Chemosensitization of carcinoma cells using epithelial cell adhesion molecule–targeted liposomal antisense against bcl-2/bcl-xL

Sajid Hussain,[1] Andreas Plückthun,[1] Theresa M. Allen,[2] and Uwe Zangemeister-Wittke[1,3]

[1]Department of Biochemistry, University of Zürich, Zürich, Switzerland; [2]Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada; and [3]Department of Pharmacology, University of Bern, Bern, Switzerland

Abstract

Nanoscale drug delivery systems, such as sterically stabilized immunoliposomes binding to internalizing tumor-associated antigens, can increase therapeutic efficacy and reduce toxicity to normal tissues compared with nontargeted liposomes. The epithelial cell adhesion molecule (EpCAM) is of interest as a ligand for targeted drug delivery because it is abundantly expressed in solid tumors but shows limited distribution in normal tissues. To generate EpCAM-specific immunoliposomes for targeted cancer therapy, the humanized single-chain Fv antibody fragment 4D5MOCB was covalently linked to the exterior of coated cationic liposomes. As anticancer agent, we encapsulated the previously described antisense oligonucleotide 4625 specific for both bcl-2 and bcl-xL. The EpCAM-targeted immunoliposomes (SIL25) showed specific binding to EpCAM-overexpressing tumor cells, with a 10- to 20-fold increase in binding compared with nontargeted control liposomes. No enhanced binding was observed on EpCAM-negative control cells. On cell binding, SIL25 was efficiently internalized by receptor-mediated endocytosis, ultimately leading to down-regulation of both bcl-2 and bcl-xL expression on both the mRNA and protein level, which resulted in enhanced tumor cell apoptosis. In combination experiments, the use of SIL25 led to a 2- to 5-fold sensitization of EpCAM-positive tumor cells of diverse origin to death induction by doxorubicin. Our data show the promise of EpCAM-specific drug delivery systems, such as antisense-loaded immunoliposomes, for targeted cancer therapy. [Mol Cancer Ther 2006;5(12):3170–80]

Received 7/14/06; revised 9/14/06; accepted 10/18/06.

Grant support: Krebsliga Zürich and the Sassella-Stiftung of the Zürich Kantonalbank.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Uwe Zangemeister-Wittke, Department of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland. Phone: 41-31-632-3290; Fax: 41-31-632-4992. E-mail: uwe.zangemeister@pki.unibe.ch

doi:10.1158/1535-7163.MCT-06-0412

Introduction

Diminished apoptosis enables cell survival under abnormal growth conditions and mediates resistance to various forms of cellular stress, including DNA damage and inhibition of growth factor signaling (1, 2). Apoptosis deficiency is thus inevitably associated with resistance to a broad spectrum of conventional and novel targeted anticancer agents (3, 4). The core cell death machinery is regulated by the opposing action of bcl-2 family members (2, 5). Antiapoptotic members include bcl-2, bcl-xL, bcl-w, and mcl-1, whereas proapoptotic proteins comprise bax, bak, and the BH3-only members bad, bid, Noxa, and Puma (6, 7). Overexpression of the partially redundant bcl-2 and bcl-xL proteins is frequent in human tumors and is correlated with drug resistance (8, 9). Therefore, targeted inhibition of bcl-2 and bcl-xL expression or function has the potential to facilitate tumor cell apoptosis and sensitize cells to a broad spectrum of anticancer agents.

Antisense technology has shown efficacy for targeted cancer therapy in preclinical and clinical studies (10–13). The promise of antisense therapy is best shown by the bcl-2 antisense oligonucleotide G3139 (oblimersen, Genasense), which, in combination with dacarbazine, achieved significantly higher response rates compared with patients treated with chemotherapy alone (14). Moreover, G3139 in combination with dexamethasone and thalidomide has also shown promising response rates in patients with relapsed multiple myeloma (15). In recent years, the functional improvements due to new chemical derivatives of oligonucleotides, the advent of small interfering RNA, and the identification of novel target genes involved in the malignant process have substantially increased the performance of antisense therapeutics (10, 11, 13). We previously described the ability of the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 to facilitate apoptosis in tumor cells of various histotypes *in vitro* and *in vivo* (16, 17). Thus, antisense oligonucleotide 4625 may be particularly advantageous for use against tumors in which both oncoproteins are overexpressed together.

On the other hand, several antisense oncology trials could not fulfill the initial expectations, which has raised doubts and speculations about the true clinical potential of this technology (11). The low performance of first-generation compounds as well as inappropriate patient selection and trial design may have all contributed to this setback. In addition, the lack of efficient drug delivery has been identified as another major reason for this failure and that of many other anticancer agents. To improve the efficacy of cancer therapy, drug delivery systems made of lipid-based vectors, such as cationic liposomes and neutral liposomes, have been used to improve the intracellular

delivery of anticancer agents, including antisense oligonucleotides (18, 19). Particularly promising are liposomes coated with lipid derivatives of polyethylene glycol (PEG), which show reduced plasma clearance and, presumably due to the extended drug half-life, improved accumulation in target tissues (20) even when not guided by a tumor-targeting ligand. These sterically stabilized liposomes can be surface modified with tumor-selective ligands, such as antibodies, to produce immunoliposomes for targeted drug delivery (21, 22). In previous studies, immunoliposomes were successfully used for the delivery of bcl-2, MDR1, and c-myc antisense oligonucleotides to tumors *in vivo* (23–25).

Various tumor-associated antigens have been validated as targets for antibody-based cancer therapy (26). The epithelial cell adhesion molecule (EpCAM) is a 40-kDa transmembrane glycoprotein involved in Ca^{2+}-independent cell adhesion and thought to trigger cell proliferation by activation of the c-*myc* oncogene (27, 28). It is abundantly expressed in many solid tumors, shows limited expression in normal epithelial tissues (29), and is a negative prognostic factor associated with disease progression and poor overall survival (30, 31). These properties suggest the use of EpCAM as a target for highly selective cancer therapy (32).

Here, we describe a new liposomal formulation that targets EpCAM and delivers the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 as a proapoptotic payload to tumor cells. The ability of this drug delivery system to enhance tumor cell apoptosis and efficiently sensitize cells to chemotherapy in an EpCAM-specific manner is shown.

Materials and Methods

Cell Lines and Reagents

The breast adenocarcinoma cell line MCF-7, the ovarian cancer cell line OVCAR-3, and the non–Hodgkin's lymphoma cell line RL were obtained from the American Type Culture Collection (Manassas, VA). The small cell lung cancer cell line SW2 was grown in our laboratory. Adherent MCF-7 and OVCAR-3 cells were grown in DMEM (Invitrogen, Carlsbad, CA), and nonadherent SW2 and RL cells were grown in RPMI 1640 (Invitrogen). Culture media were supplemented with 10% FCS (Perbio Science S.A., Lausanne, Switzerland), 2 mmol/L L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin, and cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Hydrogenated soy phosphatidylcholine, cholesterol, *N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), PEG-derivatized (M_r 2,000) distearoylphosphatidylethanolamine (DSPE-PEG_{2000}), and a coupling lipid that is a derivative of DSPE-PEG_{2000}, with a maleimide group at the distal terminus of the PEG chain (DSPE-PEG_{2000}-maleimide), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). [^{3}H]cholesteryl hexadecyl ether ([^{3}H]CHE) was purchased from Perkin-Elmer (Boston, MA). Tetramethylrhodaminethiocarbamoyl-labeled phosphatidylethanolamine (TRITC-PE) was purchased from Invitrogen. Sepharose CL-4B exclusion resin was from Amersham Biosciences (Uppsala, Sweden), and Bio-Spin 6 chromatography columns were purchased from Bio-Rad (Hercules, CA). All other reagents of analytic and molecular biology grade were obtained from Sigma-Aldrich (St. Louis, MO).

