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Anti-MUC-1 immunoliposomal doxorubicin in the treatment of murine models of metastatic breast cancer

E.H. Moase [a], W. Qi [1,a], T. Ishida [2,a], Z. Gabos [a], B.M. Longenecker [c], G.L. Zimmermann [3,b], L. Ding [4,c], M. Krantz [c], T.M. Allen [a,*]

[a] *Department of Pharmacology, University of Alberta, Edmonton, AB T6G 2H7, Canada*
[b] *Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB T6G 2H7, Canada*
[c] *Biomira, Inc., 2011-94., Edmonton, AB T6N 1H1, Canada*

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Abstract

The fate of breast cancer patients is dependent upon elimination or control of metastases. We studied the effect of antibody-targeted liposomes containing entrapped doxorubicin (DXR) on development of tumours in two models of breast cancer, pseudometastatic and metastatic, in mice. The former used the mouse mammary carcinoma cell line GZHI, which expresses the human MUC-1 gene (L. Ding, E.N. Lalani, M. Reddish, R. Koganty, T. Wong, J. Samuel, M.B. Yacyshyn, A. Meikle, P.Y.S. Fung, J. Taylor-Papadimitriou, B.M. Longenecker, Cancer Immunol. Immunother. 36 (1993) 9–17). GZHI cells seed into the lungs of *Balb/c* mice following intravenous injection. The latter used the 4T1-MUC1 cell line, a MUC-1 transfectant of the mouse mammary carcinoma cell line 4T1, which metastasizes from a primary mammary fatpad (mfp) implant to the lungs (C.J. Aslakson, F.R. Miller, Cancer Res. 52 (1992) 1399-1405). B27.29, a monoclonal antibody against the MUC-1 antigen, was used to target sterically stabilized immunoliposomes (SIL[B27.29]) to tumour cells. In vitro, SIL[B27.29] showed high specific binding to both GZHI and 4T1-MUC1 cells. The IC_{50} of DXR-loaded SIL[B27.29] was similar to that of free drug for GZHI cells. In the pseudometastatic model, mice treated with a single injection of 6 mg DXR/kg in DXR-SIL[B27.29] at 24 h after cell implantation had longer survival times than those injected with non-targeted liposomal drug. In the metastatic model, severe combined immune deficiency mice given weekly injections $\times 3$ of 2.5 mg DXR/kg encapsulated in either targeted or non-targeted liposomes were almost equally effective in slowing growth of the

Abbreviations: MPS, mononuclear phagocyte system; DXR, doxorubicin; HSPC, hydrogenated soy phosphatidylcholine; PEG-DSPE, polyethylene glycol (M_r 2000) covalently coupled to distearoylphosphatidylethanolamine; CHOL, cholesterol; PL, phospholipid; PDP-PEG-DSPE, pyridyldithiopropionamide-polyethylene glycol (M_r 2000)-distearoylphosphatidylethanolamine; MCS, ε-maleimidocaproic acid *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid; MES, 2-(4-morpholino)ethanesulphonic acid; SL, sterically stabilized (Stealth) liposomes; SIL, sterically stabilized (Stealth) immunoliposomes; mAb, monoclonal antibody; NSAb, non-specific antibody (mAb HMSA-5); MST, mean survival time; IC_{50}, 50% inhibitory concentration; mfp, No. 4 mouse mammary fatpad; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SCID, severe combined immune deficiency; ^{125}I-dUrd, 5-[^{125}I]iododeoxyuridine; FITC, fluorescein isothiocyanate; HER2, a member of the erbB family of growth factor receptors

* Corresponding author. Fax: (780) 4928078; E-mail: terry.allen@ualberta.ca
[1] Present address: Chiron Corporation, 4560 Horton St., Emeryville, CA 94608-2916, USA.
[2] Present address: Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi,1-78-1, Tokushima 770-8505, Japan.
[3] Present address: Alberta Cancer Board, Tom Baker Cancer Centre, 1331-29 St. NW, Calgary, AB T2N 4N2, Canada.
[4] Present address: Department of Neurology, Long Island Jewish Medical Center, New Hyde Park, NY 11040, USA.

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primary tumour and reducing development of lung tumours. Surgical removal of the primary tumour from mfp, followed by various chemotherapy regimens, was attempted, but removal of the primary tumour was generally incomplete; tumour regrowth occurred and metastases developed in the lungs in all treatment groups. DXR-SL reduced the occurrence of regrowth of the primary tumour, whereas neither targeted liposomal drug or free drug prevented regrowth. We conclude that monoclonal antibody-targeted liposomal DXR is effective in treating early lesions in both the pseudometastatic and metastatic models, but limitations to the access of the targeted liposomes to tumour cells in the primary tumour compromised their therapeutic efficacy in treating the more advanced lesions.

Keywords: Liposome; Breast cancer; Drug targeting; Metastasis; MUC-1; Stealth liposome; Doxorubicin

1. Introduction

One of the major problems for patients who suffer from breast cancer is the metastatic spread of cells from the primary tumour [3]. Preventing the metastatic spread of cancer cells through the circulation in an adjuvant setting may be a practical way to manage this disease. The anticancer drug doxorubicin (DXR), which is widely used in the treatment of breast cancer (either alone or in combination with other chemotherapeutics), may reduce the occurrence of micrometastasis, but bone marrow and cardiac toxicity limit its clinical value [4]. Drug carriers like liposomes can enhance the localization of chemotherapeutic drugs in solid tumours and decrease drug uptake by sensitive organs, resulting in reduced toxicity. A long-circulating (Stealth) liposomal formulation of doxorubicin (Caelyx/Doxil), which is approved for use in the treatment of Kaposi's sarcoma and ovarian cancer, is in advanced clinical trials for the treatment of metastatic breast cancer. This formulation can reduce several of the side effects that accompany administration of standard DXR therapy [5,6], while allowing the drug to accumulate in solid tumours to a higher concentration than would the free drug [7]. The liposomal encapsulation of DXR has led to an increased response rate in Kaposi's sarcoma patients compared with those treated with free DXR [8,9]. By coupling antibodies to the surface of these Stealth liposomes, the latter can be made into Stealth immunoliposomes for use as targetable drug delivery systems [10].

