Simultaneous Modulation of Multidrug Resistance and Antiapoptotic Cellular Defense by MDR1 and BCL-2 Targeted Antisense Oligonucleotides Enhances the Anticancer Efficacy of Doxorubicin

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Purpose. To enhance the anticancer efficacy of an established drug by the simultaneous suppression of pump and nonpump cellular resistance.

Methods. Multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells were used. Doxorubicin (DOX) and antisense oligonucleotides (ASO) targeted to MDR1 and BCL-2 mRNA were combined in a solution within one liposomal drug delivery system (LDDS) in different combination series. Ten series of experiments were performed. In each series cells were incubated with 12 to 45 concentrations of free DOX and different liposomal formulations over a period of 6 to 48 h. Cytotoxicity, apoptosis induction, caspases, *MDR1, BCL-2,* and *APAF-1* genes, P-glycoprotein, and BCL-2 protein were studied.

Results. The combination of DOX and ASO targeted to MDR1 and BCL-2 mRNA in one LDDS exhibited a dramatic increase in the anticancer action of DOX. As a result of the simultaneous suppression of pump and nonpump cellular resistance by the inhibition of P-glycoprotein and BCL-2 protein synthesis, a significant increase in the activation of caspases and apoptosis was observed.

Conclusions. The simultaneous suppression of multidrug resistance and antiapoptotic cellular defense significantly enhanced the anticancer activity of DOX. Therefore, the proposed DDS combination may potentially be used in the treatment of multidrug-resistant ovarian and breast cancers.

KEY WORDS: liposomes; antisense oligonucleotides; multidrug resistance; apoptosis; ovarian and breast cancer.

INTRODUCTION

Development of multidrug resistance in cancer cells is the main obstacle to the effective chemotherapy of cancer. Sensitive cancer cells, which initially respond well to a single drug treatment, significantly increase their resistance to a wide spectrum of drugs after a relatively short period of treatment (1). Two main mechanisms are responsible for this phenomenon: pump and nonpump cellular resistance (2).

The first mechanism is the overexpression of certain proteins that form membrane-bound ATP-dependent active drug efflux pumps (3,4). Efflux pumps significantly decrease the intracellular concentration of the drug below effective levels even if the extracellular drug concentration exceeds the maximal tolerated dose. We designated this type of drug resistance as "pump resistance" (2). A membrane glycoprotein, termed P-glycoprotein (P-gp), has been shown to be responsible for cross-resistance to a broad range of structurally and functionally distinct cytotoxic agents. This glycoprotein, encoded in humans by the *MDR1* gene, functions as an ATP-dependent membrane pump to remove cytotoxic agents from the resistant cells. The overexpression of the *MDR1* gene and P-gp itself is the main cause of pump resistance (1,5–7).

Several methods, including the use of antisense oligonucleotides (ASO) targeted against mRNA derived from genes encoding drug efflux pumps, were developed during the last decades to overcome or suppress multidrug resistance (1,2,5–12). However, even when those efforts led to the increase in intracellular drug concentration, that increase did not result in improvement of the treatment. The main reason for such failure is an adaptive activation of cell death defense, which we proposed to call "nonpump resistance" (2). It is known that the up-regulation of the cellular antiapoptotic system plays the main role in this defense, and BCL-2 family proteins are key players in this system (13,14). Unlike the drug efflux pump proteins, overexpression of BCL-2 protein does not interfere with the entry and accumulation of drugs in tumor cells. Instead, BCL-2 protein prevents drug-induced damage from being efficiently translated into cell death. This action of BCL-2 protein is via the prevention of cytochrome c release from mitochondria and the triggering of the caspase cascade of apoptosis execution. ASO targeted to BCL-2 mRNA (15–17) and other methods (2) can be effectively used to down-regulate the expression of BCL-2 protein and modulate antiapoptotic cellular resistance. However, the suppression of nonpump resistance alone cannot completely overcome existing multidrug resistance because its two main components — pump and nonpump resistance — act independently.

Detailed analysis of the problem led us to formulate the hypothesis that only simultaneous modulation of pump and nonpump resistance will significantly increase the efficacy of chemotherapy by traditional anticancer drugs. To verify this hypothesis we developed and manufactured a novel complex liposomal drug delivery system (LDDS), which combines ASO against *MDR1* and *BCL-2* genes with a traditional anticancer drug, doxorubicin (DOX). The first two components were designed to suppress pump and nonpump resistance, respectively, and the role of DOX was cell death induction. This paper presents the results of a series of experiments in which we have examined the efficacy of the constructed LDDS as an anticancer agent.

METHODS

Cell Lines

Experiments were carried out on multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer

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ABBREVIATIONS: DOX, doxorubicin; LDDS, liposomal drug delivery system; ASO, antisense oligonucleotides; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; dUTP, deoxyuracil triphosphate; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP-fluorescein nick end labeling; RT, reverse transcription; PCR, polymerase chain reaction; P-gp, P-glycoprotein.

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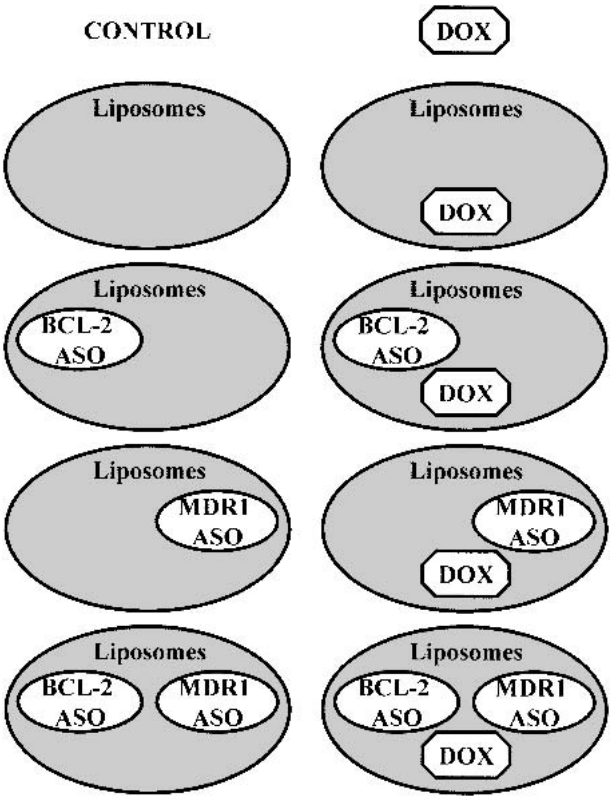

Fig. 1. Experimental design diagrammatically showing the different series of LDDS tested.

cells. A2780/AD cells were obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, PA), MCF-7/AD cells were obtained from Dr. W. N. Hait (Cancer Institute of New Jersey, NJ). Both cell lines overexpressed *MDR1* and *BCL-2* genes. Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. All experiments were performed on cells in the exponential growth phase.

