Anti-CD166 single chain antibody-mediated intracellular delivery of liposomal drugs to prostate cancer cells

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Abstract

Targeted delivery of small-molecule drugs has the potential to enhance selective killing of tumor cells. We have identified previously an internalizing single chain [single chain variable fragment (scFv)] antibody that targets prostate cancer cells and identified the target antigen as CD166. We report here the development of immunoliposomes using this anti-CD166 scFv (H3). We studied the effects of a panel of intracellularly delivered, anti-CD166 immunoliposomal small-molecule drugs on prostate cancer cells. Immunoliposomal formulations of topotecan, vinorelbine, and doxorubicin each showed efficient and targeted uptake by three prostate cancer cell lines (Du-145, PC3, and LNCaP). H3-immunoliposomal topotecan was the most effective in cytotoxicity assays on all three tumor cell lines, showing improved cytotoxic activity compared with nontargeted liposomal topotecan. Other drugs such as liposomal doxorubicin were highly effective against LNCaP but not PC3 or Du-145 cells, despite efficient intracellular delivery. Post-internalization events thus modulate the overall efficacy of intracellularly delivered liposomal drugs, contributing in some cases to the lower than expected activity in a cell line–dependent manner. Further studies on intracellular tracking of endocytosed liposomal drugs will help identify and overcome the barriers limiting the potency of liposomal drugs. [Mol Cancer Ther 2007;6(10):2737–46]

Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related death of men in industrialized countries (1, 2). Androgen deprivation and androgen receptor blockade have been the mainstays of treatment for prostate cancer. Although initially effective, hormone therapy fails for the majority of initial responders, as subpopulations of tumor cells undergo mutations and gain capacity to proliferate in an androgen-deprived environment (3, 4). Until recently, the therapeutic alternatives for hormone-refractory prostate cancer were limited (4).

Monoclonal antibodies against tumor markers can be exploited for the development of targeted therapeutics. Phage antibody display is an effective way to generate tumor-specific human antibody fragments (5, 6). For example, internalizing single chain variable fragments (scFv) targeting erbB2 and epidermal growth factor receptor (EGFR) has been identified and used to deliver liposomal drugs to breast cancer cells (7–9). Recently, we have selected a nonimmune phage antibody library containing more than 100 million members on live tumor cells and identified a panel of internalizing scFvs targeting prostate cancer cells (10). We have identified a subset of unique antibodies that (a) recognize hormone-refractory cell lines; (b) deliver therapeutic payloads efficiently and specifically to tumor cells; and (c) stain tumor cells in tissue slides by immunohistochemistry (10, 11). Using immunoprecipitation and mass spectrometry, we have identified the antigen for one of the selected scFv antibodies (H3) as CD166, also known as MEMD or activated leukocyte cell adhesion molecule (11, 12). CD166, a member of the immunoglobulin superfamily, was found to be expressed on cultured prostate cancer cells and specifically in prostate cancer tissues (13–15). Immunohistologic studies detected overexpression of CD166 in 86% of prostate carcinomas (13). In addition, CD166 was shown to undergo ligand-induced endocytosis (14). An internalizing scFv targeting CD166 could thus be used for intracellular delivery of various therapeutic agents to prostate cancer cells.

Liposomal nanocarriers represent one of the most efficient and well-characterized drug delivery strategies for small molecules (16, 17). Liposomal encapsulation of a drug can improve the toxicity profile and therapeutic efficacy of the encapsulated agent by enhancing its localization to solid tumors and optimizing the rate at which it becomes bioavailable due to release from the nanocarrier (16, 18). Therapeutic immunoliposomes contain encapsulated chemotherapeutic drugs and have antibody fragments conjugated to their surface to impart delivery specificity and preferably endocytosis, leading to improved delivery of bioavailable drug and antitumor efficacy.
In this study, we used the internalizing anti-CD166 human scFv to target immunoliposomes to prostate cancer cells. We have constructed H3-targeted immunoliposomes encapsulating small-molecule drugs that disrupt DNA metabolism (topotecan, doxorubicin, and mitoxantrone) and affect microtubule dynamics (vinorelbine) and key post-translational modifications, such as histone deacetylation (LAQ824). The internalization and cytotoxicity of the various scFv-targeted immunoliposomal drug formulations were compared with nontargeted liposomal formulations of the same drugs. We found that H3 scFv-targeted immunoliposome drugs were efficiently delivered intracellularly to prostate cancer cells. However, different immunoliposome drugs exhibited varying degrees of efficacy when compared with the corresponding nontargeted liposomes or free drugs. Anti-CD166 (H3 scFv) immunoliposome topotecan, once delivered intracellularly, was effective in killing prostate cancer cells, whereas nontargeted liposome topotecan or free topotecan were not. Other immunoliposome drugs, such as doxorubicin, however, displayed modest improvements in targeted cytotoxic activity when compared with the nontargeted liposome drugs despite significant intracellular uptake by prostate cancer cells. Thus, events after internalization may significantly modify the efficacies of immunoliposome drugs.