The MUC-1 mucin, also known as the CA27.29 antigen, is one of the antigens that may be a good candidate for use in targeting Stealth liposomal DXR to breast tumour tissues. This antigen, which is expressed on the surface of normal epithelial cells, is upregulated and aberrantly glycosylated in cancerous cells. Tumour cells shed the MUC-1 antigen; blood levels of the antigen are elevated in patients with malignant breast cancer [11,12] and ovarian cancer [13]. Serum levels of MUC-1 antigen are routinely monitored as an indicator of relapse in breast cancer patients. In this paper, a monoclonal antibody (mAb) against MUC-1, B27.29, was tested for its suitability for targeting immunoliposomes to breast cancer cell lines transfected with the human MUC-1 antigen.

It has been demonstrated previously that long-circulating liposomes of small size (approx. 100 nm in diameter) will accumulate in solid tumours via a passive targeting mechanism by taking advantage of the leaky vasculature of tumours undergoing angiogenesis [14–16]. While a passive targeting mechanism may be effective in increasing drug accumulation in tumours once they begin to establish a vasculature, liposomal drugs have no mechanism, other than a sustained release mechanism, for accumulating in metastatic cells prior to angiogenesis. We hypothesized that the accumulation of liposomal drugs in micrometastases, or in single metastatic cells migrating in blood or lymph, may be increased through the use of liposomes targeted to cell surface antigens such as MUC-1. Hence, pseudometastatic and metastatic murine breast cancer tumour models were used to investigate the therapeutic efficacy of DXR-loaded MUC-1-targeted sterically stabilized immunoliposomes (DXR-SIL[B27.29]) in the treatment of metastatic disease.

2. Materials and methods

2.1. Monoclonal antibodies

B27.29 mAb, which binds to MUC-1 and has an

IgG1 isotype [17], was provided by Biomira (Edmonton, AB). MAb HMSA-5, an isotype-matched non-specific antibody that reacts with a melanosomal component of normal and neoplastic melanocytes [18] was a generous gift of Dr. K. Jimbo (Department of Dermatology, University of Alberta, Edmonton, AB).

2.2. Mice

Six-to-eight-week-old female *Balb/c* cr Alt BM mice were purchased from Health Sciences Laboratory Animal Services, University of Alberta. Female C.B.-17/Icr severe combined immune deficiency (SCID) mice were purchased from Taconic Farms (Germantown, NY) and housed in a virus antigen-free unit in the Health Sciences Laboratory Animal Services facility, University of Alberta. All animal protocols were approved by the Health Sciences Animal Policy and Welfare Committee, University of Alberta, and were in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

2.3. Tumour cell lines

The GZHI cell line, obtained from Biomira, is derived from the murine breast adenocarcinoma cell line 410.4 by transfection with a full-sized construct of the human MUC-1 gene [1,19]. GZHI cells were cultured (in a humidified 37°C incubator with a 5% CO_2 atmosphere) in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and geneticin (400 μg/ml) (all supplied by Life Technologies, Burlington, ON). The 4T1 cell line, a metastatic, thioguanine-resistant subclone of the 410.4 cell line [2], was kindly provided by Dr. Fred Miller (Michigan Cancer Foundation, Detroit, MI). This cell line has been shown to metastasize via the blood [2] to several organs, including the lungs. The 4T1-MUC1 cell line is a MUC-1 transfectant, prepared with the same gene construct as above. 4T1-MUC1 cells were cultured in DMEM supplemented with 10% foetal bovine serum, 60 μM thioguanine, 1 mM MEM non-essential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and geneticin (400 μg/ ml), all supplied by Life Technologies. Geneticin is required as a selective agent in the growth medium for both GZHI and 4T1-MUC1 cell lines because the MUC-1 plasmid also carries geneticin resistance. However, the GZHI cell line has been grown for up to 2 months in medium without geneticin, and showed no loss of MUC1 expression on the cell surface [19].

2.4. Flow cytometry (FACS) analysis.

The expression of the MUC-1 gene product on the surface of GZHI and 4T1-MUC1 cells was analysed by flow cytometry (FACS). Briefly, cells were cultured several days until they reached log growth, and then harvested by trypsinization. Aliquots of cells (1×10^6/tube) were incubated for 30 min at 4°C with 10 μg of mAb B27.29. The cells were washed with phosphate-buffered saline (PBS), and then incubated with goat anti-mouse FITC-labelled secondary antibody (Sigma-Aldrich Canada, Oakville, ON) for another 30 min at 4°C. After washing, cells were fixed with 0.5% formalin in PBS, and then analysed on a FACScan (Becton Dickinson, San Jose, CA) using Lysis II or CellQuest software.

2.5. Liposome preparation

Hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL), methoxypolyethylene glycol (M_r 2000)-distearoylphosphatidylethanolamine (mPEG-DSPE), and pyridyldithiopropionamide-polyethylene glycol (M_r 2000)-distearoylphosphatidyl ethanolamine (PDP-PEG-DSPE) were provided by SEQUUS Pharmaceuticals (now Alza Corporation; Menlo Park, CA). ^{125}I-NaI and ^{3}H-cholesteryl hexadecylether were purchased from Mandel Scientific (Guelph, ON). Antibody-targeted liposomes were prepared by chemical coupling of the antibody to the end of the PEG chain of PDP-PEG-DSPE, according to a previously described method [20], with modifications as noted below. In some studies, liposomes were loaded with the pH-sensitive dye HPTS [21] rather than DXR, in order to determine the local pH of the liposomal environment. Doxorubicin-loaded liposomes, with or without a ^{3}H-cholesteryl hexadecylether tracer, were prepared by remote loading using an ammonium sulphate gradient [22], then activated with 20 mM DTT (dithiothreitol) for 30 min at room temperature. Excess DTT was removed

by chromatography on a Sephadex G-50 column in 25 mM HEPES, 25 mM MES, and 140 mM NaCl (pH 6.7). The mAb B27.29 was radiolabelled with ^{125}I-NaI using Iodobeads (Pierce, supplied by Bio-Lynx, Brockville, ON). MAb B27.29, with or without a ^{125}I-labelled mAb B27.29 tracer, was activated using MCS (ε-maleimidocaproic acid *N*-hydroxysuccinimide ester, Sigma-Aldrich Canada) at a molar ratio of 15:1 MCS:mAb for 30 min at room temperature; the excess MCS was removed by gel filtration on a Sephadex G-50 (Amersham-Pharmacia Biotech, Baie d'Urfe, PQ) column in 25 mM HEPES, 25 mM MES, and 140 mM NaCl (pH 6.7). DXR-loaded liposomes were coupled to mAb B27.29 (at a ratio of 75–150 μg mAb/μmol phospholipid (PL) for use during in vitro studies, or 40 μg mAb/μmol PL for use during in vivo studies) for 1 h at room temperature, followed by overnight storage at 4°C. Uncoupled mAb was separated from liposomes by chromatography on a Sepharose CL-4B (Amersham-Pharmacia Biotech) column in HEPES buffer (25 mM HEPES, 140 mM NaCl, pH 7.4). Liposome preparations were sized by dynamic light scattering using a Brookhaven B190 Particle Sizer (Brookhaven Instruments Corporation, Holtsville, NY); they had an average diameter of 90–120 nm, and a polydispersity index of less than 0.2.