Liposomal Drug Delivery Systems

Sequences of ASO used in the present study were 5′–CAGCGTGCGCCATCCTTCCC–3′ (17) (BCL-2) and 5′–TTCAAGATCCATCCCGACCTCGCG–3′ (11) (MDR1). The DNA backbone of all bases in oligonucleotides was P-ethoxy modified to enhance nuclease resistance and increase incorporation efficacy into liposomes (17). ASO were synthesized by Oligos Etc. (Wilsonville, OR). ASO were packaged with DOX in liposomes, which were prepared using a previously described lipid film rehydration method (18). 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-phosphotidylcholine:cholesterol). DOX was loaded into the liposomal formulation using the pH gradient method by adding solid DOX to the liposomes at a ratio of 3:20 (DOX:lipid, w/w) and adjusting the pH to 7.8 using 0.2 M sodium bicarbonate. ASO were loaded into the liposomes by dissolving the ASO in the rehydration buffer at a concentration of 0.5 mM. For the remaining DOX-containing formulations, DOX was loaded passively by being dissolved in the rehydration buffer with the ASO. After preparation, free DOX was separated from the liposomal DOX by passing the liposome suspension through a Sephadex G-50 column. DOX and ASO were encapsulated into the liposomes. The encapsulation efficiency ranged from 51.5 to 58.3% in different series of experiments. The mean liposome diameter was about 100 nm, and changes in size after loading DOX and ASO did not exceed 10%.

Cytotoxicity Assay and Apoptosis Detection

The cytotoxicity of drugs was assessed using a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as previously described (19). This method allows testing of drugs in a wide range of concentrations (2^{10} to 2^{44}) with acceptable reproducibility and quantitative analysis

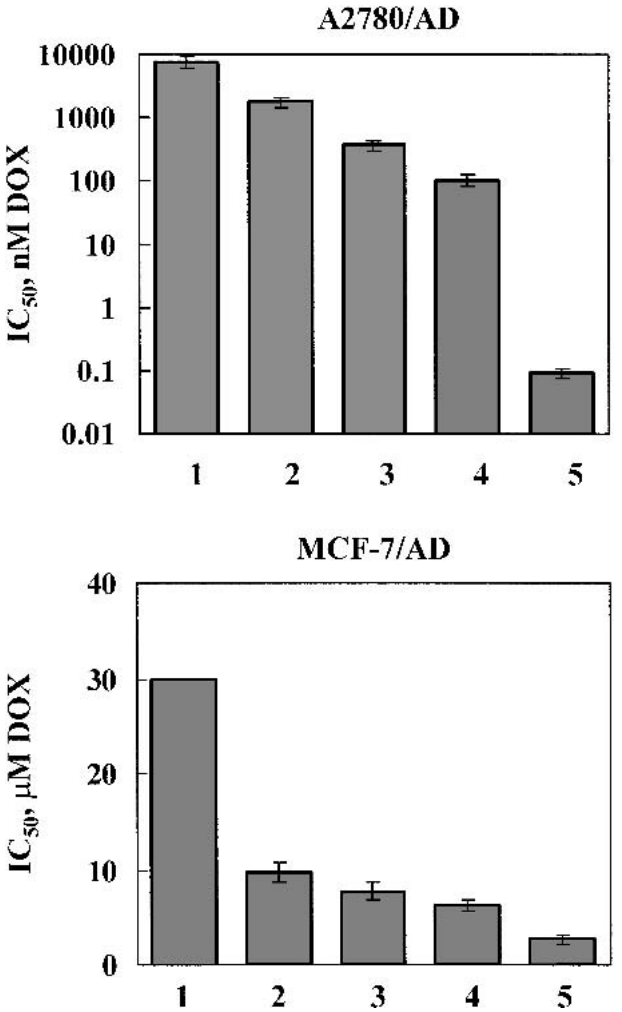

Fig. 2. Cytotoxicity of free DOX (1), liposomal DOX (2), and liposomes containing DOX and BCL-2 ASO (3), DOX and MDR1 ASO (4), and DOX and BCL-2 ASO and MDR1 ASO (5) in multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells. The ordinate shows the IC_{50} doses (drug concentrations that inhibit growth by 50% relative to untreated control cells). Cytotoxicity was measured by modified MTT assay. Means ± SD are shown.

using a conventional microtiter plate reader. The method of calculation and corresponding computer program have been developed and extensively tested (1,2,5,7). Apoptosis induction was analyzed in A2780/AD and MCF-7/AD cells separately incubated with saline (control), free DOX, and liposomes with DOX, DOX and BCL-2 ASO, DOX and MDR1 ASO, DOX and BCL-2 and MDR1 ASO. The concentration of DOX in all series was 8 μM (A2780/AD cells) or 30 μM (MCF-7/AD cells), the difference reflecting the higher resistance of the latter. Two approaches were used to assess apoptosis induction. The first approach was based on measurement of the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in the cell cytoplasm using antihistone and anti-DNA antibodies in a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as previously described (5,20). The method was used to analyze time-dependent (6, 18, 24, and 48 h) apoptosis induction. 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, Nutley, NJ) using a terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described (5,20). After 48 h of incubation with drugs, 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.