Materials and Methods

Materials

Pepsin A, 2-mercaptoethanol, 2-mercaptoethanol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich. DiIC16(3)-DS was purchased from Molecular Probes/Invitrogen. Distearoylphosphatidylcholine and poly(ethylene)glycol (PEG2000)-derivated distearoylphosphatidylethanolamine (mPEG2000-DSPE) were purchased from Avanti Polar Lipids. Cholesterol was purchased from Calbiochem and maleimide-derivatized PEG-DSPE (Mal-PEG-DSPE) from Shearwater Polymers. Topotecan was a kind gift of the Taiwan Liposome Co. and LAQ824 of Novartis Oncology. Mitoxantrone dihydrochloride was purchased from LKT Laboratories. Doxorubicin hydrochloride (Bedford Laboratories) and vinorelbine (GlaxoWelcome) were obtained from the pharmacy. Sucrose octasulfate (sodium salt) was purchased from Toronto Research Chemicals, Inc.

Cell Lines

All cell lines (PC3, Du-145, LNCaP, and SKBR3) were obtained from American Type Culture Collection. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 ºC and 5% CO2.

Production of H3 scFv

The coding region of the H3 scFv was spliced into an expression vector imparting a cysteine and a hexahistidine tag at the COOH terminus as described (10). Following IPTG induction, antibody fragments were purified from bacterial periplasmic space on Ni2+-NTA beads (Qiagen) as described (10).

Preparation of Fluorescence-Labeled and Drug-Loaded Liposomes

Fluorescently labeled unilamellar liposomes were prepared according to the repeated freeze-thawing method of Szoka and Papahadjopoulos (19). Liposomes were composed of distearoylphosphatidylcholine, cholesterol, mPEG-DSPE, and the lipophilic fluorescent marker, DiIC16(3)-DS, combined in a 200:133:1:1 molar ratio. Liposomes were subsequently extruded 10 to 15 times through polycarbonate filters with defined pore sizes of 0.1 µm, yielding liposomes of 100 to 120 nm diameter as determined by dynamic light scattering. Liposomal phospholipid concentrations were determined using a standard phosphate assay (20).

For encapsulation of doxorubicin, a gradient-based drug loading method using diethylammonium sulfate was used (21, 22). First, the dried lipids distearoylphosphatidylcholine/cholesterol/mPEG-DSPE (molar ratio, 3:2:0.015) were dissolved in ethanol and heated to 60 ºC. The ethanolic lipid solution was subsequently injected into a heated solution (60 ºC) of 200 mmol/L diethylammonium sulfate (pH 5.5), followed by extrusion of the hydrated lipid suspensions at 60 ºC through polycarbonate filters with uniform pore sizes ~0.1 µm. Free diethylammonium sulfate was removed by size-exclusion chromatography using a Sephadex G-75 column eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl (pH 6.5)]. Liposomes were then incubated with doxorubicin (150 µg drug/µmol phospholipid) for 30 min at 60 ºC, and unencapsulated doxorubicin was removed by gel filtration chromatography using a Sephadex G-75 column eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl (pH 7.2)]. The drug mitoxantrone was loaded into liposomes using the same method.