Liposomal DXR, termed DXR-SL throughout this paper, was the commercial preparation Doxil (in the USA), sold as Caelyx in the rest of the world, and was a generous gift from SEQUUS Pharmaceuticals (now Alza Corporation).

2.6. Targeting studies

Liposomes for targeting studies were prepared with high levels of the lipid tracer ^{3}H-cholesteryl hexadecylether (28 kBq/μmol PL). Six-well tissue culture plates were seeded with 5×10^4 GZHI or 4T1-MUC1 cells, then incubated for a further 2 days until the wells were approx. 80% confluent. Medium was aspirated from the cells, after which non-targeted control liposomes (SL) or targeted liposomes (SIL[B27.29]) were added to cells at different concentrations of PL. Some incubations were done at 4°C, a temperature non-permissive for endocytosis. Alternatively, incubations with SIL[B27.29] were done in wells of cells preincubated with a 20 times excess of free mAb B27.29, to block all specific sites of attachment of mAb. After 1 h incubation at 37°C in a CO_2 incubator, cells were washed 3 times with PBS and harvested by trypsinization, as follows. Flasks of adherent cells were washed with PBS containing 0.68 mM EDTA, then incubated in a small volume of 0.25% trypsin and 0.34 mM EDTA in PBS for approx. 5 min, until cells lifted off the plate, at which point the cells were resuspended in water. Cells were transferred to scintillation vials, ACS (Aqueous Counting Scintillant, Amersham Pharmacia Biotech) was added, and ^{3}H counts were used to calculate specific binding of immunoliposomes to cells.

2.7. Cytotoxicity assay

The IC_{50} concentrations of various formulations of DXR were determined using the MTT cell proliferation assay [23]. Cells were seeded into 96-well plates at 2×10^3 cells/well, and incubated for 1 day until the cells were 50% confluent. DXR-[SIL B27.29] was diluted with medium to different DXR concentrations, added to wells, and incubated for 15 min at 37°C in a CO_2 incubator. Controls were HEPES buffer (pH 7.4), non-targeted liposomal DXR (DXR-SL), or free DXR. After the drug was washed from the cells, wells were incubated for an additional 48 h before cell viability was assessed [23].

2.8. Therapeutic studies using a pseudometastatic model of breast cancer

Female *Balb/c* mice were purchased from the Health Sciences Laboratory Animal Service, University of Alberta (Edmonton, AB). FACS analysis of cells was conducted before cells were injected into *Balb/c* mice, to confirm that the cells expressed high levels of the target MUC-1 gene product. Pathology results showed that GZHI cells seeded and grew in the mouse lung after intravenous (i.v.) injection, but growth was not observed in other organs (not shown). These cells likely colonize the lung because this is the first capillary bed they encounter; we do not have any direct evidence that these cells have special affinity to lung cells. GZHI cells were harvested in log phase growth by trypsin digestion (0.25% trypsin, 0.34 mM EDTA, 137 mM NaCl, 5.7 mM KCl, and 7 mM $NaHCO_3$), and injected

via the tail vein into 6-week-old female mice, at 0.15×10^6 cells per mouse. Mice injected i.v. with GZHI cells were monitored for shedding of MUC-1 mucin in the circulation at 1 week intervals by an EIA assay and no detectable mucin was found until 4 weeks after cells were injected (data not shown). In single-treatment studies, injection of drug was 24 h after injection of GZHI tumour cells; in multiple-treatment studies, drug was injected on days 1, 8, and 15. Mice were divided into six groups of five mice each and groups received one of the following treatments: HEPES-buffered saline; free mAb B27.29; free DXR; DXR-SL; DXR-NSIL-[HMSA-5], DXR-loaded liposomes targeted with a non-specific, isotype-matched antibody; or DXR-SIL[B27.29]. Treatments were given i.v. at a dose of 6 mg DXR/kg mice weight, except for the HEPES-buffered saline group and free mAb groups. Treatment with mAb B27.29 was included as a control for the multiple-treatment study, using 8 μg antibody/mouse, an amount equivalent to that coupled to the injected dose of DXR-SIL[B27.29]. Survival time was used as the main criterion for determining treatment efficacy.

For uridine uptake experiments, mice were injected with GZHI cells via the i.v. route, then treated at 24 h with various formulations of DXR (free drug, DXR-SL, or DXR-SIL[B27.29]) at a dose of 6 mg/kg. At 25 days after treatment, mice were injected with 2 μCi ^{125}I-dUrd (5-[^{125}I]iododeoxyuridine, Mandel Scientific) in 0.2 ml of HEPES-buffered saline; 4 h later the lungs were removed and surface tumours counted. Lungs were cut into small pieces, washed with 10% trichloroacetic acid, and counted in a γ-counter.

2.9. Therapeutic studies using a metastatic model for breast cancer

When cells of the human MUC-1-transfected cell line 4T1-MUC1 were implanted into the No. 4 mammary fatpad (mfp) of female immune competent *Balb/c* mice, no lung metastases were subsequently detected. When the parent 4T1 cell line was similarly implanted into the mfp of immune competent *Balb/c* mice, metastases were observed in the lungs, as described [2] (data not shown). Metastatic colonies were detected in the lungs when the cells were implanted into the mfp of immunodeficient (SCID) mice, as described below. Therefore, all studies aimed at treating metastases with targeted liposomes were conducted in SCID mice.

