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Enhanced Cytotoxicity of Monoclonal Anticancer Antibody 2C5-Modified Doxorubicin-Loaded PEGylated Liposomes against Various Tumor Cell Lines

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Abstract

Doxorubicin-loaded long-circulating liposomes (Doxil™, ALZA Corp.) were additionally modified with the nucleosome-specific monoclonal antibody 2C5 (mAb 2C5) recognizing a broad variety of tumor cells via the tumor cell surface-bound nucleosomes. These mAb 2C5-modified PEGylated liposomes demonstrated 3-to-8-fold increase in the in *vitro* binding and internalization by multiple cancer cell lines of diverse origins (murine LLC, 4T1, C26 & human BT-20, MCF-7 and PC3), as shown by flow cytometry and epi and confocal microscopy. As a result, mAb 2C5-modified Doxil™ demonstrated significantly higher cytotoxicity towards various cancer cells, including those resistant to doxorubicin, than all control preparations. The specific internalization of the mAb 2C5-Doxil™ into cytosol, along with the nuclear localization of their drug load, inside the target cancer cells were mainly responsible the superior anticancer activity. The IC50 values of mAb 2C5-Doxil™ with various murine and human cancer cells were 5-to-8-fold lower than those of control doxorubicin-loaded liposomes, Doxil™ or Doxil™ modified with a non-specific IgG.

Keywords

Tumor targeting; Doxorubcin-loaded liposomes; Long-circulating liposomes; Anticancer nucleosome-specific antibody; Cytotoxicity

1. Introduction

Liposomal formulations of various anticancer drugs are widely used in experimental and clinical oncology with some of them, such as Doxil™, becoming drugs of choice under certain conditions(Alberts, et al. 2004, James, et al. 1994, Tejada-Berges, et al. 2002). The use of the liposomal carrier allows for decreased side effects of cancer chemotherapeutics, such as non-specific toxicity, and for enhanced drug delivery into tumors (Gabizon, et al. 2004, Safra, et al. 2000). Doxil™ represents doxorubicin incorporated into long-circulating PEGylated liposomes and demonstrates decreased cardiotoxicity and improved tumor accumulation via

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CONFLICT OF INTEREST STATEMENT

NONE DECLARED: All authors state that there is no conflict of interest, nor potential conflict of interest including any financial, personal or other relationships with other people or organizations within three (3) years of beginning the work submitted, that could inappropriately influence their work.

the enhanced permeability and retention (EPR) mechanism (Gabizon 1992, Northfelt, et al. 1996).

The further development of the concept of long-circulating liposomes involves the attempt to combine the properties of long-circulating liposomes and specifically targeted liposomes in one preparation. The accumulation of the liposomal drugs was shown to be still further improved by their specific targeting to the tumor, i.e. by attaching certain tumor-specific molecules to the liposome surface (Sapra, et al. 2005, Torchilin 2005). Specific vector molecules capable of recognizing tumors include antibodies, peptides, folate, transferrin and some other moieties (Kurohane, et al. 2000, Schiffelers, et al. 2003, Willis and Forssen 1998). With this in mind, several attempts have been made to further improve the anticancer efficiency of Doxil™ by actively targeting it to tumors with certain vector molecules specific to the receptors characteristic of cancer cells (Park, et al. 2001). On a broader picture, targeted doxorubicin-loaded liposomes were repeatedly shown to demonstrate an increased anticancer activity, as did, for example, folate-targeted liposomes loaded with doxorubicin with target cells *in vitro* (Lee and Low 1995). Doxorubicin-loaded liposomes conjugated with folic acid were shown to be internalized upon their binding with folate receptors (Goren, et al. 2000), suggesting the potential for such targeting and subsequent internalization strategy in the treatment of several MDR-tumors (Mamot, et al. 2003). This approach is additionally supported by the fact that endocytosis of the liposomal drugs is essential for bypassing multidrug resistant (MDR) efflux pumps, such as P-glycoprotein or Pgp, in drug-resistant tumor cells (Gabizon 2002, Reddy and Low 1998). Similar results have been obtained with doxorubicin-loaded long-circulating liposomes modified with RGD-peptide motif and capable of targeting the neovasculature of the angiogenic tumors (Xiong, et al. 2005). Using a small cell lung cancer cell line, it was shown that RGD-targeted liposomes were internalized much faster, delivered doxorubicin to the cell nuclei more efficiently, and were more cytotoxic compared to non-targeted liposomes (Moreira, et al. 2001). Doxorubicin-loaded liposomes modified with Fab′ fragments of anti-disialoganglioside antibodoes selectively and almost completely inhibited the metastatic growth of human neuroblastoma in nude mouse model (Pastorino, et al. 2003).

From the list of targeting moieties, monoclonal antibodies and their fragments seem to have the highest potential in terms of specificity and variability (Torchilin 2000). Monoclonal antibodies have been obtained that can recognize specific antigens from the majority of known tumors, such as antibodies against ovarian cancer, prostate cancer or colorectal cancer (Agus, et al. 2000). Earlier, we have identified a family of natural antibodies with nucleosome-restricted specificity, which are capable of effective recognition and binding of a broad variety of live cancer cells (but not normal cells) via the nucleosomes originating from the apoptotically dying neighboring cancer cells and attached to the surface of cancer (but not normal) cells via characteristic nucleosome-binding sites (Iakoubov, et al. 1995, Iakoubov and Torchilin 1998). In addition to their own broad anticancer potential (Chakilam 2004, Torchilin, et al. 2003), these antibodies and their representative, the monoclonal antibody 2C5 (mAb 2C5), being used in sub-therapeutic quantities, can serve as effective targeting molecules for tumor-specific delivery of drug-loaded pharmaceutical nanocarriers (Torchilin, et al. 2003). To attach antibodies to Doxil™ liposomes “above” the protective layer of PEG, we have used earlier developed protocol of preliminary antibody modification with p-nitrophenyl-carbonyl-PEG-phosphatidyl ethanolamine (pNP-PEG-PE) conjugate (Torchilin, et al. 2001) with the subsequent incorporation of the modified antibody molecule into the membrane of PEGylated liposomes via the hydrophobic PE moiety. Earlier, we have obtained some encouraging preliminary data on the increased cytotoxicity of Doxil™ modified by mAb 2C5 (Gupta, et al. 2005, Lukyanov, et al. 2004).