Liposomal topotecan of an identical lipid composition was prepared using a novel intraliposomal drug stabilization strategy as described (23). One modification from the published method (23) was that the drug entrapping solution was diethylammonium sucrose octasulfate [0.65 mol/L DEA (pH 5.5)]. Diethylammonium sucrose octasulfate was prepared from the commercially obtained sodium salt by ion exchange chromatography on the Dowex 50W×8-200 resin in the H+ form, immediately followed by titration with neat diethylamine. Following extrusion, unentrapped diethylammonium sucrose octasulfate was removed from a Sepharose CL-4B size exclusion column eluted with HEPES-buffered dextrose [5 mmol/L HEPES, 5% dextrose (pH 7.0)]. Topotecan was then added at 350 g drug/mol phospholipid and the pH was adjusted to 6.0 to 6.5 with 1 N HCl before initiating loading at 60 ºC for 30 min. The resulting liposomal topotecan was subsequently placed on ice for 15 min and purified on a Sephadex G-75 column to remove unencapsulated drug. The histone deacetylase inhibitor LAQ824 and the Vinca alkaloid, vinorelbine, were loaded using methods described previously (24, 25).
Purified H3 (scFv)\textsubscript{2} were reduced with 20 mmol/L mercaptoethylamine by incubation at 37°C for 45 min in PBS containing 2 mmol/L EDTA (pH 6.0) deoxygenated by bubbling argon through it. Reduced scFvs were subsequently recovered by purification on a Sephadex G-25 gel filtration column eluted with HEPES-buffered saline [5 mmol/L HEPES, 145 mmol/L NaCl, 3.4 mmol/L EDTA (pH 7.0)]. Reduction efficiencies were evaluated by SDS-PAGE. Typically >90% of scFv dimers were reduced. The liposomes were activated by incorporation of Mal-PEG-DSPE as follows: Mal-PEG-DSPE was dissolved in water and added to the preformed liposomes at 0.5 mole% of the liposome phospholipid, and the mixture was incubated at 60°C for 30 min (26, 27). The pH was increased to 7.0 by titration with a HEPES buffer [0.5 mol/L (pH 7.0)]. Reduced scFv was incubated with activated liposomes overnight at room temperature at 30 μg/μmol of phospholipids, corresponding to ~60 scFv/liposomal particle. RT, room temperature.

**Internalization Studies by Fluorescence Microscopy**
Prostate cancer cells (PC3, Du-145, and LNCaP) grown to 80% confluency in 24-well plates were coincubated with H3 scFv-targeted immunoliposomes or nontargeted liposomes labeled with DiIC\textsubscript{18}(3)-DS, at concentrations of 0 to 50 μmol/L phospholipids, for 4 h at 37°C. The cells were washed twice with PBS and examined through a Nikon Eclipse 300 inverted fluorescence microscope (Nikon) with a 540/25 nm bandpass filter for excitation and a long pass filter at 565 nm for emission.

**Flow Cytometry Analysis**
DiIC\textsubscript{18}(3)-DS–labeled liposomes were used for these studies. Cells grown to 80% confluency in 12-well plates were coincubated with DiIC\textsubscript{18}(3)-DS–labeled immunoliposomes or nontargeted liposomes, at concentrations of 0 to 50 μmol/L phospholipids, for 4 h at 37°C. After washing with either PBS or 100 mmol/L glycine/150 mmol/L NaCl (pH 2.8), cells were analyzed by fluorescence-activated cell sorting (BD LSRII). The ratios of the mean fluorescence intensity between the glycine-washed and PBS-washed cell samples were used to calculate the percentage of internalized liposomes. To reproduce conditions used in the drug studies, cells were further incubated in fresh medium without immunoliposomes for an additional 8 h to determine fraction internalized (typically >85%) by glycine treatment. The fraction released into the medium during the 8-h chase (typically <10%) was determined by comparing total cell-associated mean fluorescence intensity values before and after the 8-h chase.
Cellular Uptake of Immunoliposome Drugs