Female SCID mice were anaesthetized with methoxyflurane (Metofane, Janssen, Toronto, ON). The right No. 4 mfp was exposed by making a small (6–8 mm) incision just to the right of the midline. 10^5 4T1-MUC1 cells in 10 μl of phosphate-buffered saline were injected directly into the mfp [24]. The incision was closed with a wound clip and the mice were monitored for tumour growth by palpation of the mfp. The wound clip was removed 7 days after the original implant. Tumour diameters were measured using callipers and tumour volume was determined using the equation: volume = 0.4 ab^2, where '*a*' is the larger diameter and '*b*' is the smaller diameter [25]. At 7–28 days after mfp implant, mice were euthanized, at a point at which tumour diameters were greater than 1 cm. Metastatic cells were detected in blood, lungs, and peripheral lymph nodes using a clonogenic assay [2]. In some experiments, mfp tumours were generated by implantation of a 1 mm^3 piece of 4T1-MUC1 tumour obtained from a 14-day-old 4T1-MUC1 tumour growing in the mfp of a female SCID mice.

Treatment of primary tumours using various formulations of liposomal DXR was done by i.v. injection at 4, 10, and 17 days after implantation at a DXR dose of 2.5 mg/kg. Treatment groups (five mice per group) included DXR-SL, DXR-SIL[B27.29], HEPES-buffered saline (pH 7.4), and free DXR. Tumour volumes were measured regularly. At 28 days after implantation, mice were sacrificed and their lungs fixed in 10% formalin in PBS. Lungs were evaluated for tumour colonies by preparing mid-sagittal sections of each of the largest lobes and calculating the number of colonies per linear cm of lung.

In some animals, the primary mfp tumour was excised, under Metofane anaesthesia, at a time when tumour metastasis was expected to have already occurred (see Section 3). Mice (groups of four or five mice, as noted in the tables) subsequently received one or more i.v. injections of DXR-SL, DXR-SIL[B27.29], HEPES-buffered saline (pH 7.4), or free DXR. They were then evaluated for tumour regrowth and metastatic colonies in the lungs

at a suitable time after removal of the primary tumour.

3. Results

3.1. SIL[B27.29] binding and cytotoxicity

FACS analysis showed that the GZHI and 4T1-MUC1 cell lines, both of which are transfected with the MUC-1 gene, express a high level of the MUC-1 antigen, as shown by high binding of the mAb B27.29 (Fig. 1). In vitro experiments showed that SIL[B27.29] bound specifically to GZHI and 4T1-MUC1 cells with high binding levels, but non-targeted liposomes (SL) had low binding (Fig. 2). Lower binding of the SIL[B27.29] was observed at 4°C than at 37°C. However, attempts to show internalization of SIL[B27.29] into an acid intracellular compartment (lysosomes, endosomes) using the pH-sensitive dye HPTS [21] showed only a modest decrease in pH (from pH 7.4 to pH 6.9 in 4 h).

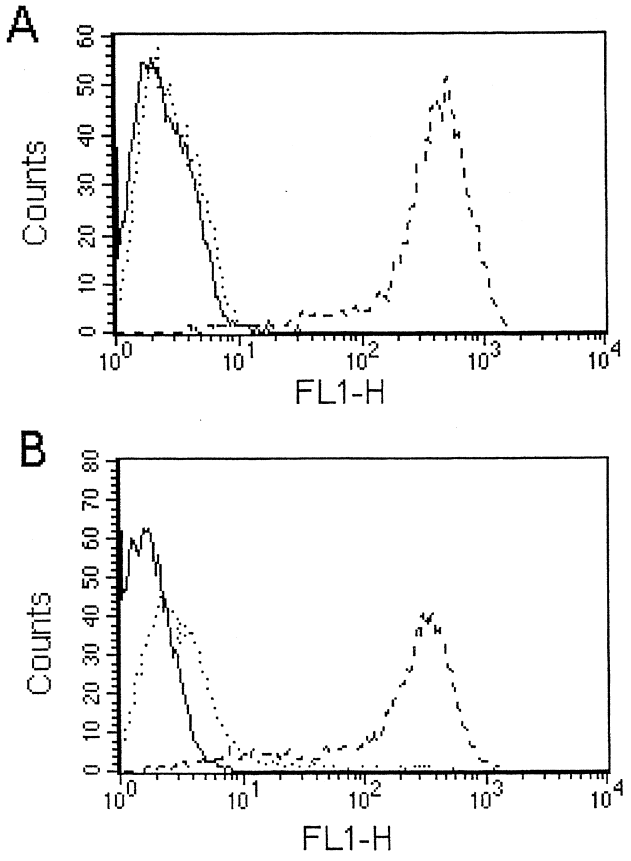

Fig. 1. Binding of mAb B27.29 to (A) GZHI and (B) 4T1-MUC1 cells, as determined by FACS. Control, solid line; secondary antibody only, dotted line; mAb B27.29, dashed line.

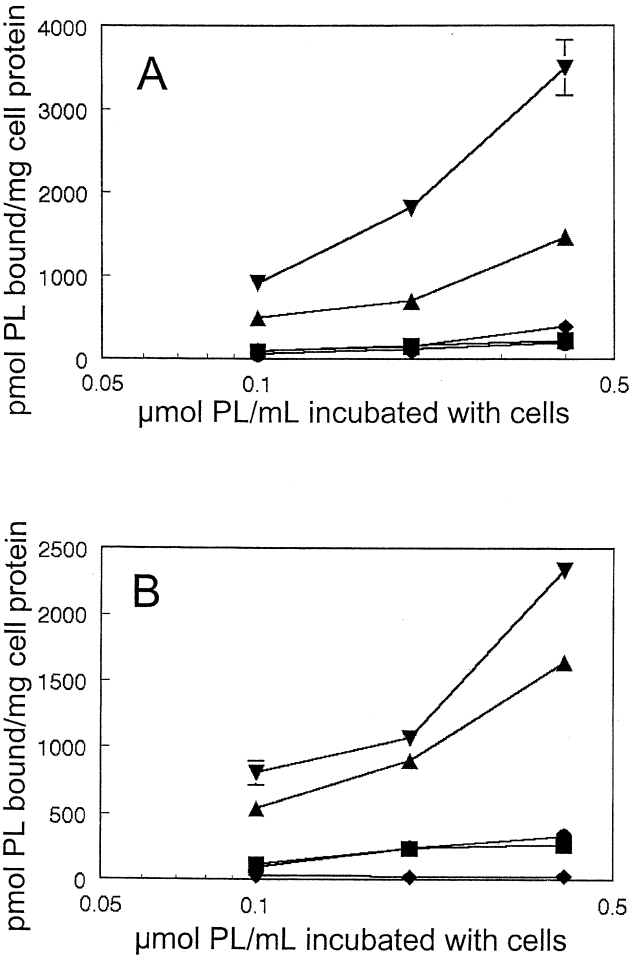

Fig. 2. Binding of antibody-targeted liposomes SIL[B27.29] to (A) GZHI cells, and (B) 4T1-MUC1 cells in vitro at various temperatures. ♦, SL 4°C; ▲, SIL 4°C; ■, SL 37°C; ▼, SIL 37°C; ●, 2-fold excess free mAb+SIL.