Gene Expression

The reverse transcriptase polymerase chain reaction (RT-PCR) was used for the analysis of gene expression in cells incubated with different reagents for 48 h. Ten different series of experiments were performed (Fig. 1). The concentration of DOX (free and liposomal) was 8 μM and 30 μM for A2780/AD and MCF-7/AD cells, respectively. The volume of solutions and the lipid and ASO concentrations were the same in all experimental series. Control cells received an equivalent volume of saline. 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 1 μg of total cellular RNA (from 1×10^7 cells) and 100 ng of random hexadeoxynucleotide primer (Amersham Biosci-

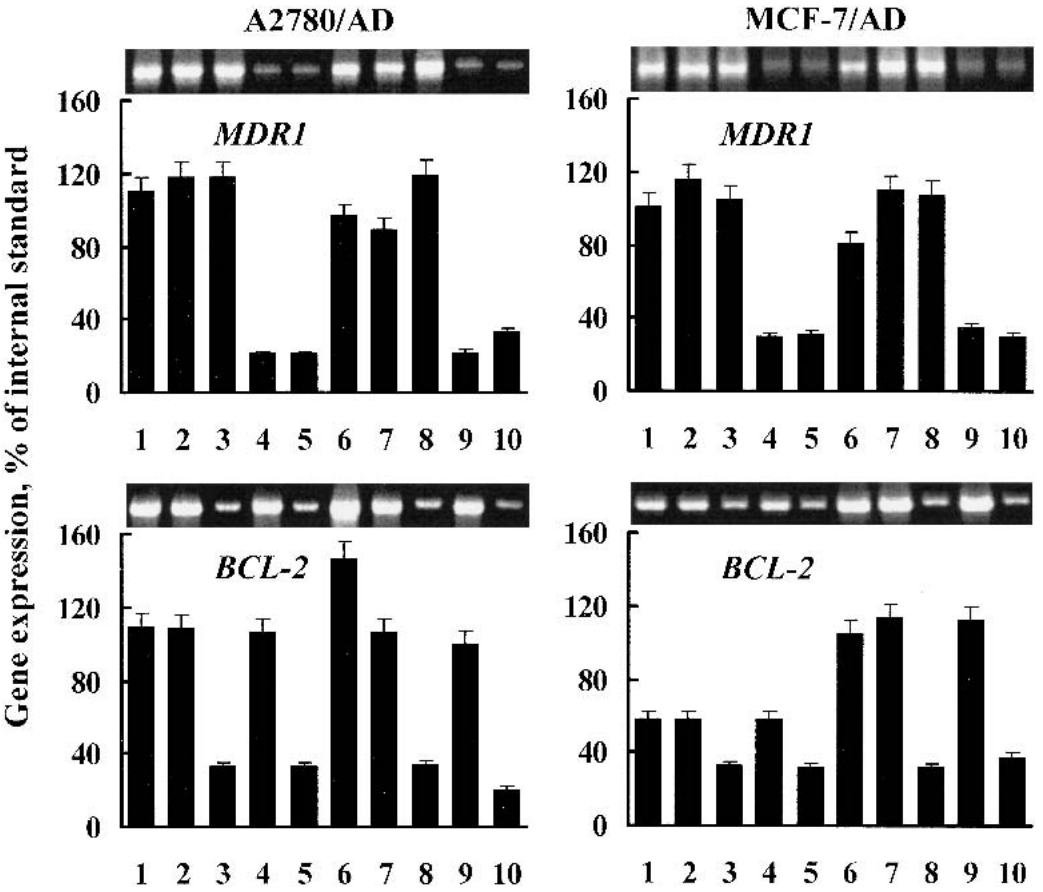

Fig. 3. Typical images of gel electrophoresis of RT-PCR products and expression of genes (percentage of internal standard, β_2-*microglobulin*) encoding P-glycoprotein *(MDR1)* and BCL-2 protein in multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells. Means ± SD are shown. Cells were incubated 48 h with DOX concentrations of 8 (A2780/AD) and 30 (MCF-7/AD) μM. The doses have been adjusted to their respective levels of resistance. 1, Control (untreated cells); 2, empty liposomes; 3, liposomes with BCL-2 ASO; 4, liposomes with MDR1 ASO; 5, liposomes with BCL-2 and MDR1 ASO; 6, free DOX; 7, liposomes with DOX; 8, liposomes with DOX and BCL-2 ASO; 9, liposomes with DOX and MDR1 ASO; 10, liposomes with DOX and BCL-2 and MDR1 ASO.

ences, Piscataway, NJ). After synthesis, the reaction mixture was immediately subjected to PCR, which was carried out using a GenAmp PCR System 2400 (Perkin Elmer, Shelton, CT). The pairs of primers used to amplify each type of cDNA and the PCR regimens have been previously described (5–7) and were: 5′–CCCATCATTGCAATAGCAGG–3′ and 5′–GTTCAAACTTCTGCTCCTGA–3′ (*MDR1*); 5′–GGATTGTGGCCTTCTTTGAG–3′ and 5′–CCAAACTGAGCAGAGTCTTC–3′ (*BCL-2*); 5′–GGGTTTCAGTTGGGAAACAA–3′ and 5′–CACCCAAGAGTCCCAAACAT–3′ (*apoptotic protease activating factor 1*, *APAF-1*); 5′–TGGAATTGATGCGTGATGTT–3′ and 5′–GGCAGGCCTGAATAATGAAA–3′ (*caspase 3*); 5′–TGACTGCCAAGAAAATGGTG–3′ and 5′–CAGCTGGTCCCATTGAAGAT–3′ (*caspase 9*); 5′–ACCCCCACTGAAAAAGATGA–3′ and 5′–ATCTTCAAACCTCCATGATG–3′ (β_2-*microglobulin*, β_2-*m*, internal standard). The PCR regimen was: 94°C/4 min, 55°C/1 min, 72°C/1 min for 1 cycle; 94°C/1 min, 55°C/50 s, 72°C/1 min for 28 cycles, 60°C for 10 min. PCR products were separated in 4% NuSieve 3:1 Reli-