Prostate cancer cells were seeded in 12-well plates at 350,000 cells per well and incubated in triplicates with liposomal drugs (immunoliposomes and nontargeted liposomes at 10 μg/mL) for 4 h at 37°C. After 4-h incubation, cells were washed thrice with PBS and lysed with 1% Triton X-100 (100 μL/well) for 10 min at 37°C. For the samples containing doxorubicin, 70% acid isopropanol was added, whereas for topotecan and vinorelbine, a mixture of acetonitrile/methanol 1:1 and acidic methanol (500 μL/well) was added. The plates were shaken for 30 min at room temperature. The samples were then centrifuged at 10,000 rpm for 2 min and the supernatants were measured by fluorescence spectroscopy (Fluorolog II, Jobin Yvon Spex) for doxorubicin (λEx/Em = 470/590 nm) and topotecan (λEx/Em = 382/530 nm). The concentration of topotecan or doxorubicin was calculated by extrapolation using a standard curve and normalized to the cell number, and cellular uptake was expressed as nanogram drug per million cells. For vinorelbine, the samples were loaded on a C18 reverse-phase silica column (Supelco C-18 column, 250 × 4 mm i.d., particle size of 5 μm) preceded by a C-18 guard column. The column was eluted isocratically with aqueous 50 mmol/L triethylammonium acetate (pH 5.5) and acetonitrile (58:42, v:v) at a flow rate of 1.0 mL/min and detected by absorbance at 280 nm. A typical retention times for vinorelbine was 9.1 min.

In vitro Cytotoxicity

Prostate cancer cells were seeded in 96-well plates (6,000 cells per well for PC3 and Du-145 cells; 10,000 cells per well for LNCaP cells) and incubated with liposomes (immunoliposomes and nontargeted liposomes) or free drugs (0–10 μg/mL) for 4 h at 37°C. Cells were washed twice with supplemented RPMI 1640 to remove drugs and incubated with fresh medium for an additional 72 h at 37°C. Cell viabilities of PC3 and Du-145 cells were assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining as described (28), and

Figure 2. Internalization of H3 scFv-targeted immunoliposomes. Du-145 and PC3 cells were treated with H3 scFv-targeted DiIC18(3)-DS–labeled immunoliposomes (25 μmol/L phospholipids) or control nontargeted liposomes at 37°C for 4 h, and uptake was examined by fluorescence microscopy. Insets, fluorescence-activated cell sorting analysis of fluorescence signals and images of phase-contrast microscopy to indicate the presence of cells.
theresultswerereadat570nmusingamicrotiterplatereader(SpectraMax190,MolecularDevices).ThecellviabilityofLNCaPcellswasdeterminedusingtheCellcountingkit-8(Dojindo)accordingtothemanufacturer’sinstructions.

**Results**

**Construction of Immunoliposomes Targeting Prostate Cancer**

Liposomes were prepared using a lipid molar ratio distearoylphosphatidylcholine/cholesterol/mPEG-DSPE 3:2:0.015, and 0.5 mole% Mal-PEG-DSPE was further inserted for antibody conjugation. Liposomes were either labeled with the fluorescent lipid DiIC18(3)-DS or loaded with a chemotherapeutic drug, such as doxorubicin, topotecan, vinorelbine, mitoxantrone, or LAQ824. The gene encoding the H3 scFv was spliced into a bacteria expression vector to create an in-frame fusion with the amino acid cysteine and the hexahistidine tag (10). For conjugation to liposomes, the (scFv)2 were purified and reduced using 2-mercaptoethamine. Approximately 90% of H3 (scFv)2 were in a reduced form as estimated from a nonreducing SDS-PAGE gel (Fig. 1A). The reduced scFv was then conjugated via its cysteine to maleimide-modified termini of PEG chains located on the external surface of the liposomes (Fig. 1B), producing 100 to 120 nm diameter liposomes bearing ~60 scFvs on the surface.

**Internalization of scFv-Targeted Immunoliposomes in Prostate Cancer Cell Lines**

Internalization of H3 scFv-targeted immunoliposomes in prostate cancer cell lines was evaluated by fluorescence microscopy and flow cytometry. For this purpose, liposomes were fluorescently labeled with DiIC18(3)-DS and conjugated with the reduced H3 scFv. Fluorescence microscopy showed significant uptake of H3 scFv-targeted immunoliposomes by prostate cancer cell lines (Fig. 2). Without scFv, nontargeted liposomes were not significantly endocytosed (Fig. 2), showing the importance of scFv on the liposome surface for targeted delivery. We further quantified liposome binding and uptake by fluorescence-activated cell sorting. H3 scFv-targeted immunoliposomes bound to prostate cancer cells in a concentration-dependent manner (Fig. 3A–C). H3-targeted immunoliposomes bound to all three prostate cancer cell lines with $K_d$ values in the range of 4.5 to 7 μmol/L phospholipids (corresponding to 75–116 pmol/L immunoliposomes). At saturating...
concentrations (50 μmol/L phospholipids), more immunoliposomes were bound by LNCaP than Du-145 and PC3 cells, suggesting a higher level of surface expression of CD166 on LNCaP cells. In all experiments, nontargeted liposomes did not show significant binding to prostate cancer cells, consistent with the results of the fluorescence microscopy studies.

