Antitumor activity of an epithelial cell adhesion molecule–targeted nanovesicular drug delivery system

Sajid Hussain,[1,3] 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

Site-specific delivery of anticancer agents to tumors represents a promising therapeutic strategy because it increases efficacy and reduces toxicity to normal tissues compared with untargeted drugs. Sterically stabilized immunoliposomes (SIL), guided by antibodies that specifically bind to well internalizing antigens on the tumor cell surface, are effective nanoscale delivery systems capable of accumulating large quantities of anticancer agents at the tumor site. The epithelial cell adhesion molecule (EpCAM) holds major promise as a target for antibody-based cancer therapy due to its abundant expression in many solid tumors and its limited distribution in normal tissues. We generated EpCAM-directed immunoliposomes by covalently coupling the humanized single-chain Fv antibody fragment 4D5MOCB to the surface of sterically stabilized liposomes loaded with the anticancer agent doxorubicin. *In vitro*, the doxorubicin-loaded immunoliposomes (SIL-Dox) showed efficient cell binding and internalization and were significantly more cytotoxic against EpCAM-positive tumor cells than nontargeted liposomes (SL-Dox). In athymic mice bearing established human tumor xenografts, pharmacokinetic and biodistribution analysis of SIL-Dox revealed long circulation times in the blood with a half-life of 11 h and effective time-dependent tumor localization, resulting in up to 15% injected dose per gram tissue. These favorable pharmacokinetic properties translated into potent antitumor activity, which resulted in significant growth inhibition (compared with control mice), and was more pronounced than that of doxorubicin alone and nontargeted SL-Dox at low, nontoxic doses. Our data show the promise of EpCAM-directed nanovesicular drug delivery for targeted therapy of solid tumors. [Mol Cancer Ther 2007;6(11):3019–27]

Introduction

Despite recent improvements in cancer therapy due to the introduction of new drug combinations and novel targeted cancer therapeutics, cancer remains a major health problem, with >1,500 deaths per day in the United States alone (1). The main problem associated with current therapies is their low efficacy due to unspecific toxicity to normal tissues, which precludes the use of curative doses. The unmet medical need for more effective anticancer agents, especially for strategies that focus toxicity to tumor cells and away from normal tissues, has led to the development of antibody-based cancer therapeutics directed against tumor-associated antigens. These promising molecules include IgGs that exploit Fc effector functions, radioimmunoconjugates, immunotoxins, and nanovesicular drug carriers, such as immunoliposomes (2, 3).

Encapsulation of anticancer agents in liposomes favorably alters their pharmacokinetic properties, resulting in increased tumor localization, improved antitumor effects, and decreased nonspecific toxicities (4, 5). In recent years, various liposome formulations have been tested with the aim to prolong their *in vivo* circulation time and enhance tumor localization. Particularly promising are liposomes containing surface-grafted lipid derivatives conjugated with polyethylene glycol (PEG; ref. 6). These sterically stabilized liposomes (also called ''Stealth'' liposomes) have long circulation times in the blood as a consequence of reduced uptake by the reticuloendothelial system (4, 5). A variety of chemotherapeutic agents, such as doxorubicin and vincristine, have been encapsulated in PEGylated liposomes and validated in preclinical models *in vitro* and *in vivo* (7–9). PEGylated liposomes achieve a higher drug load in tumors due to a passive targeting process, which exploits the ''enhanced permeability and retention effect,'' resulting from increased vascular permeability inherent to many solid tumors (4, 10). PEGylated liposomal doxorubicin (Doxil/Caelyx) has been approved for use in acquired immunodeficiency syndrome–related Kaposi's sarcoma, and refractory ovarian and breast cancers, and several other liposomal anticancer agents are currently under clinical investigation (4, 5).

With the advent of technologies for coupling specific ligands, such as proteins or peptides, to the distal terminus of PEG on liposomes (11–13), and owing to recent advances in antibody engineering, there is increasing interest in the development of antibody-guided liposomes for targeted delivery of anticancer agents to tumors (5, 14). Thus far, various tumor-associated antigens have been validated as targets for antibody-based cancer therapeutics,

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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

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including immunoliposomes (3, 15). Promising results were achieved with immunoliposomes targeting CD19, HER2/*neu*, epidermal growth factor receptor, disialoganglioside (GD_2), and prostate-specific membrane antigen expressed on various tumor cell types (8, 9, 16–18). Based on the promising preclinical data and advances made in terms of large-scale production (19), HER2-specific immunoliposomes are on their way to clinical trials.

The epithelial cell adhesion molecule (EpCAM) is a 40-kDa transmembrane glycoprotein, which is involved in Ca^{2+}-independent cell adhesion (20, 21). EpCAM triggers cell proliferation by activation of the c-myc oncogene (22). It is abundantly expressed in many solid tumors and shows limited expression in normal epithelial tissues (20, 23). In breast cancer, EpCAM overexpression was identified as a negative prognostic factor that is associated with disease progression and poor overall survival (24, 25). Recent evidence suggests EpCAM as a stem cell marker in colorectal cancer (26). The promise of EpCAM as a target for antibody therapeutics has been convincingly shown in preclinical and clinical studies (27–29).