Antisense Oligonucleotide

The 2′-*O*-(2-methoxy)ethyl–modified ''gapmer'' antisense oligonucleotide 4625 and the scrambled sequence control oligonucleotide 4626 have been described (16, 17). Both oligonucleotides are 20-mers with a phosphorothioate backbone throughout and with 2′-*O*-(2-methoxy)ethyl modifications in the five ribose residues of the flanking regions (italicized). Antisense oligonucleotide 4625 with the sequence 5′-*AAGGC*ATCCCAGCCT*CCGTT*-3′ is fully complementary to the bcl-2 mRNA and has three mismatches to the bcl-xL mRNA. The control oligonucleotide 4626 has the sequence 5′-*CACGT*CACGCGCGCA*CTATT*-3′. Italicized letters represent 2′-*O*-(2-methoxy)ethyl–modified nucleotides.

Generation of Coated Cationic Liposomes

Coated cationic liposomes (CCL) were prepared as described (24, 33) with slight modifications. In a glass tube, 1.33 μmol DOTAP dissolved in 0.25 mL $CHCl_3$ was first mixed with 0.51 mL methanol followed by addition of 500 μg oligonucleotide (70 nmol) dissolved in 0.25 mL double-distilled water. The mixture was vortexed gently and incubated at room temperature for 30 min. Following incubation, 0.25 mL $CHCl_3$ and 0.25 mL double-distilled water were added, gently vortexed, and centrifuged for 7 min at 830 × *g*. After separation of the two phases by centrifugation, the amount of oligonucleotide in the aqueous phase was measured by absorption at 260 nm. To the organic phase (~1 mL), 5.32 mol hydrogenated soy phosphatidylcholine, 2.66 mol cholesterol, and 0.26 mol DSPE-PEG_{2000} were added to result in a molar ratio of 4:2:0.2 (relative to DOTAP). For immunoliposomes (described below), only 0.213 mol DSPE-PEG_{2000} was used in addition to 0.053 mol DSPE-PEG_{2000}-maleimide to obtain the identical total molarity of PEG. Thereafter, 0.4 mL double-distilled water was added to obtain a 10 mmol/L lipid concentration, and the mixture was vortexed and sonicated in a water bath sonicator for 30 min to form a homogenous emulsion. After $CHCl_3$ was evaporated in a rotatory evaporator (Büchi, Flawil, Switzerland), the liposomes were reduced in size by extruding through 200 and 100 nm polycarbonate filters (Whatman plc, Middlesex, United Kingdom) in a Lipex Extruder (Northern Lipids, Inc., Vancouver, British Columbia, Canada). The liposome size was confirmed by dynamic light scattering using a Malvern Zetasizer 5000 (Malvern Instruments, Malvern, Worcestershire, United Kingdom). The CCLs were finally passed through a Sepharose CL-4B column to separate free from encapsulated oligonucleotides (24).

Conjugation of a Single-Chain Fv Antibody to Liposomes

The generation and tumor-targeting properties of the EpCAM-specific single-chain Fv (scFv) 4D5MOCB have been described previously (34). Purified 4D5MOCB was

engineered to contain a COOH-terminal cysteine, which was preceded by a glycine linker after the His tag and followed by the stop codon (His_5-Gly_2-Cys-Stop), and produced in *Escherichia coli* and purified as previously described for the unmodified scFv (34). The protein was then reduced with 2 mmol/L DTT for 45 to 60 min at 37°C to obtain monomeric scFv-SH yet keeping the intradomain disulfides intact. The monomeric scFv antibody fragment was separated from DTT on a Bio-Spin 6 desalting column pre-equilibrated with HBS [25 mmol/L HEPES, 150 mmol/L NaCl (pH 6.5)] and covalently linked to preformed liposomes containing maleimide groups. scFv was added to phospholipids in a molar ratio of 1:500 to 1:700 (typically 5 nmol scFv to 2.70 μmol phospholipids). The free COOH-terminal thiol group of the scFv forms a thioether linkage with the maleimide groups at the distal termini of the PEG chains (35). After overnight coupling at room temperature, unreacted maleimide was quenched by reduction with 2 mmol/L β-mercaptoethanol for 30 min at room temperature. The scFv-conjugated liposomes were separated from unconjugated scFv on a Sepharose CL-4B size exclusion column (1.5 × 30 cm) pre-equilibrated with HBS [25 mmol/L HEPES, 140 mmol/L NaCl (pH 7.4)]. The amount of scFv conjugated to the liposomes was quantified by adding trace amounts of ^{125}I-labeled scFv to the coupling reaction (33).

CCLs with surface-bound 4D5MOCB and encapsulated antisense oligonucleotide 4625 or scrambled sequence control oligonucleotide 4626 are referred to as EpCAM-targeted immunoliposomes and abbreviated SIL25 or SIL26, respectively. CCLs without surface-bound antibody are referred to as nontargeted liposomes and abbreviated CCL25 or CCL26.

Cell Binding and Internalization of Liposomes

EpCAM-positive (MCF-7, OVCAR-3 and SW2) and EpCAM-negative (RL) tumor cells were mixed with various concentrations of [^{3}H]CHE-labeled targeted or nontargeted liposomes, diluted in cell culture medium, and incubated either on ice or at 37°C for 2 h. Cells were washed with PBS and lysed with 1 mol/L NaOH before radioactivity was measured. In competition binding experiments, a 50- to 60-fold excess of free 4D5MOCB scFv was added 30 min before addition of the targeted liposomes (33).

Internalization of liposomes was monitored by fluorescence confocal microscopy as described (36) with modifications. MCF-7 cells were plated in poly-L-lysine-coated Lab-Tek chambered cover glasses (Nalge Nunc, Rochester, NY) at 1 × 10^5 cells per chamber and allowed to grow overnight. Fluorescein-labeled antisense oligonucleotide 4625, encapsulated in CCLs (nontargeted or EpCAM targeted) labeled with 0.1 mol% TRITC-PE (relative to phospholipids), was added to the cells at a final phospholipid concentration of 0.4 mmol/L and an oligonucleotide concentration of 5 μmol/L. After 3 h of incubation at 37°C, cells were washed thrice with PBS and incubated for another 2 h in complete medium without liposomes. Cells were washed again with PBS before fixation with 4% paraformaldehyde for 20 min at room temperature. Thereafter, cells were rinsed with PBS, mounted in Mowiol containing 2.5% (w/v) 1-4-diazabicyclo[2.2.2]octane (Fluka, Buchs, Switzerland) as an antifading agent, and kept in the dark at 4°C before confocal microscopy. The internalization of TRITC-PE-labeled liposomes (CCL25 or SIL25) encapsulating fluorescein-labeled antisense oligonucleotide 4625 was monitored using a confocal laser scanning microscope (TCS-SP 2, Leica, Mannheim, Germany) equipped with a Leica DM IRB/E inverted microscope and a 40× oil immersion objective (NA 1.25). Images were processed using Imaris 3D software (Bitplane AG, Zurich, Switzerland), and layouts were processed using Adobe Photoshop software.

Real-time PCR

MCF-7 and SW2 cells were cultured in six-well plates at a density of 2.5 × 10^5 cells per well. Antisense oligonucleotide 4625 or control oligonucleotide 4626 was added to the cells in the form of either nontargeted liposomes (CCL25/CCL26) or EpCAM-targeted immunoliposomes (SIL25/SIL26) at an initial oligonucleotide concentration of 40 μg/mL. Control cells were treated with the same volume of complete medium. At 4 h after treatment, cells were rinsed with PBS and transferred to liposome-free medium. After 20 h, cells were incubated with the same formulations at the same oligonucleotide concentration for 4 h before cells were washed and transferred to fresh medium. Cells were harvested for mRNA quantification after another 20 h of incubation.

Total RNA was isolated from cells by use of the RNeasy Mini kit (Qiagen, Basel, Switzerland). For cDNA synthesis, 0.5 μg of extracted RNA and Taqman Reverse Transcription Reagents was used as described in the user's manual of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). This system was used for real-time monitoring of PCR amplification of the cDNA following the Taqman Universal PCR Master Mix protocol (Perkin-Elmer Applied Biosystems). Previously described primers and probes were used for the amplification of bcl-2 and bcl-xL cDNAs (17). Relative quantification of gene expression was done by the ΔC_t method using rRNA as an internal standard as described in the user's manual.

