

Tumor-Targeted Nanomedicines: Enhanced Antitumor Efficacy *In vivo* of Doxorubicin-Loaded, Long-Circulating Liposomes Modified with Cancer-Specific Monoclonal Antibody

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Abstract Purpose: The efficacy of drug delivery systems can be enhanced by making them target specific via the attachment of various ligands. We attempted to enhance tumor accumulation and therapeutic effect of doxorubicin-loaded, long-circulating, polyethylene glycol-coated liposomes (Doxil, ALZA Corp.) by coupling to their surface the anticancer monoclonal antibody (mAb) 2C5 with nucleosome-restricted activity that can recognize the surface of various tumors but not normal cells and specifically targets pharmaceutical carriers to tumor cells *in vitro* and *in vivo*. Following earlier *in vitro* results with various cancer cell lines, the mAb 2C5 liposomes were studied *in vivo* versus plain and nonspecific-IgG liposomes.

Experimental Design: Antibody coupling to Doxil was done via the "postinsertion" technique. Using ¹¹¹In-labeled liposomes, the tissue biodistribution and pharmacokinetic profile were studied, as well as their accumulation in tumors in mice, followed by the whole-body γ -scintigraphic imaging. Therapeutic efficacy of mAb 2C5-targeted Doxil versus nonspecific IgG-modified and original Doxil controls was followed by registering live tumor growth and determining tumor weights upon mice sacrifice.

Results: mAb 2C5-targeted liposomes showed enhanced accumulation in tumors, and the *in vivo* therapeutic activity of the mAb 2C5-Doxil treatment was found to be significantly superior, resulting in final tumor weights of only 25% to 40% compared with all Doxil control treatments, when tested against the s.c. primary murine tumors of 4T1 and C26 and human PC3 tumor in nude mice.

Conclusions: Our results showed the remarkable capability of 2C5-targeted Doxil to specifically deliver its cargo into various tumors, significantly increasing the efficacy of therapy.

It is commonly recognized now that liposomal preparation of anticancer drugs should possess an extended circulation time to increase the probability of accumulating at tumor sites. For vascularized tumors, the selective accumulation and retention of liposomes is a result of the combination of "leaky" tumor neovasculature (1, 2) and malfunctioning lymphatics, integrated in the enhanced permeability and retention effect. This effect underlies the clinical efficacy of the long-circulating liposomal doxorubicin formulation (commercially known as Doxil/Caelyx). Yet, in addition to these physiologic factors, the

specificity and the homogeneous depth of penetration of the Doxil liposomes are of well-recognized importance in the clinical practice, especially in cases of solid tumors, with local variant physiology of neovasculature (3).

In general, long-circulating liposomes show dose-independent, nonsaturable, log-linear kinetics and increased bioavailability (4). These characteristics of polyethylene glycol (PEG)-coated liposomes have been highly recognized through the clinical success of Doxil/Caelyx in the treatment of skin, cervical, prostate, breast, and some other cancers, wherein this drug showed enhanced therapeutic activity compared with the free drug or drug in conventional liposomes (5-9).

Despite the superior clinical results with doxorubicin in long-circulating liposomes in several types of tumors when compared with the original free doxorubicin, the optimization of its properties is an ongoing concern. The encapsulation of doxorubicin inside PEG liposomes has increased the tumor concentration of the drug via the enhanced permeability and retention effect and decreased its volume of distribution and, thus, the therapy-associated cardiotoxicity. Despite that, the nonspecific toxicity profile of Doxil/Caelyx was changed more toward mucocutaneous reactions (10, 11), in addition to the reduced yet significant cardiomyopathy and myelosuppression (10, 12).

Although it has been shown that PEG surface modification of liposomes causes a greater accumulation of drug at the tumor

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Translational Relevance

The monoclonal antibody (mAb) 2C5, capable of binding various cancer cells via the cancer cell surface-bound nucleosomes released from apoptotically dying neighboring cancer cells, was coupled to the liposomal doxorubicin (Doxil). Earlier, mAb 2C5–Doxil was shown to recognize and kill various lung, breast, and colon cancer cells, superior to native Doxil, and was investigated now for therapeutic activity against several unrelated tumors *in vivo*. Biodistribution, γ -imaging, and pharmacokinetic data confirm the enhanced tumor accumulation of mAb 2C5–Doxil. Its therapeutic efficacy was significantly enhanced compared with control Doxil treatment when tested against primary murine tumors 4T1 and C26 and human PC3 tumor in nude mice. Combined with our earlier report on diminishing Doxil-associated mucocutaneous reactions in case of mAb 2C5–Doxil, our research showed that mAb 2C5–targeted Doxil improves therapeutic potential and safety of the original Doxil. Such targeting could improve therapeutic outcomes and decrease adverse effects of liposomal anti-cancer drugs such as anthracyclins, camptothecin analogues, and platinum compounds.

site by passive targeting, active targeting of the liposomal carrier can aid in selection of the target cells within the tumor site and internalization of the liposomes to a greater extent inside these cells. A wide variety of tumor-targeting ligands was coupled to the surface of PEGylated liposomes, including small-molecule ligands [such as folate and some vitamins (13, 14)], peptides [such as Arg-Gly-Asp (15) and sequences identified by phage display (16)], proteins (such as hormones), transferrin (17), antibodies and antibody fragments (18), and oligosaccharides (19). Regardless of the targeting moiety, the principle outcomes of active targeting are essentially the same: improved tumor cell recognition and reduced recognition at nonspecific sites, with an additional yet ligand-depending advantage to deliver a large drug payload directly inside the target tumor cells.