Here, we developed EpCAM-specific immunoliposomes loaded with the anthracycline doxorubicin and surface conjugated with the highly stable, high-affinity single-chain Fv (scFv) 4D5MOCB (30). This nanovesicular drug delivery system was characterized for tumor cell binding, internalization, and cytotoxicity *in vitro*. Its pharmacokinetic and therapeutic potential was assessed in a tumor xenograft model in mice by measurement of blood clearance, organ distribution, tumor localization, and antitumor activity. Our data show that EpCAM-targeted nanovesicles hold great promise for the effective and safe delivery of anticancer agents to solid tumors.

Materials and Methods

Materials and Reagents

Hydrogenated soy phosphatidylcholine (HSPC), cholesterol, PEG (M_r 2,000)-derivatized distearoyl phosphatidylethanolamine (PEG-DSPE), and maleimide-derivatized PEG_{2000}-DSPE (Mal-PEG-DSPE) were purchased from Avanti Polar Lipids, Inc., [^{3}H]Cholesteryl hexadecyl ether ([^{3}H]CHE), Solvable^{TM} tissue solubilizing fluid, and Ultima Gold scintillation mixture were purchased from Perkin-Elmer. Doxorubicin-HCl was a generous gift from Pfizer, Inc. Sepharose CL-4B, Sephadex G-25 and G-50 size exclusion resins were from Amersham Biosciences, and Bio-Spin 6 chromatography columns were purchased from Bio-Rad. All other reagents of analytic and molecular biology grade were obtained from Sigma-Aldrich.

Tumor Cell Lines

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

Preparation of Doxorubicin-Loaded Liposomes

Nontargeted liposomes (SL-Dox) to be loaded with doxorubicin were composed of 40 μmol HSPC, 20 μmol cholesterol, and 2 μmol PEG_{2000}-DSPE to give a molar ratio of 2:1:0.1. Liposomes were prepared by a lipid film hydration-extrusion method, as described previously (31), by sequentially extruding the hydrated liposomes in a Lipex Extruder (Northern Lipids Inc.) through a series of polycarbonate filters (Whatman plc) with pore sizes ranging from 400 to 80 nm. This extrusion procedure produces small unilamellar vesicles (32). Liposome size was verified by dynamic light scattering using a Malvern Zetasizer 5000 (Malvern Instruments Ltd.). [^{3}H]CHE was added as a nonmetabolized, nonexchangeable lipid tracer (31, 33).

Doxorubicin was loaded into liposomes by the ammonium sulfate remote loading method (34), at a doxorubicin-to-HSPC ratio of 0.2:1 (w/w). Liposome-encapsulated doxorubicin was separated from free doxorubicin over a Sephadex G-50 column. The concentration of the liposomal doxorubicin was determined by spectrophotometry (λ = 490 nm), and the phospholipid concentrations were determined from the specific activity of the [^{3}H]CHE tracer (31) whose molar ratio was 1:24,000 relative to HSPC. Previous studies have shown that this label is nonmetabolized and nonexchanged, making it a good marker for the fate of liposomes (31, 35). The loading efficiency of doxorubicin was >95%, and liposomes routinely contained 0.28 to 0.30 μmol doxorubicin per μmol phospholipid. Assuming spherical unilamellar monodisperse liposomes with 10^{13} liposomes per μmol phospholipid, each liposome would contain 2×10^4 molecules of doxorubicin and 6×10^4 molecules of HSPC and the other components at the molar ratios indicated.

EpCAM-targeted immunoliposomes (SIL-Dox) were composed of HSPC/cholesterol/PEG_{2000}-DSPE/Mal-PEG_{2000}-DSPE at a 2:1:0.08:0.02 molar ratios. These liposomes were prepared and loaded with doxorubicin as described above for nontargeted liposomes (SL-Dox). The targeting ligand was an EpCAM-specific scFv antibody 4D5MOCB (30), engineered to contain a COOH-terminal cysteine (29) and coupled to the exterior of the sterically stabilized liposomes by formation of a thioether linkage with the maleimide groups at the distal termini of the PEG chains (36). To this end, purified 4D5MOCB was reduced with 2 mmol/L DTT or 7.5 mmol/L β-mercaptoethylamine (Sigma) for 45 to 60 min at 37°C to obtain monomeric scFv-SH. The reduced scFv was desalted on a Bio-Spin 6 column preequilibrated with HBS [25 mmol/L HEPES, 150 mmol/L NaCl (pH 6.5)] and covalently linked to the preformed liposomes at a molar ratio of phospholipid to scFv of 500 to 700:1 (70–100 scFv molecules per liposome). After overnight coupling at room temperature, unreacted maleimide was quenched by reduction with 2 mmol/L β-mercaptoethanol for 30 min at room temperature (9). The

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scFv-conjugated liposomes were separated from unconjugated scFv on a Sepharose CL-4B size exclusion column (1.5 × 30 cm) preequilibrated 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 (31).

Determination of Cell Binding and Internalization

Cell binding and internalization experiments were done as described (17, 31) using EpCAM-positive (MCF-7 and SW2) and EpCAM-negative (RL) tumor cells. Various formulations of [^{3}H]CHE-labeled liposomes (EpCAM-targeted SIL-Dox or nontargeted SL-Dox) were added to the cells and incubated either on ice or at 37°C for 2 h. Cells were then washed with cold PBS and lysed with 1 N sodium hydroxide before radioactivity was measured. In competition experiments, a 50- to 60-fold molar excess of free 4D5MOCB scFv over liposome-bound scFv was added 30 min before addition of the immunoliposomes. Liposome uptake (nmol phospholipids/10^6 cells) was calculated from the specific activity of the [^{3}H]CHE-labeled liposomes and its known molar ratio to phospholipids (see above).