Western Blotting

MCF-7 and SW2 cells were plated in tissue culture flasks (25 cm^2) and treated with nontargeted liposomes (CCL25/CCL26) or EpCAM-targeted immunoliposomes (SIL25/SIL26) at an oligonucleotide concentration of 40 μg/mL. After 4 h, cells were washed with PBS and transferred to fresh medium. Treatments were repeated after 20 and 48 h for the same duration using the same formulations and oligonucleotide concentrations. At 72 h, cells were harvested and bcl-2 and bcl-xL protein levels were determined by Western blotting as described (37). Briefly, 50 μg of soluble total protein per sample, as quantified by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), were separated on 12% polyacrylamide SDS gels and transferred to polyvinylidene fluoride membranes by semidry blotting for 1 h. The membranes were blocked

in TBS containing 5% bovine serum albumin and 5% nonfat milk and then incubated overnight at 4°C with mouse anti-human bcl-2 monoclonal antibody (DAKO Diagnostics AG, Glostrup, Denmark) or rabbit anti-human bcl-xL monoclonal antibody (Transduction Laboratories, Lexington, KY). Actin staining with mouse anti-human actin antibodies (ICN Biomedicals, Aurora, OH) was used as loading control. Primary antibodies were detected by incubation with secondary polyclonal antibodies conjugated to peroxidase (Sigma-Aldrich), and visualization of immunocomplexes was done by enhanced chemiluminescence using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein bands were quantified using the NIH ImageJ software[4] and normalized to the respective β-actin bands. The band intensity of untreated control cells was arbitrarily taken as 100%.

Measurement of Cell Viability and Apoptosis

Cell viability was measured using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After a 24-h drug treatment with targeted and nontargeted liposomes at an oligonucleotide concentration of 5 μmol/L, the MTT reagent (Sigma-Aldrich) was added (10 μL/well of a 10 mg/mL solution in PBS) and allowed to react for 90 min at 37°C before the addition of 100 μL/well solubilization reagent (20% SDS in dimethyl formamide). Plates were incubated overnight, and absorbance was measured at 570 nm using a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA).

Apoptosis was measured by quantification of caspase-3-like protease activity. SW2 cells were treated with the various liposome formulations (CCL25, SIL25 or SIL26) at an oligonucleotide concentration of 5 μmol/L. After 4 h, cells were washed with PBS and transferred to fresh medium. Twenty hours thereafter, cells were again treated with the same liposomal formulations and oligonucleotide concentrations for the same duration, washed with PBS, and transferred to medium containing 0.5 μmol/L doxorubicin. Control cells were treated neither with liposomes nor with doxorubicin. Cells were harvested after a 24 h drug treatment, and caspase-3-like protease activity was measured in a colorimetric assay by use of a chromogenic peptide derivative according to the manufacturer's instructions (Alexis Biochemicals, Lausanne, Switzerland). Briefly, cells were lysed for 10 min on ice and centrifuged at 10,000 × *g* at 4°C for 1 min. Soluble cytosolic protein (50 μg) was quantified and mixed with caspase-3-specific substrate DEVD-*p*-nitroaniline in a final volume of 100 μL (38). After incubation at 37°C, substrate cleavage was monitored at 405 nm using a SpectraMax 340 microplate reader. The increase in caspase-3-like activity in antisense-treated cells was calculated relative to untreated control cells.

Determination of Chemosensitization

MCF-7 and SW2 cells were seeded in 96-well plates (1×10^4 or 2×10^4 cells per well, respectively) and cultured overnight. Treatment was done using targeted immunoliposomes (SIL25/SIL26) or nontargeted liposomes (CCL25) at an oligonucleotide concentration of 40 μg/mL. For untreated control cells, the same volume of culture medium without oligonucleotides was added. Transfection was terminated after 6 h, and cells were washed with PBS and further incubated in standard cell culture medium. After 18 h, the medium was replaced with medium containing the indicated concentrations of doxorubicin, and cells were incubated for another 24 h before viability was determined in MTT assays.

Statistical Analysis

The ΔC_t values obtained by real-time PCR analysis of the various treatment groups were statistically evaluated using repeated measure one-way ANOVA, and pairwise comparisons were made by Bonferroni's multiple comparison post test. Student's *t* test was used to determine the significance of the IC_{50} values as well as the increase in caspase-3-like activity obtained from cells exposed to different combinations of antisense-loaded liposomes and doxorubicin. Statistical evaluation of the data and *P* values were obtained using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). For all statistical evaluations, $P < 0.05$ was considered significant.

Results

Characterization of Liposome Preparations

CCLs prepared by the procedure previously described (24) had an average diameter of 125 ± 3 nm (polydispersity 0.108 ± 0.025) as determined by dynamic light scattering. The coupling efficiency of the scFv antibody fragment 4D5MOCB to the liposome surface was 50% to 65% relative to the total amount of scFv used (as determined by trace labeling the antibody with ^{125}I), which resulted in 1 to 2 nmol scFv/μmol phospholipids. The efficiency of oligonucleotide encapsulation was >80% (from A_{260}). Assuming spherical unilamellar monodisperse liposomes with 10^{13} liposomes per μmol phospholipid (39), they thus contained ~10^2 molecules of scFv and 10^3 molecules of oligonucleotide and 19,000 molecules of DOTAP (the molar ratios of the other lipid components is given in Materials and Methods). CCLs produced by this procedure were found to be stable without leakage of oligonucleotides (33), which was confirmed by our own data (not shown).

Binding and Endocytosis of Antisense-Loaded Immunoliposomes in EpCAM-Positive Tumor Cells

To serve as effective ligand-targeted delivery systems for antisense oligonucleotides and other anticancer agents, immunoliposomes must bind to the target antigen and become internalized (21). The tumor-associated antigen EpCAM is abundantly expressed in solid tumors (29), and we previously showed that the scFv 4D5MOCB specifically binds to EpCAM-positive tumor cells with high affinity (34). To determine the cellular association of [^{3}H]CHE-labeled liposomes with tumor cells, the

[4] http://rsb.info.nih.gov.

EpCAM-positive tumor cell lines OVCAR-3, MCF-7 and SW2, and the EpCAM-negative lymphoma control cell line RL were incubated with EpCAM-targeted or nontargeted liposomes at 4°C or 37°C. Cellular association was assessed by measuring the phospholipid uptake from liposomes. As shown in Fig. 1, the level of binding to EpCAM-positive cells increased with increasing liposome concentrations. With the exception of MCF-7 cells, there were no signs of saturation at the phospholipid concentrations tested. At 4°C, anti-EpCAM immunoliposomes showed a 6- to 10-fold higher binding to EpCAM-positive cells compared with nontargeted liposomes. When these target cells were incubated with the anti-EpCAM immunoliposomes at 37°C, the levels of cellular binding increased 12- to 20-fold compared with nontargeted liposomes (Fig. 1). This increase in cellular binding at 37°C indicates that the immunoliposomes were actively internalized into cells by receptor-mediated endocytosis. The lack of saturation binding suggests that the receptor was recycled back to the cell surface, as the molar concentration of scFv in the liposomes (estimated to be up to 375 nmol/L) was up to 100-fold above the K_D (34) and presumably up to 9,000-fold above the concentration of EpCAM on these cells (assuming about 0.5×10^6 EpCAM molecules per cell; ref. 40). For EpCAM-negative RL cells, there was no temperature-related difference in the levels of cellular binding for targeted and nontargeted liposomes (Fig. 1), and the level was as low as with the negative controls (no scFv on liposomes or inhibition by soluble scFv) on EpCAM-positive cells. Together, these data indicate that the increased uptake in EpCAM-positive cells was mediated by antigen binding of the scFv.

The specificity of EpCAM-targeted immunoliposomes was also determined in competition binding experiments. Figure 1 shows that the addition of a 50- to 60-fold excess of free scFv 4D5MOCB (final concentration of 20 μmol/L, ~5,000-fold above the K_D) at 30 min before the addition of immunoliposomes completely blocked their cellular association and uptake. The negligible binding of nontargeted liposomes to EpCAM-positive cells and the ability to inhibit cell binding of our immunoliposomes with free scFv 4D5MOCB confirm their high antigen specificity.