Our in vitro cytotoxicity study of DXR-SIL[B27.29] to GZHI cells is meant to more closely mimic the in vivo situation where rapid redistribution of free DXR occurs. We exposed cells to various DXR formulations, at the IC_{50} concentration of the free drug (1.6 μg/ml), for 15 min at 37°C, sufficient time for immunoliposomes to bind to the cells. DXR-SIL[B27.29] inhibited the growth of GZHI cells by 57%, compared with 14% and 8% inhibition for SL-DXR and NSIL-DXR[HMSA-5] respectively ($P < 0.01$). Thus the toxicity of SIL-DXR[B27.29]

was comparable with that of free DXR. These results suggest that not only were a large number of DXR-SIL[B27.29] bound to cells, but sufficient DXR was released from liposomes, either surface bound or internalized, to be very cytotoxic.

3.2. *In vivo therapeutic studies in a pseudometastatic model for breast cancer (GZHI)*

Therapeutic experiments were performed as single (Fig. 3A) or multiple administrations (Fig. 3B) of DXR at 6 mg/kg, the latter given on days 1, 8, and 15. The mean survival time (MST) was 29.6 days for control mice injected with HEPES-buffered saline (pH 7.4) (Fig. 3A,B). Free DXR treatment increased the MST (36.8 or 40.6 days for single or multiple injections, $P < 0.05$ or 0.01 respectively, compared with control). Treatment of mice with DXR-SL resulted in significantly longer MST compared with free DXR treatment (46.2 or 48.7 days for single or multiple injections respectively, $P < 0.05$ compared with free DXR). In mice treated with targeted liposomes, single or multiple injections of DXR-SIL[B27.29] resulted in significant increases in MST (62.4 or 64.6 days respectively, $P < 0.01$ compared with DXR-SL), but there was no statistically significant difference between single- and multiple-injection regimens. It may be that the first treatment is the critical one, when the tumours are quite small, and the SILs do not have to penetrate deep within a large tumour to reach all cells [26–28]. Further treatments with DXR-SIL[B27.29] may be ineffective as these liposomes may not effectively penetrate the multiple cell layers of the larger tumour, being mostly bound at the periphery of the tumour.

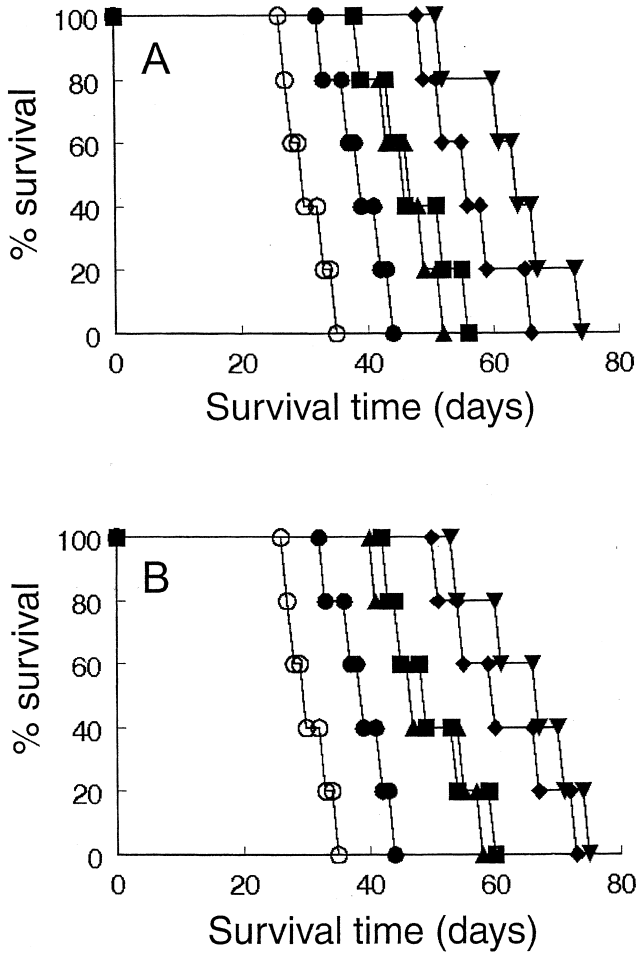

Fig. 3. Survival of *Balb/c* mice injected i.v. with 0.15×10^6 GZHI cells, and treated with either (A) a single i.v. injection of various formulations of DXR, 24 h after injection of cells, or (B) three i.v. injections of various formulations of DXR, at 24 h, 8 days and 15 days after i.v. injection of cells (five mice per group). Treatments are: ○, HEPES-buffered saline; ●, free DXR; ▲, DXR-SL; ■, DXR-NSIL[HMSA-5]; ♦, DXR-SIL-[B27.29]+DXR-SL; ▼, DXR-SIL[B27.29].

Although the MUC1 antigen is a weak immunogen to T cells and B cells [29,30], injection of liposome-mAb B27.29 conjugates in immune-competent mice may result in the complexing of circulating MUC1, which is shed by the developing tumour, with the antibody, thus initiating B- and T-cell-mediated immune responses directed against the tumour. To test whether the targeting antibody initiated an immune attack on the developing GZHI tumours, one group was treated with an amount of free mAb B27.29 equivalent to that which was attached to DXR-SIL[B27.29], in multiple injections. No difference between the life span of the HEPES-buffered saline-treated group of mice and that of mice treated with free mAb B27.29 was observed. Thus we concluded that the amount of mAb introduced on the SILs was insufficient to elicit a strong immune response from the mice.

