

# Enhancement of the Efficacy of Chemotherapy for Lung Cancer by Simultaneous Suppression of Multidrug Resistance and Antiapoptotic Cellular Defense: Novel Multicomponent Delivery System

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## ABSTRACT

The efficacy of chemotherapy of lung cancer is limited by the development of resistance in cancer cells during treatment. In most lung cancers, this resistance is associated with the overexpression of (a) multidrug resistance-associated protein (MRP) responsible for drug efflux from the cancer cells (pump resistance) and (b) BCL2 protein that activates antiapoptotic cellular defense (nonpump resistance). A novel liposomal proapoptotic anticancer drug delivery system was developed to enhance anticancer efficacy of the well-established drug doxorubicin (DOX). This multicomponent drug delivery system was tested on multidrug-sensitive and -resistant human small-cell lung cancer cells. The drug delivery system includes four components: (a) liposome as a carrier, (b) DOX as an inductor of apoptosis, (c) antisense oligonucleotides (ASOs) targeted to MRP1 mRNA as a suppressor of pump resistance, and (d) ASOs targeted to BCL2 mRNA as a suppressor of nonpump resistance. Intracellular internalization of ASOs and DOX; the influence of the proposed system on the expression of genes and proteins involved in the multidrug resistance, cytotoxicity, and apoptosis induction and antiapoptotic defense; and the activity of caspases were studied. It was found that the proposed liposomal delivery system successfully delivered ASOs and DOX to cell nuclei, inhibited MRP1 and BCL2 protein synthesis, and substantially increased the anticancer action of DOX by stimulating the caspase-dependent pathway of apoptosis in multidrug-resistant human lung cancer cells.

## INTRODUCTION

Lung cancer is the leading cancer killer in both men and women. There were an estimated 171,900 new cases of lung cancer and an estimated 157,200 deaths from lung cancer in the United States in 2003. There are two types of lung cancer: small-cell lung cancer and non-small-cell lung cancer. Non-small-cell lung cancer is a heterogeneous aggregate of at least three distinct histologies of lung cancer including epidermoid or squamous carcinoma, adenocarcinoma, and large-cell carcinoma. These histologies are often classified together because, when localized, all have the potential for cure with surgical resection. Without treatment, small-cell carcinoma of the lung has the most aggressive clinical course of any type of pulmonary tumor, with median survival time of only 2 to 4 months after diagnosis. Compared with other cell types of lung cancer, small-cell carcinoma has a greater tendency to be widely disseminated by the time of diagnosis and therefore cannot be surgically removed because of its size and distribution. This requires the use of a high dose of chemotherapy, sometimes in combination with radiation treatment.

The success of chemotherapeutic treatment of small-cell lung cancer is limited by the intrinsic and acquired resistance of cancer cells to

chemotherapy. The main mechanisms of this resistance are common to most cancers and include so-called “pump” and “nonpump” resistance (1, 2). The pump resistance is caused by membrane proteins that pump out the anticancer agents from cells or cell organelles, decreasing the intracellular drug concentration and efficacy. The main mechanism of nonpump resistance is the activation of cellular antiapoptotic defense.

P-glycoprotein and multidrug resistance-associated protein (MRP) are two main ATP-binding cassette transporter proteins that are responsible for the pump resistance in different cancer cells (3, 4). Overexpression of ATP-binding cassette transporters is the main cause of multidrug resistance. The term “multidrug resistance” is used to describe the resistance against a broad spectrum of anticancer drugs after the treatment with a single agent. It was found that most drug-resistant lung cancers overexpress both P-glycoprotein and MRPs (5–7). Therefore, the suppression of the expression of these proteins should decrease resistance, leading to increased intracellular drug concentration and thereby augmenting the efficacy of treatment. This strategy will also allow the application of lower concentrations of the toxic anticancer agent, decreasing its adverse side effects on healthy tissues and organs.

It was recently proposed that antisense oligonucleotides (ASOs) targeted to mRNA encoding drug efflux pumps might be used to overcome or suppress pump resistance (8–13). However, whereas the suppression of drug efflux pumps led to the increase in intracellular drug concentration, it did not result in a substantial improvement in the treatment. The main reason for such failure is an adaptive activation of “cell death” defense, so-called “nonpump resistance” (1, 2). It was found that the up-regulation of the cellular antiapoptotic defensive system plays the main role in the activation of this defense, and BCL2 protein is a key player in this system (14, 15). Unlike the drug efflux pump proteins, overexpression of BCL2 protein does not interfere with the entry and accumulation of drugs in tumor cells. Instead, BCL2 protein prevents the cytochrome *c* release from the mitochondrion required as a trigger for the caspase cascade in apoptosis execution. Therefore, a suppression of BCL2 protein function could substantially induce apoptosis and increase the efficacy of the toxic drug in the treatment of lung cancer.

An improved generation of antisense oligonucleotides targeted against BCL2 mRNA was described recently (16–19). In some cases, the use of these oligonucleotides led to a significant inhibition of the expression of BCL2. However, the down-regulation of BCL2 functions alone did not result in substantial apoptotic cell death. Usually for effective therapy, the combination of antisense oligonucleotides with anticancer drug as the apoptosis-inducing agent is required. However, this strategy again raises the problems related to drug efflux pumps and multidrug resistance described above. In fact, we found that the liposomal doxorubicin (DOX)-BCL2 antisense oligonucleotides combination effectively induced apoptosis in sensitive cancer cells, whereas it was almost ineffective in multidrug-resistant cells overexpressing P-glycoprotein (1).

These facts led us to formulate the hypothesis that only simultaneous suppression of multidrug resistance and antiapoptotic cellular

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defense can substantially increase the efficacy of traditional anticancer drug. To test the hypothesis, we recently developed and evaluated in ovarian and breast cancer cells a complex liposomal drug delivery system that included three main components: (a) antisense oligonucleotides targeted against MDR1 mRNA (to inhibit pump resistance); (b) antisense oligonucleotides targeted to BCL2 mRNA (to inhibit nonpump resistance); and (c) DOX, a traditional anticancer drug (to initiate apoptosis) (1). We found that simultaneous modulation of multidrug resistance and antiapoptotic cellular defense by MDR1- and BCL2-targeted antisense oligonucleotides substantially enhanced the anticancer activity of DOX. The data suggest that antisense oligonucleotides targeted to the MDR1 and BCL2 mRNA in combination with an anticancer drug may potentially be used in the treatment of ovarian and breast cancer. However, this system cannot be efficiently applied for the treatment of lung cancer because, in most cases, the main cause of pump resistance in lung cancer cells is the overexpression of both P-glycoprotein and MRPs (5–7). A different strategy was needed for lung cancer.

We proposed to use antisense oligonucleotides targeted to MRP1 mRNA as an inhibitor of pump resistance combined in one liposomal drug delivery system with an inhibitor of antiapoptotic defense (antisense oligonucleotides targeted to BCL2 mRNA) and apoptosis inducer (a traditional anticancer drug, DOX). The proposed drug delivery system utilizes a novel two-tier approach, simultaneously targeting two molecular targets: (a) drug efflux pumps to enhance drug retention by lung cancer cells and increase drug concentration inside the cells, decreasing the need for high drug dose and thus limiting adverse drug side effects; and (b) intracellular controlling mechanisms of apoptosis to suppress cellular antiapoptotic defense. In this work, we present the results of the experiments that were designed to verify the hypothesis and test the efficacy of this novel anticancer drug delivery system in multidrug-resistant lung cancer cells that overexpress MRP. We hypothesize that such a multicomponent drug delivery system, which combines an anticancer drug with suppressors of pump (MDR1- and/or MRP-targeted ASOs) and nonpump resistance, could be effectively used in chemotherapy of small-cell lung cancer.

## MATERIALS AND METHODS

### Cell Culture

The human drug-sensitive H69 and multidrug-resistant H69AR small-cell lung cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (HyClone, Logan, UT). All experiments were performed on cells in the exponential growth phase. The strain of small-cell lung cancer cells used overexpresses MRP, does not express P-glycoprotein, and is therefore likely to have the greatest effect from the suppression of MRP mRNA by the proposed complex drug delivery system.

### Liposomal Delivery of Antisense Oligonucleotides

Fourteen series of experiments were carried out according to the schema presented in Fig. 1. Multidrug-resistant H69AR small-cell lung cancer cells were separately incubated with saline (control), free DOX, empty liposomes, liposomes containing DOX, BCL2 sense or antisense oligonucleotides, BCL2 sense or antisense oligonucleotides and DOX, MRP1 sense or antisense oligonucleotides, MRP1 sense or antisense oligonucleotides and DOX, BCL2 antisense oligonucleotides and MRP1 antisense oligonucleotides, and BCL2 antisense oligonucleotides and MRP1 antisense oligonucleotides in combination with DOX.