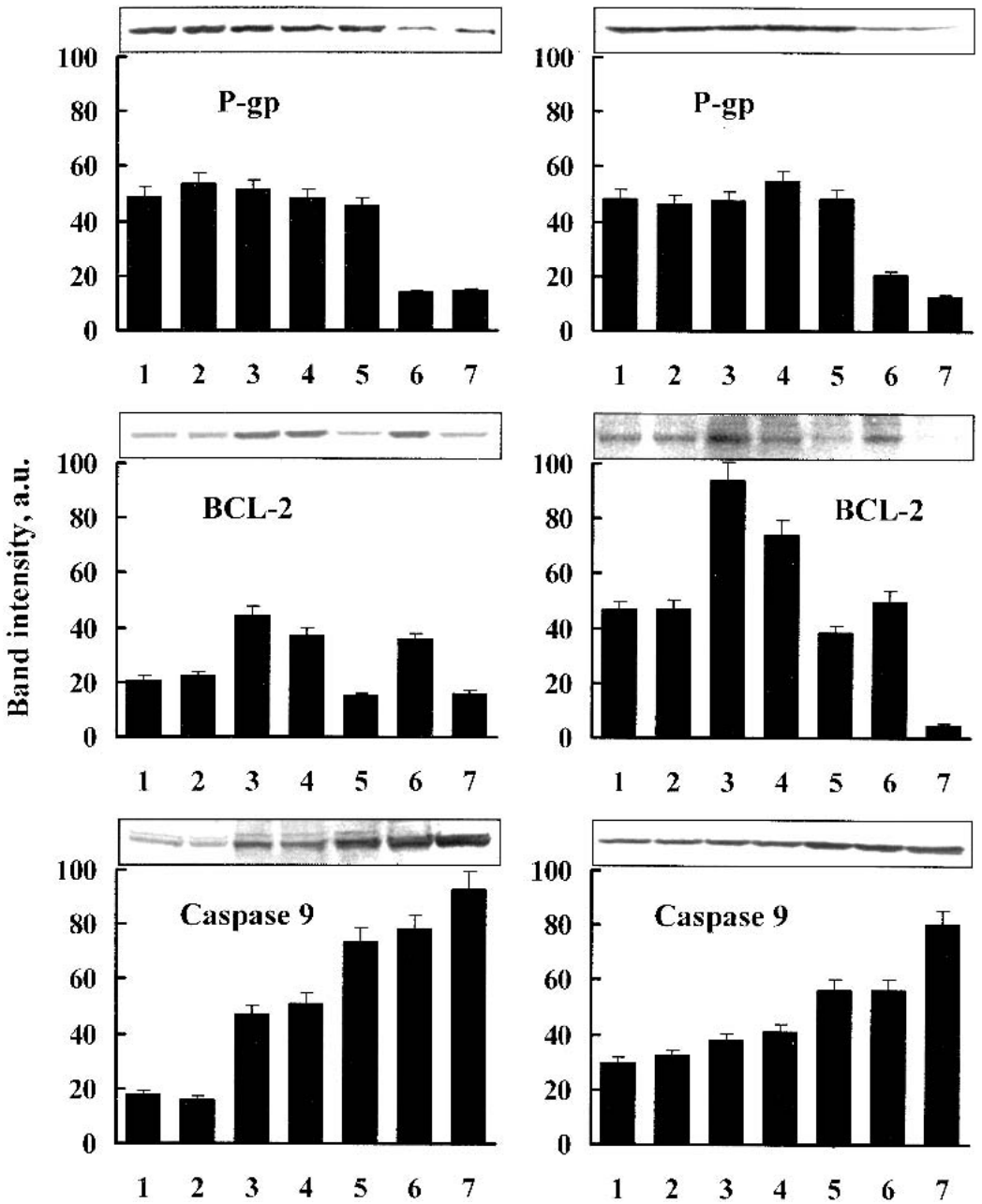

Fig. 4. Typical images of Western blots and average band intensities of P-glycoprotein (P-gp), BCL-2, and caspase 9 proteins in multidrug resistant human ovarian (A2780/AD, left panel) and breast (MCF-7/AD, left panel) cancer cells. Means ± SD are shown. Cells were incubated 48 h with DOX concentrations of 8 (A2780/AD, right panel) and 30 (MCF-7/AD, right panel) μM. The doses have been adjusted to their respective levels of resistance. 1, Control (untreated cells); 2, empty liposomes; 3, free DOX; 4, liposomes with DOX; 5, liposomes with DOX and BCL-2 ASO; 6, liposomes with DOX and MDR1 ASO; 7, liposomes with DOX and BCL-2 and MDR1 ASO.

ant® agarose gels (BMA, Rockland, ME) in 1× TBE buffer (0.089 M Tris/Borate, 0.002 M EDTA, pH 8.3; Research Organics Inc., Cleveland, OH) by submarine electrophoresis. The gels were stained with ethidium bromide, digitally photographed, and scanned using the Gel Documentation System 920 (NucleoTech, San Mateo, CA). Gene expression was calculated as the ratio of mean band density of analyzed RT-PCR product to that of the internal standard (β_2-*m*).

Protein Expression

To confirm RT-PCR data we measured the expression of P-glycoprotein, BCL-2 protein, and caspase 9. The identification of the above proteins was made by Western immunoblotting analysis and processed using scanning densitometry (Gel Documentation System 920, NucleoTech, San Mateo, CA) to quantify the expressed protein.