Cytotoxicity Assay

The cytotoxic effect of free doxorubicin and liposome-encapsulated doxorubicin was tested on EpCAM-positive (MCF-7 and SW2) and EpCAM-negative (RL) cells as described (31). Briefly, MCF-7 cells (1×10^4 cells per well), SW2 cells (5×10^4 per well), or RL cells (5×10^4 cells per well) were plated in 96-well culture plates and allowed to grow overnight. Cells were incubated with either free doxorubicin or doxorubicin encapsulated in EpCAM-targeted immunoliposomes (SIL-Dox) or nontargeted liposomes (SL-Dox) for 2 or 24 h at 37°C. Then, cells were washed twice with PBS before addition of growth medium and incubated for another 70 and 48 h, respectively. Cell viability was measured using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 72 h from the start of drug treatment, MTT reagent (Sigma) 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).

Tumor Xenograft Model

Six- to 8-week-old female CD-1 (ICR *nu/nu*) mice weighing 25 to 30 g were purchased from Charles River Laboratories and kept under specific pathogen-free conditions. All experiments were done according to the institutional guidelines of the Swiss Federal Veterinary Office of the Kanton Zürich. Tumors were grown at the lateral flank by s.c. injection of 10×10^6 SW2 lung cancer cells and randomized to constitute groups with an average tumor size of 85 to 100 mm^3.

Analysis of Tumor Localization and Biodistribution *In vivo*

To investigate the pharmacokinetic properties of the doxorubicin liposomes *in vivo*, mice bearing established SW2 tumor xenografts were injected i.v. via the tail vein with a single dose of 4 mg doxorubicin/kg (0.66 μmol phospholipids per mouse) containing ~1×10^6 cpm of the lipid label [^{3}H]CHE. At selected time points (0.5, 2, 12, 24, and 48 h) after injection, mice (three per time point) were sacrificed. Blood samples (80–100 μL) were collected by heart puncture using a heparinized syringe, and organs (liver, spleen, heart, lungs, and kidney) and tumors were resected and weighed to measure radioactivity in the tissues as described (17). Briefly, in a glass scintillation vial, 1 to 2 mL of Solvable™ tissue solubilization fluid were added to 100 to 200 mg tissue. Vials were heated to 60°C for 1 to 3 h, cooled to room temperature before the addition of 0.1 mL of 200 mmol/L EDTA followed by oxidation overnight with 0.2 to 0.4 mL hydrogen peroxide (30%, v/v). The following day, 0.1 mL of 1 N HCl and 10 to 15 mL of Ultima Gold scintillation mixture were added, and samples were counted in a Betamatic V liquid scintillation counter (Kontron).

To determine the total radioactivity in the blood, the total blood volume was assumed to constitute 7.3% of the body weight (37). Pharmacokinetic variables were calculated using the WinNonLin software version 5.0.1 (Pharsight) based on a noncompartmental analysis model 201 (i.v. bolus input).

The final organ distribution of the liposomes was calculated as percentage of the total injected lipid dose per gram (%ID/g). The amount of radioactivity per gram tissue is given as percentage of the total injected dose. Analyses were done in triplicates and data represent the mean ± SD.

Measurement of Antitumor Activity *In vivo*

Mice bearing established SW2 tumor xenografts were randomly assigned to various treatment groups (five to seven mice per group). Doxorubicin-containing EpCAM-targeted or nontargeted liposomes were administered i.v. via the tail vein at doses of either 4 mg doxorubicin/kg or 7.5 mg/kg doxorubicin every week for 3 weeks. The total doxorubicin dose was 12 or 22.5 mg/kg, and the corresponding phospholipid dose was approximately 2 or 3.75 μmol, respectively (0.66 or 1.25 μmol per dose). In additional control groups, mice received HEPES-buffered saline [25 mmol/L HEPES, 140 mmol/L NaCl (pH 7.4)] or free doxorubicin (Adriblastin). Free doxorubicin was given i.v. at its maximum tolerated dose (MTD) of 7.5 mg/kg using the same schedule used for the liposomes or the saline control. Tumors were measured twice weekly by caliper measurement and mice were routinely examined for weight loss as a sign of toxicity. Tumor size was calculated by measurement of the shortest and longest perpendicular diameter according to the following formula: (short diameter)^2 × (long diameter) × 0.4.

Statistical Analysis

The IC_{50} values obtained from the *in vitro* cytotoxicity experiments were statistically evaluated by one-way ANOVA, and pairwise comparisons were made by Tukey's multiple comparison post test. Student's *t* test was used to determine the significance of the pharmacokinetic and biodistribution data. Statistical significance of the

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differences in antitumor activity measured in the various treatment groups was determined by the nonparametric Kruskal-Wallis test with Dunn's multiple comparison test as the post test for pairwise comparisons. Evaluation of the data and P values were obtained using GraphPad Prism version 4.0 for Windows (GraphPad Software). For all calculations, $P < 0.05$ was considered significant.

