmentation (both well-established markers for apoptosis). Fig. (**9**) shows induction of apoptosis in MCF-7 cells at 48 h post transfection by these three siRNAs as measured by caspase-7 activity (Fig. **9A**) and DNA fragmentation (Fig. **9B**).

Our results demonstrated that transfection of MCF-7 cells with E1 siRNA caused a significantly higher level of apoptosis determined by these two assays than with E5 siRNA (Wilcoxon $p<0.05$), in agreement with our growth inhibition results. This effect is specific, as the scrambled siRNA sequence did not induce apoptosis in transfected MCF-7 cells. The combination of E1+E5 siRNA sequences does augment apoptosis compared to each sequence alone at the same concentration; however, the effect is less than additive. In summary, growth inhibition correlates well with induction of apoptosis.

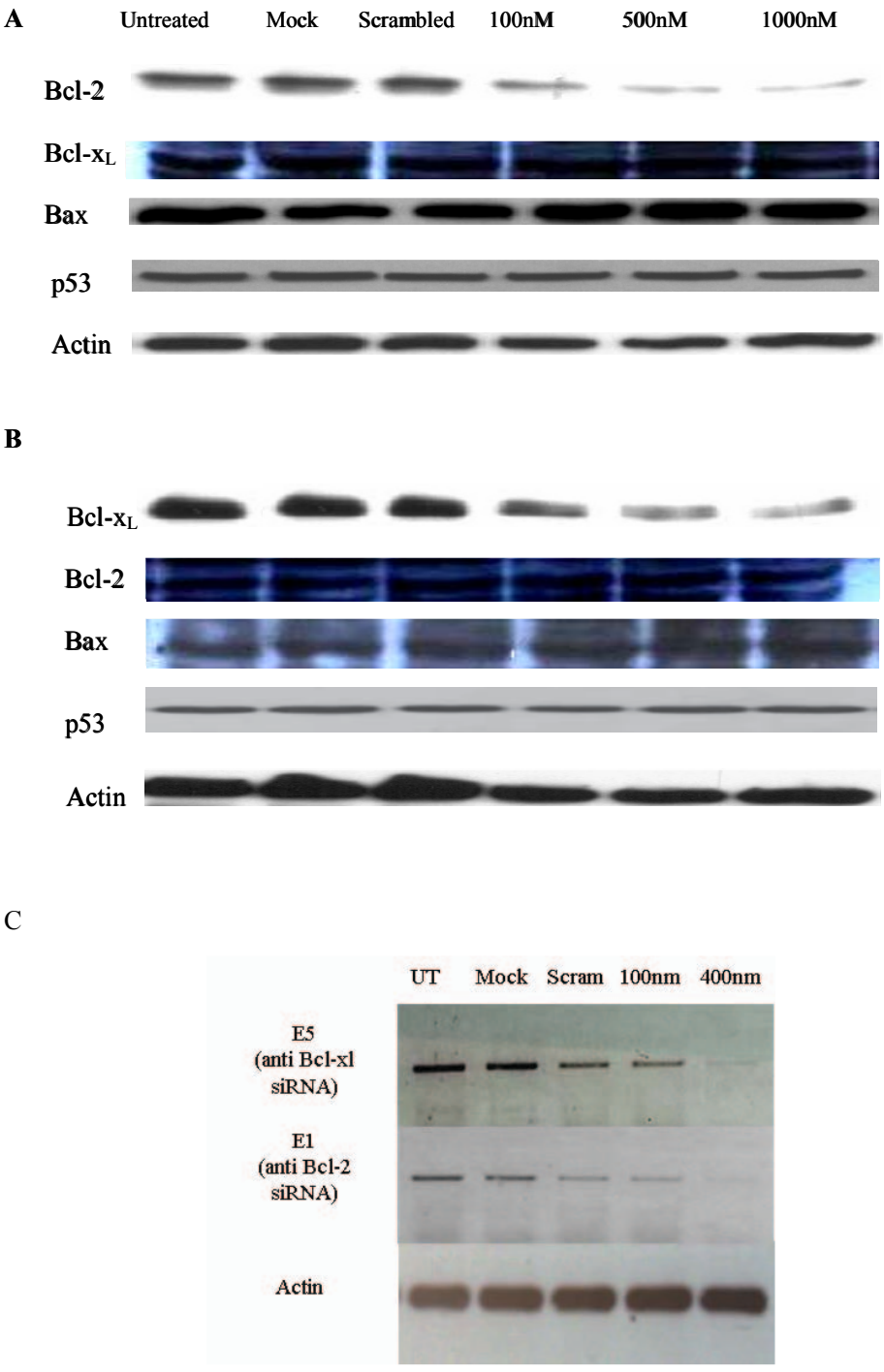


Fig. (8). Expression of Bcl-2, Bcl-xL, Bax, p53 and actin proteins in transfected MCF-7 cells by Western blot (A, B) or by RT-PCR (C). MCF-7 cells (semiconfluent monolayer) were transfected with the indicated concentrations of E1 Bcl-2 (**A**) or E5 Bcl-xL siRNA (**B**) duplexes for 24 h. Western blot analysis was carried out as described in Materials and Methods. **(C)** Cells were transfected with 100 or 400 nM of either E1 siRNA (anti Bcl-2) or E5 (anti Bcl-xL), or with 400 nM of scrambled siRNA (scram). Controls included cells treated with transfection agent only (mock) or untreated cells (UT). 24 hours after transfection cells were harvested and total RNA was extracted. RNA was subjected to RT-PCR with specific primers to ether Bcl-2, Bcl-xl or actin as indicated. The PCR products were run on 1% agarose gel.

4. DISCUSSION

4.1. Physicochemical Characterization

Transfection of cells by nucleic acids using lipoplexes as the delivery system requires complexation of the nucleic acids with cationic liposomes, followed by lipoplex uptake by the target cells. We therefore studied these two steps.

Our results of EtBr intercalation assay and of zeta potential measurement show good correlation, which indicates that lipoplexes composed of mono- or polycationic lipids with