Here, we present the results of our extended studies on the cytotoxicity of mAb 2C5-modified Doxil™ towards a broad variety of tumor cell lines as well as on the mechanism of the internalization of mAb 2C5-PEG-liposomes by cancer cells.

2. Materials and Methods

2.1. Materials

Cholesterol (Chol), fully hydrogenated soy phosphatidylcholine (HSPC), N-(carbonyl-methoxy poly (ethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine sodium salt (MPEG2000-DSPE), and phosphatidylehtanolamine (PE) were from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Doxorubicin was purchased from Sigma Chem., Inc. (St. Louis, MO). Triethylamine (TEA), octyl glucoside (OG) and diethylenetriaminepentaacetic acide anhydride (DTPA) were products of Sigma. Poly-oxyethylene3400-bis (p-nitrophenyl carbonate ($PEG(pNP)_2$) was purchased from SunBio (Orinda, CA). Cell culture media, DMEM, EMEM, and RPMI 1640, along with fetal bovine serum (FBS) and concentrated solutions of Na-pyruvate, non-essential amino acids, L-glutamine and penicillin/streptomycin stock solutions and serum-free media (SFM), were purchased from CellGro (Kansas City, MO). Female Balb/C and C57/BL mice were purchased from Charles River Laboratories (Cambridge, MA). All solvents and other chemicals were analytical grade preparations. The mAb 2C5 was produced in ascites via I.P. injection of 1.5×10^6 hybridoma cells/ml into pristine primed 4 week old Balb/C male mice. The production and the purification of the mAb 2C5 were carried out by Harlan Bioproducts (Indiannapolis, IL) using the cell line from our laboratory.

2.2. Methods

2.2.1. Synthesis of pNP-PEG-PE—Synthesis of PEG-PE conjugate activated on the free PEG terminus with p-nitrophenylcarbonyl group, pNP-PEG-PE, was performed as in (Torchilin, et al. 2001). Briefly, approx. 5.3-fold molar excess of PEG3400-$(pNP)_2$ over PE were dissolved in chloroform and then supplemented with approx. 2-fold molar excess of TEA over PE. Finally, PE in chloroform was added to the mixture with heating on an oil bath (80° C) for 5 minutes until clear solution was formed. Then, after incubating overnight at room temperature with stirring under argon, the organic solvent was removed using a rotary evaporator. Afterwards, the pNP-PEG-PE micelles formed were separated from the unconjugated PEG and released pNP on a CL-4B column using 0.01 M HC1, 0.15 M NaCl as an eluent, freeze-dried, and stored as chloroform solution at −80°C.

2.2.2. Antibody modification—To prepare antibodies (mAb 2C5 or non-specific IgG) modified with PEG3400-PE, a 40 molar excess of pNP-PEG3400-PE dispersed in 10 mg/ml solution of OG in 5 mM Na-citrate, 150 mM NaCl, pH 5.0, was added to an equal volume of 1 mg/ml solution of a protein (mAb 2C5 or IgG) in Tris-buffered saline (TBS), pH 8.5. The mixtures were incubated for 24 hr at pH 8.5 at 4°C (Lukyanov, et al. 2004). Micelles of modified antibodies were separated from other reaction products by the size exclusion gel-chromatography using a CL-4B column (Torchilin, et al. 2001).

2.2.3. Preparation of antibody-modified liposomes—To obtain doxorubicin-loaded liposomes modified with mAb 2C5 or non-specific IgG, proteins conjugated with PEG3400-PE (2C5-PEG-PE or IgG-PEG-PE) were co-incubated with commercial Doxil™ preparation. For this purpose, the products from the modification step (see the previous paragraph on antibody modification) were mixed in equal volumes with Doxil™ suspension and incubated for 8 h at 4°C. Following the incubation, the remaining OG and free, non-incorporated immunoglobulins were removed by dialysis using cellulose ester dialysis tubes with a cutoff size of 250,000 Da. The complete removal of the non-incorporated immunoglobilins (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 antibody-PEG-PE or antibody-PEG-PE micelles) (Lukyanov, et al. 2004).

2.2.4. Preparation of control liposomes—Control PEGylated liposomes mimicking Doxil™ composition (LMD) 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 the chloroform solution of MPEG2000-DSPE (3.19 mg/ml), HSPC (9.58 mg/ml), and Chol (3.19 mg/ml). The lipid film was suspended in HEPES-buffered saline (HBS), pH 7.4, and sonicated with a probe-type sonicator at 11 Watts power for 10 min, followed by several passages through a mini-extruder with 100 nm pore size polycarbonate filter, until approx. 100 nm liposomes with narrow size distribution were obtained (Lukyanov, et al. 2004).

2.2.5. Liposome size and zeta-potential—Liposome size and size distribution for different samples were determined by the dynamic light scattering using a Coulter N4 MD Submicron Particle Analyzer (Beckman Coulter, Inc., Fullerton, CA).

Zeta potentials of the liposomal preparations were determined using 90 PLUS particle size analyzer with ZETA PALS System, Brookhaven Corp. (Holtsville, NY) at 25 °C.

2.2.6. In vitro release of doxorubicin from liposomes—The *in vitro* release of doxorubicin from the different Doxil™ formulations over a 48 hr period, was investigated in DMEM cell culture medium with 10% FBS. One ml aliquots of liposomes at doxorubicin concentration of 0.5 mg/ml, diluted in the media, were sealed into dialysis tubes with the cutoff size of 12,000-to-14,000 Da. Then, the liposomes-loaded dialysis tubes were incubated in 50 ml of the media for 48 h at 37°C, with continuous stirring at medium speed. At various time points, aliquots were withdrawn, and replaced with equal volume of the media. The doxorubicine concentrations were then measured at 485 nm (Xiong, et al. 2005) using a Hitachi U-1500 spectrophotometer, Hitachi Instruments (Schaumburg, IL). The validity of the used protocol and the minimal influence of the medium components on the doxorubicin fluorescence have been shown earlier (Eliaz and Szoka 2001).