From this list, monoclonal antibodies (mAb), mainly of IgG class, and their fragments have the highest potential target specificity and thus provide the most promising opportunity as targeting moieties (20). There are antibodies available that can recognize specific antigens from most known tumors. Among these are antibodies against ovarian cancer, prostate cancer, and colorectal cancer (21). It was shown earlier that doxorubicin-loaded Fab' fragments of antidiisialoganglioside immunoliposomes selectively and almost completely inhibited the metastatic growth of human neuroblastoma in the nude mouse model (22). The modification of Doxil with anti-HER2 mAb fragments resulted in a formulation that showed marked anticancer efficiency against tumor lines overexpressing HER2, far superior to that of control nontargeted liposomes in cell-culture and in *in vivo* models (23).

Earlier studies have shown that among antibodies with anticancer specificity, monoclonal antinuclear autoantibodies with nucleosome-restricted specificity are of particular interest (24, 25). Findings by our group have shown that the monoclonal, nucleosome-specific, nonpathogenic antinuclear autoantibody 2C5, derived from healthy aged BALB/c mice, was

able to recognize the surface of numerous lymphoid and nonlymphoid tumor cells of murine and human origin, but not of normal cells (24, 25). Tumor cell surface-bound intact nucleosomes, originating from neighboring apoptotic tumor cells, are their molecular targets (24–26). In addition to their own antibody-dependent cellular cytotoxicity-mediated anti-cancer activity, such antibodies, specifically the mAb 2C5, when used in subtherapeutic quantities, can serve as effective targeting moieties for the tumor-specific delivery of various drug-loaded pharmaceutical nanocarriers (27, 28). Earlier, we have obtained promising data on the increased *in vitro* cytotoxicity of Doxil modified with mAb 2C5 (29, 30). In our design, the mAb 2C5 is attached outside the protecting polymer layer by coupling it with the *p*-nitrophenylcarbonyl group-activated terminus of PEG-phosphatidylethanolamine polymer grafted on the liposome surface. Following a single-step postinsertion approach, the antibody (mAb 2C5) was first modified with a lipid derivative of PEG (PEG3400-phosphatidylethanolamine) and then incorporated into the liposomes by coincubating the loose micelles of PEG3400-phosphatidylethanolamine-modified antibody with Doxil (27, 29). The molecular weight of PEG derivative was intentionally chosen to be higher than the molecular weight of PEG in the composition of Doxil to prevent a possible “shielding” effect of the liposomal PEG coating onto the liposome-incorporated antibody (31, 32). Moreover, it was shown in our research that Doxil modified with mAb 2C5 undergoes active endocytic uptake into cancer cells, which can be useful for bypassing multidrug resistance efflux pumps, namely, P glycoprotein, in resistant tumor cells (30).

We present here the results of our extended *in vivo* studies on the broad-spectrum, tumor-targeting capacity of mAb 2C5-modified, doxorubicin-loaded, PEGylated liposomes and their significantly enhanced therapeutic efficacy against various tumors.

Materials and Methods

Cholesterol, fully hydrogenated soy phosphatidylcholine, *N*-carbonyl-methoxy-poly(ethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (MPEG2000-DSPE), and phosphatidylethanolamine were from Avanti Polar Lipids, Inc. and used without further purification. Doxil was purchased from Pharmaceuticals, Inc. Doxorubicin was purchased from Sigma Chemicals, Inc. Triethylamine, octyl glucoside, and diethylenetriaminepentaacetic acid (DTPA) anhydride were products of Sigma. Poly-oxyethylene3400-bis(*p*-nitrophenyl carbonate) was purchased from SunBio. Cell culture media, DMEM, Eagle's minimal essential medium, RPMI 1640, fetal bovine serum, and concentrated solutions of Na-pyruvate, nonessential amino acids, L-glutamine, and penicillin/streptomycin stock solutions were from CellGro. Female BALB/c and C57/BL mice were purchased from Charles River Laboratories. All solvents and other chemicals were analytic grade preparations. The mAb 2C5 was produced in ascites via the i.p. injection of 1.5×10^6 hybridoma cells/mL into pristinely primed BALB/c 4-wk-old male mice. The production and the purification of the mAb 2C5 were carried out by Harlan Bioproducts using the cell line from our laboratory. Control IgG2a isotype-matching mAb UPC10 was from Sigma.

Preparation of Liposomes

Attachment of antibody to liposomes. To prepare antibody (mAb 2C5 or UPC10) conjugates with PEG3400-phosphatidylethanolamine, an

excess of *p*-nitrophenylcarbonyl–PEG-phosphatidylethanolamine dispersed in 10 mg/mL micellar solution of octyl glucoside in 5 mmol/L Na-citrate and 150 mmol/L NaCl (pH 5.0) was added to an equal volume of 1 mg/mL solution of a protein (mAb 2C5 or UPC10) in TBS (pH 8.5). The mixtures were incubated for 24 h (pH 8.5) at 4°C (33). To obtain doxorubicin-loaded liposomes modified with mAb 2C5 or nonspecific UPC10, the reaction mixtures form the mAb–to–PEG-phosphatidylethanolamine conjugation reactions (aforementioned) were mixed in equal volumes with Doxil and incubated for 5 h at 4°C. Then, the remaining octyl glucoside and free nonincorporated proteins were removed by dialysis using cellulose ester dialysis tubes with a cutoff size of 300,000 Da. The complete removal of the nonincorporated mAb (if such fraction did exist at all) was confirmed by the presence of only one peak of liposomes on the gel chromatogram of the final sample (no peaks of free mAb–PEG-phosphatidylethanolamine or mAb–PEG-phosphatidylethanolamine micelles; ref. 29). This procedure does not cause any significant loss of doxorubicin from liposomes (33).