Mice treated with DXR-NSIL[HMSA-5] had a survival time similar to that of the DXR-SL group, indicating that removal of SILs from circulation is not significantly more rapid than that of SLs. However, we tried to keep the amount of mAb coupled to the surface of SILs about 40 μg/μmol phospholipid

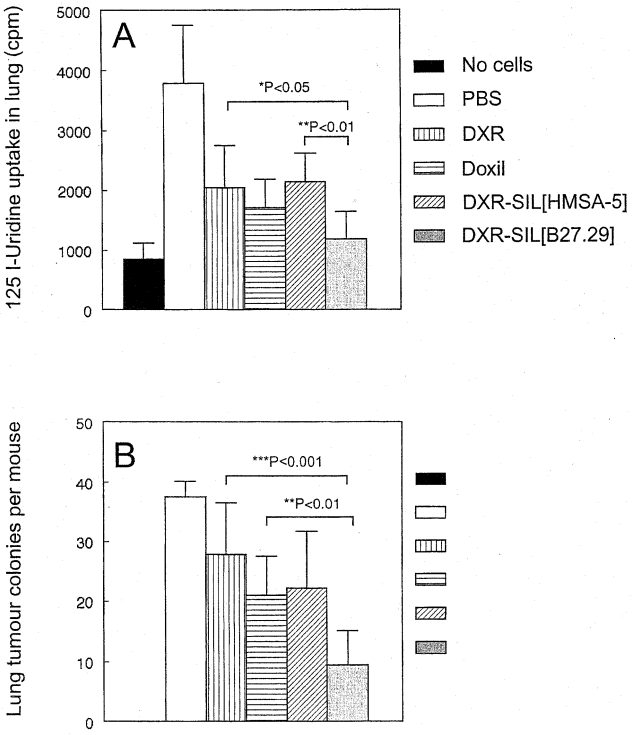

Fig. 4. Evaluation of lung tumours in mice 25 days after injection with 0.15×10^6 GZHI cells i.v., and treated at 24 h with a single injection of various formulations of DXR at 6 mg/kg. (A) ^{125}I-dUrd counts in lungs. ^{125}I-dUrd was injected via the tail vein; 4 h later mice were sacrificed. Lungs were weighed and surface tumours counted. The lungs were then minced, washed with 10% TCA, and counted in a γ-counter. (B) Number of tumour nodules per lung.

(approx. 25 mAb/liposome) in order to prevent Fc-mediated clearance of immunoliposomes from the circulation [20].

We also treated one group of mice with a combination of 3 mg DXR/kg as DXR-SL plus 3 mg DXR/kg as DXR-SIL[B27.29]. The results showed a MST that was intermediate between that obtained with DXR-SIL[B27.29] versus DXR-SL (Fig. 3A,B). Because of possible differences in penetration of SILs vs. SLs into solid tumours, we anticipated that for a well-established tumour the DXR-SL could diffuse further through the tumour interstitial space than targeted liposomes, which would experience a 'binding site barrier' [27]. However, in this study, combination treatment showed no improvement over the targeted DXR-SIL[B27.29] alone.

To determine the effect of the various drug treatments on growth of GZHI tumours in the lung, ^{125}I-dUrd uptake experiments were performed in tumour-bearing mice (Fig. 4A). Uptake into lung of ^{125}I-dUrd has been established to be directly proportional to the number of seeded tumour cells [31]. HEPES-buffered saline-treated tumour-bearing control mice had significantly higher uridine uptake than tumour-free mice ($P<0.01$). Mice treated with DXR-SIL[B27.29] had a significant decrease in uridine uptake compared with either untreated tumour-bearing mice ($P<0.01$) or mice treated with either free DXR ($P<0.05$) or DXR-NSIL[HMSA-5] ($P<0.01$). The number of tumour nodules per lung in the various treatment groups was also compared. DXR-SIL[B27.29] was able to reduce the number of tumour nodules compared with DXR ($P<0.001$) or DXR-SL ($P<0.01$) treatment (Fig. 4B).

3.3. *In vivo therapeutic studies in a metastatic model for breast cancer (4T1-MUC1)*

The number of metastatic cells located in the lung at various time points after implantation of 10^5 cells into the mfp of SCID mice was determined by a clonogenic assay. Although the number of clones isolated from lungs was highly variable, metastatic cells were located in the lungs of all mice by 14 days after injection of tumour cells into the mfp.

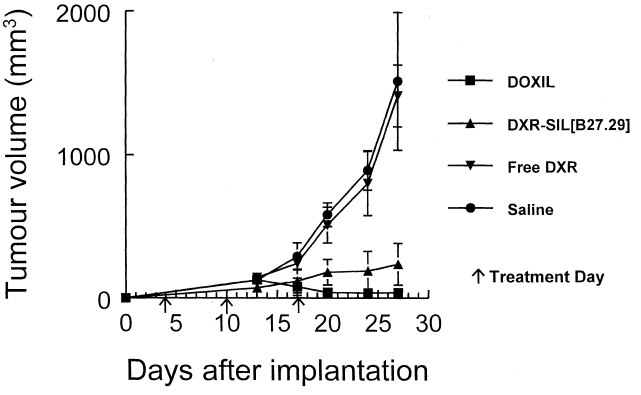

Fig. 5. Volume of 4T1-MUC1 mfp tumours in SCID mice treated with various formulations of doxorubicin at a DXR dose of 2.5 mg/kg, on days 4, 10 and 17 after implant of 10^5 tumour cells into the mfp. Volumes were calculated using the formula volume = 0.4 ab^2, where 'a' is the larger diameter and 'b' is the smaller diameter.

By 25 days after injection of cells into the mfp, tumours were visible on the surface of lungs.

Various treatments (free DXR, DXR-SL, DXR-SIL[B27.29] or HEPES-buffered saline, at a DXR dose of 2.5 mg/kg) were injected i.v. on days 4, 10 and 17 after implantation of 4T1-MUC1 cells into the mfp of SCID mice. Tumour volumes for the various treatment groups were determined (Fig. 5). DXR-SL and DXR-SIL[B27.29] were equally effective in preventing growth of the primary tumour, while free DXR was ineffective. Histological examination of the lungs at 28 days after implantation showed that both DXR-SIL[B27.29] and DXR-SL were able to completely prevent the seeding of metastases into the lungs. By comparison, treatment with free DXR or HEPES-buffered saline resulted in 3.2 ± 1.8 and 8.2 ± 10.2 colonies/linear cm respectively.