2.2.7. Cell cultures—In order to investigate the broad tumor-specificity of the mAb 2C5-modified liposomes and their *in vitro* anticancer activity, several unrelated tumor cell lines have been used. Murine Lewis lung carcinoma (LLC), murine breast adenocarcinoma (4T1), murine colon cancer (C26), human mammary adenocarcinoma (BT-20), human prostate cancer (PC3), and estrogen receptor-sensitive human breast carcinoma (MCF-7) were purchased from the American Type Culture Collection (Manassas, VA). LLC, PC3 and MCF-7 cells were maintained in DMEM cell culture medium supplemented with 10% FBS, 1 mM Na pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin. BT-20 cells were maintained in EMEM cell culture medium supplemented with 10% FBS, 1 mM Na pyruvate, and non-essential amino acids and L-glutamine to 1 mM each. 4T1 and C26 cells were maintained in RPMI-1640 cell culture medium supplemented with 10% FBS, 1 mM Na pyruvate, and penicillin and streptomycin to 50 units/ml and 50 μg/ml, respectively

2.2.8. Epi-fluorescence microscopy—The enhanced tumor-cell association of the mAb 2C5-Doxil™ was demonstrated using the fluorescence microscopy. After an initial passage in tissue culture flasks, C26 and PC3 cells randomly chosen for this experiment were grown in 6-well tissue culture plates. After reaching 70–80% confluence, the plates were washed with Hank's balanced buffer, pH 7.4, then treated with 1% BSA in DMEM (1–2 ml/well) and incubated for 1h at 37°C, 5% CO_2. To these cells, different (plain/immuno)-Doxil™ formulations (total lipid concentration of 0.5 mg/ml) were added and incubated for 1h at 4°C, in 5%CO_2. After the incubation, the cells were washed with Hank's balanced buffer and stained

with DAPI nuclear stain after fixation in NBF for 30 min. Then, individual cover slips were mounted cell-side down on glass slides using the fluorescence-free glycerol based mounting medium (Fluoromount-G, Southern Biotechnology Associates, Inc.), and cells were viewed with a Nikon Eclipse E400 microscope under the bright light or epi-fluorescence with rhodamine/TRITC filter (excitation/emission: 543/570) and DAPI filter (excitation/emission: 345/661) (Torchilin, et al. 2001).

2.2.9. FACS analysis—To asses the target cell binding of the mAb 2C5-modified Doxil™ compared to control Doxil™ formulations, flow cytometry analysis of different cell types incubated with the different liposomal formulations was performed. Selected cells (C26, 4T1 and PC3) were grown in 25 ml cell culture flasks (BD Biosciences, Bradford, MA) until they reached a confluence of 70%. The cells were detached by the repetitive pipetting, and then washed twice with 1% bovine serum albumin in PBS, pH 7.4. For the evaluation of both target cell binding and uptake, LMD labeled with 0.5 wt % of FITC-PE (total lipid concentration was 1 mg/ml) were added to the washed cells, and the samples were incubated for 45 min at both 4°C and 37°C, in 5% CO_2. After the incubation, the cells were washed twice with PBS, fixed with 4% paraformaldehyde solution in PBS, live-gated using forward vs. side scatter to exclude debris and dead cells and analyzed (10,000 cells in average count) using FACScan™ (Beckton Dickinson Biosciences, San Jose, CA) (Lukyanov, et al. 2004).

2.2.10. Confocal fluorescence microscopy—Confocal fluorescence microscopy was used to confirm the active internalization/uptake of mAb 2C5-modified Doxil™ by target cancer cells. After an initial passage in tissue culture flasks, C26, and PC3 cells were grown in 6-well tissue culture plates. After reaching 70–80% confluence, the plates were washed with Hank's balanced buffer, pH 7.4, then treated with 1% BSA in DMEM (2 ml/well) and incubated for 1h at 37°C, 5% CO_2. To these cells, mAb 2C5-modified Doxil™ (total lipid concentration of 1 mg/ml) was added and cells were incubated for both 1 and 2 h at 37°C, in 5%CO_2. After the incubation, cells were washed with Hank's balanced buffer and stained with DAPI nuclear stain (20 μl, 100 μM) for 10 min, after fixation in neutral buffered formalin (NBF) for 30 min. Following this, individual cover slips were mounted cell-side down on glass slides using the fluorescence-free glycerol based mounting medium (Fluoromount-G, Southern Biotechnology Associates, Inc.) and cells were viewed with a an inverted Zeiss confocal laser scanning microscope (Ziess Meta 510 LSM; Carl Zeiss, Jena, Germany). A pinhole of around 1 airy unit was used with an oil immersion objective, resulting in optical slices of around 0.8 μm. To visualize doxorubicin, the excitation was performed at 530 nm by the internal He-Neon laser and the fluorescence emission was observed above 570 nm. For DAPI, the excitation was performed at 350 nm line by the internal Argon laser, and the fluorescence emission was observed above 650 nm (Goren, et al. 2000).

2.2.11. Time-dependent association of doxorubicin with cells—Doxorubicin association with cells was determined by FACS analysis (Lukyanov, et al. 2004) as described above. After the incubation of C26 and PC3 cells with different Doxil™ formulations in SFM (lipid concentration 0.5 mg/ml) at 37°C for 0-to-4 h, cells were washed twice with PBS, fixed with 4% paraformaldehyde solution in PBS, live-gated using forward vs. side scatter to exclude debris and dead cells and analyzed (20,000 cells in average count) using FACScan™ (Beckton Dickinson Biosciences, San Jose, CA).

2.2.12. Cytotoxicity assay—The cytotoxicity of various preparations of the liposomal doxorubicin against LLC, BT20, 4T1, C26, PC3 and MCF-7 cells was studied using a MTS test. A ready-for-use CellTiter 96® Aqueous One solution of MTS (Promega, Madison, WI) was used according to a protocol suggested by the manufacturer. Formulations with doxorubicin concentration of up to 200 μg/ml or equivalent concentrations of lipid and/or

protein components dispersed in Hank's buffer were added to cells grown in 96-well plates to about 75% confluence, in duplicates. After 24 h incubation at 37°C, 5% CO_2, plates were washed three times with Hank's buffer followed by the addition of 20 μl of CellTiter 96® Aqueous One solution. After 1 h incubation at 37°C, 5% CO_2, the cell survival rate was estimated by measuring the color intensity of the MTS degradation product at 492 nm using an ELISA plate reader. Assay was performed twice for each cell line. Full concentration dependences have been obtained for 2C5-Doxil™ and non-specific IgG-Doxil™ samples. Additional control with doxorubicin-free 2C5-LMD was done at the fixed lipid concentration equivalent to that of 100 μg/ml Doxil™ liposomes, since no difference from the "no treatment" sample or any concentration dependence was expected in this case. Corresponding IC50 values were determined from the cytotoxicity data using Microcal Origin software (Microcal Software, Inc., Northampton, MA)(Lukyanov, et al. 2004).