Control liposomes. Control PEGylated liposomes mimicking Doxil composition but containing no doxorubicin were prepared using the same lipid components and in the same concentrations as in Doxil. A lipid film was obtained from *N*-(carbonyl-methoxyPEG 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (3.19 mg/mL), fully hydrogenated soy phosphatidylcholine (9.58 mg/mL), and cholesterol (3.19 mg/mL). The lipid film was suspended in HEPES-buffered saline (pH 7.4) and sonicated with a probe-type sonicator at 10-W power for 15 mins, followed by several passages through the mini-extruder with 100-nm pore size polycarbonate filter, until ~100 nm liposomes with narrow size distribution were obtained (29, 30).

¹¹¹In radiolabeling of liposomes. Doxil-mimicking liposomes containing the membrane-incorporated amphiphilic chelate DTPA-phosphatidylethanolamine (hydrogenated soy phosphatidylcholine:cholesterol:MPEG2000-DSPE:DTPA-phosphatidylethanolamine in 3:2:0.3:0.3 molar ratios, respectively) were prepared along with the UPC10 and 2C5 immuno-analogues. The loading of the liposome-incorporated DTPA-phosphatidylethanolamine with ¹¹¹In was done via the transchelation mechanism. DTPA-phosphatidylethanolamine-containing liposomes were incubated for 1 h with ¹¹¹In chloride in 1 mol/L citrate buffer at room temperature and then dialyzed overnight against HEPES-buffered saline at 4°C to remove the free label (31).

Tumor Mouse Models

Cell cultures. Murine breast adenocarcinoma (4T1), murine colon cancer (C26), and human prostate cancer (PC3), all frequently used in preclinical studies on anticancer drugs and their delivery systems, were purchased from the American Type Culture Collection. PC3 cells were maintained in DMEM cell culture medium supplemented with fetal bovine serum to 10%, Na pyruvate to 1 mmol/L, and penicillin and streptomycin to 50 units/mL and 50 µg/mL, respectively. 4T1 and C26 cells were maintained in RPMI 1640 cell culture medium supplemented with fetal bovine serum to 10%, Na pyruvate to 1 mmol/L, and penicillin and streptomycin to 50 units/mL and 50 µg/mL, respectively.

Tumor growth in mice. For therapeutic and γ -imaging studies, 4T1 and C26 tumors were implanted in 8-wk-old BALB/c mice by the s.c. injection of ~10⁵ cancer cells into fat pads in the lower abdominal region. Similarly, PC3 human tumors were s.c. implanted into 6- to 8-wk-old *nu/nu* mice (10⁷ cells/mouse). The time for the appearance of the tumor varies from one cell line to another and usually takes 10 to 14 d. Mice were constantly monitored and allowed for free access to food and water (following the animal care protocol R01210 approved by Northeastern University Institutional Animal Care and Use Committee, in accordance with the "Principles of laboratory animal care," NIH publication 85-23, revised in 1985), and tumor volumes were calculated using the formula 0.5 (length \times width²) by measuring the dimensions of the tumor at regular time intervals.

In vivo γ -Tracking of Liposomes

Tumor accumulation of ¹¹¹In-labeled liposomes in mice. When the tumor diameter reached 5 to 8 mm, mice were injected with 0.1 mL of 4 mg/mL ¹¹¹In-radiolabeled, Doxil-mimicking liposomal formulations via the lateral tail vein. At 24 and 48 h postinjections, blood was collected using a Pasteur pipette from the retro-orbital plexus of the eye, and then, the mice were euthanized with carbon dioxide, followed by the excision of the tumor and surrounding muscle. The amount of radioactivity in tissue samples was quantified as counts per minute using a Beckman 5500B gamma counter. The amount of the accumulated radioactivity per gram of tissue and tumor-to-normal ratios were calculated as in ref. 31. The accumulation of ¹¹¹In-labeled, Doxil-mimicking liposomal formulations in the developed tumors was also visualized using an Ohio Nuclear radio-isotope camera (Ohio-Nuclear, Inc.) equipped with a high energy collimator and NU Mac computer (NC systems) at 2, 4, and 6 h postinjection after anesthetizing the mice by injecting a mixture of xylazine and ketamine i.p. The same gating parameters of the capture device, in terms of collection time of the radio-signal and sensitivity level of the camera, have been applied to all test samples. Digital pictures of the tumor-bearing mouse were taken using a Kodak digital camera (Eastman Kodak Company; ref. 27).

Tissue biodistribution of ¹¹¹In-labeled liposomes. *In vivo* biodistribution studies of ¹¹¹In-radiolabeled, Doxil-mimicking liposomal formulation and their UPC10 and mAb 2C5 analogues were done in 4T1 tumor-bearing, 8-wk-old female BALB/c mice. Mice were injected with 0.1 mL of 4 mg/mL ¹¹¹In-radiolabeled, Doxil-mimicking liposomal formulations via the lateral tail vein. At time points of 15, 30, 120, 360, 720, and 1,440 mins postinjection, blood was collected using a Pasteur pipette from the retro-orbital plexus of the eye, and then, the mice were euthanized with carbon dioxide, followed by organ collection. There at least four mice for each time point, and the organs collected were liver, kidney, spleen, lung, muscle, and tail. The amount of the radioactivity was quantified as counts per minute using a Beckman 5500B gamma counter (Beckman Instruments), and the amount of the accumulated radioactivity per gram of tissue was calculated (27, 31). Pharmacokinetic data analysis, based on noncompartmental statistical model, was calculated using PK Solutions 2.0 (Summit research Services).