Intracellular Distribution of Antisense-Loaded Immunoliposomes in EpCAM-Positive Tumor Cells

Internalization of the EpCAM-targeted immunoliposomes was also evaluated by confocal fluorescence microscopy. To this end, fluorescein-labeled oligonucleotides were encapsulated into targeted or nontargeted liposomes, which were fluorescently labeled with the lipid label TRITC-PE. EpCAM-positive MCF-7 cells were incubated with the liposomes for 3 h at 37°C. Cells treated with immunoliposomes showed internalization of both the oligonucleotides (green fluorescence; Fig. 2A) and the labeled phospholipids (red fluorescence; Fig. 2B). In contrast, cells treated with the nontargeted control liposomes

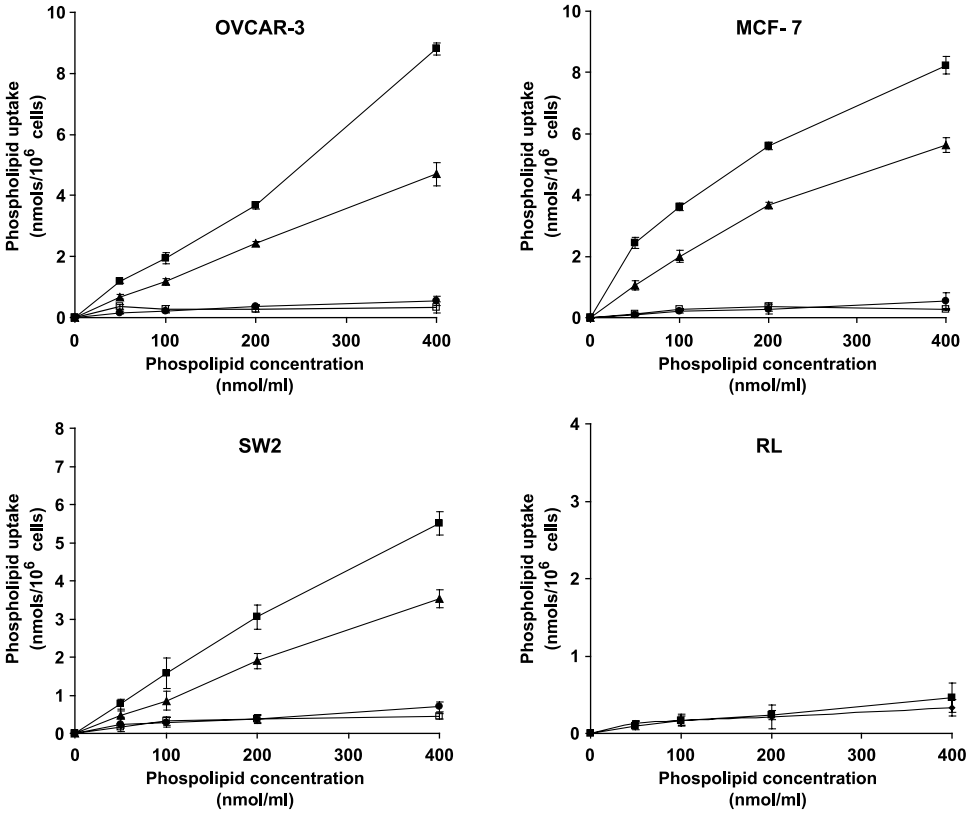

Figure 1. Binding and uptake of [^{3}H]CHE-labeled antisense-loaded immunoliposomes and nontargeted liposomes in EpCAM-positive (OVCAR-3, MCF-7 and SW2) and EpCAM-negative (RL) cells as a function of liposome concentration. Binding and cellular uptake of EpCAM-targeted immunoliposomes encapsulating antisense oligonucleotide 4625 were measured after incubation of cells for 2 h at 4°C (▲) or 37°C (■). Nontargeted liposomes (●) were incubated with tumor cells for 2 h at 37°C. In competition binding experiments, EpCAM-positive tumor cells were incubated with a 50- to 60-fold excess of free scFv 4D5MOCB for 30 min before addition of the targeted immunoliposomes (□). *Points,* nmol [^{3}H]CHE-phospholipids/10^6 cells (n = 3); *bars,* SD.

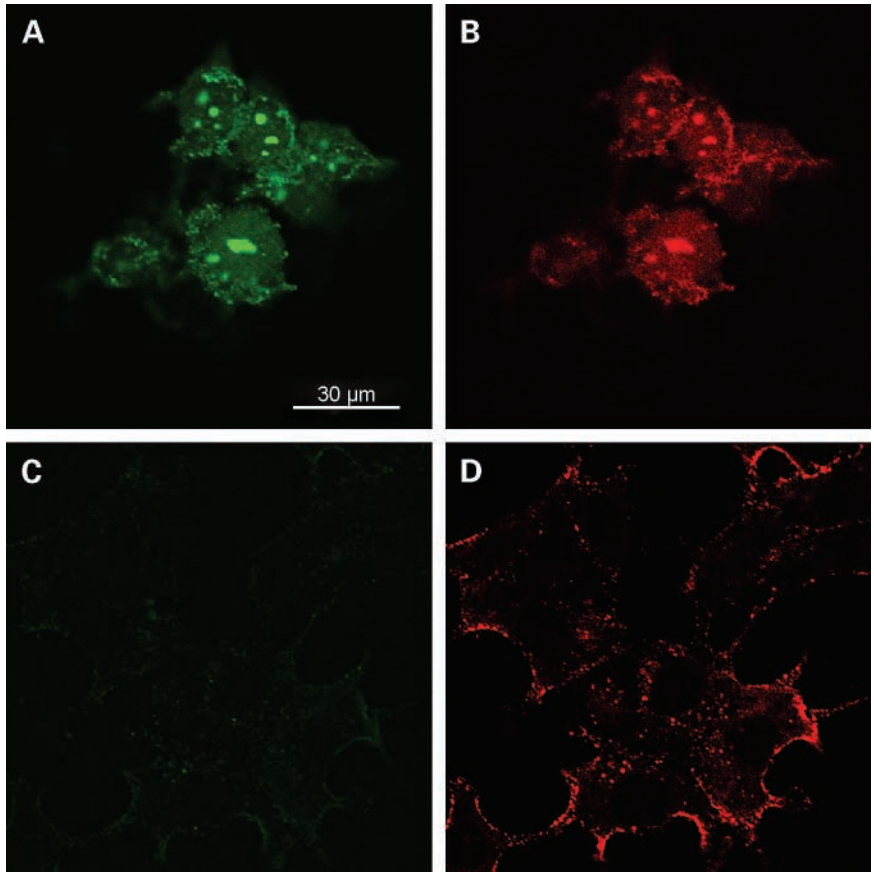

Figure 2. Confocal microscopy images of antisense-loaded immunoliposome internalization in EpCAM-positive MCF-7 cells. Tumor cells were incubated with TRITC-PE-labeled liposomes encapsulating fluorescein-labeled antisense oligonucleotide 4625 for 3 h at 37°C. The confocal micrographs are shown for EpCAM-targeted immunoliposomes (**A** and **B**) and for nontargeted control liposomes (**C** and **D**). The lipid label TRITC-PE on liposomes is displayed as red fluorescence, and the fluorescein-labeled antisense oligonucleotide is displayed as green fluorescence.

showed no significant uptake or internalization as shown by the faint green background fluorescence and the red fluorescence limited to the cell surface (Fig. 2C and D). On superimposing the images of Fig. 2A and B, the green oligonucleotide fluorescence and the red liposome fluorescence colocalized within the cell (data not shown), which is consistent with an endosomal/lysosomal staining pattern. Some cytoplasmic staining also occurred, indicating that the immunoliposomes were able to deliver intact bcl-2/bcl-xL antisense oligonucleotides into cells, with some part of it escaping from the endosomal/lysosomal compartment.