The sequence of the sense oligonucleotides targeted to MRP1 and BCL2 mRNA was 5'-CGGCGGGGGCACGAACAGCA-3' and 5'-GGGAAGGATGGCGCACGCTG-3', respectively. The sequence of the antisense oligonucleotides targeted to MRP1 and BCL2 mRNA was 5'-TGCTGTTCGTGC-

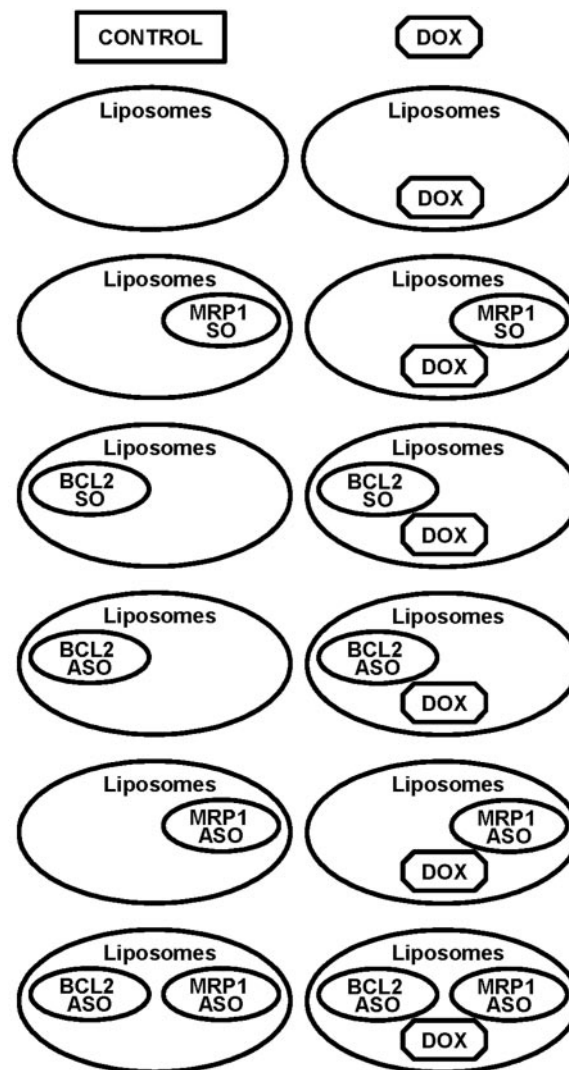


Fig. 1. Experimental schema diagrammatically showing the different series of experiments. SO, sense oligonucleotides; ASO, antisense oligonucleotides.

CCCCGCCG-3' and 5'-CAGCGTGCGCCATCCTTCCC-3', respectively (12, 16). The DNA backbone of all bases in oligonucleotides was P-ethoxy modified to enhance nuclease resistance and increase incorporation efficacy into liposomes. ASOs were synthesized by Oligos Etc. (Wilsonville, OR). ASOs and DOX (Sigma) were packaged in liposomes, which were prepared using a previously described lipid film rehydration method (1, 20). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, evaporated to a thin film in a rotary evaporator, and rehydrated with citrate buffer. The lipid ratio for all formulations was 7:3:10 (egg phosphatidylcholine:1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine:cholesterol). ASOs were loaded into the liposomes by dissolving the ASOs in the rehydration buffer at a concentration of 0.5 mmol/L when MRP1 and BCL2 ASOs were used separately and 0.25 mmol/L of each type of ASO when MRP1 and BCL2 were used in combination. The mean liposome diameter was about 100 nm. Empty liposomes and liposomes containing oligonucleotides with sequences similar to the sense strand of corresponding mRNA were used as negative controls.

### Release of Antisense Oligonucleotides and Doxorubicin from Liposomes and Their Intracellular Localization

To analyze release of ASOs from liposomes and their intracellular localization, a portion of the oligonucleotides were labeled with fluorescein isothiocyanate (FITC) before incorporation into the liposomes. These labeled ASOs were used only in ASO release and localization experiments. In other experimental series, ASOs were not labeled. The fluorescent substances were

visualized by fluorescence microscopy (Zeiss Axiostar Plus fluorescence microscope) using the following filters: excitation wavelength of 470 nm and emission wavelength of 525 nm, FITC; excitation wavelength of 546 nm and emission wavelength of 590 nm, DOX; and excitation wavelength of 360 nm and emission wavelength of 420 nm, Hoechst 33258. The fluorescence of labeled ASOs and DOX inside liposomes in aqueous solutions and inside cells is quenched by the outer membranes of the liposomes (21). Therefore, the intensity of FITC and DOX fluorescence is proportional to the concentration of free ASOs or DOX released from the liposomes. The ASO and DOX release from liposomes in saline was monitored for 2 weeks. Fluorescence in aliquots of incubation solution was measured every hour during the first day and once per day for the next 13 days by a fluorescent microtiter plate reader. Intracellular localization and release of ASOs and DOX were studied by fluorescence microscopy. In these experiments, cell nuclei were additionally stained by Hoechst 33258 nuclear dye (Sigma). To quantitatively estimate release of ASOs and DOX, images from the fluorescence microscope were digitally photographed after incubation of cells with liposomal drug delivery system for 2, 5, 15, and 30 minutes and 1, 2, 3, 24, and 48 hours. Visible light and fluorescent images were simultaneously recorded for each field. The total fluorescence intensity of each field was then calculated and normalized to the total absorbance of visible light images. This allowed us to take into account the number of cells in the field. These normalized intensities were then plotted against time to characterize the release of ASOs and DOX inside the cells.

**Reverse Transcription-Polymerase Chain Reaction**

**Conventional Reverse Transcription-Polymerase Chain Reaction.** Reverse transcription-polymerase chain reaction (RT-PCR) was used for the analysis of gene expression as described previously (1, 20, 22–26). Total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) with 2 µg of total cellular RNA (from 1 × 10<sup>7</sup> cells) and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences). After synthesis, the reaction mixture was immediately subjected to polymerase chain reaction (PCR), which was carried out using GenAmp PCR System 2400 (Perkin-Elmer, Shelton, CT). The pairs of primers used to amplify each type of cDNA are shown in Table 1. *β<sub>2</sub>-Microglobulin* was used as an internal standard. PCR regimen was as follows: 94°C for 4 minutes; 55°C for 1 minute and 72°C for 1 minute for 1 cycle; 94°C for 1 minute, 55°C for 50 seconds, and 72°C for 1 minute for 28 cycles; and 60°C for 10 minutes. The mentioned PCR regimen was selected after linearity between the amount of PCR product and the amount of RNA used for RT-PCR was established in a series of preliminary experiments (26). PCR products were separated in 4% NuSieve 3:1 Reliant-agarose gels (BMA, Rockland, ME) in 1 × Tris-borate EDTA buffer [0.089 mol/L Tris-borate, 0.002 mol/L EDTA (pH 8.3); Research Organics Inc., Cleveland, OH] by submarine electrophoresis. The gels were stained with ethidium bromide and digitally photographed.

**Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction.** For reverse transcription, first-strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase. All reagents for reverse transcription were purchased from Invitrogen (Carlsbad, CA), and the reaction was performed according to the manufacturer’s protocol. Briefly, 1 µg of total RNA in 5 µl was added to 12 µl of Master Mix 1 containing 2 µl of random hexamer (111 ng/µl), 1 µl of 10 nmol/L deoxynucleotide triphosphate mix, and 4 µl of water per reaction. The reaction mixture was heated at 65°C for 5 minutes to denature RNA and chilled on ice. Then 8 µl of Master Mix

2 containing 4 µl of 5 × first-strand buffer, 2 µl of 0.1 mol/L dithiothreitol, 1 µl of water, and 1 µl (200 units) of Moloney murine leukemia virus reverse transcriptase were added, and the mixture was incubated at 25°C for 10 minutes to allow random hexamers to anneal. Then samples were incubated at 37°C for 50 minutes to allow reverse transcriptase to generate cDNA. Finally, the enzyme was inactivated at 70°C for 15 minutes.

Reverse transcription reaction mixture was diluted 1:1 with water, and 4 µl of the diluted mixture were placed in a 0.1-ml PCR tube (Pyrosequencing Inc., Westborough, MA) and mixed with 20 µl of Master Mix 3. The latter contained 11 µl of water, 2.5 µl of 10 × PCR buffer (Corbett Research, Mortlake, New South Wales, Australia), 2.5 µl of 2 mmol/L deoxynucleotide triphosphates, 2 µl of primer mix (5 µmol/L each), 0.75 µl of MgCl<sub>2</sub>, 1.25 µl of SYBR Green I (1:1,000 dilution from the stock obtained from Molecular Probes, Inc., Eugene, OR), and 0.125 µl of platinum Taq DNA polymerase (Invitrogen). The pairs of primers used to amplify each type of cDNA in real-time PCR are shown in Table 1. Fluorescent real-time PCR was performed in Rotor Gene 2000 Real-Time Cycler (Corbett Research). The real-time PCR regimen was as follows: 94°C for 180 seconds; and 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 s for 40 cycles. Direct detection of PCR product was monitored in each cycle by measuring the increase in fluorescence (excitation wavelength of 470 nm, emission wavelength of 585 nm) caused by the binding of SYBR Green dye to double-stranded DNA. The cycling was followed by dissociation curve analysis performed at each experiment to verify the specificity of the target amplification by incubation of reaction mixture as temperature changed from 55°C to 99°C for 5 seconds at each degree and acquiring fluorescence of the dye at each point.

The threshold cycle was determined for each gene by the real-time PCR computer program (Rotorgene Version 4.4; Corbett Research). The threshold cycle values were converted to copy number using a standard curve obtained with gel-purified PCR product as a template. A housekeeping gene, *β-actin*, was used as an internal standard to normalize the quantification of the target and minimize variability in the initial concentration, quality of total RNA, and conversion efficiency of the reverse transcription reaction. The expression of each gene mRNA is presented as the ratio between copy numbers of the analyzed gene and those of the internal standard.