We further did a dose-response experiment at 37°C, which permits receptor endocytosis (Fig. 3D). Below ~10 μmol/L phospholipids, the amount of immunoliposomes associated with prostate cancer cells was linearly related to the amount of liposomes added. Above 15 μmol/L, a plateau was observed for all prostate cancer cells studied (Fig. 3D). There was no association of nontargeted liposomes to any of the prostate cancer cells studied across all the concentrations tested (Fig. 3D).

**Uptake of H3 scFv-Targeted Immunoliposome Drugs by Prostate Cancer Cells**

Three liposomal drugs were studied for cell uptake: doxorubicin, topotecan, and vinorelbine. In all experiments, nontargeted liposome drugs were poorly taken up by prostate cancer cells (Fig. 4A and B). In contrast, delivery of drugs by H3 scFv-targeted immunoliposomes to prostate cancer cells was efficient, and high uptake ratios of immunoliposome drugs versus nontargeted liposome drugs were obtained. The level of uptake varied depending on the cell lines and specific liposomal drugs. LNCaP cells took up more liposomal doxorubicin than PC3 and Du-145 cells (1,802 ng drug/million LNCaP cells versus 277 ng drug/million cells for Du-145 cells and 177 ng drug/million cells for PC3 cells; Fig. 4A). The uptake of H3 scFv-targeted immunoliposomes encapsulating topotecan was similar in the three cell lines (between 731–1,299 ng drug/million cells; Fig. 4B) and ranged from 20- to 38-fold greater than observed for the corresponding nontargeted liposomes. Similarly, H3 scFv-targeted immunoliposome vinorelbine was efficiently taken up by prostate cancer cells, with uptake reaching 559 ng drug/million Du-145 cells, 1,173 ng drug/million PC3 cells, and 2,670 ng drug/million LNCaP cells (data not shown). The targeting by H3 scFv thus promoted efficient drug delivery to prostate cancer cells. All these data are consistent with the results obtained using Dil-labeled H3 scFv-targeted immunoliposomes (see Fig. 2).