Antisense-Loaded Immunoliposomes Down-regulated bcl-2 and bcl-xL Expression in EpCAM-Positive Tumor Cells

We previously showed that oligonucleotide 4625 efficiently inhibits the expression of the bcl-2 and bcl-xL mRNA and protein in tumor cells of diverse histotypes (16, 17). To determine the ability of liposomal-encapsulated oligonucleotide 4625 (EpCAM targeted and nontargeted) to down-regulate both bcl-2 and bcl-xL mRNA, MCF-7 and SW2 cells were treated for 4 h with liposomes containing a dose of 40 μg/mL liposome-encapsulated oligonucleotide 4625. Cells were then transferred to oligonucleotide-free medium, and the same dose of encapsulated oligonucleotide was used for a repeat treatment at 20 h. The sequence specificity of the antisense effect was controlled by delivery of the scrambled sequence control oligonucleotide 4626 in identical liposome formulations. As shown by real-time PCR, in MCF-7 cells, the EpCAM-targeted immunoliposomes loaded with oligonucleotide 4625 (SIL25) down-regulated bcl-2 and bcl-xL expression by approximately 70 ± 2.5% and 60 ± 5% ($n = 3$; $P < 0.001$), respectively (Fig. 3A). In SW2 cells, down-regulation of the two mRNA species was 75 ± 3% and 60 ± 3.5% ($n = 3$; $P < 0.001$), respectively (Fig. 3B). In contrast, EpCAM-targeted immunoliposomes loaded with 4626 (SIL26) and the nontargeted liposomes loaded with 4625 (CCL25) or 4626 (CCL26) did not down-regulate bcl-2 and bcl-xL expression in the cell lines (Fig. 3A and B). These findings show the ability of SIL25 to deliver and release fully functional antisense oligonucleotide 4625 into the cytoplasm from where it translocates to the nucleus to hybridize to the target mRNAs.

The ability of EpCAM-targeted immunoliposomes SIL25 to down-regulate the bcl-2 and bcl-xL protein in EpCAM-positive tumor cells was examined by Western blotting, and band intensities in treated cells relative to control untreated cells were quantified by using the NIH ImageJ software. As shown in Fig. 4, treatment with SIL25 significantly reduced bcl-2 protein levels in MCF-7 and SW2 cells to 42 ± 7% and 36 ± 5%, respectively, and bcl-xL to 40 ± 6% and 41 ± 4%, respectively. This effect on protein

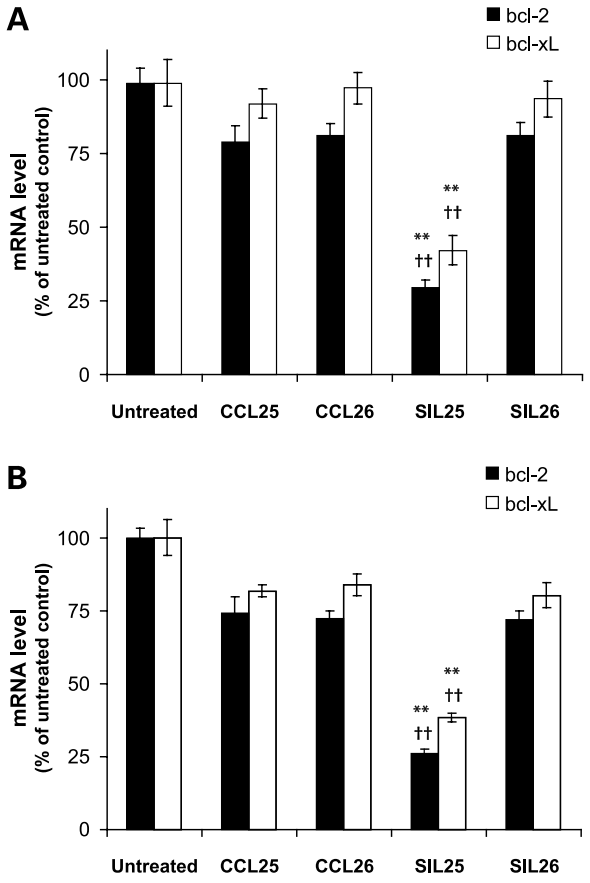

Figure 3. Effect of antisense-loaded immunoliposomes on bcl-2 and bcl-xL mRNA levels in MCF-7 (**A**) and SW2 cells (**B**). Cells were treated with 40 μg/mL antisense oligonucleotide 4625 or control oligonucleotide 4626, encapsulated in either EpCAM-targeted immunoliposomes (SIL25 and SIL26) or nontargeted liposomes (CCL25 and CCL26) for 4 h. Cells were then washed and transferred to oligonucleotide-free medium, and treatments were repeated after 20 h. After the second treatment, cells were harvested and the total RNA was isolated. mRNA levels were quantified by real-time PCR analysis as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD. The level of target mRNA relative to rRNA in untreated cells maintained under identical experimental conditions was taken as 100%. **, $P < 0.001$, compared with untreated controls (one-way ANOVA); ††, $P < 0.001$, compared with SIL26 treatment (Bonferroni's post test).

levels correlated with the antisense activity measured on the mRNA level shown in Fig. 3. Again, SIL26 and the nontargeted CCL25 control liposomes hardly reduced bcl-2 and bcl-xL protein levels (Fig. 4).

Antisense-Loaded Immunoliposomes Sensitize EpCAM-Positive Tumor Cells to Doxorubicin

Up-regulated expression of the antiapoptotic proteins bcl-2 and/or bcl-xL in tumors inhibits or delays cell death induced by various cytotoxic stimuli. Therefore, targeted inhibition of bcl-2 and bcl-xL expression using antisense technology has the potential to sensitize tumor cells also to the proapoptotic effect of chemotherapy. To investigate this possibility, MCF-7 and SW2 cells were treated with SIL25 followed by treatment with varying concentrations of doxorubicin. Cell viability measured in MTT assays is shown in Fig. 5. The degree of chemosensitization was calculated by determining the ratio of the IC_{50} values of the combination treatments relative to those of untransfected (no liposome) and the respective liposome-only (no doxorubicin) treated cells, and data are summarized in Table 1. At the dose used in this experiment (5 μmol/L oligonucleotide), antisense oligonucleotide 4625 alone reduced cell viability by 25%. In the presence of various doses of doxorubicin, chemosensitization by SIL25 was 4-fold for SW2 cells ($n = 3$; $P < 0.005$ versus doxorubicin alone; Fig. 5A) and 10-fold for MCF-7 cells ($n = 3$; $P < 0.005$ versus doxorubicin alone; Fig. 5B), relative to cells treated with doxorubicin only. When IC_{50} values were normalized to cells treated with liposomes only, chemosensitization by SIL25 was 2-fold for SW2 cells and 5-fold for MCF-7 cells (Table 1). In contrast to SIL25, in either cell line, combinations of control SIL26 or CCL25 liposomes with doxorubicin did not result in chemosensitization.