Assessment of Tumor Growth in Mice

In vivo therapeutic efficacy of 2C5-modified Doxil. Therapeutic treatment of 4T1, C26, and PC3 tumor-bearing mice started 5 d after tumor implantation with the dose intensity of 2 mg doxorubicin/kg every 5 d. Each treatment group consisting of 8 to 10 mice received the corresponding doxorubicin liposomal formulation for four times. Tumor diameters were measured, and approximate tumor volume was calculated as 0.5 (length \times width²; ref. 22) every 3 d during the treatment period to monitor tumor growth. Thirty-five days after the first injection, mice were sacrificed and tumors were excised and weighed.

Statistical analysis. Differences in the apparent tumor volumes during treatment and postmortem tumor weights were compared using the Student's *t* test for two independent samples and Kruskal-Wallis analysis with Tukey's honestly significant differences *post hoc* test for all pair mean comparisons for three or more independent samples. These tests were analyzed using the kaledagraph software, ver. 3.6 (Synergy Software). For easy representation of the differences in tumor volumes during the treatment between the control and treated groups, the average tumor volume (cubic millimeters) of the group versus days was plotted. Tumor weights were plotted as box plots, which represent the median, quartiles, and extremes in a group.

Results

The modification of the mAb 2C5 with the *p*-nitrophenyl-carbonyl-activated PEG-phosphatidylethanolamine lipid derivative with the formation of stable carbamate (urethane)

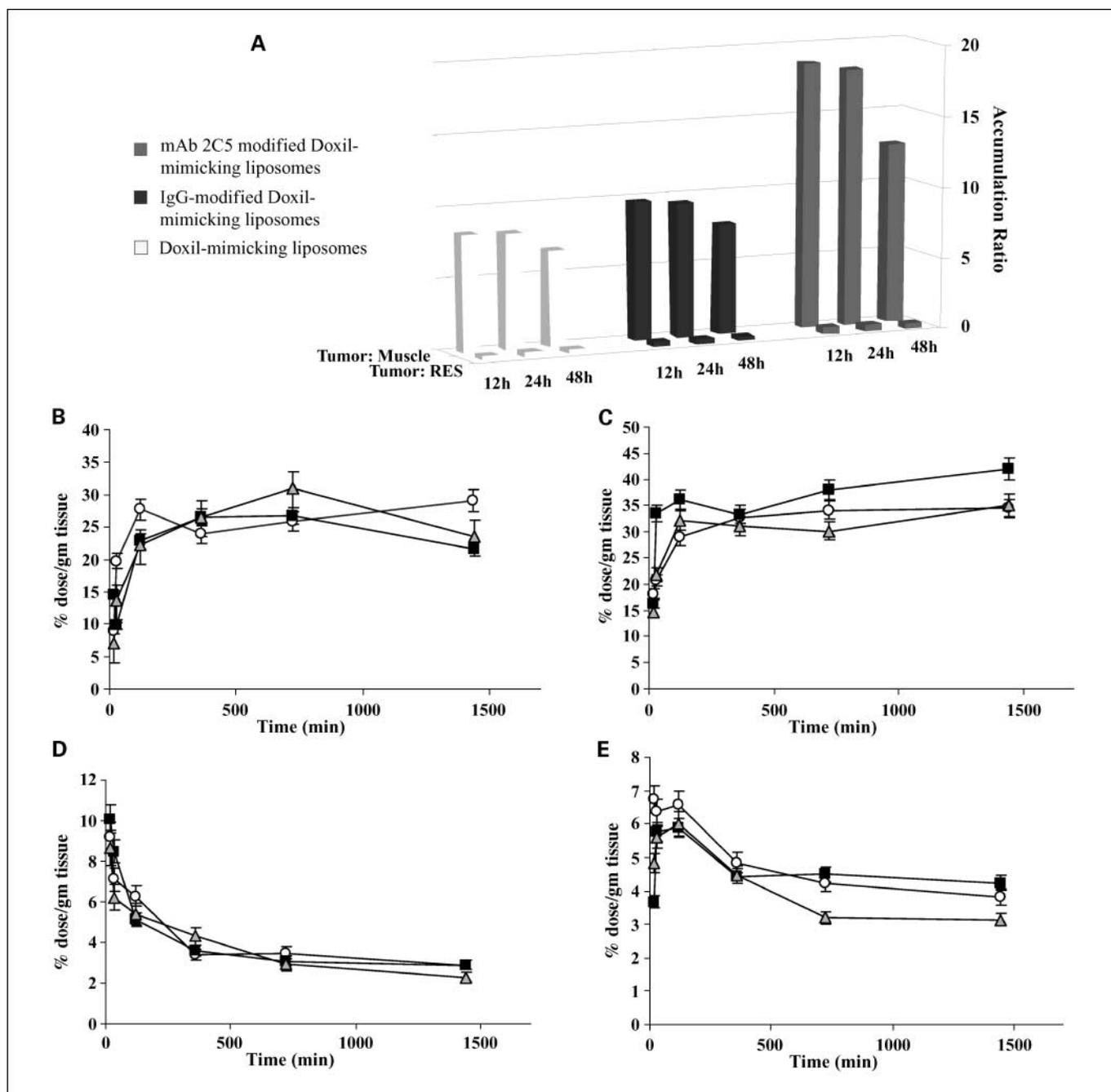


Fig. 1. Tumor accumulation of the liposomal preparations (A) and biodistribution profile of ^{111}In -labeled liposomal formulations in liver (B), spleen (C), lungs (D), and kidneys (E) using murine breast carcinoma 4T1 tumor model ($n = 5$; mean \pm SD).