**In vitro Cytotoxicity Studies**

Five anticancer drugs were studied, targeting various cellular functions. Topotecan, doxorubicin, and mitoxantrone were studied for cell uptake: doxorubicin, topotecan, and vinorelbine. In all experiments, nontargeted liposome drugs were poorly taken up by prostate cancer cells (Fig. 4A and B). In contrast, delivery of drugs by H3 scFv-targeted immunoliposomes to prostate cancer cells was efficient, and high uptake ratios of immunoliposome drugs versus nontargeted liposome drugs were obtained. The level of uptake varied depending on the cell lines and specific liposomal drugs. LNCaP cells took up more liposomal doxorubicin than PC3 and Du-145 cells (1,802 ng drug/million LNCaP cells versus 277 ng drug/million cells for Du-145 cells and 177 ng drug/million cells for PC3 cells; Fig. 4A). The uptake of H3 scFv-targeted immunoliposomes encapsulating topotecan was similar in the three cell lines (between 731–1,299 ng drug/million cells; Fig. 4B) and ranged from 20- to 38-fold greater than observed for the corresponding nontargeted liposomes. Similarly, H3 scFv-targeted immunoliposome vinorelbine was efficiently taken up by prostate cancer cells, with uptake reaching 559 ng drug/million Du-145 cells, 1,173 ng drug/million PC3 cells, and 2,670 ng drug/million LNCaP cells (data not shown). The targeting by H3 scFv thus promoted efficient drug delivery to prostate cancer cells. All these data are consistent with the results obtained using Dil-labeled H3 scFv-targeted immunoliposomes (see Fig. 2).
target DNA metabolism, vinorelbine disrupts microtubule function, and LAQ824 inhibits histone deacetylation. We first studied sensitivity of prostate cancer cells to free drugs. Other than topotecan, which has a poor membrane permeability (29, 30), the other drugs in their free forms showed potent cytotoxicities toward prostate cancer cells (Supplementary Table S1). The cytotoxic effects (IC_{50}) of the same drugs in liposomal forms, either H3 scFv targeted or nontargeted, are shown in Table 1. In most instances, drug-loaded nontargeted liposomes showed a weak cytotoxicity toward the cells studied. Some nontargeted liposomes showed cytotoxicity at the highest concentrations tested, probably due to a small amount of weakly associated drug or leakage of drug from the liposomes during incubation with prostate cancer cells. H3 scFv-targeted immunoliposome drugs showed various levels of *in vitro* cytotoxicity. Treatment with H3 scFv-targeted immunoliposome topotecan was 5-fold more efficient in killing Du-145 cells than nontargeted liposome topotecan (IC_{50}, 0.13 versus 0.6 µg/mL; Fig. 4D; Table 1). Similar improvements in cytotoxicity were obtained for LNCaP cells (0.029 µg/mL immunoliposomes versus 0.14 µg/mL nontargeted liposomes) and PC3 cells (1.6 versus >10 µg/mL; Table 1). There was no significant improvement (immunoliposome topotecan versus nontargeted liposomes) in cytotoxicity on the control cell line SKBR3 (Table 1; the CD166 expression on SKBR3 was shown in Supplementary Fig. S1). The immunoliposome topotecan is thus a promising therapeutic for the treatment of prostate cancer.

Treatment with H3 scFv-targeted immunoliposome doxorubicin was 39-fold more potent in killing LNCaP cells than nontargeted liposome doxorubicin (IC_{50}, 0.19 versus 7.4 µg/mL; Fig. 4C; Table 1). However, H3 scFv-targeted immunoliposome doxorubicin did not show significant improvement in cytotoxicity in PC3 or Du-145 cells, when compared with nontargeted liposome doxorubicin, although both cell lines were sensitive to free doxorubicin (Table 1). The sensitivity of LNCaP cells to free doxorubicin was similar to that of Du-145 cells (Table 1). It is interesting to note that LNCaP cells took up about six times more immunoliposome doxorubicin (see Fig. 4A) than Du-145 cells, possibly due to a higher level of CD166 expression on those cells (see Fig. 3). No improvement in cytotoxicity (immunoliposomes versus nontargeted liposomes) was observed in the control cell line SKBR3 (Table 1).

The improvement of other immunoliposomes over nontargeted liposome drugs is rather moderate. H3 scFv-targeted immunoliposome vinorelbine showed a 4-fold improvement in cytotoxicity compared with nontargeted liposome vinorelbine in Du-145 cells, 2-fold in PC3 cells, and 3.5-fold in LNCaP cells (data not shown). The improvement in cytotoxic activity for immunoliposome mitoxantrone and LAQ824 was about 1.5- to 3-fold over nontargeted liposome drugs (data not shown).

The above studies show that although H3 scFv-targeted immunoliposome drugs were efficiently delivered intracellularly into prostate cancer cells, their potencies vary depending on the drug and the cell line used. The immunoliposome topotecan was the most effective among the various drugs that we have studied for cytotoxic activity in all three prostate cancer cell lines.

### Potency of a Liposomal Drug versus a Free Drug

The aforementioned studies revealed discrepancies between cytotoxicity profile and uptake for some of the drugs studied. For example, H3 scFv-targeted immunoliposome doxorubicin was readily taken up by PC3 and Du-145 cells but its cytotoxicity was not significantly improved compared with nontargeted liposomes. To understand the relationship between the amount of liposomal drug accumulated in the cells and its cytotoxic effects, we compared viabilities of cells taking up equal amounts of free versus immunoliposome drug. We first measured concentration-dependent uptake of free doxorubicin in Du-145 cells (Fig. 5A). For example, when incubated with 1.0 µg/mL free doxorubicin, 277 ng doxorubicin was taken up per 10^6 cells. This amount of cellular uptake of free drug was sufficient to kill 91% of Du-145 cells (Fig. 5B and C). Approximately the same amount of liposomal doxorubicin/10^6 cell was delivered to the Du-145 cells after 4-h incubation of H3 scFv-targeted immunoliposome doxorubicin (at 10 µg drug/mL; Fig. 4A). If doxorubicin is efficiently liberated from its liposomal carrier on internalization, then one liposomal doxorubicin molecule should be as potent as one free doxorubicin molecule, and the amount delivered should theoretically be sufficient to cause similar cytotoxic effects in Du-145 cells. However, only 58% killing of Du-145 cells was achieved (Fig. 5B), indicating that at equal intracellular concentration, liposome-encapsulated doxorubicin was less efficient in cell killing compared with free drug. Thus, to achieve similar levels of cell killing, more immunoliposome doxorubicin was needed to be delivered into target cells than free drug.