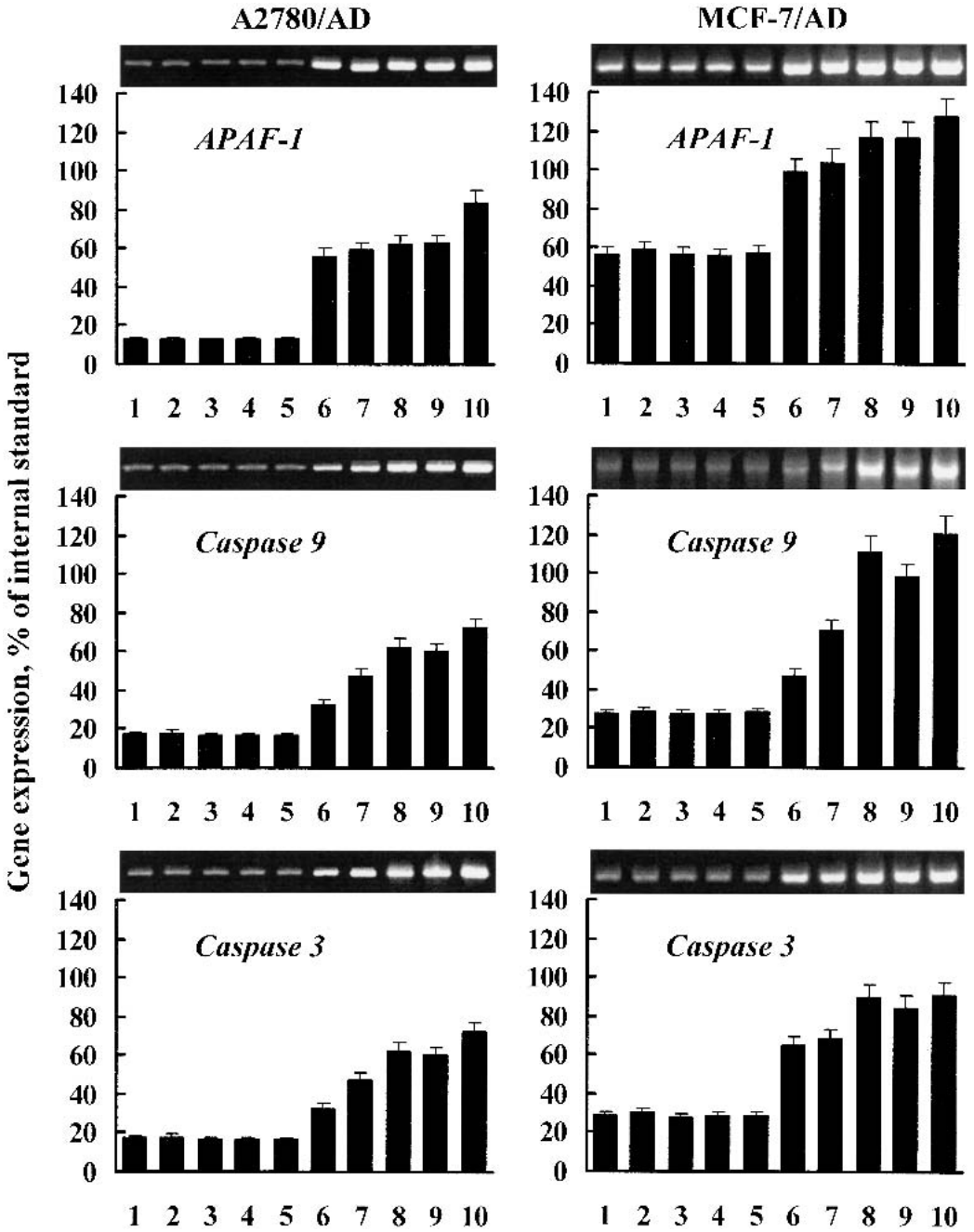

Fig. 5. Typical images of gel electrophoresis of RT-PCR products and expression of genes (percentage of internal standard, β_2-microglobulin) encoding APAF-1, caspase 9, and caspase 3 proteins in multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells. Means ± SD are shown. Cells were incubated 48 h with DOX concentrations of 8 (A2780/AD) and 30 (MCF-7/AD) μM. The doses have been adjusted to their respective levels of resistance. 1, Control (untreated cells); 2, empty liposomes; 3, liposomes with BCL-2 ASO; 4, liposomes with MDR1 ASO; 5, liposomes with BCL-2 and MDR1 ASO; 6, free DOX; 7, liposomes with DOX; 8, liposomes with DOX and BCL-2 ASO; 9, liposomes with DOX and MDR1 ASO; 10, liposomes with DOX and BCL-2 and MDR1 ASO.

To this end, harvested cells were lysed in Ripa buffer (Santa Cruz Biotechnologies) using a needle and syringe. Following incubation on ice for 45 min, the cells were centrifuged at 10,000 *g* for 10 min. Protein content in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), and 50 μg of protein was run on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel immersed in Tris/glycine/SDS buffer (BioRad, Hercules, CA) for 90 min at 70 V. Proteins were transferred to an Immobilon-P nitrocellulose membrane (Millipore, Bedford, MA) in a Tris/glycine buffer (Cat. # 161-0771, BioRad, Hercules, CA) for 90 min at 100 V. The membrane was blocked in nonfat milk for 30 min at room temperature on a rotating shaker to prevent nonspecific binding, washed, and incubated overnight with the rabbit primary antibody, anti-P-glycoprotein (Code No. Z5116, 1:50 dilution, DAKO, Carpinteria, CA), anti-BCL-2 (Cat # AAP 070, 1:280 dilution, Stress Gen Biotechnologies, Victoria State, BC Canada), and anti-caspase 9 (Cat # AAP 070, 1:2000 dilution, Stress Gen Biotechnologies, Victoria State, BC Canada) at 4°C. Following further washing, the membrane was immersed in a goat antirabbit IgG biotinylated antibody (Cat # 1706412, 1:3000 dilution, BioRad, Hercules, CA) at room temperature for 1 h on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (Cat # 1706412, BioRad, Hercules, CA).