bond resulted in an active antibody-PEG-phosphatidylethanolamine conjugate (29). Then, via the coincubation with liposomes, spontaneous process allowed for the transfer of PEG-phosphatidylethanolamine-modified antibodies from their loose micelles onto the liposome surface. This postinsertional liposome modification method yields ~70 to 80 antibody molecules per single 100 nm liposome (29, 30). The antibody incorporation was not accompanied by any significant loss of the liposomal doxorubicin, and practically no difference in the preparation stability over time and drug release was estimated between the original Doxil and the

immuno-Doxil formulations (modified with either the UPC10-PEG-phosphatidylethanolamine or the mAb 2C5-PEG-phosphatidylethanolamine) in 48 h *in vitro* release experiment (29, 30, 32).

Moreover, previous reports have shown that liposomes, including doxorubicin-containing PEGylated liposomes, modified with mAb 2C5-PEG-phosphatidylethanolamine show strong and specific binding with mAb 2C5 antigen, nucleosomes (29), whereas nonspecific IgG-modified liposomes and nonmodified liposomes do not bind with the nucleosome monolayer (30).

In vivo Tumor Accumulation of mAb 2C5–Modified Liposomes

The investigation of tumor accumulation of ^{111}In -labeled, Doxil-mimicking liposomes and their IgG and mAb 2C5 immuno-analogues in 4T1 murine tumor models clearly showed an enhanced tumor accumulation of 2C5-modified liposomes. In the studied tumor models, the tumor accumulation ratios of mAb 2C5–modified liposomes compared with the neighboring muscle were almost double that of the nonspecific IgG–bearing or plain liposomes formulations after 24 hours and even after 48 hours. At the same time, the ratio between tumor accumulation and reticuloendothelial system (RES) clearance remained virtually the same for all liposomal formulations at all times (Fig. 1A).

The whole body direct γ -imaging of 4T1 tumor-bearing mice, as shown in Fig. 2 for 4 hours postinjection time point, clearly confirmed the enhanced accumulation of mAb 2C5–targeted preparations in both tumors.

Single-Dose Pharmacokinetics and Biodistribution of ^{111}In -Labeled Liposomes

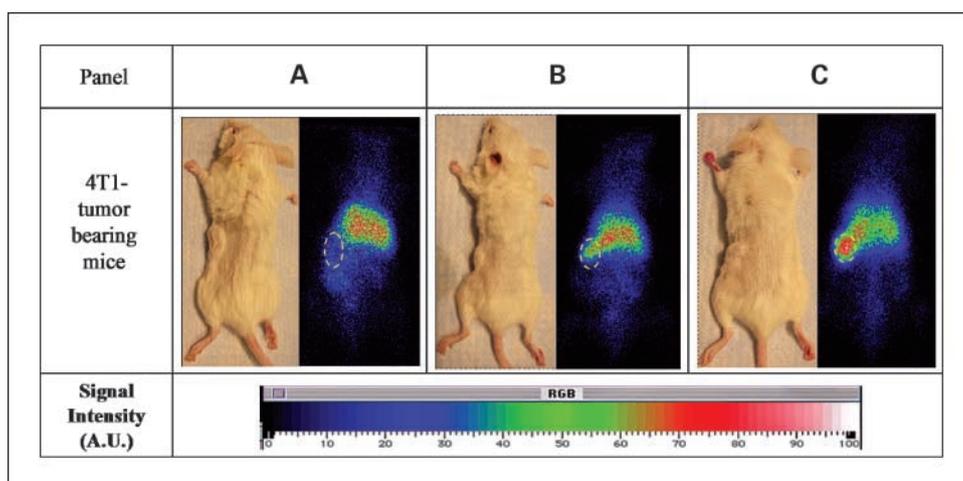
The pharmacokinetic profile of analogous Doxil-mimicking liposomes was studied in 4T1 tumor-bearing mice (31), wherein blood clearance data show that UPC10 and mAb 2C5 immuno-analogues of the ^{111}In -labeled, Doxil-mimicking liposomes expectedly cleared somewhat faster from the body compared with plain liposomes. Still, both formulations retained the ability to circulate long in the blood, with half-lives of about 12 and 13 hours, respectively, compared to 17 hours ($K_{el} = 0.045\text{h}^{-1}$) in case of the nonmodified formulation. This corresponds well to results reported earlier with other targeted liposomes in tumor-bearing mice (22), wherein tumors were found responsible for “attracting” targeted liposomes out of blood, which does not take place in healthy animals. In addition, the observed slight increase in the clearance of the antibody-modified liposomes from the blood can be explained by the presence of whole antibodies on the liposomal surface, which makes them more liable to be taken up by RES via the Fc-mediated uptake. The use of smaller mAb 2C5 fragments such as Fab’ to prepare cancer-targeted immunoliposomes could minimize this type of clearance by RES, although this was not specifically studied in our set of experiments.

The biodistribution of the radiolabeled liposomes in various organs over 24 hours, indicated that, although immuno(UPC10 or mAb 2C5 modified)-liposomes are mainly excreted by liver and spleen, they do have negligible accumulation in nontarget tissues. For example, in the 4T1 tumor-bearing mice, after 12 hours, the tumors had ~6% and 3% of the 2C5-liposome and the UPC10-analogue administered doses, respectively, in comparison with ~0.3% in muscle and 0.25% in skin (not shown) for both immunoformulations. On the other hand, both immunoformulations had higher accumulation in RES organs (liver and spleen combined), about 55% of injected dose, at 12 hours postinjection compared with <48% of plain Doxil-mimicking liposome even after 24 hours postinjection (see the summary data in Fig. 1B-E).