Results

EpCAM-Specific Binding and Internalization of Immunoliposomes in Tumor Cells

Immunoliposomes were generated using the EpCAM-specific high-affinity scFv 4D5MOCB (30). A prerequisite for efficient tumor-specific drug delivery by ligand-targeted nanovesicles involves binding to the target antigen followed by internalization (2). To examine the target specificity of the immunoliposomes, *in vitro* cell binding and internalization analyses were done on EpCAM-positive (MCF-7 and SW2) cells and the EpCAM-negative control cell line RL. Cellular association/binding was quantified by measuring the uptake of phospholipid from liposomes based on the specific activity of [^{3}H]CHE counts associated with the cells (17, 31), which are in a known molar fraction to phospholipid. To discriminate between nonspecific cell surface binding/association (which occurs whenever cells are exposed to liposomes) and internalization, presumably by receptor-mediated endocytosis because these are nonfusogenic liposomes, cell binding was measured both at 37°C and 4°C (nonpermissive for endocytosis). As shown in Fig. 1, the EpCAM-targeted immunoliposomes (SIL-Dox) bound specifically to SW2 and MCF-7 cells at a level that was 10- to 20-fold higher than the nontargeted control liposomes (SL-Dox). Moreover, at 37°C, the cell-associated radioactivity was higher than at 4°C. This might suggest that to a certain degree EpCAM receptor recycling with multiple uptake events may have occurred following internalization of SIL-Dox or that internalization was due to another unknown mechanism (Fig. 1). EpCAM-overexpressing cells showed considerable uptake of EpCAM-targeted immunoliposomes, reaching 7,000 to 9,000 nanovesicles/cell at saturating liposome concentrations. No difference in cellular binding of SIL-Dox and SL-Dox was found on EpCAM-negative RL cells (Fig. 1).

The specificity of cell binding was confirmed by competition experiments with the addition of a 50- to 60-fold excess of free 4D5MOCB scFv over liposome-bound scFv at 30 min before the addition of anti-EpCAM immunoliposomes. The excess EpCAM-scFv blocked the specific cellular binding and uptake of the immunoliposomes, resulting in a decrease in radioactivity associated with the cells to levels similar to that measured for the nontargeted liposomes (Fig. 1). These findings show that cellular binding and internalization of the immunoliposomes was mediated by the specific interaction of the scFv on the liposome surface with its target antigen EpCAM on the tumor cells.

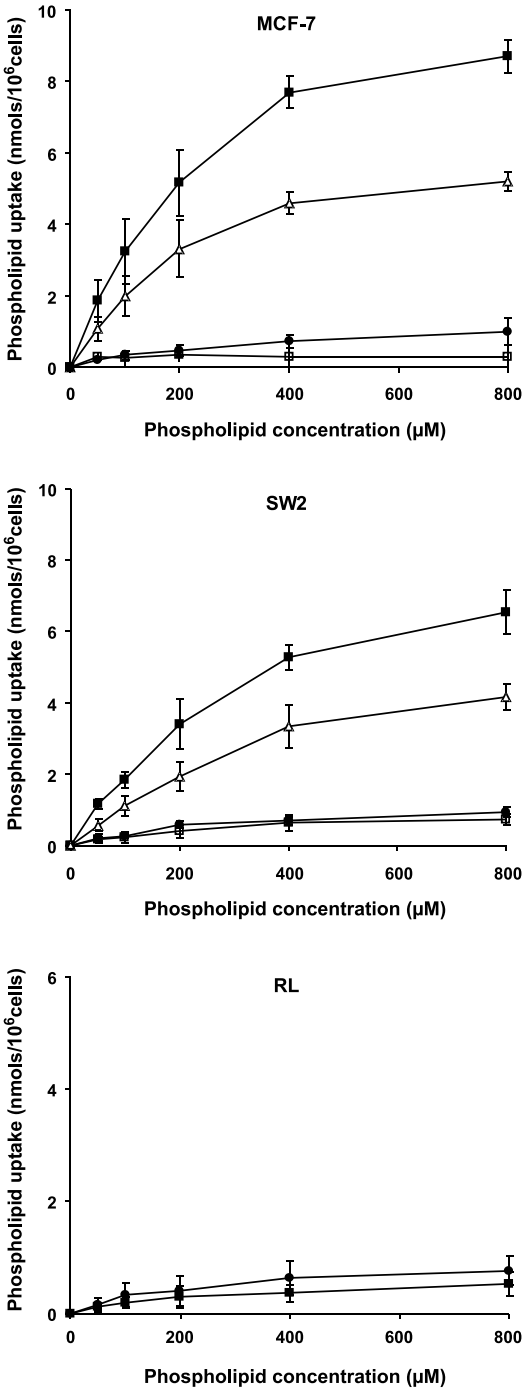

Figure 1. Binding and uptake of [^{3}H]CHE-labeled doxorubicin-loaded immunoliposomes and nontargeted liposomes in EpCAM-positive (MCF-7 and SW2) and EpCAM-negative (RL) cells as a function of phospholipid concentration (phospholipid and [^{3}H]CHE are in a known molar ratio). Binding and cellular uptake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

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Cytotoxicity of EpCAM-Targeted Immunoliposomes