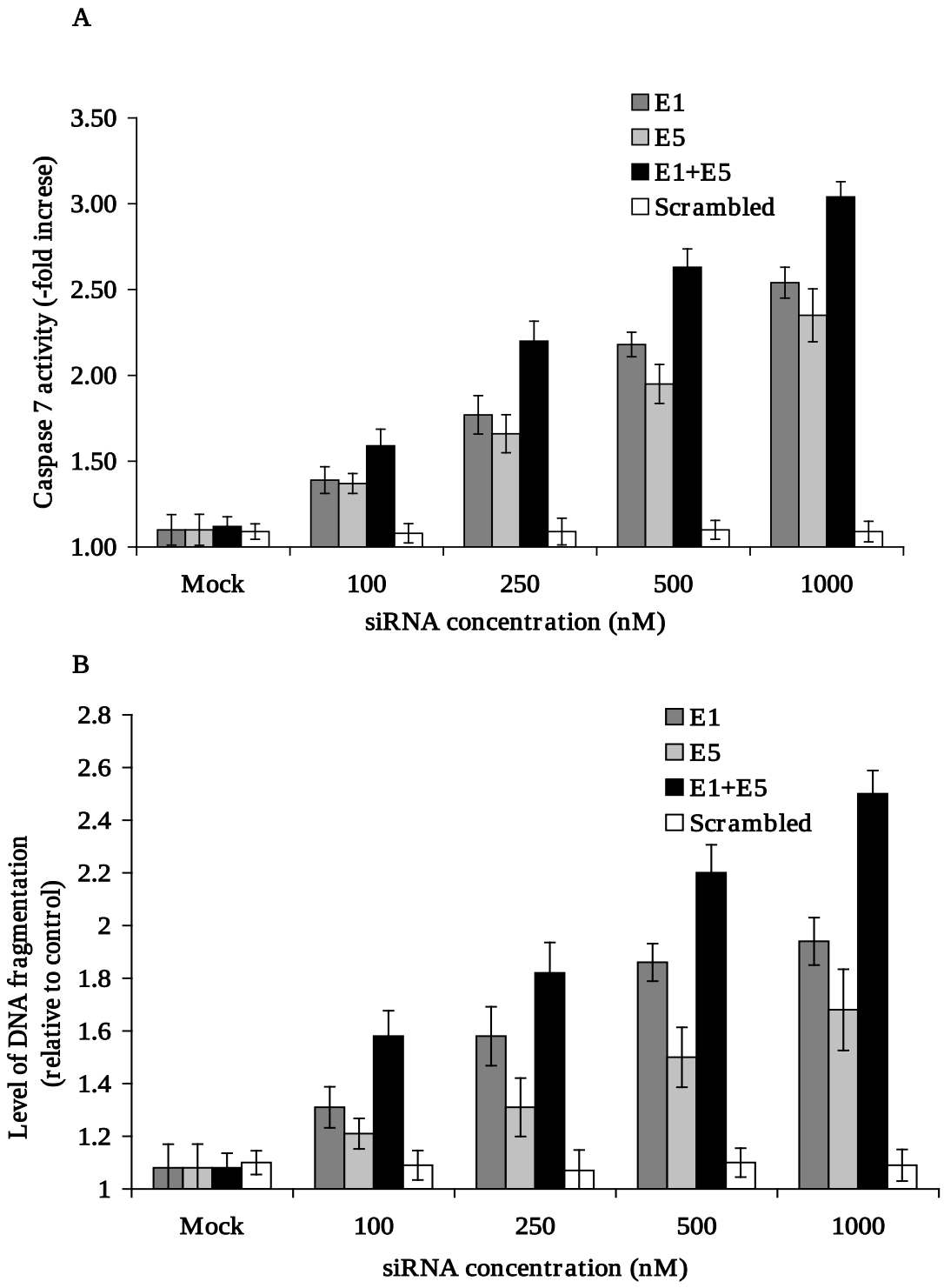


Fig. (9). Quantitation of apoptosis in MCF-7 cells transfected with various Bcl-2 siRNAs. MCF-7 cells were transfected by the indicated concentrations of siRNA duplexes. **A** – Caspase-7 activity was measured in MCF-7 cells at 48 h after transfection by Apo-ONE^{tm} Homogenous Caspase-3/7 assay. **B** – 48 h after transfection cells were lysed and assayed for DNA fragmentation. E1 siRNA: dark grey, E5 siRNA: light grey, E1+E5 siRNAs: black, Scrambled siRNA: white.

Bcl-2 siRNA are formed. Efficiency of siRNA binding to cationic lipid UHV is high (80-100%).

Size distribution measurements by DLS showed that addition of siRNA to either the monocationic DOTAP-based UHV or the polycationic CCS-based UHV resulted in their size increase (2-fold for the DOTAP systems and 10-fold for the CCS systems). These results are consistent with our previous results on cationic DOTAP/Chol-based liposomes complexed with ODN, showing that combining of ODN with cationic liposomes induces liposome aggregation and lamellar condensation [45]. That study showed that cationic

membranes of the liposomes are adsorbed to each other by the ODN to form paired membranes. Close examination of cryogenic temperature transmission electron microscopy (cryo-TEM) images of paired membranes reveals that the plane of contact between bilayers has higher contrast than their outer edges, suggesting electron-dense ODN molecules sandwiched between the membranes (data not shown). This phenomenon can also explain liposome aggregation and size increase, as ODN molecules could "glue" adjacent liposomes together [45]. The much larger size increase in the CCS (compared to the DOTAP) system is related to the fact that CCS is polycationic while DOTAP is monocationic (see Scheme **1** with structural formulas).

4.2. Transfection Efficiency