3.4. Treatment of metastases after surgical removal of primary tumour

Complete surgical removal of the large primary tumour at 14 days without tumour regrowth was not possible when suspensions of cells were injected into the mfp. The 4T1-MUC1 cell line grows very aggressively in the mouse mfp (see Fig. 5, HEPES-buffered saline treatment group). Surgical removal of the primary tumour at 8 days after implantation (the earliest time point at which lung metastases could be detected), followed by treatment on days 1, 8, and 15 with different formulations of DXR (2.5 mg/kg), was then attempted in order to see if the growth of lung metastases could be prevented. The mice were also monitored for regrowth of the primary tumour. At 22 days after the surgery (30 days post implantation), the presence of metastases in lung was evaluated by histopathology. Regrowth of the primary tumour occurred in all mice in the groups treated with HEPES-buffered saline, DXR-SIL[B27.29] or free DXR. Only in the group treated with DXR-SL were there any animals in which the primary tumour did not regrow (50% tumour-free, $n = 4$). Although it was our intention to treat only the metastatic disease following removal of the primary tumour (adjuvant model of chemotherapy), the regrowth of the primary tumour prevented this. It was not possible to remove a wide margin of normal tissue from around a mouse mfp tumour, partly because mice are quite small and partly because the tumours that develop in this model are relatively large at 8–14 days.

As an alternative approach, we implanted a 1 mm^3 explant of 4T1-MUC1 tumour into the mfp of SCID mice. It was hoped that the primary tumour, which developed from such an implant, would have cleaner margins and be easier to remove than the tumour that developed from the implantation of a cell suspension. 4T1-MUC1 colonies in lungs were first detected on day 8; three of three mice had tumour cells in their lungs by day 11. This observation is similar to the time for detection of metastases after implant of a cell suspension in the mfp, but the standard deviations of colony numbers were smaller, implying more reproducible metastases.

The therapeutic efficacy of DXR-SIL[B27.29] in treatment of metastases following surgical removal of the primary tumour 8 days after implantation of 1 mm^3 explants was examined (Tables 1 and 2). Regrowth of the primary tumour was observed in all mice treated with either DXR-SIL[B27.29], free

Table 1
Regrowth of primary tumour after surgical removal of tumours grown from 1 mm^3 explants

Type of treatment (number of mice)	Number of mice with no regrowth	Number of mice with regrowth	% regrowth
DXR-SIL[B27.29] ($n = 5$)	0	5	100
DXR-SL ($n = 5$)	2	3	60
Free DXR ($n = 5$)	0	5	100
HEPES-buffered saline ($n = 4$)	0	4	100

Explants of 4T1-MUC1 tumours (1 mm^3) were implanted into the mfp of 20 female SCID mice. Eight days after implantation, primary tumours were removed. The mice were injected i.v. with various formulations of DXR at 2.5 mg DXR/kg 24 h after surgery, then at weekly intervals for a total of three treatments per group. The mice were monitored for regrowth of the primary tumour; at 20 days after surgery (28 days post implantation), lung metastases were evaluated by histopathology.

Table 2
Number of metastatic colonies in lungs at day 20 (28 days after implantation of primary tumour)

Type of treatment (number of mice)	Number of metastatic colonies per linear cm of lung, in left lobe	Number of metastatic colonies per linear cm of lung, in largest right lobe
DXR-SIL[B27. 29] ($n=5$)	0.251 ± 0.293	0.504 ± 0.597
DXR-SL ($n=5$)	0.530 ± 0.292	0.950 ± 0.376
Free DXR ($n=4$)	0.413 ± 0.223	1.073 ± 0.93
HEPES-buffered saline (control) ($n=3$)	0.438 ± 0.49	0.220 ± 0.296

The primary tumour was surgically removed on day 8 after implantation of a 1 mm^3 explant of 4T1-MUC1 tumour into the mfp of SCID mice. Mice were treated i.v. 24 h after surgery, then at weekly intervals for a total of three treatments per group, at a DXR dose of 2.5 mg/kg.

DXR, or HEPES-buffered saline (Table 1). Only mice treated with DXR-SL showed a reduction in tumour regrowth (40% tumour regrowth, $n=5$). At 20 days after surgical removal of the primary tumour and subsequent treatment (28 days after implant of tumour explants into the mfp) histopathological staining was used to identify tumour colonies in the lungs. All animals, in all groups (including those mice in which the primary tumour did not regrow, i.e., the DXR-SL group), were found to have tumour colonies in their lungs (Table 2). The numbers of colonies varied from 0.25 to 1.1 per linear cm of lung, with no significant differences between the various treatment groups.

4. Discussion

Several researchers have used targeted long-circulating liposomes to deliver anti-tumour drugs in experimental animal models of disease [32–39]. Targeting moieties may be attached at the surface of the liposome or at the terminus of the hydrophilic PEG-lipid anchor conjugate; various coupling mechanisms can be used, resulting in random or specific orientation of the targeting molecule [40,41]. All these methods affect efficiency of coupling, targeting in vivo and clearance of liposomes from the circulation. The coupling method chosen for use in this paper is one which gives good coupling efficiency, long-circulating pharmacokinetics in vivo, and retention of binding after coupling [42].

Treatment of solid tumours with targeted liposomal drug presents unique problems. The penetration of liposomes into a tumour may be limited by high internal hydrostatic pressure [15,43]. However, there is ample evidence that non-targeted Stealth liposomes do penetrate solid tumours [44] [15], so this phenomenon does not completely prevent delivery of drug. Targeted liposomes may bind to cell surface receptors at the tumour periphery, preventing them from reaching cells in the interior of the tumour [27]. In the models used in this study, we wanted to evaluate the usefulness of immunoliposomes in the treatment of solid tumours at a stage at which the tumours are pre-angiogenic, such as would be found at the early stage of metastasis. The pseudometastatic model is designed to test the feasibility of this approach, i.e., the treatment of lung micrometastases soon (1 day) after they seed in the lung following i.v. injection.