2.2.13. Statistics—Differences in cancer-cell killing by different formulations were compared using the Kruskal-Wallis analysis with Tukey's HSD Post-Hoc test for all mean comparisons, for three or more independent samples. These tests were analyzed using Kaliedagraph software, ver. 3.6 (Synergy Software, Reading, PA).

3. Results

The method of antibody attachment to liposomes by transferring PEG-PE-modified antibodies from their loose micelles onto the liposome surface used in this study results in attaching approx. 70–80 antibody molecules per single liposome (Gupta, et al. 2005, Lukyanov, et al. 2004). The antibody incorporation was not accompanied by any significant loss of the liposomal doxorubicin, and no drug release was registered in both the original Doxil™ and the immuno-Doxil™ formulations (modified with either the IgG-PEG-PE or the mAb 2C5-PEG-PE) in 48 h *in vitro* release experiment.

It was also repeatedly shown that liposomes, including doxorubicin-containing PEGylated liposomes, modified with mAb 2C5-PEG-PE demonstrate strong and specific binding with mAb 2C5 antigen, nucleosomes (Lukyanov, et al. 2004), while IgG-modified liposomes, and non-modified liposomes do not bind with the nucleosome monolayer.

3.1. Specific binding and uptake of mAb 2C5-modified liposomes into cancer cells in vitro

The ability of mAb 2C5-modified liposomes to selectively recognize target cancer cells was confirmed using flow cytometry (FACS) analysis (Fig. 1). Two different cell lines of murine origin; namely C26 colon cancer, and 4T1 breast cancer cell lines, and human PC3 prostate cancer cell line used in this experiment, were incubated with the LMD labeled with 0.5 wt % of FITC-PE. At 4°C, the higher binding of mAb 2C5-liposomes was observed compared to controls, although a certain binding of IgG-liposomes to some cells was also observed, which can be attributed to presence of a variable number of Fc receptors on the surface of various cancer cells. Some variability in mAb 2C5 binding to various tumor cell lines can be explained by the variation in the amount of the cell surface-bound nucleosomes in different cell lines together with the variation in the quantity of released nucleosomes in different cell cultures (Chakilam 2004). Still, mAb 2C5-liposomes demonstrated 3-to-6-fold increase in cell surface association at both 4°C and 37°C compared to IgG-analogues.

The increase in cell-associated fluorescence noted at 37°C for mAb 2C5-liposomes compared to that at 4°C for all cell lines (see **panels B** on Fig. 1), could be explained by the active uptake (endocytosis) of immuno-liposomes by the cancer cells. As one could expect, control LMD did not demonstrate any noticeable increase in cell-associated binding (fluorescence increase) at 37°C compared to that at 4°C, indicating the absence of active cell uptake in this case (Sapra and Allen 2002,Xiong, et al. 2005).

Moreover, the epi-fluorescence microscopy pictures of obtained for C26 and PC3 cell lines at 37°C (Fig. 2) as well as confocal microscopy images of the same cancer cell lines (Fig. 3) provide conclusive evidences that support the enhanced uptake of mAb 2C5-modified Doxil™ by cancer cells by demonstrating strongly increased doxorubicin concentration inside the cells (doxorubicin fluorescence) in case of mAb 2C5-modified Doxil™, compared to both non-specific controls. The confocal images obtained after 2 h incubation (Fig. 3) also demonstrate an increased nuclear localization of doxorubicin in case of 2C5-Doxil™, compared to controls as could be in Figure 2.

Figure 4 describes the kinetics of the cellular uptake of different liposomal formulations at 37° C for two different cell lines, murine C26 and human PC3. It clearly shows the superior increase in the cellular accumulation of doxorubicin with time in case of mAb 2C5-modified Doxil™ compared to both the IgG-modified and original formulations.

3.2. *In vitro* cytotoxicity assay

The cytotoxicity of different liposomal preparations (Doxil™, IgG-Doxil™ and mAb 2C5-Doxil™ at the same doxorubicin concentration; and 2C5-modified empty LMD - at the same lipid concentration) was investigated using several diverse cancer cell lines: murine 4T1, LLC and C26, and human MCF-7, BT20, and PC3 cells. Representative results of cytotoxicity experiments are shown in Figure 5, for three different cell lines, 4T1, C26 and PC3. This figure presents the results obtained at various doxorubicin concentrations (up to 200 μg/ml) with IgG-Doxil™ and 2C5-Doxil™ as well as cytotoxicity “cross-section” at doxorubicin concentration of 100 μg/ml. Empty 2C5-liposomes did not show any cytotoxic effect.

In all tested cell lines, 2C5-targeted Doxil™ formulation demonstrated the far highest toxicity against all cancer cell lines studied. After 24 hours, the formulation modified with mAb 2C5 killed approx. 90% of cells in the case of PC3, 80% of cells in the case of C26, and 83% in case of 4T1 cells. The most cytotoxic of all control formulations, non-specific IgG-modified Doxil™, killed only ca. 43% of PC3, 40% of C26 cells, and 45% of 4T1 cells. The differences between targeted formulation and other controls were even greater. There was no significant difference in cytotoxicity between the original Doxil™ and the IgG-modified Doxil™, in all studied cell lines. The IC50 values for different preparations of the liposomal doxorubicin were also determined with all cell lines (Table 1). Thus, for example, in 4T1 cell line, the IC50 of 2C5-Doxil™ was calculated as 22±1.8 μg/ml compared to 118±7 μg/ml in case of non-specific IgG-Doxil™; in PC3 cells, the IC50 values were 23±3 μg/ml and 122±7 μg/ml for 2C5-Doxil™ and IgG-Doxil™ respectively.