Caspase Activity

Direct measurements of caspase activity were made using colorimetric protease assay kits (MBL International, Watertown, MA) as previously described (7,20) after the incubation of cells with saline (control), free DOX, and liposomes with DOX, DOX and BCL-2 ASO, DOX and MDR1 ASO, or DOX and BCL-2 and MDR1 ASO. The concentration of DOX in all series was 8 μM (A2780/AD cells) or 30 μM (MCF-7/AD cells). The assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the substrates X-pNA, where X stands for an amino acid sequence recognized by the specific caspase (DEVD and LEHD for caspases 3 and 9, respectively). The increase in the caspase activity was determined by comparing these results with the level of the untreated control incubated with saline.

Statistical Analysis

Data obtained were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA), and presented as mean value ± SD from four to eight independent measurements.

RESULTS

Delivery of DOX by LDDS Containing BCL-2 and MDR1 ASO Significantly Enhanced Drug Cytotoxicity

Different formulations of LDDS were manufactured and tested (Fig. 1): "empty" liposomes, liposomes containing DOX, BCL-2 ASO, MDR1 ASO, and their combinations. These LDDS in combination with free DOX and saline (control) were used in ten series of experiments to assess cytotox-

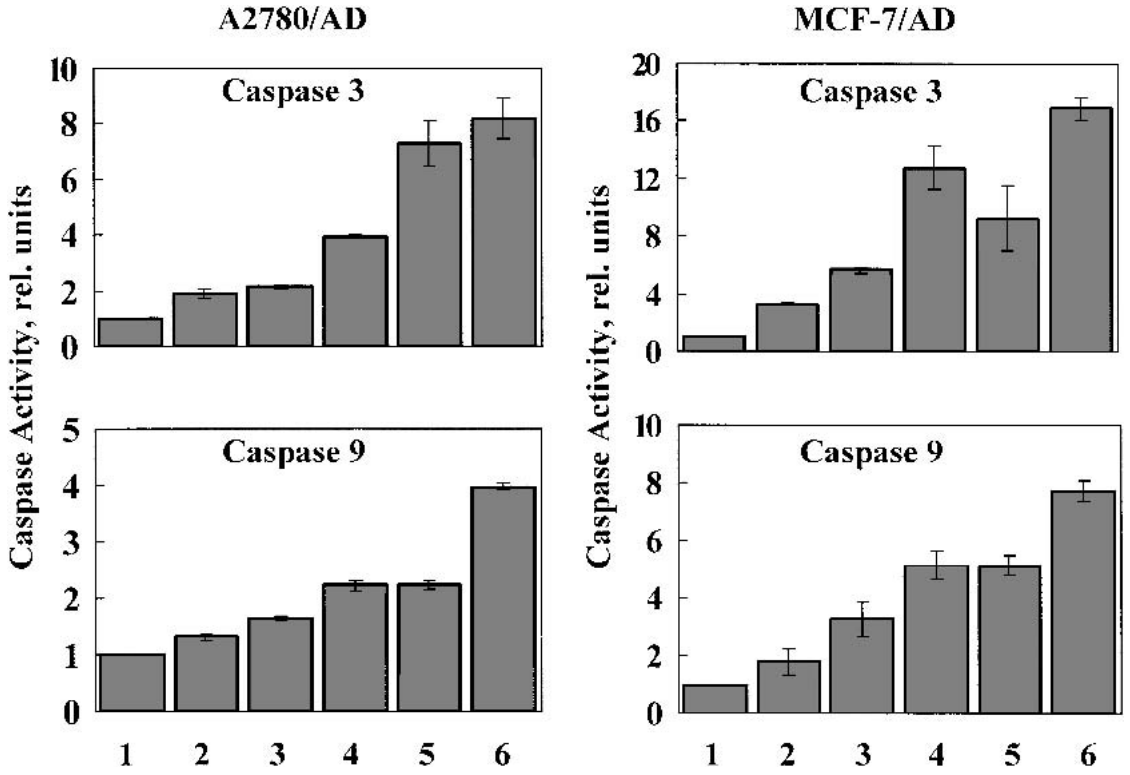

Fig. 6. Activity of caspases 3 and 9 in multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells. Activities in the control (untreated cells) were set to a relative unit of 1. Cells were incubated 48 h with DOX concentrations of 8 (A2780/AD) and 30 (MCF-7/AD) μM. The doses have been adjusted to their respective levels of resistance. Means ± SD are shown. 1, Control (untreated cells); 2, free DOX; 3, liposomes with DOX; 4, liposomes with DOX and BCL-2 ASO; 5, liposomes with DOX and MDR1 ASO; 6, liposomes with DOX and BCL-2 and MDR1 ASO.

icity of individual components and their combinations. Our preliminary experiments showed that free ASO in maximal available concentration did not induce cell death (data not shown). Analysis of cytotoxicity of empty liposomes, liposomes containing DOX, BCL-2 ASO, MDR1 ASO, and their combinations without DOX in maximal concentrations used in the present study were also not toxic in human ovarian and breast cancer cells (data not shown). Encapsulation of DOX in liposomes led to a significant increase in toxicity (3.1- to 4.3-fold) in both types of cancer cells used (Fig. 2). The enhancement in toxicity was achieved by encapsulating ASO against BCL-2 or MDR1 genes separately or in combination in DOX-containing liposomes. The effect was more pronounced in A2780/AD ovarian cancer cells.

Liposomal BCL-2 and MDR1 ASO Suppressed Pump and Nonpump Resistance

To study the influence of different components of LDDS on the pump and nonpump cellular resistance, we analyzed the expression of *MDR1* and *BCL-2* genes and the corresponding proteins — P-gp and BCL-2 — the main players in those types of cellular resistance against anticancer drugs. The data obtained show that empty liposomes did not change the expression of studied genes and proteins (Fig. 3, Lane 2; Fig. 4, Lane 2). ASO targeted to *BCL-2* and *MDR1* genes incorporated separately or in combination into a liposomal delivery system without DOX significantly suppressed the expression of targeted genes in both human cancer cells studied (Fig. 3, Lanes 3, 4, 5). Exposure of human ovarian and breast cancer cells to free DOX activated antiapoptotic cellular defense by the up-regulation of the *BCL-2* gene expression (Fig. 3, Lane 6), leading to the overexpression of BCL-2 protein (Fig. 4, Lane 3). Encapsulation of DOX into liposomes slightly limited this activation (Fig. 3, Lane 7; Fig. 4, Lane 4). Addition of antisense oligonucleotides to LDDS containing DOX led to the suppression of the expression of corresponding genes (Fig. 3, Lanes 8, 9, 10) and proteins (Fig. 4, Lanes 5, 6, 7). It seems that the presence of DOX in the drug delivery system did not significantly change the degree of this suppression (compare Lane 5 to 10 in Fig. 3).