To show that cell death on treatment with SIL25 and doxorubicin was due to the induction of apoptosis, caspase-3-like protease activity was measured. Nontargeted liposomes CCL25 and the immunoliposomes SIL26 were tested as controls. Figure 6 shows that treatment of SW2 cells with SIL25 in combination with doxorubicin resulted in a 3-fold increase in caspase-3-like activity compared with untreated cells (no liposome, no doxorubicin), which was significantly higher than in cells treated with doxorubicin only ($n = 3$; $P = 0.003$) or SIL26 in combination with

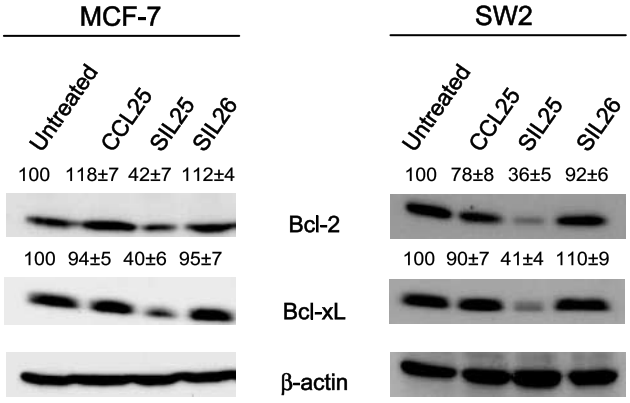

Figure 4. Effect of antisense-loaded immunoliposomes on bcl-2 and bcl-xL protein expression in MCF-7 and SW2 cells. Except for untreated control experiments, antisense oligonucleotide 4625 encapsulated in EpCAM-targeted immunoliposomes (SIL25) or in nontargeted liposomes (CCL25) and control oligonucleotide 4626 encapsulated in EpCAM-targeted immunoliposomes (SIL26) were added to the cells at an oligonucleotide concentration of 40 μg/mL for 4 h. Cells were washed and transferred to fresh medium. After 20 and 48 h, treatments were repeated and cells were harvested 72 h after the start of treatment. Protein levels were analyzed by Western blotting. β-Actin is shown as control for equal protein loading. Protein bands were quantified using the NIH ImageJ software and normalized to the respective β-actin bands. The band intensity of untreated control cells was arbitrarily taken as 100%. Bands from one representative experiment are shown. Numbers indicate the mean ± SD ($n = 3$) given as percentage of the bcl-2 and bcl-xL levels in untreated cells.

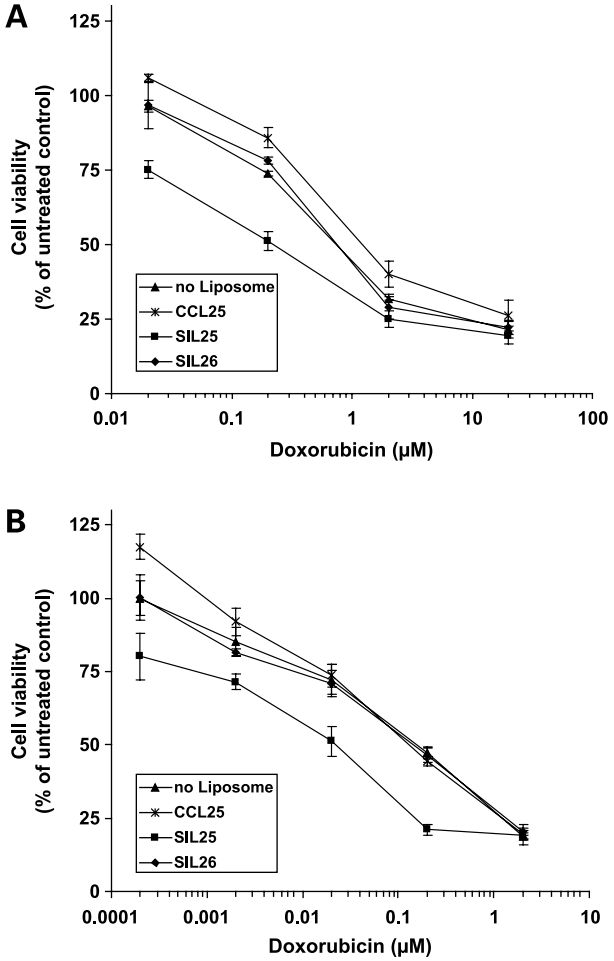

Figure 5. Chemosensitization of SW2 (**A**) and MCF-7 (**B**) cells after combination treatment with antisense-loaded immunoliposomes and doxorubicin. Cells were treated with 40 μg/mL antisense oligonucleotide 4625 or control oligonucleotide 4626 in various liposomal formulations (CCL25, SIL25 and SIL26), washed and incubated in culture medium. After 18 h, cells were incubated with medium containing doxorubicin at the indicated concentrations. Cell viability was determined in MTT assays after 48 h. *Points,* mean of at least three independent experiments done in triplicates; *bars,* SD.

doxorubicin ($n = 3$; $P = 0.001$). The effect of liposomes CCL25 and SIL26 in combination with doxorubicin on caspase-3-like activity was similar to that observed with doxorubicin alone (Fig. 6). This shows that down-regulation of bcl-2 and bcl-xL protein expression by SIL25 facilitated cell death induction via the classic mitochondrial apoptosis pathway controlled by these antiapoptotic proteins.

Discussion

With the aim of developing a new drug delivery system with high specificity for solid tumors, we generated sterically stabilized EpCAM-targeted immunoliposomes encapsulating the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 (17). The ability of this drug delivery system to selectively facilitate and/or enhance tumor cell apoptosis and sensitize cells to chemotherapy was investigated. To this end, the EpCAM-specific high-affinity scFv antibody fragment 4D5MOCB (34) was linked to the surface of sterically stabilized CCLs.

Various types of anticancer agents have been encapsulated in liposomes with the aim of improving their pharmacokinetic and therapeutic performance (21, 22). Sterically stabilized liposomes, which carry PEG linked to phospholipids on their surface, offer the advantage of a long plasma half-life and the ability to release the encapsulated drug over an extended time period (22). However, because they only passively target diseased tissues, mainly due to differences in vascular architecture and permeability, a significant amount of the therapeutic payload may fail to become internalized into target cells, particularly those compounds unable to cross cell membranes. Sterically stabilized liposomes can be surface-modified with tumor-selective ligands, such as antibodies, to produce immunoliposomes, which discriminate between normal and malignant tissues (21, 22). Among the various target antigens tested, those rapidly internalized by receptor-mediated endocytosis are most promising for the delivery of effector molecules having intracellular targets, such as antisense oligonucleotides. For example, disialoganglioside(GD2)-targeted immunoliposomes loaded with myb or myc antisense oligonucleotides were shown to induce apoptosis in neuroblastoma and melanoma cells, and inhibit the growth of melanoma tumors *in vivo* (25, 33).

EpCAM is another interesting target for antibody-based cancer therapy due to its abundant expression in most solid tumors and its comparatively low expression in normal epithelial tissues (29, 41). On ligand binding, it is rapidly internalized by receptor-mediated endocytosis and thus well suited to deliver cytotoxic payloads to intracellular targets. The EpCAM-specific humanized scFv antibody fragment 4D5MOCB has high binding affinity and thermal stability and shows excellent tumor-targeting properties *in vivo* (34). In a previous study, 4D5MOCB was used to generate an immunotoxin that potently inhibited the growth of established tumor xenografts of various histotypes *in vivo* (32). This EpCAM-directed immunotoxin, under the name Proxinium™ is currently under investigation in phase II oncology trials for head and neck tumors and in a phase I/II study, under the name of Vicinium™ for bladder carcinomas. To covalently link 4D5MOCB to the exterior of the CCLs, we introduced a COOH-terminal cysteine for conjugation via a thioether linkage to the maleimide groups at the terminal ends of PEG chains on the liposomal surface (35). The cysteine-modified scFv can be prepared in *E. coli* at ~60% of the yield of the unmodified scFv (34), and the modified scFv shows a coupling efficiency of 50% to 65% to the preformed CCLs. The resulting immunoliposomes were of small size (~125 nm) and showed high efficiency of oligonucleotide encapsulation (>80%). These properties are in good agreement with other recently reported liposome preparations

Table 1. Antisense-loaded immunoliposomes sensitize EpCAM-positive tumor cells to doxorubicin

Cell line	Treatment	IC_{50} (μmol/L)	Fold sensitization*	Fold sensitization†
SW2	No liposomes (untransfected)	0.8 ± 0.2	—	—
	CCL25	1 ± 0.5	0.8	0.8
	SIL25	0.2 ± 0.05‡	4	2
	SIL26	0.8 ± 0.2	1	1
MCF-7	No liposomes (untransfected)	0.2 ± 0.05	—	—
	CCL25	0.18 ± 0.08	1.1	1
	SIL25	0.02 ± 0.01‡	10	5
	SIL26	0.15 ± 0.05	1.3	1

*Fold sensitization was determined as the ratio of the IC_{50} values after treatment with doxorubicin only (no liposomes) and doxorubicin in combination with liposomes (IC_{50} of untransfected/IC_{50} of combination treatment).
†Fold sensitization was determined as the ratio of the IC_{50} values of untransfected cells and cells treated with antisense-doxorubicin combinations. The IC_{50} values for the combination treatments are expressed relative to cells treated with the respective liposomes without doxorubicin (IC_{50} values not shown).
‡$P < 0.005$, compared with untransfected cells as calculated by Student's t test (two tailed).