It is worth noting that cytotoxicity profile of the liposomal formulations in C26 colon cancer cell line (Fig. 5 II), a Pgp-expressing doxorubicin-insensitive cancer(Huang, et al. 1992,Papahadjopoulos, et al. 1991), clearly show that 2C5-targeted formulation demonstrated the highest toxicity, evidently bypassing the Pgp-mediated resistance mechanism. After 24 hours, the formulation modified with mAb 2C5 killed approx. 80% of C26 cells, while the best control formulation, non-specific IgG-modified Doxil™, killed only about 40% of cancer cells. The IC50 of 2C5-Doxil™ with CT26 cells was calculated as 19 ± 2 μg/ml compared to 117 ± 8 μg/ml in case of non-specific IgG-Doxil™. Hence, the mAb 2C5-modified Doxil™ formulation was approx. 6-fold more toxic than the most toxic control, the non-specific IgG-modified Doxil™ formulation, in this drug-resistant cancer cells (Table 1).

4. Discussion

The toxic side effects of anti-cancer drugs, resulting from the lack of specificity of conventional therapies, usually limit the increase in the dose often required to eradicate the cancerous growth. One of the most evolved strategies that have been developed in the last two decades was the

enhancement of the tumor-specific delivery of the chemotherapeutic drug, mainly anthracyclins, through actively targeting them via monoclonal antibodies capable of selectively binding antigens that are over-expressed on cancer cells. The active targeting of anthracycline (mainly Doxorubicin)-loaded liposomes via monoclonal antibodies and their fragments has received extensive research attention in the last decade (Allen, et al. 2005, Eliaz and Szoka 2001, Sapra, et al. 2005, Torchilin 2005) and allowed for the facilitated delivery of these liposomes with their drug cargos into the site of drug action and minimized undesired side effects.

Based on the ability of mAb 2C5 to specifically react with a variety of lymphoid and non-lymphoid tumor cells of murine and human origin (Iakoubov and Torchilin 1997), but not with the surface of normal cells, we aimed to employ this mAb as a targeting moiety to actively target Doxil™ toward diverse tumors, both *in vitro* and *in vivo*. The targeting moiety is usually attached outside ("above") the protecting polymer layer, by coupling it with the distal ends of activated liposome-grafted polymeric molecules, specifically via the p-nitrophenylcarbonyl group (pNP)-activated terminus of PEG-PE polymer in this particular case (Gupta, et al. 2005, Lukyanov, et al. 2004). Following this approach, in a simple attachment scheme, the antibody (mAb 2C5 or IgG) was first modified the activated PEG-lipid derivative and then incorporated into liposomes by co-incubating the loose micelles of PEG3400-PE-modified proteins with Doxil™ (Gupta, et al. 2005, Lukyanov, et al. 2004).

The preservation of a sufficient specific activity of PEG-PE-modified mAb 2C5 samples was repeatedly confirmed by ELISA using nucleosomes as a binding substrate (Chakilam 2004, Lukyanov, et al. 2004). It was found that antibody-PEG-PE conjugates incorporate into the liposomal membrane without inducing a significant loss of encapsulated doxorubicin or producing substantial alteration in the release profile for the original Doxil™. This immuno-modification of Doxil™ did not noticeably change the liposome size (remained in the range of approx. 90 to 120 nm) or the net charge at the liposomes surface (Zeta potential was approx. −25 to −23) (Elbayoumi and Torchilin 2006).

Through fluorescence microscopy and flow cytometry analysis, FITC-labeled mAb 2C5-modified LMD displayed significantly higher cancer cell binding, 3-to-6-fold, than analogous non-targeted IgG-formulations, in all tested cell lines of both murine and human origins when measured at 4°C.

Furthermore, FACS analysis have demonstrated that the temperature increase from 4°C to 37° C did not result in any significant increase in cell-associated liposome fluorescence in case of plain liposomes. At the same time, in the case of mAb 2C5-modified liposomes, there was a significant increase in the cell-associated liposome fluorescence, by 6-to10 folds compared with different controls. This can serve as indication of much more active uptake (endocytosis) of mAb 2C5 antibody-modified liposomes by cells compared to non-specific liposomes in good agreement with (Eliaz and Szoka 2001, Sapra and Allen 2002).

Supporting this observation, the fluorescence microscopy pictures of two investigated for this purpose cell lines, C26 and PC3 cells, at 37°C, together with the confocal microscopy images of cancer cells, demonstrated a significantly higher quantity of doxorubicine inside cancer cells (enhanced intracellular doxorubicin fluorescence) in the case of mAb 2C5 modified-Doxil™, compared to both non-specific controls. These studies have also revealed the marked nuclear localization of doxorubicin in case of 2C5-Doxil™. This may also contribute to the enhanced activity of 2C5-Doxil™, since nuclear localization is essential for the activity of doxorubicin, which acts through DNA intercalation and topoisomerase poisoning (Goren, et al. 2000, Xiong, et al. 2005, Xiong, et al. 2005). Moreover, the improved doxorubicin influx inside the

doxorubicin-resistant C26 colon cancer (Huang, et al. 1994, Papahadjopoulos, et al. 1991) was also confirmed.

Consequently, the *in vitro* cytotoxicity assay of the different Doxil™ formulations showed that the cytotoxicity of 2C5-modified Doxil™ was remarkably higher, with 5–6 times lower IC_{50} values, than that of even the most cytotoxic of control formulations, non-specific IgG-Doxil™. When compared with the original Doxil™, the increase in the toxicity was even higher, up to 8-fold. In addition to the enhanced cell binding, the evidently superior internalization of the 2C5-Doxil™ into cancer cells also may play an important role in enhancing the anticancer action, in particular, against the doxorubicin-resistant C26 colon cancer, probably by the endocytosis-mediated bypass of the P-gp efflux effect.

In conclusion, the present study clearly demonstrates that a simple modification of Doxil™ with the anti-nucleosome mAb 2C5 resulted in obtaining a pan-specific tumor-targeted doxorubicin delivery system. The mAb 2C5-Doxil™ formulation showed a remarkable *in vitro* anticancer activity against several cancer cells of diverse origins, including a doxorubicin-resistant cancer cells.