**Protein Expression**

To confirm RT-PCR data, the expression of MRP1 and BCL2 proteins and caspase 9 was measured. The identification of the above-mentioned proteins was made by Western immunoblotting analysis and processed using scanning densitometry to quantify the expressed protein. To this end, harvested cells were lysed in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Santa Cruz, CA) using a needle and syringe. After incubation on ice for 45 minutes, the cells were centrifuged at 10,000 × g for 10 minutes. Protein content in the supernatant was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL), and 50 µg of protein were run on a 15% SDS-polyacrylamide gel immersed in Tris/glycine/SDS buffer (Bio-Rad, Hercules, CA) for 90 minutes at 70 V. Proteins were transferred to an Immobilon-P nitrocellulose membrane (Millipore, Bedford, MA) in a Tris/glycine buffer (Bio-Rad) for 90 minutes at 100 V. The membrane was blocked in nonfat milk for 30 minutes at room temperature on a rotating shaker to prevent nonspecific binding, washed, and incubated overnight with anti-MRP1 mouse primary antibody (1:250 dilution; Chemicon International, Temecula, CA), anti-BCL2 rabbit primary antibody (1:250 dilution; Stress Gene Biotechnologies, Victoria State, British Columbia, Canada), anti-caspase 9 rabbit primary

Table 1 List of primers used in conventional RT-PCR and real-time RT-PCR

Genes	Primers (5' to 3')	
	Conventional RT-PCR	Real-time RT-PCR
<i>MRP1</i>	atgtcacgctggaataaccagcgaagactgaaactccttctct	atattcctggccccagtggtgaaactccttctctctctctc
<i>BCL2</i>	ggattgtgcccctctcttgagccaaactgagcagagctcttc	tttgagttcgggtggggtcatgccgggttcaggtaactcagtc
<i>APAF1</i>	gggtttcagttgggaaacaaccccagagctcccaaacat	accacagagggccagttggtttatgggtgacccatcctgggt
<i>SMAC</i>	ggagccagagctgagatgaccagcttgggttctgcttcc	tggatgactgcagttgggtctccagctgaatgtgattcctg
<i>Caspase 3</i>	tggaaatgatgctgctgatgttggcaggcctgaataatgaaa	tttttcagaggggatcggttctgtctcaatgccacagctcc
<i>Caspase 9</i>	tgactgccaaagaaaatggtgcagctgggtcccatgaaat	gttgagacccctggagacataaaatccttccaccgaaaca
<i>β<sub>2</sub>-microglobulin</i> (internal standard)	acccccactgaaaaagatgaatcttcaaacctccatgatg	N/A
<i>β-actin</i> (internal standard)	N/A	gcatacctcaccctgaagatgtggtgcccagatattgtctc

Abbreviation: N/A, not applicable.

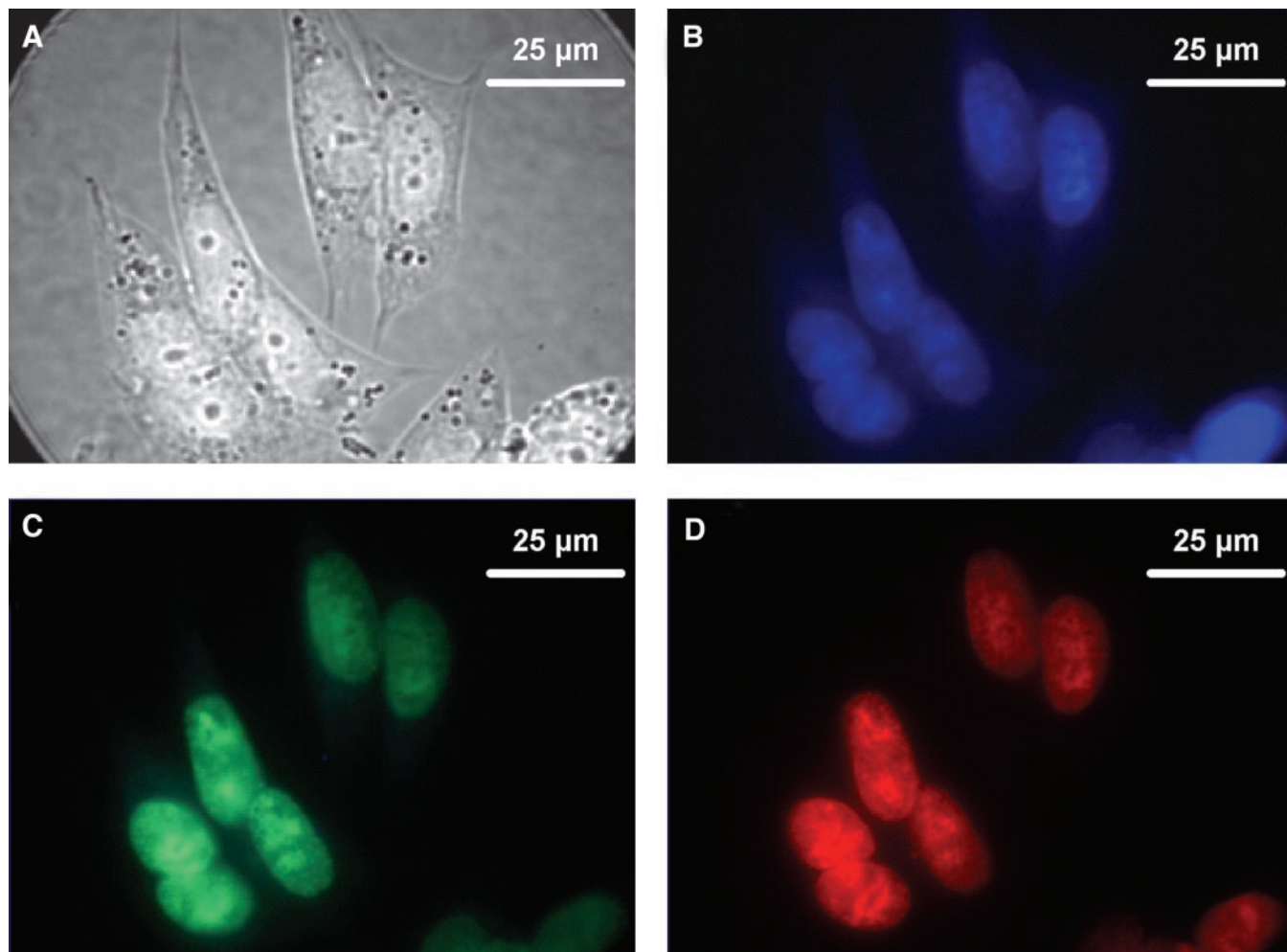


Fig. 2. Intracellular localization of ASOs and the anticancer drug DOX. Typical images of H69AR human lung cancer cells incubated for 48 hours with FITC-labeled ASOs targeted to BCL2 mRNA and DOX in liposomes. A, light microscopy; B, fluorescence microscopy (Hoechst 33258 nuclear staining); C, fluorescence microscopy (FITC-labeled ASOs); D, fluorescence microscopy (DOX).

antibody (1:500 dilution; Stress Gen Biotechnologies), or anti- $\beta$ -actin mice primary antibody (1:2,000 dilution; Oncogene Research, San Diego, CA) at 4°C. After further washing, the membrane was immersed in goat antirabbit and goat antimouse IgG biotinylated antibody (1:3,000 dilution and 1:1,000 dilution, respectively; Bio-Rad) at room temperature for 1.5 hours on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (Bio-Rad). The bands were digitally photographed and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA).  $\beta$ -Actin was used as an internal standard to normalize protein expression. Band intensities of MRP1, BCL2 proteins, and caspase 9 are expressed as the percentage of the  $\beta$ -actin band intensity, which was set at 100%.

### Caspase 3 Activity

The direct measurements of caspase 3 activity were made using a colorimetric protease assay kit (MBL International, Watertown, MA) as described previously (1, 20, 23–25). The assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide after cleavage from the substrates *X-p*-nitroanilide, where *X* stands for amino acid sequence recognized by the specific caspase (DEVD for caspases 3). The increase in the caspase activity was determined by comparing these results with the level of the untreated control incubated with saline.

### Apoptosis

Two approaches were used to assess apoptosis induction. The first approach was based on measuring the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in the cell cytoplasm using anti-histone and

anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as described previously (1, 20, 22–25, 27). The second approach was based on the detection of single- and double-stranded DNA breaks (nicks) by an *in situ* cell death detection kit (Roche) using terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) as described previously (1, 20, 23–25, 27). Briefly, cells were fixed, permeabilized, and incubated with the TUNEL reaction mixture. The label incorporated at the damaged sites of the DNA was visualized by a fluorescence microscope.

### Cytotoxicity

Cytotoxicity of different preparations was measured using CytoTox-ONE Homogenous Membrane Integrity Assay (Promega, Madison, WI) according to the manufacturer's protocol. Briefly,  $10^4$  cells in 100  $\mu$ l of media were seeded into wells of a 96-well microtiter plate. After a 24-hour incubation at 37°C, the medium was aspirated and substituted with fresh media containing a studied cytotoxic agent. Cells were separately incubated with 12 different concentrations of each studied preparation in the cell growth medium (1:2 serial dilutions) within 48 hours. After the incubation, plates were equilibrated at room temperature, the medium was aspirated, 100  $\mu$ l of CytoTox-ONE reagent were added in each well, and plates were incubated for 10 minutes at room temperature. The reaction was stopped by adding 50  $\mu$ l of Stop Solution, and plates were shaken. The fluorescence was recorded with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The IC<sub>50</sub> dose (the dose that kills 50% of cells) for each agent was calculated according to a previously described method (26) using a computer program (VPIC50 V. 4.3; Olympus Research, Salt Lake City, UT).