(25, 33). The EpCAM-targeted immunoliposomes showed significant binding and internalization only in antigen-positive tumor cells, whereas binding to EpCAM-negative cells was negligible, as was the binding of nontargeted control liposomes.

Recent improvements in oligonucleotide chemistry, the advent of small interfering RNA, and the identification of novel target genes involved in the malignant process have enhanced the efficacy of antisense therapeutics (10, 11, 13). Apoptosis inhibitors could prove to be particularly interesting antisense targets for enhancing the efficacy of conventional chemotherapy. In human tumors, the two major apoptosis inhibitors bcl-2 and bcl-xL are often overexpressed together and they may redundantly or nonredundantly contribute to chemoresistance (9, 42). In this situation, it is difficult to predict which of the two antiapoptotic proteins is more crucial for cell survival under a given proapoptotic condition. Moreover, solid tumors represent extremely heterogeneous cell populations in which the relative expression and function of bcl-2 and bcl-xL is unpredictable. For example, single tumor cells are able to switch expression from bcl-2 to bcl-xL (42), confirming the high plasticity of the resistance machinery. As we have learned from the clinical use of kinase inhibitors (43, 44), focusing the effect of a single agent on more than one tumor-relevant target is a rational approach to more effective cancer therapy.

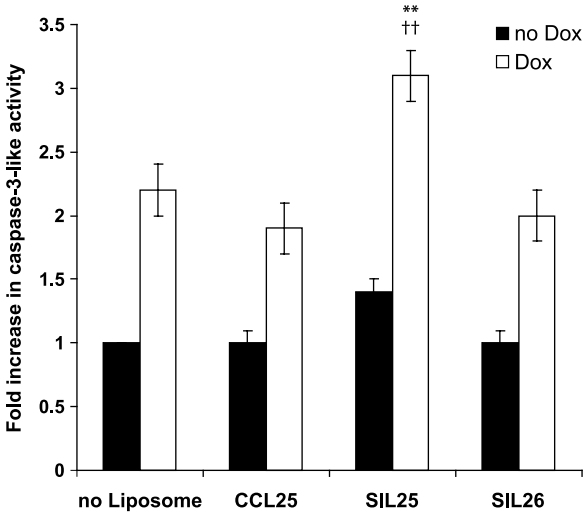

Figure 6. Caspase-3-like protease activity in lysates of SW2 cells following a combination treatment with antisense-loaded immunoliposomes and doxorubicin. SW2 cells were treated with EpCAM-targeted immunoliposomal 4625 (SIL25), nontargeted liposomal antisense oligonucleotide 4625 (CCL25), or EpCAM-targeted immunoliposomal control oligonucleotide 4626 (SIL26) for 4 h at an oligonucleotide concentration of 5 μmol/L. Treatment was repeated after 20 h for the same duration. After the second treatment, cells were incubated with medium containing 0.5 μmol/L doxorubicin for 24 h and harvested before caspase activity was measured as described in Materials and Methods. The caspase-3-like activity is expressed relative to untreated cells (no liposome, no doxorubicin). *Columns,* mean of at least three independent experiments done in triplicates; *bars,* SD. **, $P < 0.005$, relative to cells treated with doxorubicin only; ††, $P < 0.005$, relative to cells treated with SIL26-doxorubicin combination. Statistical significance was evaluated by the Student's t test (two tailed).

Antisense oligonucleotide 4625 is a functionally improved second-generation compound able to facilitate apoptosis in tumor cells of various histotypes *in vitro* and to inhibit the growth of solid tumors *in vivo* (16, 17). To investigate the possibility that targeted delivery of 4625 has the potential to further increase its therapeutic performance, oligonucleotide 4625 was encapsulated in EpCAM-targeted immunoliposomes, and its ability to down-regulate bcl-2 and bcl-xL expression and facilitate tumor cell apoptosis in an EpCAM-specific manner was determined. Antisense oligonucleotide 4625 was encapsulated with an efficiency of >80%, which compares well with other state-of-the-art liposome preparations (25, 33), and the immunoliposomes showed a 10- to 20-fold higher binding to EpCAM-positive cells compared with nontargeted liposomes. Moreover, in agreement with previous reports (24, 33), we found that our immunoliposome preparation remained stable in human serum without detectable loss of oligonucleotides.

On target cell binding, liposomes normally enter cells by endocytosis (45) to become fully effective. Here, we found

that internalization of oligonucleotide 4625 was at least in part mediated by an active transport mechanism, as it was significantly increased at 37°C compared with 4°C. Similar findings of receptor-mediated endocytosis were reported for immunoliposomes targeting disialoganglioside GD2 (25), HER2 (ErbB2; ref. 46), and the transferrin receptor (47). Compared with small drugs or protein toxins, therapeutic nucleic acids, such as antisense oligonucleotides and small interfering RNAs, may not readily escape from endosomes/lysosomes on receptor-mediated endocytosis. This restriction in intracellular translocation significantly hampers the efficiency of the delivery approach and hence may reduce therapeutic efficacy. Moreover, it was reported that high intracellular concentrations of phosphorothioate oligonucleotides can lead to enhanced exocytosis (48, 49), although it remains questionable whether these concentrations can be reached under physiologic conditions *in vivo*. Nevertheless, treatment of EpCAM-positive SW2 and MCF-7 cells with our immunoliposomes efficiently inhibited bcl-2 and bcl-xL expression on the mRNA and protein level by 60% to 75% and 70% to 80%, respectively. This finding suggests that, despite suboptimal translocation, a substantial amount of active antisense oligonucleotide could escape lysosomal degradation to reach its site of action in the nucleus.

Due to their ability to facilitate and/or enhance tumor apoptosis, antisense oligonucleotides targeting apoptosis inhibitors could prove to be particularly useful in combination with chemotherapy (15, 50, 51). The ability of oligonucleotide 4625 to synergize with chemotherapy (38, 52) and growth factor inhibitors (53), and to inhibit tumor angiogenesis (54) was reported. Here, we provide evidence that the simultaneous inhibition of bcl-2 and bcl-xL expression using immunoliposomal antisense oligonucleotide 4625 can lead to a 2- to 5-fold increase in the sensitivity of tumor cells to death induction by a chemotherapeutic agent. In contrast to free antisense oligonucleotide 4625, this effect was strictly dependent on EpCAM expression on the tumor cells, emphasizing the high target specificity of our liposomal drug delivery system.

In conclusion, our data show the promise of EpCAM-specific immunoliposomes loaded with bcl-2/bcl-xL antisense oligonucleotides to specifically sensitize tumor cells to chemotherapy and suggest their use in targeted therapy of drug-resistant tumors.

Acknowledgment

We thank Heather Vandertol-Vanier (Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada) for technical assistance and helpful advice in the preparation of CCLs and Pasquale Stano (Materials Science Department, ETH, Zürich, Switzerland) for assistance with the dynamic light scattering measurements.

References

1. Green DR, Evan GI. A matter of life and death. Cancer Cell 2002;1:19–30.

2. Green DR. Apoptotic pathways: ten minutes to dead. Cell 2005;121:671–4.

3. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002;2:277–88.

4. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell 2002;108:153–64.

5. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647–56.

6. Nunez G, Clarke MF. The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol 1994;4:399–403.

7. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 1997;91:627–37.

8. Sartorius UA, Krammer PH. Upregulation of Bcl-2 is involved in the mediation of chemotherapy resistance in human small cell lung cancer cell lines. Int J Cancer 2002;97:584–92.

9. Simonian PL, Grillot DA, Nunez G. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. Blood 1997;90:1208–16.

10. Zangemeister-Wittke U. Antisense to apoptosis inhibitors facilitates chemotherapy and TRAIL-induced death signaling. Ann N Y Acad Sci 2003;1002:90–4.