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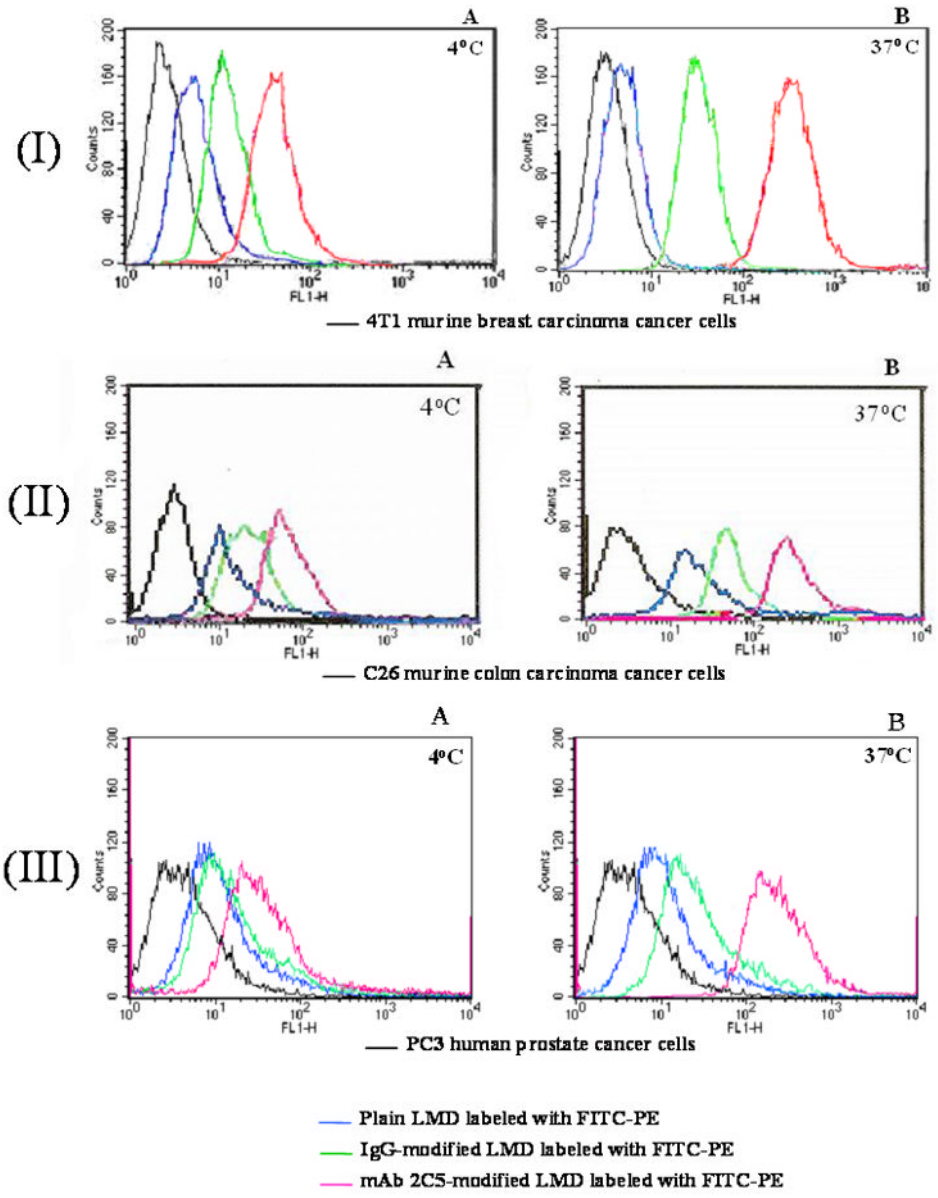

Figure 1.

Flow cytometry analysis of the surface binding (A) and uptake (B) of mAb 2C5-targeted and control liposomes, labeled with FITC-PE, in murine breast carcinoma cells, 4T1 (I); murine colon carcinoma cells, C26 (II); and human prostate carcinoma cells, PC3 (III).

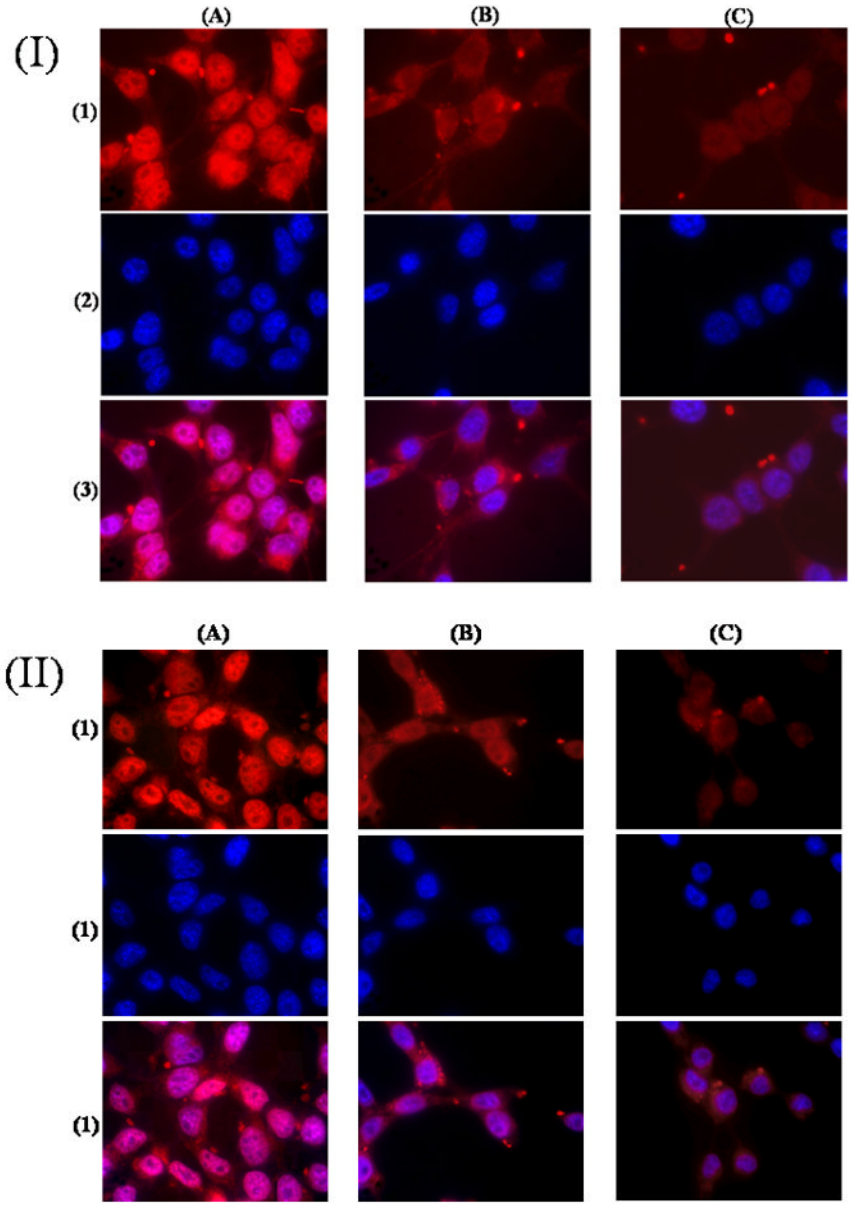

Figure 2.