### Table 1. Cytotoxic effects (IC_{50}, µg/mL) of various drugs in free, liposomal, or H3 scFv-targeted immunoliposomal forms, on prostate cancer and control cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>PC3</th>
<th>Du-145</th>
<th>LNCaP</th>
<th>SKBR3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50} Ratio</td>
<td>IC_{50} Ratio</td>
<td>IC_{50} Ratio</td>
<td>IC_{50} Ratio</td>
</tr>
<tr>
<td>TPT</td>
<td>Free</td>
<td>3.4</td>
<td>0.50</td>
<td>3.2</td>
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<tr>
<td></td>
<td>NT-Ls</td>
<td>&gt;10</td>
<td>0.6</td>
<td>0.14</td>
</tr>
<tr>
<td>H3-iLs</td>
<td>&gt;10</td>
<td>&gt;6</td>
<td>0.13</td>
<td>0.029</td>
</tr>
<tr>
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<td>Free</td>
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<td>0.047</td>
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<tr>
<td></td>
<td>NT-Ls</td>
<td>&gt;10</td>
<td>9.0</td>
<td>7.4</td>
</tr>
<tr>
<td>H3-iLs</td>
<td>7.3</td>
<td>&gt;1.5</td>
<td>6.4</td>
<td>0.19</td>
</tr>
</tbody>
</table>

NOTE: The IC_{50} ratios (nontargeted liposomes/immunoliposomes) are calculated to indicate enhancement in cytotoxicity of H3-targeted immunoliposome over nontargeted liposome drugs.

Abbreviations: TPT, topotecan; Dox, doxorubicin; NT-Ls, nontargeted liposomes; iLs, immunoliposomes.

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6 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
For example, to achieve 58% killing (42% cell viability), 277 ng of immunoliposome doxorubicin were needed to be delivered per 10^6 cells, whereas only 30 ng of free doxorubicin per 10^6 cells were needed to achieve the same effect, a difference in efficacy of about 9-fold (Fig. 5D).

**Discussion**

Targeted cancer therapy requires the combination of a tumor recognition and a tumor killing function in a single therapeutic entity. We have previously identified an internalizing scFv, H3, which targets prostate cancer cells. The H3 scFv binds to an internalizing epitope of CD166 (12), which is overexpressed in both primary and metastatic prostate carcinomas (13, 31, 32). We developed H3 scFv-targeted immunoliposomes and studied cytotoxicity profiles of a panel of liposomal small-molecule drugs following intracellular delivery to prostate cancer cells. The use of scFv instead of whole IgG molecule prevents Fc receptor-mediated clearance in vivo (33, 34), protecting liposomes from rapid clearance by macrophages of the reticuloendothelial system (25, 35).

We constructed immunoliposomes encapsulating a panel of small-molecule drugs and studied their cytotoxic activity in prostate cancer cells following H3 scFv-targeted delivery. These drugs target different cellular functions. Topotecan, doxorubicin, and mitoxantrone disrupt DNA metabolism, whereas vinorelbine and LAQ824 disrupt microtubule assembly and inhibit histone deacetylation, respectively. We first studied uptake of fluorescently labeled immunoliposomes and found a significant increase in intracellular delivery compared with fluorescently labeled nontargeted liposomes. We next quantified uptake of liposomal drugs by prostate cancer cells. We found that, like the fluorescently labeled immunoliposomes, H3 scFv-targeted immunoliposome drugs were efficiently internalized by the three prostate cancer cells studied. Nontargeted liposome drugs were not efficiently taken up by prostate cancer cells, showing the importance of a targeting function in mediating efficient payload delivery to tumor cells.