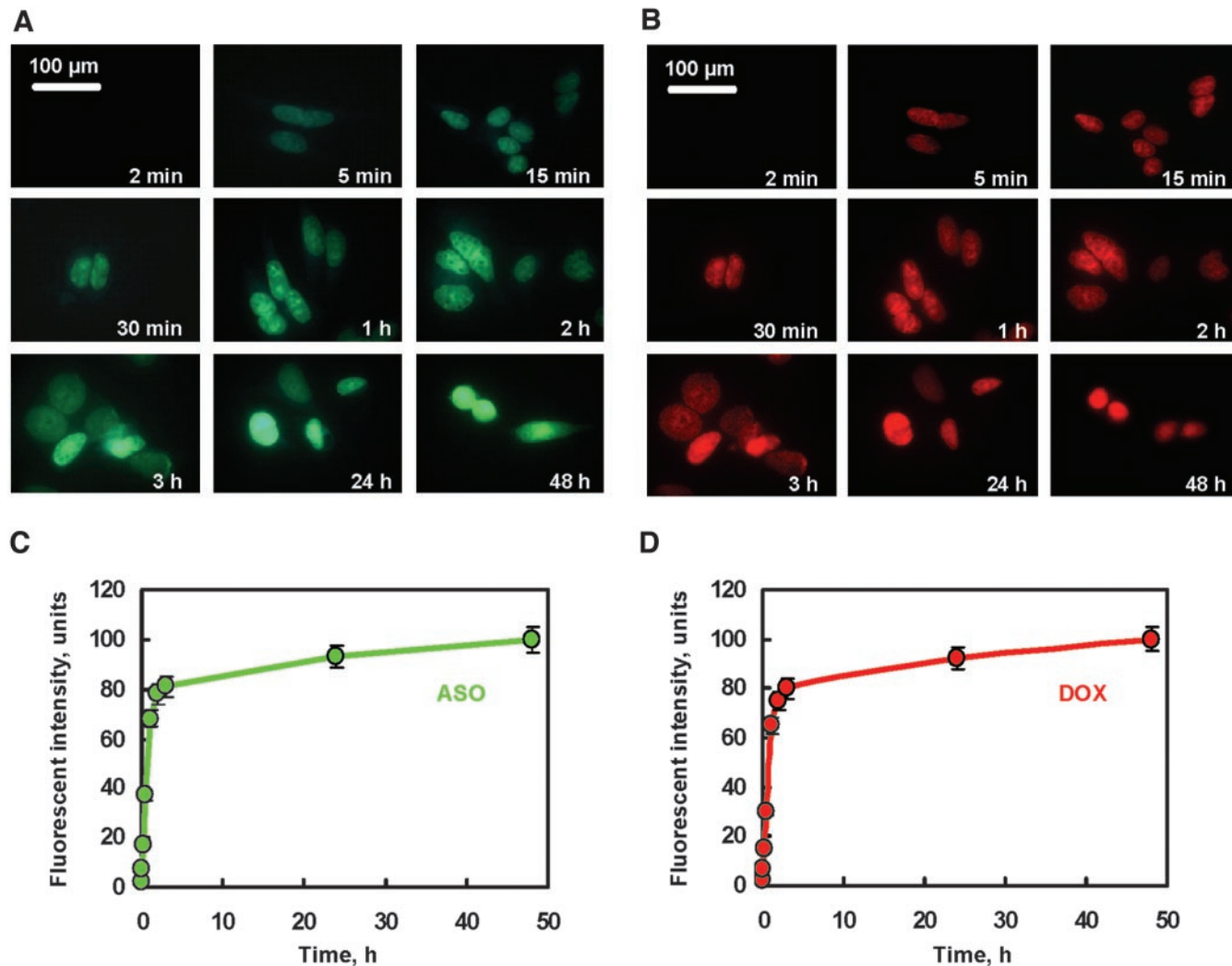


Fig. 3. Intracellular release of FITC-labeled BCL2 ASOs and DOX from liposomes. *A* and *B*, typical images of H69AR human lung cancer cells incubated at different periods of time with FITC-labeled BCL2 ASOs and DOX, respectively, in liposomes. *C* and *D*, time course of fluorescence intensity of FITC-labeled ASOs and DOX released from liposomes. Means  $\pm$  SD are shown.

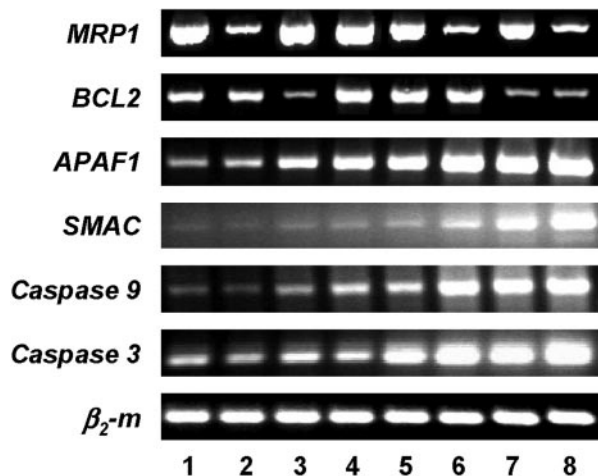


Fig. 4. Typical images of gel electrophoresis of RT-PCR products of the *MRP1*, *BCL2*, *APAF1*, *SMAC*, *caspase 9*, *caspase 3*, and  $\beta_2$ -microglobulin ( $\beta_2$ -m; internal standard) genes in H69AR human lung cancer cells. Cells were incubated for 48 hours with the indicated formulations. 1, control (fresh media); 2, liposomal MRP1 ASOs; 3, liposomal BCL2 ASOs; 4, free DOX; 5, liposomal DOX; 6, liposomal DOX + MRP1 ASOs; 7, liposomal DOX + BCL2 ASOs; 8, liposomal DOX + MRP1 and BCL2 ASOs.

Statistical Analysis

Data obtained were analyzed using descriptive statistics and single-factor analysis of variance and are presented as mean  $\pm$  SD from four to eight independent measurements in separate experiments.

RESULTS

**Intracellular Localization and Release of Antisense Oligonucleotides and Doxorubicin.** The analysis of intracellular localization by fluorescence microscopy showed that ASOs and DOX released from the liposomal drug delivery system and accumulated in nuclei (Fig. 2). In this respect, similar results were obtained for both types of ASOs targeted to MRP1 and BCL2 mRNA, respectively. We did not observe visible fluorescence in the cytosol. This suggests that free ASOs and free DOX did not accumulate in the cytoplasm and that transfer of these substances was achieved within endosome vesicles. The liposomal drug delivery system substantially increased the transfection efficacy of ASOs. Substantial ASO fluorescence was observed in 75–90% of cells after 1 hour of incubation (Fig. 2). In contrast, substantial fluorescence attributable to free ASOs was not seen inside cell nuclei even when they were used at the highest attainable concentration (about 1 mg/ml), which was limited by their solubility (data not shown).

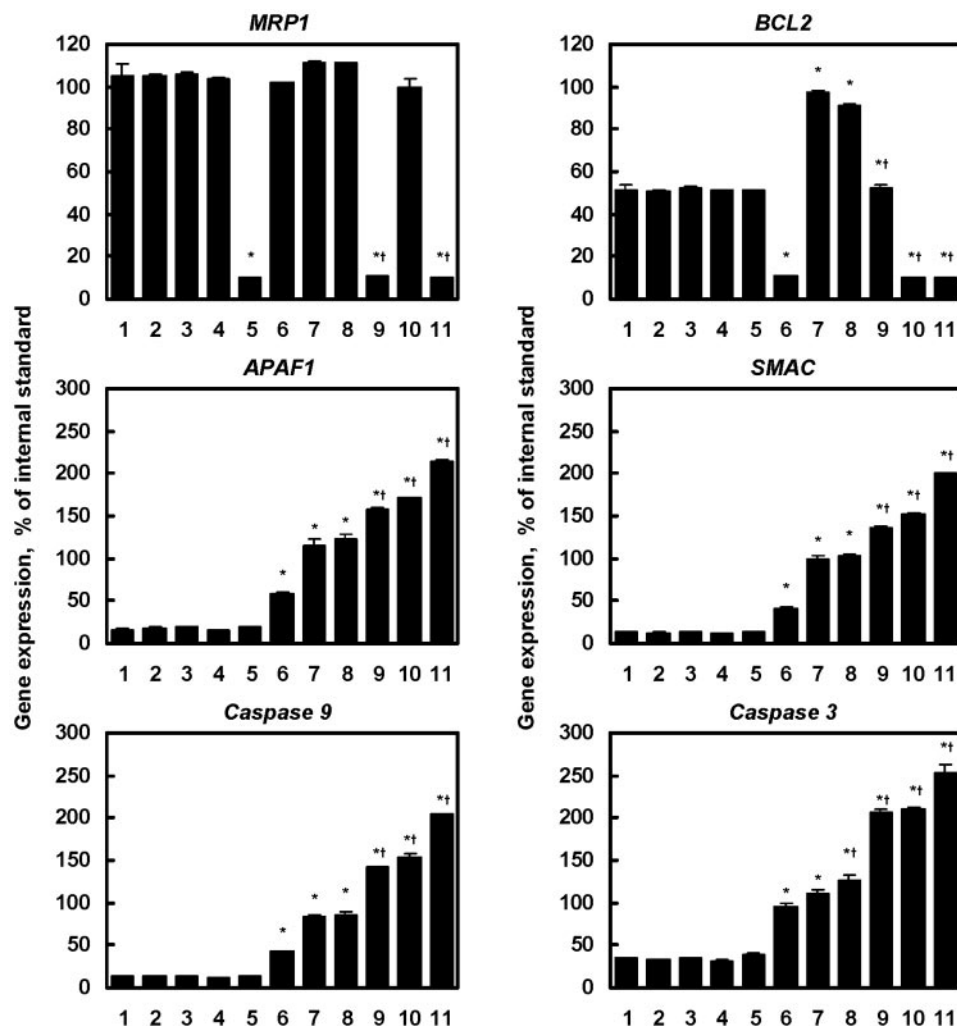


Fig. 5. *MRP1*, *BCL2*, *APAF1*, *SMAC*, *caspase 9*, and *caspase 3* gene expression levels in H69AR human lung cancer cells determined by quantitative real-time reverse transcription-PCR. Messenger RNA levels are expressed as the percentage of the  $\beta$ -actin (internal standard) level, which was set at 100%. Cells were incubated for 48 hours with the indicated formulations. Means  $\pm$  SD are shown. 1, control (fresh media); 2, empty liposomes; 3, liposomal *MRP1* sense oligonucleotides; 4, liposomal *BCL2* sense oligonucleotides; 5, liposomal *MRP1* ASOs; 6, liposomal *BCL2* ASOs; 7, free DOX; 8, liposomal DOX; 9, liposomal DOX + *MRP1* ASOs; 10, liposomal DOX + *BCL2* ASOs; 11, liposomal DOX + *MRP1* and *BCL2* ASOs. \*,  $P < 0.05$  when compared with control. †,  $P < 0.05$  when compared with free DOX.