For successful liposomal drug delivery into tumors, a key issue is the release of the encapsulated drug from the liposomes after cell surface–specific binding and internalization in order for the bioavailable (released) drug to gain access to its intracellular target (2). The ability of our targeted nanovesicles to deliver doxorubicin and induce cell death in an EpCAM-specific manner was examined on EpCAM-positive SW2 and MCF-7 cells for EpCAM-targeted liposomal doxorubicin (SIL-Dox), free doxorubicin, and nontargeted liposomes (SL-Dox) in an *in vitro* cytotoxicity assay. As shown in Table 1, the IC_{50} values for SIL-Dox (dose at which cell viability was reduced by 50%), after a 2-h incubation, were 12-fold lower (i.e., more cytotoxic) and, after a 24-h incubation, 10-fold lower for MCF-7 cells ($P < 0.001$ for both time points) than for SL-Dox. For SW2 cells, after 2 and 24 h, the IC_{50} for SIL-Dox was ~3-fold lower ($P < 0.001$ at both time points) than for SL-Dox. After 24 h, the IC_{50} of SIL-Dox and SL-Dox started to become more similar. This probably reflects the fact that, during longer incubation times, more and more free drug was present in the medium due to drug release from both the nontargeted and the targeted liposomes. This gradual attenuation of the difference in cytotoxicity between targeted and nontargeted liposomes at longer time points is in good agreement with previously published reports (16, 31). As expected, there was no significant difference in the cytotoxicity of SIL-Dox and SL-Dox against EpCAM-negative RL cells after 2 and 24 h ($P > 0.05$; Table 1), indicating that the cytotoxic effect was most likely mediated by binding of the immunoliposomes to EpCAM on the cell surface followed by receptor-mediated endocytosis and intracellular delivery of doxorubicin. Although free doxorubicin killed the cells more effectively than the liposome encapsulated formulations, this cytotoxicity was not specific for EpCAM-expressing cells and is an effect of the *in vitro* culture system, which lacks the drug redistribution phenomenon that is seen *in vivo*.

Table 1. Cytotoxicity of EpCAM-targeted immunoliposomes (SIL-Dox), nontargeted liposomes (SL-Dox), and free doxorubicin against EpCAM-positive (MCF-7 and SW2) and EpCAM-negative (RL) tumor cells

Cell line	Formulation	IC_{50} (μmol/L)*	
		2 h	24 h
MCF-7	Free doxorubicin	0.6 ± 0.2	0.15 ± 0.1
	SIL-Dox (anti-EpCAM)	15 ± 3†	5 ± 1†
	SL-Dox (nontargeted)	>175	55 ± 10
SW2	Free doxorubicin	5 ± 2	1.5 ± 0.5
	SIL-Dox (anti-EpCAM)	60 ± 10†	25 ± 5†
	SL-Dox (nontargeted)	>175	80 ± 10
RL	Free doxorubicin	9 ± 1	2 ± 1
	SIL-Dox (anti-EpCAM)	>175	100 ± 10
	SL-Dox (nontargeted)	>175	120 ± 10

*Cells were plated in 96-well culture plates and incubated with free doxorubicin or doxorubicin encapsulated in liposomes as described in Materials and Methods. Data are presented as mean IC_{50} ± SD ($n = 3$).
†$P < 0.001$, compared with nontargeted SL-Dox (one-way ANOVA, Tukey's post test).

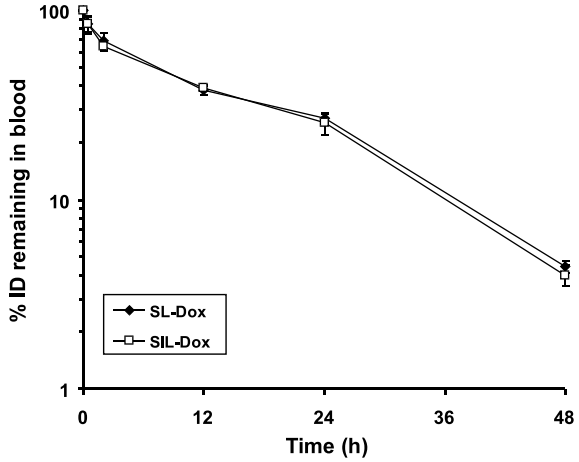

Figure 2. Blood clearance of EpCAM-targeted immunoliposomes versus nontargeted liposomes in CD-1 nude mice bearing SW2 tumor xenografts. Doxorubicin-containing sterically stabilized liposomes (EpCAM targeted or nontargeted) were radiolabeled with [^{3}H]CHE. Mice (three per time point) were injected i.v. via the tail vein with anti-EpCAM immunoliposomal doxorubicin (SIL-Dox) or nontargeted liposomal doxorubicin (SL-Dox), with a single bolus dose of 4 mg doxorubicin/kg (0.66 μmol phospholipid per mouse). At selected time points after injection (0.5, 2, 12, 24, and 48 h), mice were euthanized and whole blood was analyzed for radioactivity. Results are expressed as the percent of the total injected phospholipid dose, set to 100 %, remaining in the blood at various time points after the injection of liposomes. *Points,* mean ($n = 3$); *bars,* SD.

Pharmacokinetics of EpCAM-Targeted Immunoliposomes *In vivo*

The pharmacokinetic profile of the EpCAM-targeted immunoliposomes (SIL-Dox) was evaluated in mice bearing SW2 tumor xenografts. SIL-Dox and nontargeted SL-Dox liposomes were labeled with [^{3}H]CHE (18, 38) and injected i.v. at a single dose of 4 mg doxorubicin/kg (0.66 μmol phospholipids per mouse). The blood clearance is expressed as a function of the total dose of injected lipid (17, 38). As shown in Fig. 2, the dose of the lipid remaining in the blood after 24 h was similar for SIL-Dox (27 ± 1.6%) and SL-Dox (25.4 ± 3.6%) as has been observed for other targeted liposomes in mice bearing solid tumors (8). Moreover, absolute concentrations in blood were identical and not significantly different for SIL-Dox and SL-Dox. Both showed long circulation times in the blood with half-lives of 11.2 ± 0.4 and 11.4 ± 0.2 h, respectively, which is the same within experimental error (Table 2). This indicates that the conjugation of the scFv 4D5MOCB to the liposome surface did not alter the pharmacokinetic properties of the liposomes. Almost identical blood clearance values (5 ± 0.3 versus 4.8 ± 0.2 mL/h/kg) and area under the blood concentration versus time curves (3,358 ± 239 versus 3,526 ± 144 μg·h/mL) were observed for SIL-Dox and SL-Dox, respectively (Fig. 2; Table 2), and for both types of liposomes, the volume of distribution equaled the total blood volume.