11. Gleave ME, Monia BP. Antisense therapy for cancer. Nat Rev Cancer 2005;5:468–79.

12. Jansen B, Zangemeister-Wittke U. Antisense therapy for cancer—the time of truth. Lancet Oncol 2002;3:672–83.

13. Stein CA, Benimetskaya L, Mani S. Antisense strategies for oncogene inactivation. Semin Oncol 2005;32:563–72.

14. Kirkwood JM, Bedikian AY, Millward MJ, et al. Long-term survival results of a randomized multinational phase 3 trial of dacarbazine (DTIC) with or without Bcl-2 antisense (oblimersen sodium) in patients (pts) with advanced malignant melanoma (MM). Proc Am Soc Clin Oncol 2005;24:7506.

15. Badros AZ, Goloubeva O, Rapoport AP, et al. Phase II study of G3139, a Bcl-2 antisense oligonucleotide, in combination with dexamethasone and thalidomide in relapsed multiple myeloma patients. J Clin Oncol 2005;23:4089–99.

16. Gautschi O, Tschopp S, Olie RA, et al. Activity of a novel bcl-2/bcl-xL-bispecific antisense oligonucleotide against tumors of diverse histologic origins. J Natl Cancer Inst 2001;93:463–71.

17. Zangemeister-Wittke U, Leech SH, Olie RA, et al. A novel bispecific antisense oligonucleotide inhibiting both bcl-2 and bcl-xL expression efficiently induces apoptosis in tumor cells. Clin Cancer Res 2000;6:2547–55.

18. Bennett CF, Chiang MY, Chan H, Shoemaker JE, Mirabelli CK. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. Mol Pharmacol 1992;41:1023–33.

19. Zelphati O, Zon G, Leserman L. Inhibition of HIV-1 replication in cultured cells with antisense oligonucleotides encapsulated in immunoliposomes. Antisense Res Dev 1993;3:323–38.

20. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. Biochim Biophys Acta 1991;1066:29–36.

21. Allen TM. Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2002;2:750–63.

22. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4:145–60.

23. Gutierrez-Puente Y, Tari AM, Stephens C, et al. Safety, pharmacokinetics, and tissue distribution of liposomal P-ethoxy antisense oligonucleotides targeted to Bcl-2. J Pharmacol Exp Ther 1999;291:865–9.

24. Stuart DD, Kao GY, Allen TM. A novel, long-circulating, and functional liposomal formulation of antisense oligodeoxynucleotides targeted against MDR1. Cancer Gene Ther 2000;7:466–75.

25. Pastorino F, Brignole C, Marimpietri D, et al. Targeted liposomal c-myc antisense oligodeoxynucleotides induce apoptosis and inhibit tumor growth and metastases in human melanoma models. Clin Cancer Res 2003;9:4595–605.

26. Zangemeister-Wittke U. Antibodies for targeted cancer therapy—technical aspects and clinical perspectives. Pathobiology 2005;72:279–86.

27. Litvinov SV, Velders MP, Bakker HA, Fleuren GJ, Warnaar SO. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. J Cell Biol 1994;125:437–46.

28. Munz M, Kieu C, Mack B, et al. The carcinoma-associated antigen

EpCAM upregulates c-myc and induces cell proliferation. Oncogene 2004; 23:5748 – 58.

29. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17-1A antigen (Ep-CAM). J Mol Med 1999;77:699 – 712.

30. Gastl G, Spizzo G, Obrist P, Dunser M, Mikuz G. Ep-CAM overexpression in breast cancer as a predictor of survival. Lancet 2000;356: 1981 – 2.

31. Spizzo G, Went P, Dirnhofer S, et al. High Ep-CAM expression is associated with poor prognosis in node-positive breast cancer. Breast Cancer Res Treat 2004;86:207 – 13.

32. Di Paolo C, Willuda J, Kubetzko S, et al. A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. Clin Cancer Res 2003;9:2837 – 48.

33. Pagnan G, Stuart DD, Pastorino F, et al. Delivery of c-myb antisense oligodeoxynucleotides to human neuroblastoma cells via disialoganglioside GD(2)-targeted immunoliposomes: antitumor effects. J Natl Cancer Inst 2000;92:253 – 61.

34. Willuda J, Honegger A, Waibel R, et al. High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. Cancer Res 1999;59:5758 – 67.

35. Kirpotin D, Park JW, Hong K, et al. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro*. Biochemistry 1997;36:66 – 75.

36. Hu Q, Bally MB, Madden TD. Subcellular trafficking of antisense oligonucleotides and down-regulation of bcl-2 gene expression in human melanoma cells using a fusogenic liposome delivery system. Nucleic Acids Res 2002;30:3632 – 41.

37. Ziegler A, Luedke GH, Fabbro D, et al. Induction of apoptosis in small-cell lung cancer cells by an antisense oligodeoxynucleotide targeting the Bcl-2 coding sequence. J Natl Cancer Inst 1997;89:1027 – 36.

38. Hopkins-Donaldson S, Cathomas R, Simoes-Wust AP, et al. Induction of apoptosis and chemosensitization of mesothelioma cells by Bcl-2 and Bcl-xL antisense treatment. Int J Cancer 2003;106:160 – 6.

39. Lopes de Menezes DE, Pilarski LM, Allen TM. *In vitro* and *in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma. Cancer Res 1998;58:3320 – 30.

40. Rao CG, Chianese D, Doyle GV, et al. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. Int J Oncol 2005;27:49 – 57.

41. Went PT, Lugli A, Meier S, et al. Frequent EpCam protein expression in human carcinomas. Hum Pathol 2004;35:122 – 8.

42. Han Z, Chatterjee D, Early J, et al. Isolation and characterization of an apoptosis-resistant variant of human leukemia HL-60 cells that has switched expression from Bcl-2 to Bcl-xL. Cancer Res 1996;56: 1621 – 8.

43. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med 2005;353:172 – 87.

44. Pao W, Miller VA. Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. J Clin Oncol 2005;23:2556 – 68.

45. Zelphati O, Szoka FC, Jr. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. Pharm Res 1996; 13:1367 – 72.

46. Park JW, Hong K, Kirpotin DB, et al. Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. Clin Cancer Res 2002; 8:1172 – 81.

47. Chiu SJ, Liu S, Perrotti D, Marcucci G, Lee RJ. Efficient delivery of a Bcl-2-specific antisense oligodeoxyribonucleotide (G3139) via transferrin receptor-targeted liposomes. J Control Release 2006;112:199 – 207.

48. Remaut K, Lucas B, Braeckmans K, et al. Protection of oligonucleotides against nucleases by pegylated and non-pegylated liposomes as studied by fluorescence correlation spectroscopy. J Control Release 2005; 110:212 – 26.

49. Fisher TL, Terhorst T, Cao X, Wagner RW. Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. Nucleic Acids Res 1993; 21:3857 – 65.

50. Jansen B, Schlagbauer-Wadl H, Brown BD, et al. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat Med 1998; 4:232 – 4.

51. Simoes-Wust AP, Olie RA, Gautschi O, et al. Bcl-xl antisense treatment induces apoptosis in breast carcinoma cells. Int J Cancer 2000;87:582 – 90.

52. Yamanaka K, Rocchi P, Miyake H, et al. A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells. Mol Cancer Ther 2005;4:1689 – 98.

53. Milella M, Trisciuoglio D, Bruno T, et al. Trastuzumab down-regulates Bcl-2 expression and potentiates apoptosis induction by Bcl-2/Bcl-XL bispecific antisense oligonucleotides in HER-2 gene-amplified breast cancer cells. Clin Cancer Res 2004;10:7747 – 56.

54. Del Bufalo D, Trisciuoglio D, Scarsella M, Zangemeister-Wittke U, Zupi G. Treatment of melanoma cells with a bcl-2/bcl-xL antisense oligonucleotide induces antiangiogenic activity. Oncogene 2003;22: 8441 – 7.