Analysis of the intracellular release of ASOs and DOX (Fig. 3) showed that a substantial amount of active substances was observable in the cell nuclei even 5 minutes after the beginning of the incubation. The time profiles of the release of both types ASO and DOX were very similar. Nearly 80% of the fluorescence could be detected within the cells in the first 3 hours. Within 48 hours, nearly all of the fluorescence found in the cells was localized in the nuclei (Fig. 3). A series of *in vitro* experiments established the stability of the liposomes containing fluorescent ASOs and DOX. Liposomes incubated in saline for several weeks did not release a substantial amount of labeled ASOs.

**Liposomal Delivery of Antisense Oligonucleotides Targeted to *MRP1* and *BCL2* mRNA Prevented the Overexpression of *MRP1* and *BCL2* Genes and Proteins.** To analyze the effect of the active components of the proposed liposomal drug delivery system on pump and nonpump cellular resistance, we measured the expression of *MRP1* and *BCL2* mRNA and corresponding proteins that play central roles in pump and nonpump resistance, respectively (Figs. 4-6). It was found that liposomal ASOs significantly suppressed the expression of corresponding genes and proteins. At the same time, oligonucleotides with sense sequence did not show an inhibitory effect. Whereas incubation of lung cancer cells with free DOX did not lead to statistically significant overexpression of the *MRP1* gene, it produced a significant increase in the expression of *MRP1* protein. At the same time, free DOX significantly increased the expression of the *BCL2* gene and protein. Liposomal DOX did not significantly change the

expression of the *MRP1* gene and protein and slightly decreased the expression of the *BCL2* gene and protein when compared with free DOX. It should be stressed that empty liposomes did not change the expression of the *MRP1* and *BCL2* genes as well as the expression of other studied genes (Fig. 5) and proteins (Fig. 6). Similarly, liposomes containing *MRP1* and *BCL2* sense oligonucleotides did not influence the expression of studied proteins (Fig. 6, 3 and 4). Therefore, the observed increase in the expression of *MRP1* protein and *BCL2* gene and protein after incubation of cells with liposomal DOX (Figs. 5 and 6) was caused by DOX, not by liposomes. Antisense oligonucleotides targeted to the corresponding mRNA prevented such increase. Combination of DOX with the two ASOs targeted to *MRP1* and *BCL2* mRNA in one liposomal delivery system prevented the activation of antiapoptotic resistance by DOX and significantly suppressed both pump and nonpump resistance by down-regulation of the expression of *MRP1* and *BCL2* proteins.

**Antisense Oligonucleotides Targeted to *MRP1* and *BCL2* Enhanced the Induction of the Caspase-Dependent Pathway of Apoptosis by Doxorubicin.** We analyzed the influence of different liposomal preparations on the caspase-dependent pathways of apoptosis by measuring the expression of genes encoding two main caspase activators, apoptotic protease-activating factor 1 (*APAF1*) and second mitochondria-derived activator of caspase (*SMAC*), and caspases 9 and 3, the main proteins responsible for the activation of caspase-executors of apoptosis and apoptotic execution respectively. In addition, we measured the expression of procaspase 9 and active caspase

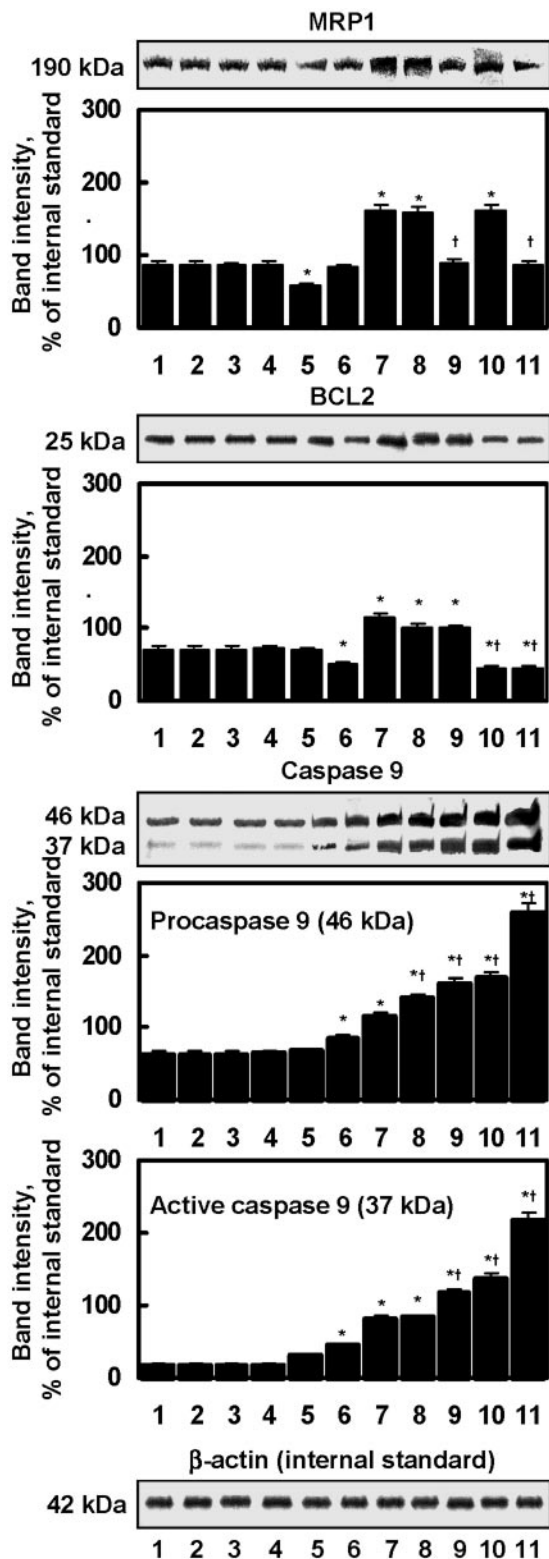


Fig. 6. Typical images of Western blots of MRP1, BCL2, caspase 9, and  $\beta$ -actin (internal standard) proteins and densitometric analysis of bands. Band intensities of MRP1, BCL2 proteins, and caspase 9 are expressed as the percentage of the  $\beta$ -actin band intensity, which was set at 100%. Cells were incubated for 48 hours with the indicated formulations. Means  $\pm$  SD are shown. 1, control (fresh media); 2, empty liposomes; 3, liposomal MRP1 sense oligonucleotides; 4, liposomal BCL2 sense oligonucleotides; 5, liposomal MRP1 ASOs; 6, liposomal BCL2 ASOs; 7, free DOX; 8, liposomal DOX; 9, liposomal DOX + MRP1 ASOs; 10, liposomal DOX + BCL2 ASOs; 11, liposomal DOX + MRP1 and BCL2 ASOs. \*,  $P < 0.05$  when compared with control. †,  $P < 0.05$  when compared with free DOX.

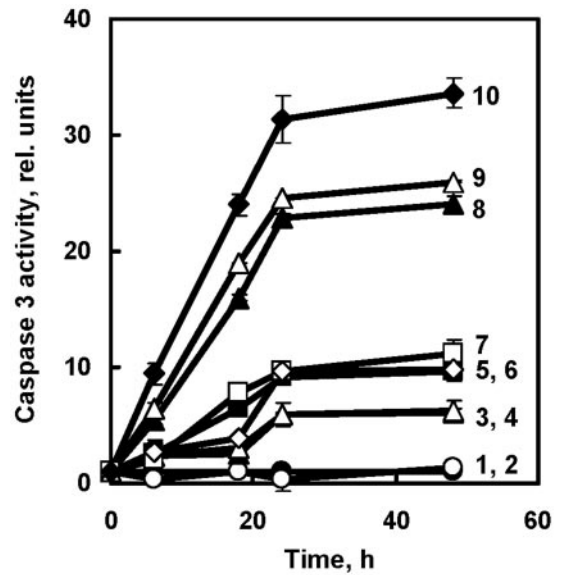


Fig. 7. Time-dependent activity of caspase 3 in H69AR human lung cancer cells. Activities in the control (untreated cells) were set to a relative unit of 1. 1, control (fresh media); 2, empty liposomes; 3, liposomal MRP1 ASOs; 4, liposomal BCL2 ASOs; 5, liposomal MRP1 and BCL2 ASOs; 6, free DOX; 7, liposomal DOX; 8, liposomal DOX + MRP1 ASOs; 9, liposomal DOX + BCL2 ASOs; 10, liposomal DOX + MRP1 and BCL2 ASOs.