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Table 2. Pharmacokinetic and blood clearance of EpCAM-targeted immunoliposomes (SIL-Dox) and nontargeted liposomes (SL-Dox) in tumor-bearing mice

Liposome	Half-life (h)	Clearance (mL/h/kg)	$AUC_{t=0\rightarrow\alpha}$ (μg•h/mL)	V_d (mL/kg)
SL-Dox	11.2 ± 0.4	4.8 ± 0.2	3,526 ± 144	77.9 ± 3.6
SIL-Dox	11.4 ± 0.2	5 ± 0.3	3,358 ± 239	79.8 ± 2.0

NOTE: Mice bearing SW2 tumor xenografts (85–100 mm^3) were injected i.v. with a single dose of 4 mg doxorubicin/kg (0.66 μmol phospholipid per mouse) encapsulated in liposomes labeled with [^{3}H]CHE. Pharmacokinetic variables were determined using WinNonLin software. Data represent the mean ± SD (n = 3 mice per time point).
Abbreviations: AUC, area under the blood concentration versus time curve; V_d, volume of distribution.

Tumor Localization and Biodistribution of EpCAM-Targeted Immunoliposomes

For successful ligand-targeted drug delivery, the immunoliposomes should preferentially localize to the tumor and spare normal tissues from unspecific toxicity. The biodistribution and tumor localization of the SIL-Dox immunoliposomes and the nontargeted SL-Dox liposomes was assessed in mice bearing SW2 tumor xenografts using the same protocol as above. Tumor localization of the EpCAM-targeted immunoliposomes SIL-Dox was approximately double that of the nontargeted liposomes (13.0 ± 0.5 versus 7.7 ± 0.7 %ID/g; P = 0.004), respectively (Table 3). At 48 h after injection, the amount in the tumor remained roughly the same as after 24 h (15.0 ± 1.0 for SIL-Dox versus 7.1 ± 1.7 %ID/g for SIL-Dox, respectively; Table 3). This increased tumor accumulation for SIL-Dox versus SL-Dox shows the significant improvement that is obtained by introducing an antigen recognition function into the liposomes, achieved by conjugation of the 4D5MOCB scFv to the surface of SIL-Dox. Because the tumor is small, the increased distribution of targeted liposomes to tumor tissue did not materially affect the blood clearance of the liposomes. At 24 and 48 h after injection, the blood levels (%ID/g) of SIL-Dox and SL-Dox were similar to the previous experiment.

Table 3. Biodistribution of EpCAM-targeted immunoliposomes (SIL-Dox) and nontargeted liposomes (SL-Dox) loaded with doxorubicin in mice bearing SW2 tumor xenografts

Tissue	SIL-Dox (%ID/g)		SL-Dox (%ID/g)	
	24 h	48 h	24 h	48 h
Blood	11.8 ± 1.7*	1.6 ± 0.5	12.2 ± 0.9	2.6 ± 0.4
Liver	14.1 ± 0.6	14.5 ± 1.1	12.0 ± 1.3	14.7 ± 1.9
Spleen	19.3 ± 2.2	22.7 ± 2.1	18.3 ± 1.6	22.0 ± 1.6
Heart	2.4 ± 0.9	2.6 ± 0.4	1.5 ± 0.1	1.7 ± 0.4
Lung	2.5 ± 0.8	1.8 ± 0.6	1.4 ± 0.2	1.7 ± 0.3
Kidney	6.1 ± 1.4	10.3 ± 1.2	6.4 ± 1.5	9.1 ± 1.1
Tumor	13.0 ± 0.5†	15.0 ± 1.0†	7.7 ± 0.7	7.1 ± 1.7

*Values were determined after i.v. injection of [^{3}H]CHE-labeled liposomes. Data represent the mean %ID/g tissue ± SD (n = 3 mice per time point).
†P < 0.005, compared with SL-Dox. Statistical significance of the difference between SIL-Dox and SL-Dox was evaluated with a two-tailed, unpaired Student's t test.

As shown in Table 3, there was only very little uptake in the other major organs, such as heart, lungs, and kidneys, for either liposome formulation. However, with increasing time, both SIL-Dox and SL-Dox accumulated in the liver and spleen (Table 3). This reflects the ultimate clearance of both targeted and nontargeted liposomes by the reticuloendothelial system, as the PEG-containing phospholipids may gradually be metabolized, and is in good agreement with the findings of others (39, 40).

Antitumor Activity of EpCAM-Targeted Immunoliposomes

The therapeutic potential of the doxorubicin-loaded immunoliposomes (SIL-Dox) and the nontargeted (SL-Dox) liposomes was assessed in mice bearing SW2 tumor xenografts of 85 to 100 mm^3 in size. To this end, mice were injected i.v. with the liposomes either at a dose of 4 mg doxorubicin/kg (~0.66 μmol phospholipid per mouse) or 7.5 mg doxorubicin/kg (~1.25 μmol phospholipid per mouse) for 3 weeks at weekly intervals (total dose of 12 mg or 22.5 mg doxorubicin/kg, approximately 2 or 3.75 μmol phospholipid, respectively). Mice in other control groups received HEPES-buffered saline or free doxorubicin at its MTD of 7.5 mg/kg.