In vivo Therapeutic Efficacy of 2C5-Modified Doxil in Murine and Human Tumor Models in Mice

The choice of murine 4T1 breast carcinoma and C26 colon carcinoma models in “normal” mice, as well as of human prostate cancer PC3 in nude mice, for our *in vivo* studies was based on previous reports (34, 35) in which they were proven to be good models for studying therapeutic activity of doxorubicin-containing formulations. Based on the single-dose pharmacokinetics data obtained earlier along with the reported therapeutic profile of Doxil in 4T1 tumor animal model (36), the dose of 2.2 mg of doxorubicin/kg every 5 days (corresponds to about six half-lives for the original Doxil and approximately seven half-lives of the antibody-modified Doxil formulations, which means almost complete elimination of drug by the time of the next dose) for four consecutive administrations was chosen (i.e., total dose given is just about 9 mg/kg). Some previous studies with Doxil in similar models used similar dosing intensity of 9 mg/kg every 2 weeks for two doses or 4.5 mg/kg every 3 days for four doses (i.e., total dose equals 18 mg/kg; ref. 36). Using the selected treatment regimen, the therapeutic efficacy of mAb 2C5–modified Doxil was compared with that of the nonspecific UPC10–modified Doxil and the original Doxil formulations, in addition to the negative control PBS injections. The effect was assessed in terms of the average tumor volume observed during the treatment period of 32 to 35 days and in terms of the average postmortem tumor weight at the end of the treatment.

Fig. 2. Whole body γ -imaging of s.c. 4T1 tumor-bearing mice 4 h after the injection of ^{111}In -labeled liposomes: control Doxil-mimicking liposomes (A), nonspecific UPC10–modified, Doxil-mimicking liposomes (B), and mAb 2C5–modified, Doxil-mimicking liposomes (C). Circles, tumor locations.



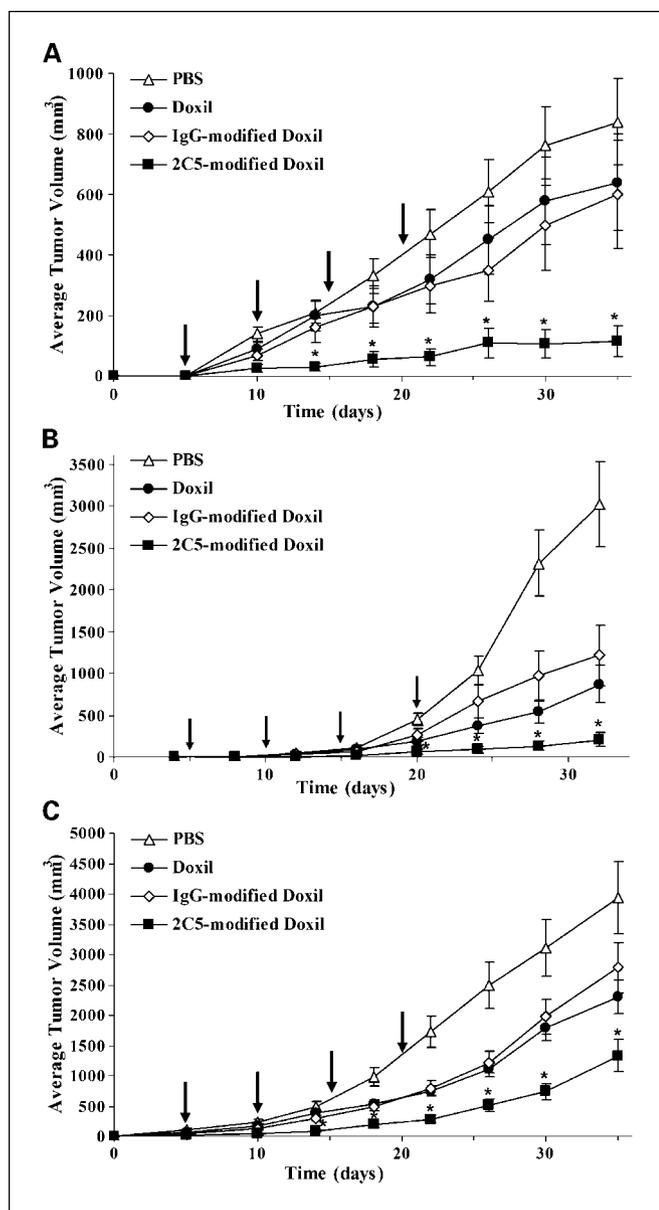


Fig. 3. Therapeutic activity, expressed as tumor volumes, of 2C5-modified Doxil against control preparations in 4T1- (A), C26- (B), and PC3-implanted (C) mice. Arrows, treatment schedule, 2 mg/kg every 5 d ($n = 8-10$). * $P < 0.05$ versus corresponding IgG-Doxil, nonparametric Kruskal-Wallis with Tukey's honestly significant differences *post hoc* test (mean \pm SD).