ASO Increased the Ability of DOX to Induce the Caspase-Dependent Pathway of Apoptosis

Despite the suppression of pump and nonpump resistance, liposomal MDR1 and BCL-2 ASO did not induce the caspase-dependent pathway of apoptosis (Fig. 5, Lanes 3, 4, 5). However, in the presence of the apoptosis inducer DOX, ASO increased the degree of activation of this pathway by the drug (compare Lines 8, 9, 10 to 3, 4, 5 on Fig. 5; see Fig. 4, Lines 5, 6, 7). It should be stressed that the combination of ASO targeted to BCL-2 and MDR1 mRNA with DOX led to the significant overexpression of apoptotic protease activation factor-1 (APAF-1) and caspases 3 and 9 themselves. This level of expression cannot be achieved by DOX or combinations of the drug with only one ASO. Similar data were obtained in both types of studied human cancer cells. Quantitative assessment of these observations is best seen in the direct measurement of caspase activity that supports the results of the gene and protein expression study (Fig. 6).

Simultaneous Modulation of Pump and Nonpump Cellular Resistance Enhances the Ability of DOX to Induce Apoptosis

Apoptosis was measured by two independent methods. The results are presented in Figs. 6 and 7. Both methods of apoptosis detection showed that delivery of DOX by liposomes slightly increased its ability to induce apoptosis in human ovarian and breast cancer cells (compare Lines 1 and 2 in Fig. 7 and "DOX" to "Lip-DOX" in Fig. 8). The addition of ASO targeted to the *BLC-2* and *MDR1* genes to LDDS led to the further enhancement of apoptosis (compare Lines 2 to 5 in Fig. 7 and "Lip-DOX" to "Lip-DOX-BCL-2-MDR1 ASO in Fig. 8). The maximum effect was achieved by the combination in one liposomal drug delivery system the anticancer drug with suppressors of pump and nonpump resistance.

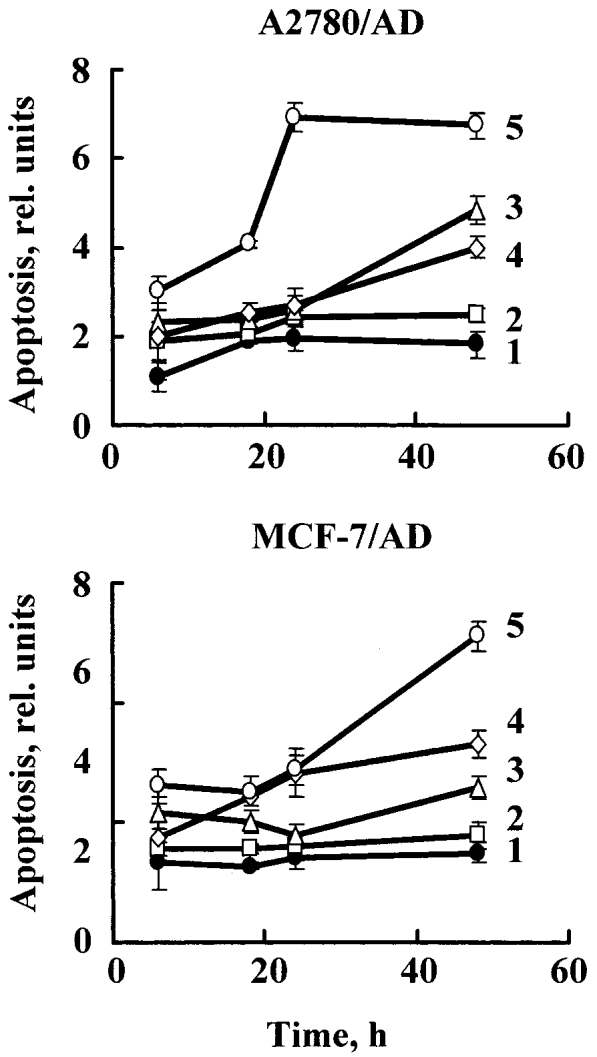

Fig. 7. Time-dependent apoptosis induction by free DOX (1), liposomal DOX (2), and liposomes containing DOX and BCL-2 ASO (3), DOX and MDR1 ASO (4), and DOX, BCL-2 ASO, and MDR1 ASO (5), in multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells. Cells were incubated 6, 18, 24, and 48 h with DOX concentrations of 8 (A2780/AD) and 30 (MCF-7/AD) μM. The doses have been adjusted to their respective levels of resistance. The enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in control cells was set to unit 1, and the degree of apoptosis was expressed in relative units. Means ± SD are shown.