9 as well as the activity of caspase 3 (Figs. 5–7). The results of all measurements corroborate each other. Liposomal ASOs targeted to BCL2 mRNA to a certain extent activated the caspases themselves (Figs. 5–7). However, the proapoptotic activity of both MRP1 and BCL2 ASOs when combined with DOX produced the full extent of apoptosis observed. Such a combination significantly enhanced the proapoptotic activity of this traditional anticancer drug by suppression of pump and nonpump cellular resistance. This suppression led to a significant overexpression of APAF1 and SMAC genes (Fig. 6), which in turn converted procaspase 9 into the active form (Fig. 5). Active caspase 9 triggered downstream caspases including the executor of apoptosis, caspase 3 (Fig. 6). Finally, the activity of this caspase increased dramatically, reaching a plateau starting 24 hours after initiation of the experiment (Fig. 7).

**Simultaneous Modulation of Pump and Nonpump Cellular Resistance Enhances the Ability of Doxorubicin to Induce Apoptosis and Significantly Enhances Its Cytotoxicity.** The two methods of apoptosis analysis used showed similar results. Suppression of pump and nonpump resistance significantly enhanced the apoptosis induction activity of DOX (Figs. 8 and 9). Although enhancements were observed after the separate suppression of pump or nonpump cellular resistance, a dramatic increase in apoptosis induction was achieved only after simultaneous suppression of both types of resistance accompanied by apoptosis induction by DOX. As a result, cytotoxicity of DOX was significantly enhanced by the suppression of cellular pump and nonpump resistance.

The comparison of multidrug-resistant H69AR cells with their H69 drug-sensitive parent cell line showed that induction of apoptosis by free DOX in the drug-sensitive cell line was significantly higher when compared with apoptosis in the multidrug-resistant cell line (Fig. 8, compare 1 on the top and bottom panels). Further analysis demonstrated that this difference is attributed mainly to overexpression of MRP, which is responsible for pump resistance in the multidrug-resistant H69AR lung cancer cell line. In fact, the suppression of MRP1 protein in the multidrug-resistant cell line by a liposomal combination of DOX with ASOs targeted to MRP1 mRNA signifi-

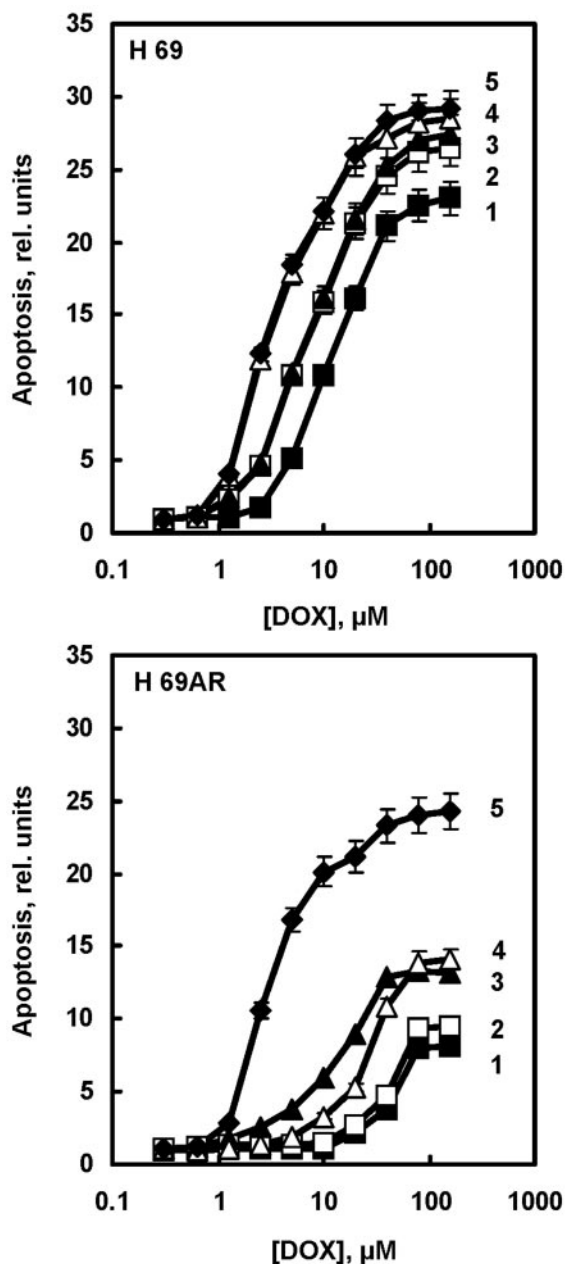


Fig. 8. Apoptosis induction in drug-sensitive H69 (*top panel*) and multidrug-resistant H69AR (*bottom panel*) human lung cancer cells. The enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in control untreated cells was set to unit 1, and the degree of apoptosis was expressed in relative units. Cells were incubated for 48 hours with the indicated formulations. Means  $\pm$  SD are shown. 1, free DOX; 2, liposomal DOX; 3, liposomal DOX + MRP1 ASOs; 4, liposomal DOX + BCL2 ASOs; 5, liposomal DOX + MRP1 and BCL2 ASOs.

cantly enhanced apoptosis induction when compared with free and liposomal DOX (Fig. 8, compare 1–3 in the *bottom panel*). In the drug-sensitive cell line, encapsulation of DOX into liposomes led to an increase in the ability of DOX to induce apoptosis (Fig. 8, compare 1 and 2 in the *top panel*). At the same time, incubation of H69 cells with a liposomal formulation containing DOX and MRP1 ASOs did not lead to a significant increase in apoptosis induction when compared with liposomal DOX (Fig. 8, compare 2 and 3 in the *top panel*). The substantial differences in apoptosis induction between drug-sensitive and -resistant cell lines confirmed the importance of MRP1 protein in the development of resistance in the H69AR human lung cancer cell line. Down-regulation of BCL2 protein expression in the

multidrug-resistant cell line led to substantially less enhancement of apoptosis when compared with the suppression of MRP1 protein under intermediate DOX concentrations (Fig. 8, compare 3 and 4 in the *bottom panel*). In contrast, down-regulation of BCL2 protein after incubation with high concentrations of DOX significantly enhanced apoptosis induction in the multidrug-resistant cell line (Fig. 8, 3 and 4 in the *bottom panel*). Combination of DOX and ASOs targeted to MRP1 and BCL2 mRNA in one liposomal formulation enhanced apoptosis induction when compared with free and liposomal DOX (Fig.8, compare 1, 2, and 5 in the *bottom panel*). Liposomal DOX combined with MRP1 ASOs showed similar efficacy to liposomal DOX in terms of apoptosis induction in drug-sensitive cell line (Fig.8, compare 2 and 3 in the *top panel*), whereas suppression of BCL2 protein enhanced the efficacy of liposomal DOX in the H69 drug-sensitive human lung cancer cell line (Fig. 8, compare 3 and 4 in the *top panel*).

Data obtained showed that liposomal delivery of DOX increased the cytotoxicity of DOX by 1.3-fold when compared with free DOX (compare 8 and 9 on Fig. 10B and 8 and 9 on Fig. 10C). Modulation of pump resistance by ASOs targeted to MRP1 mRNA further increased cytotoxicity of liposomal DOX by 4-fold (compare 9 and 12 on Fig. 10B). At the same time, suppression of nonpump resistance (using liposomes containing DOX and ASOs targeted to BCL2 mRNA) led to an almost 2-fold decrease in the  $IC_{50}$  dose when compared with liposomal DOX (compare 9 and 13 on Fig. 10B and C). Finally, simultaneous suppression of pump and nonpump resistance (using liposomes containing DOX and ASOs targeted to MRP1 and BCL2 mRNA) increased the cytotoxicity of DOX by 10-fold when compared with both free and liposomal DOX (compare 8 and 9 with 14 on Fig. 10B and C). Such an increase in toxicity was attributed mainly to the enhancement of DOX toxicity by simultaneous suppression of pump (MRP1) and nonpump (BCL2) cellular resistance. Formulations without DOX did not show significant toxicity (empty liposomes, liposomes with sense oligonucleotides, and liposomes containing MRP1 ASOs) or showed only a slight decrease in the cellular viability (using liposomal BCL2 ASOs; Fig. 10A). Liposomes containing DOX in combination with MRP1 or BCL2 sense oligonucleotides showed similar toxicity as liposomal DOX without oligonucleotides (compare 9 with 10 and 11 on Fig. 10B and C). Therefore, enhancement of the cytotoxicity was attributed to the sequence of ASOs, not to the presence of oligonucleotides in the liposomal formulations.

## DISCUSSION

The proposed liposomal drug delivery system fulfills four main tasks: (a) it provides for the delivery of antisense oligonucleotides and an anticancer drug into the cell nucleus with a high transfection efficacy; (b) it induces apoptosis in lung cancer cells; (c) it suppresses pump resistance; and (d) it restrains nonpump resistance. The main result of the application of this complex delivery system is the substantial enhancement of the anticancer efficacy of a traditional anticancer drug, DOX. Similar approaches can be applied to other anticancer agents that induce apoptosis and simultaneously activate cell defensive mechanisms.