Although scFv targeting the CD166 epitope was effective in mediating intracellular delivery of liposomal drugs, the
cytotoxic effects of the drugs differed considerably between drugs. H3 scFv-targeted delivery of liposomal topotecan achieved significantly greater cell killing than either non-targeted liposome or free topotecan, showing the benefit of a targeting mechanism. Unlike the other drugs studied, free topotecan did not cross cell membranes readily, and there was no measurable uptake in any of the prostate cancer cells studied even at 10 μg/mL (data not shown). This is consistent with the poor membrane permeability of the carboxylate form of the drug at neutral pH and thus poor cellular uptake and cytotoxicity (29, 30). Therefore, the use of a liposome carrier for topotecan is particularly relevant to its therapeutic effects.

For other drugs, however, efficient intracellular delivery did not readily predict their potency in cell killing. For example, immunoliposome doxorubicin was efficiently delivered to all three prostate cancer cells, but it was only in LNCaP cells that we observed a significant increase in potency compared with its nontargeted liposome counterpart. The total uptake of immunoliposome doxorubicin by LNCaP cells was several times higher than that of Du-145 and PC3 cells, possibly due to higher surface expression of CD166 on LNCaP cells as revealed by our fluorescein-activated cell sorting binding studies. Nonetheless, the amount of doxorubicin molecules delivered to Du-145 cells by H3 scFv-targeted immunoliposomes reached the level that should have been sufficient to kill those cells, as predicted from the uptake and cytotoxicity profile of free drug. It is thus evident that intracellularly delivered liposomal doxorubicin, on a mole-per-mole basis, is less potent in cell killing than free doxorubicin. We have shown that for Du-145 cells, 9-fold more immunoliposome doxorubicin than free drug needs to be delivered intracellularly to achieve the same level (58%) of cell killing.

Several factors may account for this observed discrepancy. For example, subcellular localization of endocytosed immunoliposomes and the release rate of encapsulated drugs may all have profound influence on the efficiency of liposomal drugs. Endocytosed immunoliposomes are often routed through the endosome and lysosome pathway (9, 36, 37). For encapsulated doxorubicin, release from liposomes and further escape from endosomes/lysosomes are required for translocation to the nucleus to disrupt DNA metabolism (38). Chloroquine and omeprazole increase endosome pH and have been reported to increase the rate of release of free doxorubicin trapped inside the endosome (39). Treatment of prostate cancer cells with chloroquine or omeprazole, either concurrently to or immediately after liposomal drug delivery, however, did not lead to significant changes in cytotoxicity of immunoliposome doxorubicin (data not shown), indicating that doxorubicin molecules remained encapsulated inside the liposomes. Further development of pH-sensitive liposome formulations may facilitate the collapse of endosome-trapped liposomes, allowing more efficient release of encapsulated drug (38, 39).

In conclusion, conjugating liposomes with a scFv targeting the internalizing epitope of CD166 is an effective strategy to deliver small-molecule drugs to prostate cancer cells. Once delivered inside the cells, liposomal drugs exhibited different cytotoxic effects compared with equal amounts of their corresponding free drugs. It is evident that post-internalization events modify the potency of liposomal drugs and in many cases reduce their efficacies compared with their free drug counterparts. This reduced efficacy, however, helps create a threshold effect that may increase the specificity of targeted killing by liposomal drugs as many tumor cell surface antigens are also expressed on normal cells at lower densities. In practice, the threshold is likely to vary depending on cell types, drugs used, and the formulation of liposomes. From the perspective of tumor cell surface antigens, the threshold hypothesis predicts that simultaneously targeting multiple epitopes will achieve greater potency than targeting a single epitope alone. Constructing such multitargeting immunoliposomes may offer one way to improve efficacy and specificity of tumor cell killing. Nonetheless, the demonstrated levels of prostate cancer uptake and cytotoxicity of the anti-CD166 scFv-targeted immunoliposome drugs warrant further investigation in vivo using tumor models, as targeting may further improve efficacy by increasing intratumoral distribution of the immunoliposome drugs (40).

References