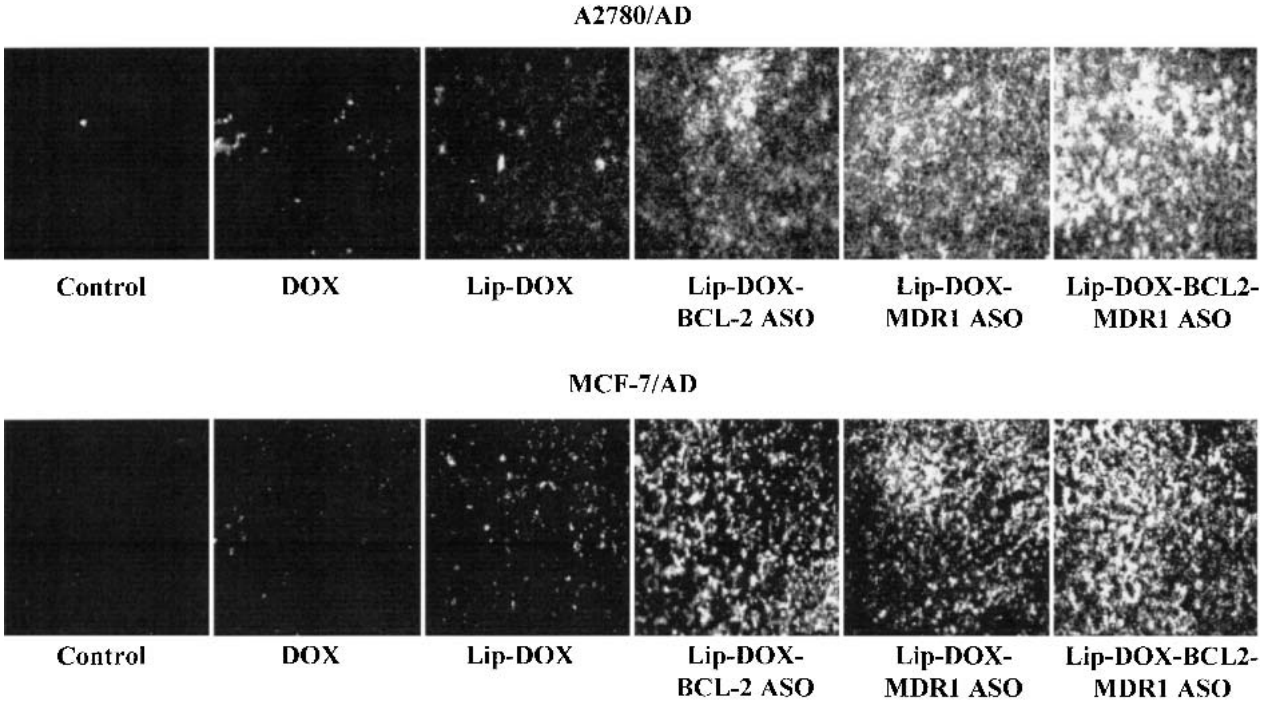

Fig. 8. Typical fluorescence microscopy images of TUNEL-labeled multidrug resistant human ovarian (A2780/AD) and breast (MCF-7/AD) cancer cells after exposure to free DOX (DOX), liposomal DOX (Lip-DOX), and liposomes containing DOX and BCL-2 ASO (Lip-DOX-BCL-2 ASO), DOX and MDR1 ASO (Lip-DOX-MDR1 ASO), and DOX, BCL-2 ASO, and MDR1 ASO (Lip-DOX-BCL-2-MDR1 ASO). Cells were incubated 48 h with DOX concentrations of 8 (A2780/AD) and 30 (MCF-7/AD) μM. The doses have been adjusted to their respective levels of resistance.

DISCUSSION

Three conclusions can be drawn from the present study. First, we confirmed our previous results (2,5–7), which showed that DOX initiates two main effects in cancer cells: (1) induction of cell death by activating of caspase-dependent pathway of apoptosis and (2) simultaneous activation of defensive mechanisms against cell death. In fact, we found that free DOX activated both caspase-dependent pathway of apoptosis and antiapoptotic defense by the overexpression of the *BCL-2* gene and corresponding protein. The latter limits apoptosis induction. This dual action of DOX, which is a feature of almost all traditional chemotherapeutic agents, decreases the anticancer efficacy of the drug and ultimately leads to the development of multidrug resistance.

The second main conclusion from the present study is that the delivery of DOX by the designed liposomal drug delivery system enhanced the cytotoxicity and apoptosis-inducing ability of DOX even without ASO. Although the mechanisms of this effect are unknown and require additional mechanistic study, one can hypothesize that it may be linked to the more efficient lipid-to-lipid fusion and internalization of liposomes across the plasma membrane of the multidrug resistant cells. We have also reported a similar increase in the efficiency of internalization when multidrug resistant human ovarian cancer cells were exposed to water-soluble polymeric DOX (5–7,19). We found that endocytotically internalized DOX attached to a macromolecular platform overcomes the drug efflux pumps, increasing the concentration of the anticancer agent inside the cells, when compared with free drug permeation by simple diffusion. However, this increase in the drug concentration inside the cells did not result in a significant boost of apoptosis because antiapoptotic cellular defenses cannot be suppressed by the delivery of DOX loaded on liposomes.

The third main conclusion from the present study is that only the simultaneous suppression of pump and nonpump resistance is capable of significantly enhancing the anticancer efficacy of a traditional chemotherapeutic drug. It was found that the constructed LDDS containing the anticancer drug and ASO targeted to BCL-2 and MDR1 mRNA led to a multifold increase in cytotoxicity of DOX and its ability to induce apoptosis in multidrug resistant human ovarian and breast cancer cells. It was demonstrated that this was a result of the simultaneous down-regulation of *MDR1* and *BCL-2* genes, which encode a P-glycoprotein drug efflux pump and the BCL-2 protein that is the main player in cellular antiapoptotic defense.

Therefore, the proposed liposomal drug delivery system utilizes a novel three-tier approach, simultaneously attacking three targets: (1) the gene encoding drug efflux pumps to suppress cellular pump resistance and enhance drug retention by cancer cells, (2) mechanisms of apoptosis initiation in order to induce a cell death, and (3) controlling mechanisms of apoptosis in order to suppress cellular antiapoptotic defense. Our approach is dramatically different from previous approaches even though known elements such as DOX, liposomal drug delivery, and antisense oligonucleotides have been used in nonrelated studies. There have been no previous attempts to use LDDS, a delivery system that combines in one drug delivery system an anticancer drug with suppressors of antiapoptotic cellular defense and drug efflux pumps. This is a cellular- and molecular-based two-pronged attack on can-

cer. It prevents multidrug resistance and promotes the cellular pathways leading to death in cancer cells. We expect that the use of this novel complex LDDS will increase the efficacy of ovarian and breast cancer therapy to an extent that cannot be achieved by individual components applied separately.

AKNOWLEDGMENT

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