As shown in Fig. 3, there was a significantly greater tumor growth inhibition, compared with free doxorubicin, in mice treated with SL-Dox or SIL-Dox, at doses of either 4 mg or 7.5 mg/kg. As expected, the tumor growth inhibition effect was more pronounced at the MTD of 7.5 mg/kg free doxorubicin equivalent, although at this dose there was a failure to distinguish between the two treatments as both inhibited tumor growth almost completely. The antitumor activity of SIL-Dox was significantly higher than that of the SL-Dox liposomes when given at 4 mg/kg (P < 0.01). At a dose of 7.5 mg doxorubicin/kg, mice in the SL-Dox group showed signs of toxicity, including weight loss, although this was not apparent in the SIL-Dox group. At a dose of 4 mg doxorubicin/kg, SL-Dox showed increased activity over the free drug (P < 0.05), and free doxorubicin also inhibited tumor growth compared with the saline control. Even at the end of the observation period, >50 days after the start of treatment, the median tumor volume of the SIL-Dox group did not exceed the initial volume by >2-fold at 7.5 mg doxorubicin/kg and by >6-fold at 4 mg doxorubicin/kg group. In contrast, at >50 days, the nontargeted liposomes only inhibited tumor growth at the MTD.

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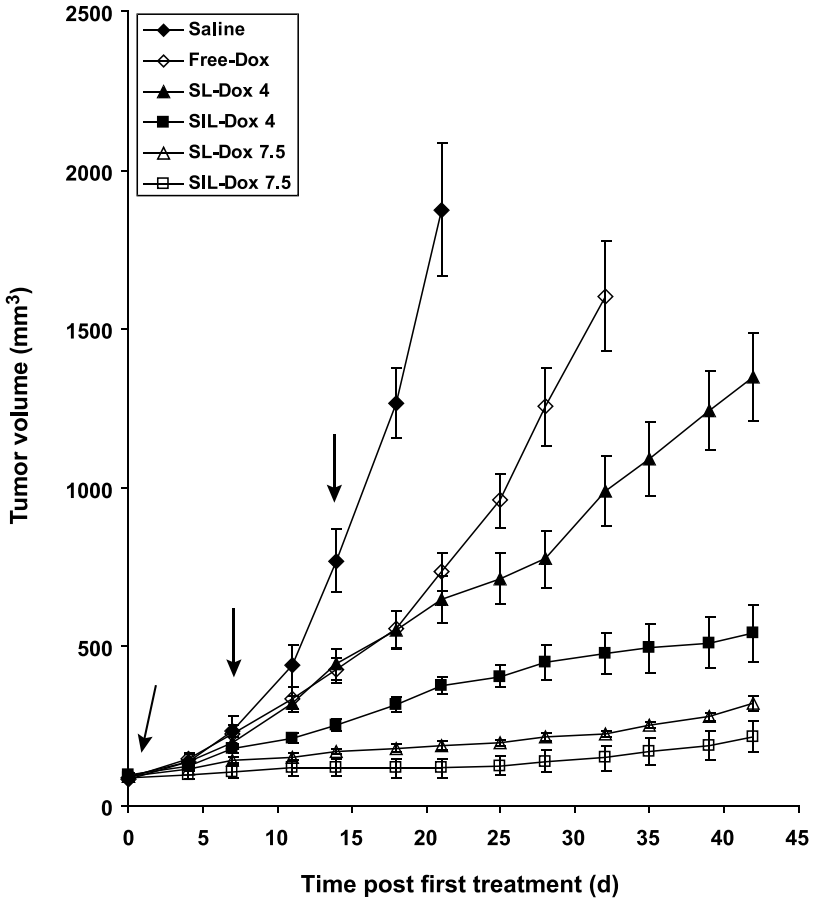

Figure 3. Therapeutic efficacy of EpCAM-targeted immunoliposomal doxorubicin in a tumor xenograft model. CD-1 athymic mice (five to seven per group) bearing SW2 tumor xenografts (85–100 mm^3) received i.v. injections of anti-EpCAM immunoliposomal doxorubicin (SIL-Dox) at a dose of either 4 or 7.5 mg/kg (MTD of the free doxorubicin equivalent). Other treatment groups included HEPES-buffered saline, nontargeted liposomal doxorubicin (SL-Dox) given at the same dose, and free doxorubicin given at its MTD of 7.5 mg/kg. All treatment groups received a total of three treatments at weekly intervals for 3 wk. Liposomal doxorubicin was injected to a total dose of 12 mg/kg and free doxorubicin was 22.5 mg/kg. *Arrows in the tumor growth curve,* treatment schedule. The median tumor size at the start of treatment was 90 mm^3. *Points,* mean values of the treatment groups; *bars,* SE.

Discussion

In this study, we generated EpCAM-specific immunoliposomes (SIL-Dox) encapsulating the anticancer agent doxorubicin. Targeted delivery of doxorubicin using liposomes conjugated with the internalizing EpCAM-specific scFv 4D5MOCB showed improved therapeutic efficacy compared with the nontargeted SL-Dox formulation, and this advantage was particularly pronounced when the injected dose was well below the MTD. This clearly shows the potential of EpCAM-targeted nanovesicles to enhance the antitumor effect of doxorubicin at low, well-tolerated dose levels and suggests further investigations to assess the clinical potential of this nanovesicular drug delivery system for cancer therapy.