Fluorescence microscopy of 2 different carcinoma cell lines (murine C26, I; and human PC3, II), incubated for 1 hr at 37°C, with different Doxil™ formulations: mAb 2C5-mododified Doxil™ (A); IgG-modified Doxil™ (B); original Doxil™ (C). Row (1) represents doxorubicin distribution, images taken using Rh/TRITC filter; row (2) represents nuclei of C26 cells stained with DAPI stain; row (3) represents merged images.

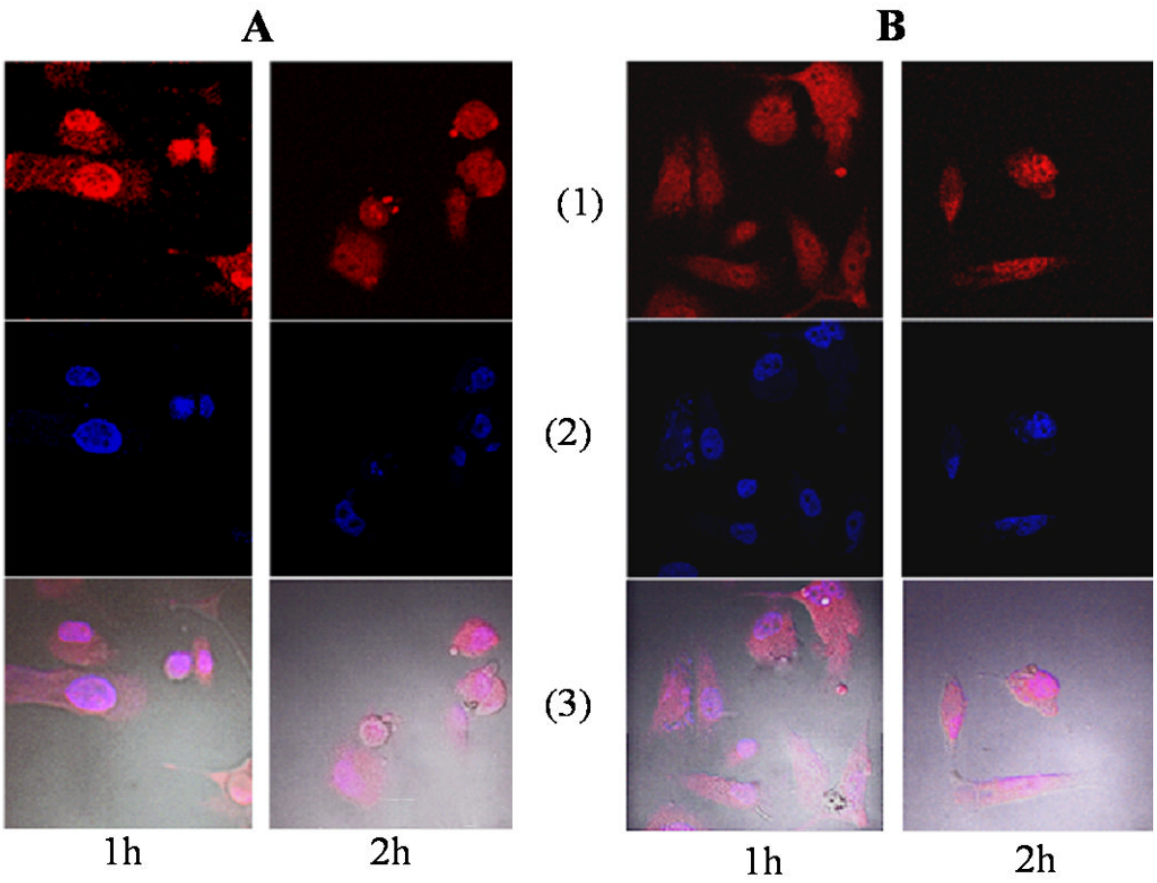

Figure 3.
Confocal fluorescence microscopy of C26 (**A**) and PC3 (**B**), cells incubated with mAb2C5-modified Doxil™ formulation for 1 and 2 hours respectively. Red color represents doxorubicin distribution, images taken using Rh/TRITC filter, row (1); Blue color represents nuclei of cells stained with DAPI stain, row (2); merged images with contrast bright light, row (3).