Data obtained showed that the delivery mechanism of active components by the drug delivery system was most likely achieved by endocytosis in membrane-limited organelles. This suggestion is supported by the fact that the observed fluorescence of ASOs and DOX appeared only in nuclei and the perinuclear region. In fact, if liposomes fused with the cellular plasma membrane, fluorescent active component would release in the cytoplasm and produce substantial fluorescence. It is most likely that liposomes were taken by cells as a



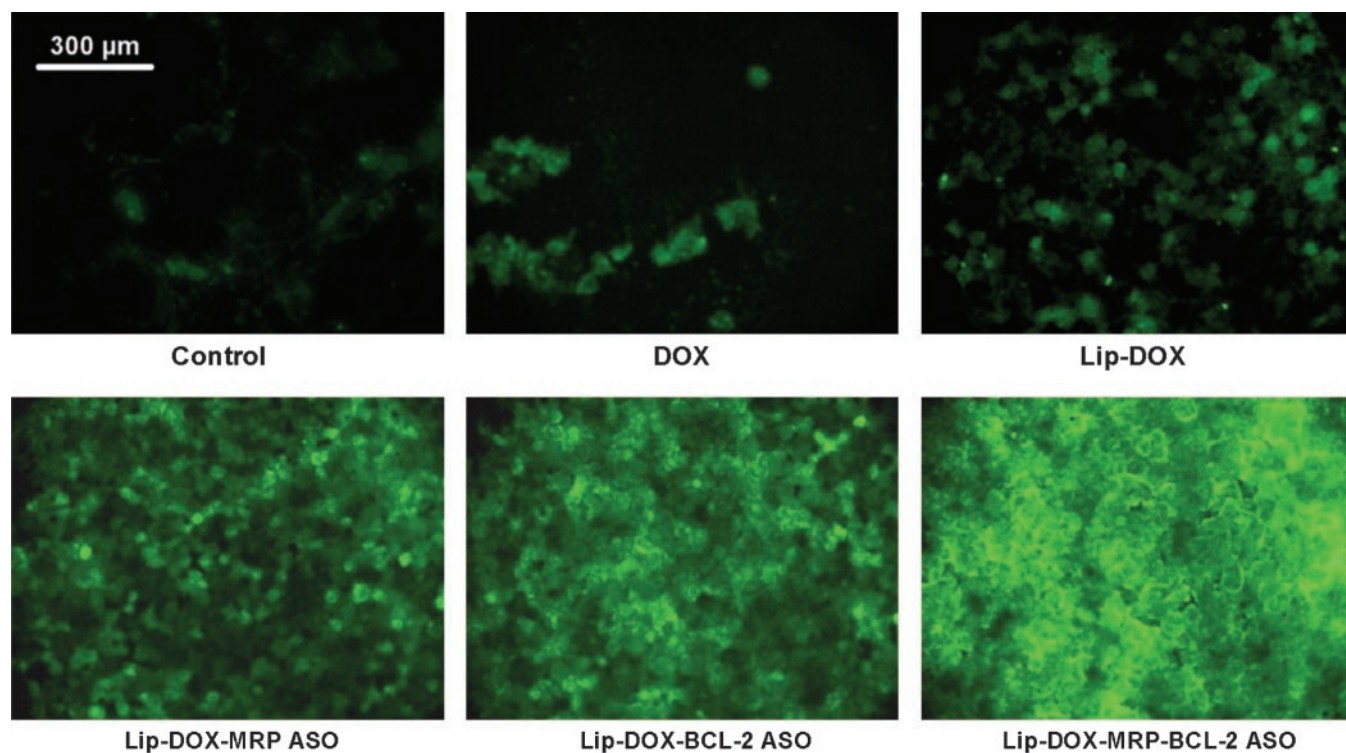


Fig. 9. Typical fluorescence microscopy images of TUNEL-labeled H69AR human lung cancer cells. Cells were incubated for 48 hours.

whole complex and transported in endosomes; only in this case, liposomal membrane can quench the fluorescence of labeled ASOs and DOX (21). The active components of the liposomes were released after the fusion of endosomes with lysosomes in the perinuclear region of the cells. This type of delivery also provides for a high degree of transfection of ASOs and preserves the anticancer activity of DOX.

We found that ASOs targeted to BCL2 protein to a certain extent were able to induce cell death in lung cancer cells. However, the inclusion of a specific cell death-inducing agent, DOX, in the drug delivery system provides a distinct advantage, substantially increasing the ability of the whole delivery system to initiate apoptosis. The main mechanisms of apoptosis induction by DOX include activation of the caspase-dependent cell death signal. However, the efficacy of this activation is substantially limited by the simultaneous stimulation of cellular antiapoptotic defense by DOX. We found that the incubation of lung cancer cells with DOX led to an increase in the expression of BCL2 protein, a central player in cellular antiapoptotic defense. Similar dual action of DOX was found in ovarian, breast, and prostate cancer cells (1, 23, 28). We also observed an increase in cellular antiapoptotic defense after the incubation of cancer cells with camptothecin (25, 27). Therefore, it is reasonable to hypothesize that such a dual effect (induction of cell death and simultaneous activation of cellular antiapoptotic defense) is attributed to many (if not all) anticancer drugs and might be found in different cancer cells. Consequently, the suppression of cellular antiapoptotic defense should substantially increase the efficacy of anticancer drugs. In addition to the present study, we used several approaches to this end, including ASOs targeted to BCL2 protein (1) and synthetic BCL2 homology 3 peptide (24, 25). These studies, as well as the present results, support our concept and showed that simultaneous suppression of cellular antiapoptotic defense substantially increased the proapoptotic efficacy of anticancer drugs.

The second factor that limits the efficacy of cancer chemotherapy is

the development of multidrug resistance. Active drug efflux pumps play the main role in such drug resistance. We proposed to suppress drug efflux pumps by incorporating in complex drug delivery system antisense oligonucleotides targeted to mRNA encoding proteins that are the main players in multidrug resistance. We have already successfully blocked P-glycoprotein-dependent multidrug resistance in ovarian and breast cancer cells by using ASOs targeted to the MDR1 gene as a part of a complex drug delivery system. The present study showed that ASOs targeted to MRP can be used successfully in cancer cells that overexpress this protein. The blockade of drug efflux pumps prevents a decrease in drug concentration inside the cells and therefore increases the efficacy of cancer treatment.

The study of cell death enhancement by the suppression of pump and nonpump cellular resistance under different concentrations of apoptosis induction by DOX showed that the inhibition of nonpump (antiapoptotic) resistance in multidrug resistant cells is effective only under high concentrations of DOX. Because of this, it is not surprising that a substantial activation of antiapoptotic defense is observed only under high concentrations of DOX. Consequently, suppression this antiapoptotic defense would be most effective only in combination with the simultaneous suppression of pump resistance, which in turn increases the intracellular concentration of the apoptosis-inducing agent. Therefore, the combination of anticancer agent with suppressor of pump resistance and antiapoptotic cellular defense (nonpump resistance) should be most effective in terms of cell death induction and anticancer activity in multidrug-resistant cancer cells. The direct measurement of separate cytotoxicity of individual components and complex liposomal drug delivery system showed that liposomes, DOX, and ASOs targeted to MRP1 and BCL2 mRNA work together to enhance the cytotoxicity of the whole complex.

The present data showed the distinct advantages of the proposed complex drug delivery system, which includes (a) an apoptosis inducer, (b) a suppressor of drug efflux pumps, and (c) an inhibitor of cellular antiapoptotic defense. Substantial enhancement in cytotoxic-