Cytotoxic anticancer agents have been encapsulated in liposomes with the aim to improve their pharmacokinetic properties and therapeutic window by decreasing dose-limiting side effects (4, 5). Nontargeted sterically stabilized liposomes, such as Doxil/Caelyx, which carry PEG-phospholipid derivatives on their surface, offer the advantage of a long plasma half-life and the ability to release the encapsulated drug over an extended period of time (5, 41, 42). However, nontargeted liposomes only passively target diseased tissues mainly due to differences in their vascular architecture and permeability. Moreover, drug uptake by tumor cells occurs only following release of the encapsulated drug from liposomes into the tumor interstitial space from where drug uptake into nontarget cells may also occur as well as by drug redistribution out of the tumor. Nontargeted liposomes preferentially localize in the tumor stroma and in tissue macrophages, but not inside the tumor cells (8, 43). In contrast to this, targeted immunoliposomes are predominantly found internalized in tumor cells within a broad area of distribution in the tumor tissue (8). Tumor-targeted drug delivery, mediated by the binding of the liposomes to a tumor-associated antigen with a high rate of endocytosis such as EpCAM, represents a rational approach not only to get more of the encapsulated drug into target cells, but also to increase the specificity of drug uptake.

Immunoliposomes have successfully been used in preclinical studies to deliver anticancer agents to different tumor types by binding to various antigens on the cell surface (8, 16–18, 36). The successful large-scale production of HER2-specific immunoliposomes (19) has finally paved the way also for the clinical use of tumor-targeted nanovesicles. In addition to the aforementioned targets, EpCAM also holds promise as a target for liposomal drug delivery because it is abundantly expressed in many solid tumors and shows limited expression in normal epithelial

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tissues (20, 23, 44, 45). On antibody binding, it is rapidly internalized by endocytosis and thus well suited to deliver anticancer agents that act intracellularly. We previously reported the high antigen-binding affinity and the excellent *in vivo* tumor-targeting properties of the EpCAM-specific scFv 4D5MOCB (30). Owing to these favorable properties, the scFv 4D5MOCB has also been used for the generation of an immunotoxin (4D5MOCB-ETA; ref. 27), which under the names Proxinium™ and Vicinium™ is currently under investigation in phase II and III clinical trials (28, 46).

The finding that the addition of free scFv could block cell binding and that the level of uptake in EpCAM-positive cells was higher at 37°C than at 4°C indicates that internalization on binding occurred by active receptor-mediated endocytosis or by another unknown mechanism. As expected, on EpCAM-negative RL cells, no difference in cell binding and inhibition of proliferation was observed for EpCAM-targeted and nontargeted liposomes. These results are in line with previous reports showing that the uptake of targeted liposomes by receptor-mediated endocytosis is obligatory for the cytotoxicity of encapsulated drugs (31, 36, 47).

The EpCAM-targeted immunoliposomes generated with scFv antibodies showed long circulation times comparable to nontargeted liposomes, likely due to the lack of an Fc portion of the antibody, which would accelerate catabolism (16, 17, 48). Long-circulating liposomes seem to be needed for improved tumor localization (17, 18, 48) and increased antitumor activity (9, 17, 18, 49, 50). The EpCAM-targeted immunoliposomes described here, with their long circulation half-lives, achieved double the uptake of nontargeted liposomes into tumors 24 h after injection, although both types of liposomes showed similar biodistribution patterns for the other major organs. Other investigators have reported that the tumor localization was similar for either targeted or nontargeted liposomes (18, 40). Given that both types of liposomes reach solid tumors by the passive targeting mechanism, the reason for the increased tumor localization of the EpCAM-targeted liposomes versus the nontargeted liposomes may be due to both the high affinity of the scFv fragment, for the antigen, its rapid internalization, and its stability, as a high percentage of scFv remains intact after coupling and during 48 h in the body (30).

It is notable that the antitumor effect of EpCAM-targeted SIL-Dox immunoliposomes was superior to that of nontargeted liposomes when given at a lower dose, although at the MTD the effect of the two liposomal preparations was similar. The phenomenon that at high doses nontargeted SL-Dox was as effective as tumor-targeted SIL-Dox in inhibiting tumor growth is well known and has been reported previously also in other *in vivo* tumor models (51, 52). One explanation may be that at the MTD the accumulation of SIL-Dox at the tumor site becomes limited by receptor saturation. On the other hand, the nontargeted SL-Dox continues to passively localize in the tumor and finally reach a similar level of drug concentration. Because tumor growth is very low for both preparations at the MTD, the high dose is not capable of distinguishing the efficacy of one treatment versus the other. In this situation, a commonly accepted means of distinguishing between the two treatments is to lower the dose of each.

In conclusion, we describe the development of EpCAM-targeted liposomal nanovesicles and show their ability to efficiently deliver a cytotoxic payload to tumor cells in an antigen-dependent manner *in vitro* and *in vivo*. In view of the currently still unmet medical need for strategies that focus toxicity to tumor cells and away from normal tissues, EpCAM-specific nanovesicular drug delivery systems hold promise to successfully expand our arsenal of highly selective and effective tumor-targeted cancer therapeutics.

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