Compared to all controls, the mAb 2C5-modified Doxil showed a significantly enhanced therapeutic efficacy in all used models in mice, as shown by markedly smaller tumor volumes, starting 2 weeks after tumor implantation ($P \leq 0.05$). The final average tumor weight in animals with 4T1 tumors treated with this preparation was only ~ 0.3 g compared with ~ 1 g in animals treated with nonspecific UPC10-modified Doxil or original Doxil and ~ 2 g in animals receiving only the buffer injections. Similarly, the final average tumor weight in animals with C26 tumors treated with mAb 2C5-modified Doxil was only ~ 0.43 g compared with ~ 1.7 g in animals treated with UPC10-modified Doxil or original Doxil and ~ 4.5 g in animals receiving only the buffer injections. In case of human

PC3 tumor-implanted nude mice, the administration of mAb 2C5-modified Doxil also resulted in significantly smaller tumor volumes starting 20 days after tumor implantation ($P \leq 0.05$). In addition, the final average tumor weight in animals with PC3 tumors treated with this preparation was only ~ 0.7 g compared with ~ 1.7 g in animals treated with UPC10-modified Doxil or plain Doxil and ~ 3.3 g in animals receiving only buffer injections (see the corresponding data in Figs. 3 and 4).

Discussion

Nontargeted sterically stabilized liposomes extravasate into perivascular areas and adjacent extracellular space, especially in tumors, where they then accumulate in tumor stroma. Eventually, liposome breakdown or phagocytic uptake occur, resulting in the release of an encapsulated drug and its subsequent diffusion to nearby cancer cells (37). Although the permeability-dependent mechanism serves as the basis for the antitumor effect of Doxil/Caelyx, it is at the same time the main cause underlying the drug-related mucocutaneous side effects occurring in healthy tissues (38, 39). Hence, although the Doxil/Caelyx formulation have shown a substantial reduction of the toxicity profile of the free doxorubicin, these long-circulating liposomes suffered from the introduction of new side effects, mainly skin toxicity, manifested as hand-foot syndrome and mucositis (40, 41). These adverse effects are virtually inherent to the long-circulating liposomal carriers and can be overcome by modifying the liposome composition (32).

Therefore, attaching certain ligands, first of all, mAbs, as active targeting moieties onto these PEG liposomes influences not only the specificity of the drug delivery but also the penetration and distribution of the preparation within the tumor via rendering them capable of selectively binding certain antigens overexpressed on cancer cells. Earlier, we have investigated the potential of i.v. administration of drug-loaded mAb 2C5 immunomicelles to enhance the delivery of sparingly soluble anticancer agent, paclitaxel, into experimental mice bearing murine Lewis lung carcinoma. Our results showed an increased accumulation of paclitaxel by in the tumor by immunomicelles compared with free drug or drug in nontargeted micelles and an enhanced tumor growth inhibition *in vivo* (28). We have also attempted to use the mAb 2C5 as a targeting ligand to actively target liposomal drug preparations such as Doxil/Caelyx toward various tumors *in vivo* (32). From our earlier *in vitro* experiments (27, 29, 30), the remarkable cytotoxicity of mAb 2C5-Doxil against the various tumor cell lines of diverse origins was evident, mainly due to mAb 2C5 proven specific tumor cell-surface binding and subsequent specific uptake inside the cancer cells. The internalization of mAb 2C5-Doxil was previously shown to be mediated by the antibody-nucleosome/receptor complex (30) because immunoliposomes bearing irrelevant IgG failed to bind or internalize in various cancer cells with surface-bound nucleosome *in vitro*.

The biodistribution data (using the 4T1 murine *in vivo* model) showed that tumor accumulation ratios of ¹¹¹In-labeled, 2C5-modified, Doxil-mimicking liposomes (prepared by adding 0.5 mol % of amphiphilic chelate, DTPA-phosphatidylethanolamine, to the lipid composition) compared with the neighboring muscle were almost double that of the

nontargeted formulations after 24 hours and even after 48 hours. The whole body γ -imaging of 4T1 tumor-bearing mice at 4 hours postadministration confirms the tumor accumulation of the 2C5-modified, Doxil-mimicking liposomes compared with control formulations. Based on the pharmacokinetics and the biodistribution profile of the immunoliposomes, it is warranted to assume that this mAb 2C5 modification of the Doxil-mimicking liposomes have made them significantly less enhanced permeability and retention dependent, and a fewer number of passages of the 2C5 liposomes in a relatively short period of time (\sim 4-6 hours) has resulted in an increase in the tumor accumulation of these liposomes (a markedly stronger tumor signal) compared with that of the enhanced permeability and retention-dependent, plain, Doxil-mimicking liposomes.

Our earlier study has also indicated that IgG and mAb 2C5 immuno-analogues of the ^{111}In -labeled, Doxil-mimicking liposomes showed faster elimination from the body (31), mainly through the uptake by RES organs, liver and spleen. However, it is still fair to consider that both liposomal formulations modified with whole antibodies on their surface still retained the ability to circulate sufficiently long in the blood (half-life of about 12 hours compared with 17 hours in the case of the nonmodified formulation in 4T1 tumor-bearing mice). The results obtained showed that the decrease in the circulation half-life of the radiolabeled liposomes due to the immunomodification with mAb 2C5 had an only minor effect on the development of a sharper and faster tumor-specific signal compared with the nonmodified control. The use of antibody fragments such as Fab' to prepare cancer-targeted immunoliposomes should also be possible and should probably improve the blood residence time of the immunoliposomes to the level close to that of plain liposomes (42). Yet, one may hypothesize based on earlier results (32) that keeping the circulation time of such immunoliposomes on the shorter side could be actually beneficial in reducing their nonspecific extravasations in healthy tissues, hence their skin side effects, with virtually no effect on their therapeutic activity.