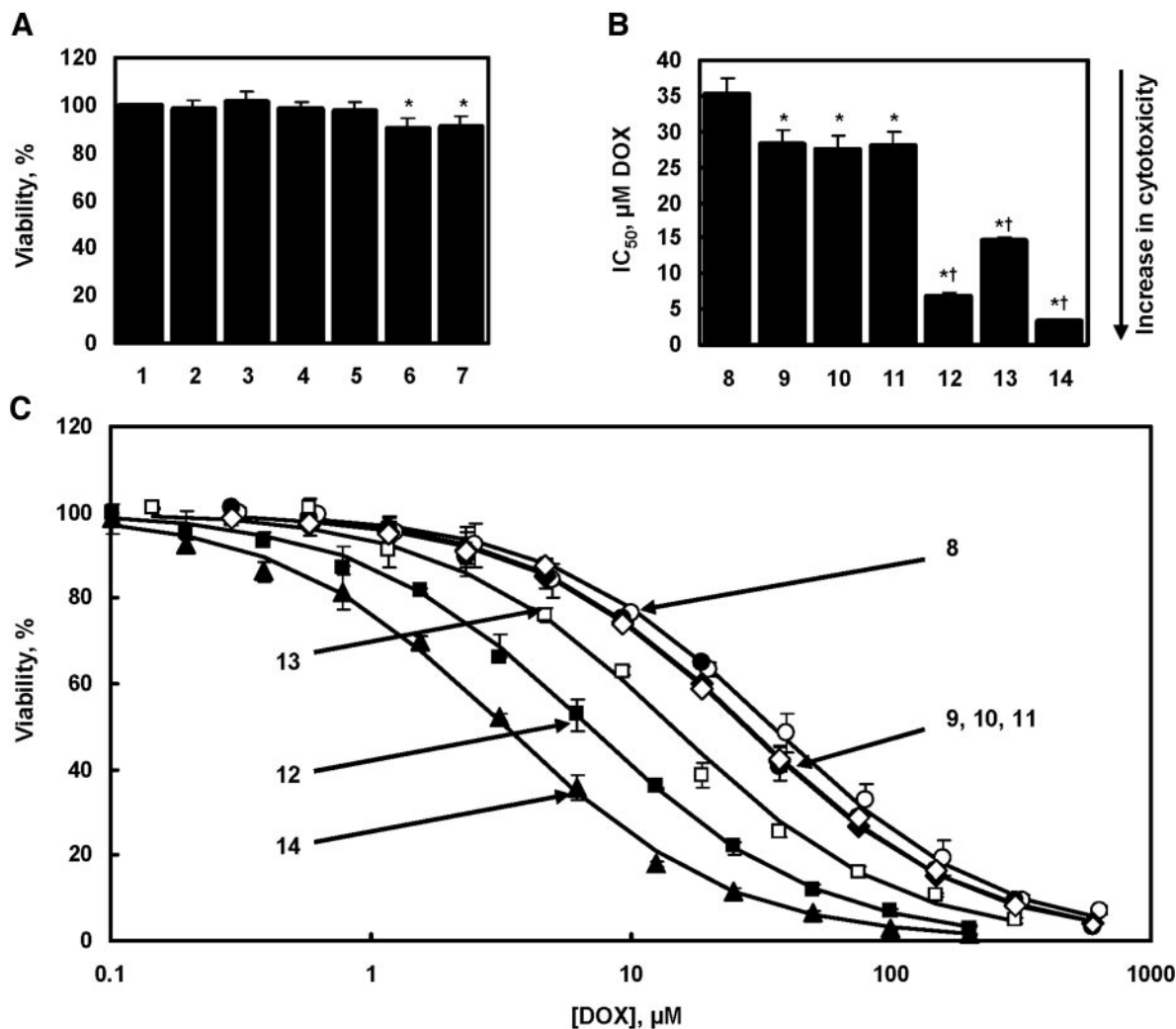


Fig. 10. Viability of multidrug-resistant H69AR human lung cancer cells incubated for 48 hours with the indicated formulations. A, cytotoxicity of formulations that do not contain DOX; B, cytotoxicity of formulations that contain DOX; C, actual dose-response curves of formulations that contain DOX. The concentration of oligonucleotides and composition of liposomes in all formulations were the same. Means  $\pm$  SD are shown. 1, control (fresh media); 2, empty liposomes; 3, liposomal MRP1 sense oligonucleotides; 4, liposomal BCL2 sense oligonucleotides; 5, liposomal MRP1 ASOs; 6, liposomal BCL2 ASOs; 7, liposomal MRP1 and BCL2 ASOs; 8, free DOX; 9, liposomal DOX; 10, liposomal DOX + MRP1 SO; 11, liposomal DOX + BCL2 SO; 12, liposomal DOX + MRP1 ASOs; 13, liposomal DOX + BCL2 ASOs; 14, liposomal DOX + MRP1 and BCL2 ASOs. \*,  $P < 0.05$  when compared with free DOX. †,  $P < 0.05$  when compared with liposomal DOX.

ity and anticancer action of a traditional anticancer drug is achieved by simultaneously attacking two additional molecular targets: the genes encoding drug efflux pumps and the main player in cellular antiapoptotic defense. Although elements of such a drug delivery system have previously been applied separately in nonrelated studies, only simultaneous use of all of the components in one complex drug delivery system allows for realization of the full potential of the approach: simultaneous inhibition of pump and nonpump cellular defenses to enhance the efficacy of cancer chemotherapy.

We are aware that the development of clinical multidrug resistance in lung cancer might be caused by several different drug efflux pumps, not only by MRP1. Consequently, the suppression of only MRP1 protein will not be effective in such multifactorial clinical multidrug resistance. Therefore, suppression of several proteins might be required in such cases. Previously, we developed a drug delivery system that utilizes ASOs targeted to MDR1 mRNA to suppress P-glycoprotein (1, 2). Other ASOs targeted to corresponding drug efflux pumps can be included in the proposed complex liposomal drug delivery system as required. Theoretically, analysis of the expression of genes/proteins responsible for the pump resistance in the patient's tumor tissues might help in the individual selection of one or several ASOs

targeted to the required types of mRNA. The present investigation provides proof of concept for the use of a complex drug delivery system including an anticancer drug combined with suppressors of pump and nonpump cellular resistance for the enhancement of chemotherapy of multidrug-resistant lung cancer.

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**REFERENCES**

1. Pakunlu RI, Cook TJ, Minko T. Simultaneous modulation of multidrug resistance and antiapoptotic cellular defense by MDR1 and BCL2 targeted antisense oligonucleotides enhances the anticancer efficacy of doxorubicin. *Pharm Res* 2003;20:351-9.
2. Minko T, Dharar SS, Pakunlu RI, Wang Y. Molecular targeting of drug delivery systems to cancer. *Curr Drug Targets* 2004;5:389-406.
3. Szakacs G, Jakab K, Antal F, Sarkadi B. Diagnostics of multidrug resistance in cancer. *Pathol Oncol Res* 1998;4:251-7.
4. van Veen HW, Konings WN. The ABC family of multidrug transporters in microorganisms. *Biochim Biophys Acta* 1998;1365:31-6.

5. Scagliotti GV, Novello S, Selvaggi G. Multidrug resistance in non-small-cell lung cancer. *Ann Oncol* 1999;10(Suppl 5):S83–6.
6. Nakamura M, Abe Y, Katoh Y, et al. A case of pulmonary adenocarcinoma with overexpression of multidrug resistance-associated protein and p53 aberration. *Anticancer Res* 2000;20:1921–5.
7. Hsia TC, Lin CC, Wang JJ, Ho ST, Kao A. Relationship between chemotherapy response of small cell lung cancer and P-glycoprotein or multidrug resistance-related protein expression. *Lung* 2002;180:173–9.
8. Alahary SK, DeLong R, Fisher MH, et al. Novel chemically modified oligonucleotides provide potent inhibition of P-glycoprotein expression. *J Pharmacol Exp Ther* 1998;286:419–28.
9. Corrias MV, Tonini GP. An oligomer complementary to the 5' end region of MDR1 gene decreases resistance to doxorubicin of human adenocarcinoma-resistant cells. *Anticancer Res* 1992;12:1431–8.
10. Motomura S, Motoji T, Takanashi M, et al. Inhibition of P-glycoprotein and recovery of drug sensitivity of human acute leukemic blast cells by multidrug resistance gene (mdr1) antisense oligonucleotides. *Blood* 1998;91:3163–71.
11. Rebowski G, Wojcik M, Boczkowska M, et al. Antisense hairpin loop oligonucleotides as inhibitors of expression of multidrug resistance-associated protein 1: their stability in fetal calf serum and human plasma. *Acta Biochim Pol* 2001;48:1061–76.
12. Stewart AJ, Canitrot Y, Baracchini E, et al. Reduction of expression of the multidrug resistance protein (MRP) in human tumor cells by antisense phosphorothioate oligonucleotides. *Biochem Pharmacol* 1996;51:461–9.
13. Yang JY, Luo HY, Lin V, et al. Subcellular daunorubicin distribution and its relation to multidrug resistance phenotype in drug-resistant cell line SMMC-7721/R. *World J Gastroenterol* 2002;8:644–9.
14. Gross A, McDonnell JM, Korsmeyer, S. BCL2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899–911.
15. Reed J. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999;17:2941–53.
16. Konopleva M, Tan AM, Estrov Z, et al. Liposomal BCL2 antisense oligonucleotides enhance proliferation, sensitize acute myeloid leukemia to cytosine-arabioside, and induce apoptosis independent of other antiapoptotic proteins. *Blood* 2000;15:3929–38.
17. de Mendes DEL, Hudon N, McIntosh N, Mayer LD. Molecular and pharmacokinetics properties associated with the therapeutics of BCL2 antisense oligonucleotide G3139 combined with free and liposomal doxorubicin. *Clin Cancer Res* 2000;6:2891–902.
18. Poulaki V, Mitsiades N, Romero ME, Tsokos M. Fas-mediated apoptosis in neuroblastoma requires mitochondrial activation and is inhibited by FLICE inhibitor protein and BCL2. *Cancer Res* 2001;61:4864–72.
19. Tari A, Tucker S, Deisseroth A, Lopez-Berestein GF. Liposomal delivery of methylphosphonate antisense oligonucleotides in chronic myelogenous leukemia. *Blood* 1994;84:601–7.
20. Wang Y, Pakunlu RI, Tsao W, Pozharov V, Minko T. Bimodal effect of hypoxia in cancer: the role of hypoxia inducible factor in apoptosis. *Mol Pharmacol* 2004;1:156–65.
21. Tsukioka Y, Matsumura Y, Hamaguchi T, et al. Pharmaceutical and biomedical differences between micellar doxorubicin (NK911) and liposomal doxorubicin (Doxil). *Jpn J Cancer Res* 2002;93:1145–53.
22. Minko T, Kopeckova P, Kopecek J. Efficacy of the chemotherapeutic action of HPMA copolymer-bound doxorubicin in a solid tumor model of ovarian carcinoma. *Int J Cancer* 2000;86:108–17.
23. Minko T, Dharap SS, Fabbriatore AT. Enhancing the efficacy of chemotherapeutic drugs by the suppression of antiapoptotic cellular defense. *Cancer Detect Prev* 2003;27:193–202.
24. Dharap SS, Minko T. Targeted proapoptotic LHRH-BH3 peptide. *Pharm Res* 2003;20:889–96.
25. Dharap SS, Qiu B, Williams G, et al. Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides. *J Controlled Release* 2003;91:61–73.
26. Minko T, Kopeckova P, Pozharov V, Kopecek, J. HPMA.copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line. *J Controlled Release* 1998;54:223–33.
27. Minko T, Paranjpe P, Qiu B, et al. Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethylene glycol) conjugates in sensitive and multidrug resistant human ovarian carcinoma cells. *Cancer Chemother Pharmacol* 2002;50:143–50.
28. Minko T, Dharap SS, Pakunlu RI, Colaizzi JL. Advanced drug delivery systems in cancer chemotherapy. *Dis Management Clin Outcomes* 2001;3:48–54.