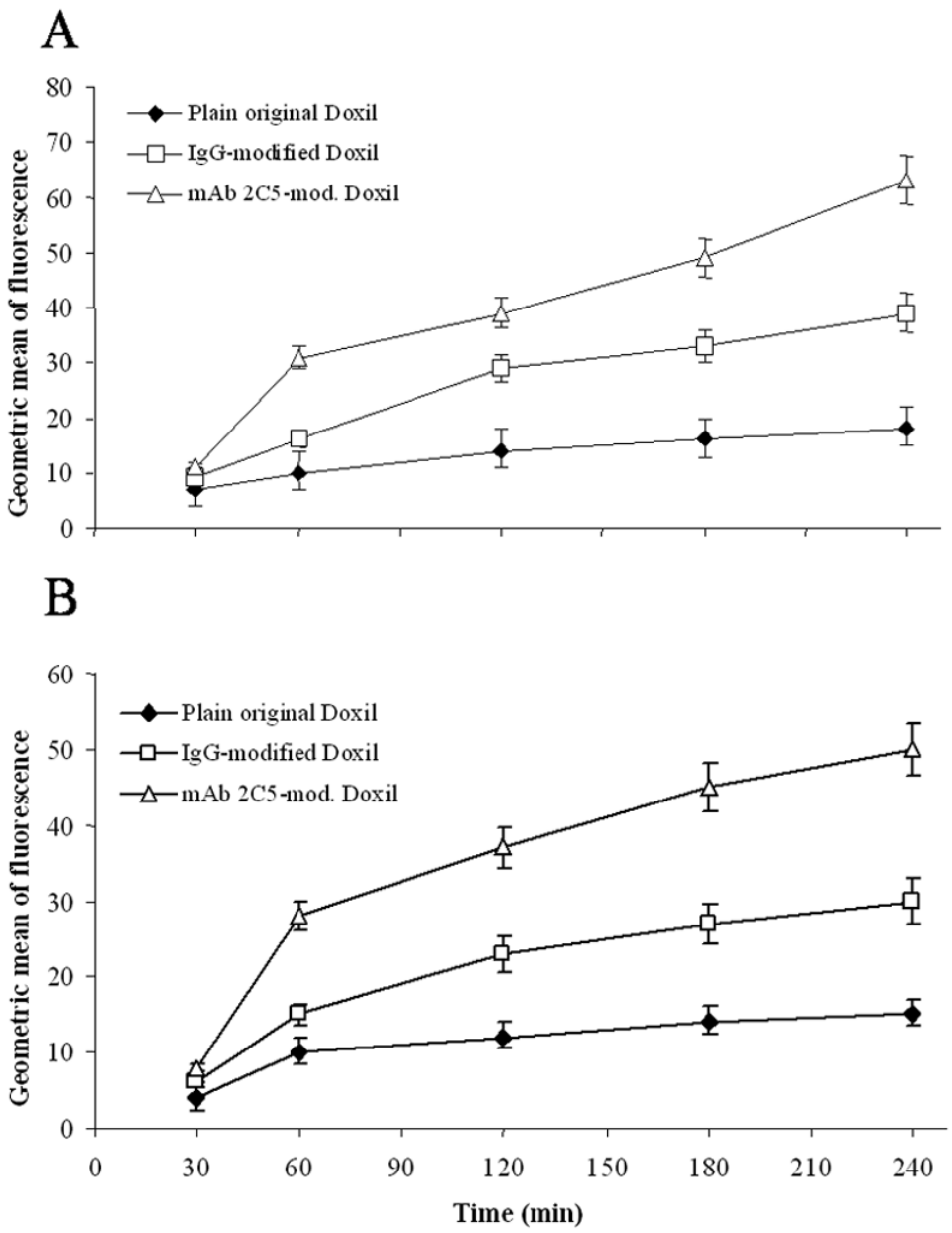

Figure 4.
Time-dependent cell-associated doxorubicin fluorescence of cells incubated with various liposomal formulations at 37°C (lipid concentration is 0.25mg/ml), murine colon cancer (C26), panel A; human prostate carcinoma (PC3), panel B; (n=3)

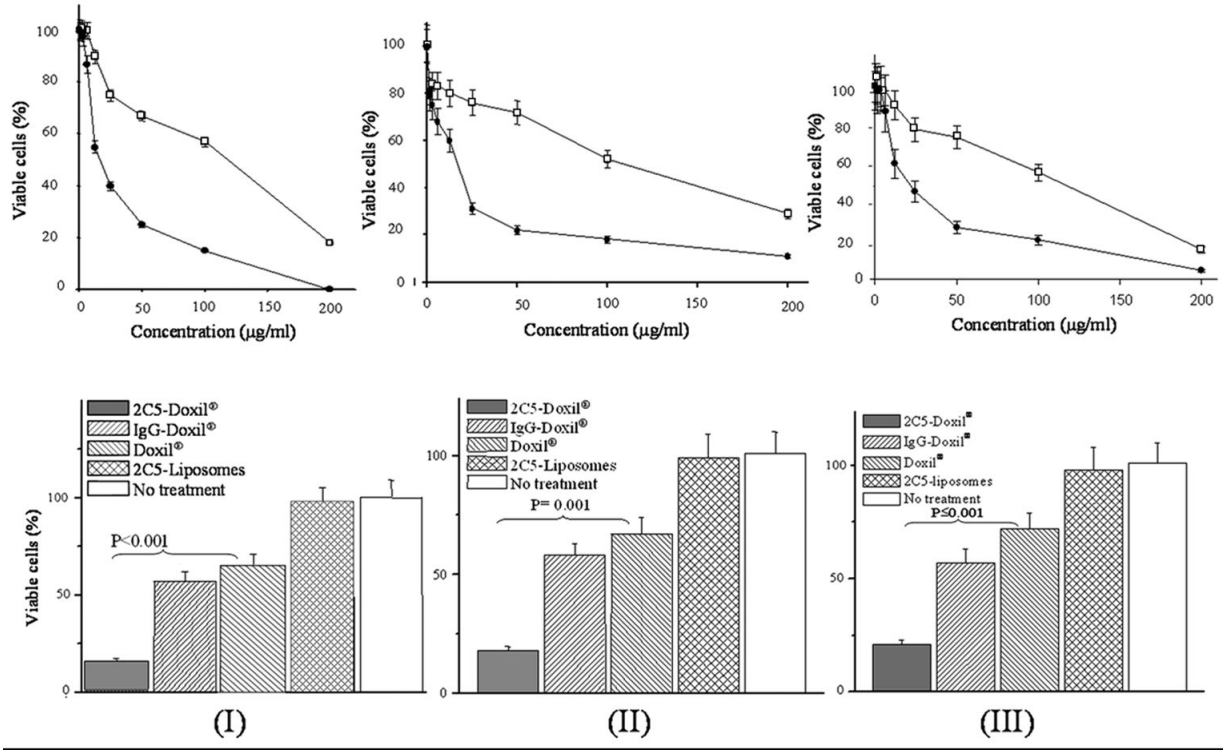

Figure 5.

In vitro cytotoxicity results using different cancer cell lines. Upper panels -cytoxic effect of different concentrations of IgG-Doxil™ (□) and mAb 2C5-Doxil™ (●) on cancer cells; lower panels – cytotoxicity of various preparations at the fixed concentration of IgG-Doxil™ and 2C5-Doxil™ (as 100 μg/ml free doxorubicin) and same concentration of 2C5-liposomes (as lipid). 4T1 murine cell line, I; murine C26 cell line, II; and human PC3 cell line, III.

Table 1

IC50 values of the different Doxil™ formulations in various cell lines

Cancer cell line	IC50 (μg/ml)		
	Doxil™	IgG-modified Doxil™	mAb 2C5-modified Doxil™
LLC	146±12	125±8	15±2
BT-20	149±14	100±6.5	20±1.7
MCF-7	155±17	122±11.5	25±2.3
4T1	145±11	118±7	22±2
C26	163±14	117±8	19±2
PC3	152±15	123±9	23±3