The selection of the dose regimen, 2.2 mg/kg every 5 days for four doses with total cumulative dose of 9 mg/kg, was based upon the pharmacokinetic data of the liposomal formulations and the effective Doxil therapeutic regimen used in similar tumor models (total cumulative dose of 18 mg/kg). It was chosen as a subtherapeutic dose of Doxil to be able to investigate the statistical significance of the therapeutic difference between the various Doxil formulations. Considering the primary s.c. solid tumor growth in different types of murine tumor models (4T1 breast carcinoma and C26 colon carcinoma), the mAb 2C5-modified Doxil showed a significantly improved therapeutic efficacy in mice compared with all controls, resulting in markedly smaller tumor volumes starting 2 to 3 weeks after tumor implantation, and significantly lower final average tumor weights ($P \leq 0.05$). These significant therapeutic results confirm the important role of the tumor-specific accumulation of the mAb 2C5-modified Doxil in achieving high drug concentration in the tumor. Although the diffusion and deep tumor penetration are not expected for the particles of the size of liposomes, it is quite likely that the encapsulated drug, being much more diffusible than the carrier itself, can reach distant tumor cells surrounding the area of extravasated liposomes and exert such a significant antineo-

plastic effect in addition to the effect produced via the internalization of drug-loaded targeted liposomes by tumor cells surrounding the regions of liposome extravasation.

Finally, compared with all controls, the mAb 2C5-modified Doxil showed a significantly improved therapeutic efficacy in human PC3 tumor-bearing nude mice, also expressed as markedly smaller tumor volumes starting 3 weeks after tumor implantation, and significantly lower final average tumor weights compared with control nonspecific treatments ($P \leq 0.05$). These therapeutic outcomes, in case of aggressive human tumor, indeed substantiate the key role of the tumor-specific binding and internalization of the mAb 2C5-modified Doxil

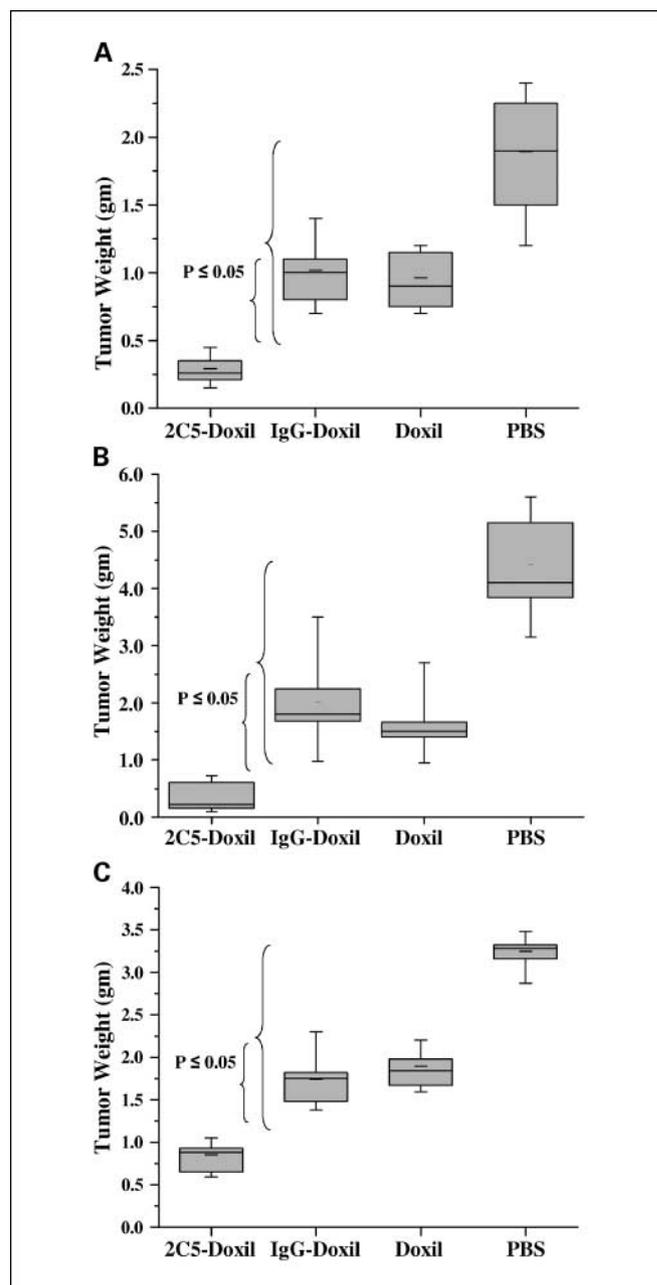


Fig. 4. Therapeutic activity, expressed as postmortem tumor weights, of 2C5-modified Doxil against control preparations in 4T1- (A), C26- (B), and PC3-implanted (C) mice ($n = 8-10$; mean \pm SD).

in achieving elevated local concentration of the chemotherapeutic agent inside the tumor interstitium.

Conclusions

The present study provides clear evidence that a simple immunomodification of Doxil with the cancer-specific antinucleosome mAb 2C5 resulted in a successful development of a rather universal tumor-targeted doxorubicin delivery system, showing prolonged circulation in the body and specific recognition of various tumors. The substantial and rapid accumulation of the 2C5-targeted formulation of Doxil specifically into tumors resulted in a prominent tumor

growth regression in various tumor models. The mAb 2C5-modified Doxil provides a promising opportunity to further enhance the therapeutic index of the original formulation and broaden the spectrum of its antitumor activity. It should also encourage further research to expand the application of this tumor-specific ligand to target more drugs used in the current medical oncology community, including paclitaxel derivatives, platinum derivatives, and novel phase I agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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