Regarding cellular uptake of the lipoplexes, there were no significant differences between lipoplexes of various compositions, as 60-70% of MCF-7 cells took up either E1 or scrambled siRNA. Transfection efficiency of cells that express high levels of apoptosis-inhibiting Bcl-2 protein was quantified by determining if, and to what extent, the specific siRNAs reduce the level of Bcl-2 and induce cell death. Overall, our results demonstrate that the effect of siRNA is specific, and that scrambled and mock siRNAs lack this effect. In addition, only siRNAs delivered *via* carrier lipoplexes or polyplexes were efficacious in promoting cell death and reducing Bcl-2 protein level. From the therapeutic point of view, it is very important that only ~5% E1 siRNA lipoplex is needed to exert the same cytotoxicity as Bcl-2 antisense oligonucleotide lipoplexes, which indicates higher efficacy and lower toxicity of siRNA lipoplexes. Comparing lipoplexes based on our own cationic lipid/siRNA (DOTAP- or CCS-based) to commercial kits for siRNA delivery, we found that cytotoxic activity of our lipoplexes was comparable, and in some cases superior. Comparing the influence of size of DOTAP- and CCS-based lipoplexes, no significant differences were found in growth inhibition of MCF-7 cells. However, comparing our cationic lipid-based formulations, we found that DOTAP-based liposomes were less toxic than CCS-based liposomes and, more important, less toxic than commercial transfectants. Our results correlate with results of several other research groups. Sioud and Sorensen showed that cationic lipid complexes with siRNA can deliver siRNAs into various cell types [46].

The process of the lipoplex formation plays an important role in their transfection efficiency [22]. Particle size and surface charge of the lipoplexes are some of the important parameters affecting transfection efficiency [22]. We used $CaCl_2$ as a transfection enhancer and found that, indeed, calcium(II) ions improve transfection efficiency as shown by the additional reduction of Bcl-2 protein level. According to our previous results with pDNA lipoplexes, addition of $CaCl_2$ did not change particle size and zeta potential of the liposomes and lipoplexes (unpublished data). We proposed, in agreement with previous publications [24, 47] that calcium ions enhance DNA transfection by condensing DNA through neutralization of the phosphate groups of the DNA backbone.