The nature of the cellular epitope to which the liposomes are targeted will greatly influence the delivery of the liposomal drug. Drug may directly diffuse out of the targeted liposomes bound to the target cell surface and be taken up by the target cells, in a process dependent upon the drug release rate of the liposome formulation, as well as factors such as cell permeability to the drug. Alternatively, the drug-liposome package may be taken up by the cell after binding to an internalizing receptor [35,37,45]. In the case of an internalized receptor-immunoliposome complex, targeted liposomes will be processed by the cell's lysosomal or endosomal systems so the drugs that escape degradation may be released inside the cell. Internalizing epitopes are thought to be more efficient at increasing intracellular drug concentrations because entry of the drug into the cells is not dependent upon leakage and passive diffusion. It has been observed by Litvinov and Hilkens [46] that the

incompletely glycosylated intermediate of the MUC-1 epitope is internalized and then recycled to the surface of the ZR-75-1 human mammary carcinoma cell line as additional sialyl groups are attached to the mucin. These authors show that 0.9% of the surface episialin (also known as MUC-1) is recycled per minute, probably via a cellular compartment containing sialyltransferase, presumably the trans-Golgi and trans-Golgi network [47]. In the study reported here, MUC-1 expression on the GZHI and 4T1-MUC1 cell lines has been induced by transfection of mouse cells with the human MUC-1 gene. It is not clear whether the mechanism of MUC-1 sialylation in mouse cells will proceed in a similar fashion to that observed in human cells. If receptor recycling does occur in our model system, it is unlikely that the rate of MUC-1 recycling is rapid enough to result in effective internalization of DXR-SIL[B27.29] bound to the cell surface. When GZHI cells were incubated with B27.29-targeted immunoliposomes loaded with the pH-sensitive dye HPTS rather than DXR, we observed only a slight drop in the internal pH of the liposomes. The higher amount of binding to GZHI and 4T1-MUC1 cells observed for SIL[B27.29] at 37°C versus 4°C suggests that some internalization of the liposomes may occur, since 4°C is a non-permissive temperature for receptor-mediated internalization; the additional binding at 37°C may be due to differences in the binding kinetics at the two temperatures, and likely does not reflect a significant amount of internalization.

In the metastatic process, the primary tumour sheds cells that have the capacity to reattach and develop into secondary tumours in locations distant from the primary tumour. Our goal was to test the hypothesis that targeted liposomal DXR could be useful, as an adjuvant to the surgical removal of the primary tumour, in the treatment of secondary tumours that had already seeded at the time of surgical removal, but were still very small and poorly vascularized.

The finding that non-targeted liposomal doxorubicin was as effective as targeted liposomal DXR in preventing growth of primary mfp tumours (Fig. 5) is supported by results in another solid tumour model studied by our group, the human ovarian cancer cell line Caov.3, grown in nude mice as a subcutaneous implant [10]. In our experiments in the ovarian cancer model, targeted liposomal DXR was slightly less effective in preventing growth of the subcutaneous tumour than non-targeted liposomal DXR.

In our metastatic model, metastasis occurs via the blood, with the main site of seeding of secondary tumours being the lungs [2]. The number of lung colonies increased with time after implant of the primary tumour. These lung tumours were expected to be amenable to treatment with targeted liposomal DXR, as were the small tumours in the pseudometastatic model. In the true metastatic model, the seeding of secondary tumours is a more continuous process than an i.v. bolus of cells, although the actual tumour burden is considerably smaller (0.15×10^6 cells seeded i.v. in the pseudometastatic model vs. a finding of in the order of 100 colony-forming units in the lungs at 7 days in the metastatic model. We hypothesize that successful treatment of micrometastases in the lungs of mice following mfp implantation would depend on treating the mice at a time when the secondary tumours are small, poorly vascularized, and still in contact with the lung's blood circulation.

A major limitation of the metastatic model described in this paper is our inability to completely remove the primary tumour. This made it impossible to treat only the secondary metastases with targeted liposomal drug, because the regrowing primary tumour continually shed more tumour cells into the circulation. The dosing regimen of once a week may not be sufficient to intercept single circulating tumour cells and small, non-vascularized tumours, both of which appeared to be amenable to treatment by targeted liposomes in the pseudometastatic model. Targeted Stealth liposomes are themselves a continuous delivery system, with a plasma half-life of in the order of 7–9 h [20]; therefore, a more practical delivery regimen would possibly be twice weekly, given what we know about the pharmacokinetics of these liposomes. In the pseudometastatic model, we would not expect multicellular tumours to have developed in the mouse lung at 24 h after i.v. injection of a cell suspension.

Differences in response to targeted liposomal treatment, between immune-competent mice treated with GZHI cells and immunocompromised SCID mice, may reflect differences in the immune status of the mice. In the immune-competent mice, the immune

system may play a role in rejection of GZHI tumours when targeted liposomes bind to these cells, as the GZHI cells express a foreign (human) MUCI gene.

There are considerable differences in DXR tolerance between conventional and SCID mice [25]. Conventional mice can readily tolerate a doxorubicin dose of 6 mg/kg, whereas SCID mice, which are deficient in DNA repair, cannot tolerate more than 2.5–3 mg/kg without dose-limiting toxicities [25]. In SCID mice bearing the metastatic model, the dose of liposomal DXR may be below the dose required for a successful therapeutic outcome. This could also help to explain the differential success in treating the pseudometastatic model vs. the metastatic model.

One intriguing observation was that both targeted and non-targeted liposomal DXR were able to prevent the seeding of metastases into lungs when the primary tumour was left in place. Treatment with free DXR was not able to achieve this result. This suggests that liposomal DXR may be useful in adjuvant chemotherapy when complete surgical removal of the primary tumour is possible. These results should be pursued further.

The use of immunoliposomes in another mouse model for breast cancer has been described [48]. In their study, Park et al. found that anti-HER2 immunoliposomes accumulated in Her2 (c-erB-2, neu)-overexpressing tumours growing in nude mice, when injected i.v., and that DXR-loaded anti-HER2 immunoliposomes were significantly more cytotoxic to the tumours than were untargeted DXR-loaded liposomes. This probably reflects their finding that anti-HER2 immunoliposomes are avidly internalized by HER2-overexpressing cells, delivering drug into the cell. In our system, if internalization does take place, it is probably at a significantly slower rate than internalization in the HER2 system.

In conclusion, liposomal DXR targeted against the MUC-1 epitope appeared to be effective in treating early lung metastases in a pseudometastatic model of breast cancer and in preventing the growth of lung metastases in a metastatic breast cancer model when no attempt was made to surgically remove the primary tumour. However, limitations to the access of the targeted liposomes to tumour cells in the primary tumour compromised their therapeutic efficacy in treating the more advanced lesions and did not give them a selective advantage over non-targeted liposomes.

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