The present study showed that siRNA directed towards Bcl-2 and Bcl-xL inhibited MCF-7 cell growth and induced apoptosis. Suppression of Bcl-2 expression was more effective in growth inhibition, relative to suppression of Bcl-xL expression, despite achieving similar suppression of the respective gene product. This is consistent with the results of Leung and Wang, who found that, generally, estrogen-dependent breast cancer cells have higher Bcl-2 levels and lower Bcl-xL levels [48]. Therefore, cells expressing ER are appropriate targets, especially for Bcl-2 gene silencing.

The efficiency of the various lipoplexes in suppression of the relevant gene product expression was variable: E1 and E5 were the most effective siRNAs towards Bcl-2 and Bcl-xL, respectively. The heterogeneity among different siRNA duplexes is well documented using multiple mRNA targets [49, 50]. It is the result of several confounding factors, including the secondary conformation of the target mRNA and associated proteins, which may interfere with the access of the RISC complex to its target. The experiments in which two siRNAs were used simultaneously proved that this approach is feasible but not always advantageous. Therefore, the efficacy should be examined individually for each pair of siRNAs. We observed enhancement of gene silencing when Bcl-2 and Bcl-xL siRNAs were added together. This indicates that with the relatively low (less than 500 nM) concentrations employed, we have not yet reached saturation, and different siRNA duplexes were not competing for loading into RNA-induced silencing complex (RISC). The antagonistic effect observed in the combination of two Bcl-2 siRNAs may be the result of a spatial configuration that prevents the RISCs of E1 and E3 siRNAs to access the target area together. If we consider that the two target sequences are only 248 base pairs apart, we can understand how such a disturbance can occur.

The effect of siRNA, which culminated in the reduction of Bcl-2 and Bcl-xL, was specific to each protein, and there were no off-target effects. The unchanged levels of actin, p53 and Bax proteins are additional evidence for specificity. We found that reduction in the levels of Bcl-2 and Bcl-xL correlates with induction of apoptosis. The induction of apoptosis shows the promise of this technique, but it should be kept in mind that *in vitro* conditions differ greatly from *in vivo*.

CONCLUSION

Our studies show that siRNA delivered to tumor cells in the form of siRNA lipoplexes of various lipid compositions and in the form of PEI polyplexes resulted in specific growth inhibition of cancer cells *in vitro*. This was associated with reduction in the level of Bcl-2 and Bcl-xL proteins and with induction of apoptosis. We found that E1 siRNA lipoplexes were at least 20-fold more efficacious in growth inhibition than Bcl-2 antisense oligonucleotide. Moreover, selection of better (more efficacious and more stable) siRNA sequences than those used by us in this study, has the potential to improve further the silencing efficiency of siRNA. According to our results, Bcl-2 is a valid target for gene silencing in MCF-7 breast cancer cells. Based on experience with *in vivo* delivery of nucleic acids [22, 48], this study opens the road for efficient delivery of siRNA *in vivo* for treatment of various malignancies. Further investigation requires experiments to be done using optimized siRNA Bcl-2 and Bcl-xL sequences with additional cell lines and subsequently in animal models. Bcl-2 and Bcl-xL siRNA should also be combined

with chemotherapeutic drugs (especially when loaded into liposomes) and apoptosis inducers such as ceramides [51] to explore the sensitization effects of gene silencing.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BH	=	Bcl-2 homology
Chol	=	Cholesterol
CCS	=	D-*erythro*-*N*-palmitoyl-sphingosyl-carbamoyl spermine
cryo-TEM	=	Cryogenic temperature transmission electron microscopy
DAPI	=	4,6-Diamino-2-phenylindole
DLS	=	Dynamic light-scattering
DMEM	=	Dulbecco's modified Eagle medium
DOPE	=	Dioleoylphosphatidyl ethanolamine
DOTAP	=	1,2-Dioleyloxy-3-(trimethylamino)-propane
dsRNA	=	Double stranded RNA
EtBr	=	Ethidium bromide
ER	=	Estrogen receptor
FAM	=	5'-Carboxy-fluorescein
GSRT	=	Gene silencing RNAi technology
Hepes	=	N-(2-Hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
LUV	=	Large unilamellar vesicles
MB	=	Methylene blue
ODN	=	Oligonucleotides
PBS	=	Phosphate buffered saline
PEI	=	Linear polyethyleneimine
RISC	=	RNA-induced silencing complex
RT-PCR	=	Reverse transcription-polymerase chain reaction
siRNA	=	Short interfering RNA
UHV	=	Unsized heterolamellar vesicles
TNBS	=	Trinitrobenzene sulfonic acid

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