Immunoliposomal Delivery of ^{213}Bi for Alpha-emitter Targeting of Metastatic Breast Cancer

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ABSTRACT

Current treatment for late stage metastatic breast cancer is largely palliative. Alpha-particles are highly potent, short-range radiation emissions capable of sterilizing individual cells with one to three traversals of the cell nucleus. The alpha-emitter, ^{213}Bi (T ½ = 45.6 min), was conjugated to a 100 nm-diameter liposomal-CHX-A″-DTPA construct, upon which the rat HER2/*neu* reactive antibody, 7.16.4 was grafted. A conjugation time of 10 min was achieved giving a specific activity corresponding to 0.1 ^{213}Bi atom per liposome; stablility *in-vitro* and *in-vivo* was confirmed. Efficacy in a rat/*neu* transgenic mouse model of metastatic mammary carcinoma was investigated. Three days after left cardiac ventricular injection of 10^5 rat HER-2/*neu* expressing syngeneic tumor cells, macrophage depleted Neu-N mice were treated, by IV injection, with: (a) 19.2 MBq (520 μCi) liposome-CHX-A″-DTPA-^{213}Bi, (b) 19.2 MBq of liposome-CHX-A″-DTPA-^{213}Bi-7.16.4, (c) 4.44 MBq (120 μCi) ^{213}Bi-7.16.4 and (d) cold (non-radioactive) liposome-CHX-A″-DTPA-7.16.4 as control. Treatment with (a) increased median survival time to 34 days compared with 29 days for the untreated controls (P = 0.013) and 27 days for treated cold controls. Treatment with the radiolabeled antibody-conjugated liposome (b) increased median survival time to 38 days (p = 0.0002 relative to untreated controls). The radiolabeled antibody treated group (c) gave a median survival of 39 days similar to that for radiolabeled antibody-conjugated liposome-treated group (p = 0.5). We have shown that the ^{213}Bi radiolabeled immunoliposomes are effective in treating early stage micrometastases, giving median survival times similar to those obtained with antibody-mediated delivery of ^{213}Bi in this animal model.

INTRODUCTION

Disseminated metastatic breast cancer is classed as a stage four cancer. Treatment at this late stage is generally palliative (*1*) and the 5-year relative survival rate of patients is 23% (*2*). Alpha-particle emitters are being investigated as therapeutics against metastatic disease. Alpha-particles travel a very short distance (≈ 50 to 80 μm) and deposit highly focused energy along their path. The average linear energy transfer of alpha-particles is approximately 400 to 800 times greater, on average than that of beta particles (*3*). As a result, α-particles can efficiently kill single cells and micrometastases with limited toxicity to surrounding normal tissues. Furthermore, the high prevalence of DNA double-strand breaks caused by α-particle radiation reduces the possibility of repair of sublethal damage, thereby making targeted α-particle therapy less susceptible to the majority of tumor resistance mechanisms. The short half-life of ^{213}Bi is well suited to targeting hematologic malignancies and prevascularized micrometastases. Monoclonal antibody mediated delivery of the alpha-emitter ^{213}Bi to target cells expressing the HER-2/*neu* antigen was effective in treating HER-2/*neu* -expressing micrometastases in a pre-clinical model (*4-6*). The efficacy of targeted alpha-emitter therapy has been shown to critically depend upon the antibody specific activity and the target cell antigen density (*7*). The number of alpha-particle emitting atoms that may be delivered to target cells per carrier may be increased by using nano scale carrier systems. Several radiolabeled multifunctional nanocarriers have been effective in detecting and treating cancer in animal models (*8*). Nanoparticles, including liposomes and other nanoscale constructs, can be used to deliver drugs to tumors (*9, 10*).

Liposomes have been studied for more than 30 years, in particular as vehicles for drug delivery (*11, 12*). Their application in drug delivery has been made possible by the development of sterically stabilized structures that use polyethylene glycol (PEG) chains to reduce uptake and catabolism of intravenously administered liposomes by the reticuloendothelial system, thereby increasing circulatory half-life. Such liposomes are typically 100–150 nm in diameter since this size range reduces reticuloendothelial system uptake while retaining adequate aqueous volume for drug delivery. Liposome tumor localization is dependent on the differential permeability of normal- versus tumor-tissue capillaries (*13-15*). Liposomes have also been used to deliver radionuclides, for tumor diagnosis, therapy and infectious site imaging (*16, 17*). The possibility of using liposomes to deliver the alpha-particle emitter, ^{225}Ac, and its daughters to target cells has been examined (*18, 19*).

In this paper, the toxicity and therapeutic efficacy of engineered liposomal vesicles that deliver a greater number of ^{213}Bi atoms per carrier than can be achieved by antibody conjugation is examined.

METHODS

Liposome Preparation

Mixtures of 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine (DMPE), cholesterol (Sigma) (1:1 molar ratio) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] DSPE-PEG labeled lipids (4-mol percentage of total lipid) in $CHCl_3$ were dried in a rotary evaporator at 55°C (Avanti Polar Lipids, Alabaster, AL), dried under N_2 with few glass beads and were hydrated in chelexed phosphate buffered saline (PBS) (Sigma). The lipid suspension was

then annealed to 55°C for 2.5 h (*20*). To make unilamellar small liposomes, the lipid suspension was then taken through 21 cycles of extrusion (LiposoFast; Avestin, Ottawa, Ontario, Canada) through 2 stacked polycarbonate filters (100 nm filter pore diameter) at 55°C and were stored at 4°C until further use.

Preparation of macrophage depleting liposomes: Mixture of egg PC, cholesterol and DSPE PEG-2000 (4-mol percentage of total lipid) were dried as explained above and hydrated with 0.5M solution of dicholoro methylene diphosphonate (DMDP) in PBS (*21*). Unentrapped DMDP was removed by size exclusion chromatography (SEC) passing liposome-DMDP through Sephadex G-25 PD-10 desalting column (Biorad, Hercules CA).

CHX-A″-DTPA Conjugation to Liposome for ^{213}Bi Chelation:

To conjugate CHX-A"-DTPA *N*-[2-amino-3-(*p*-isothiocyanatophenyl)propyl]-*trans*-cyclohexane-1,2-diamine-*N,N*',*N*',*N*",*N*"-pentaacetic acid (Macrocyclics, Dallas TX) to the liposome, the NH_3 group of DMPE lipid in the liposome was deprotonated with 10 times (10X) concentrated conjugation buffer (80.44 g $NaHCO_3$, 4.50g Na_2CO_3, 175.32g NaCl, 2L ddH_2O, treated with Chelex-100) (Sigma). CHX-A″-DTPA solution in DMSO was added 10X molar excess to DMPE lipid concentration, pH was adjusted to ~8-9 using 1X conjugation buffer and allowed to react at room temperature overnight on a mixer. Unbound CHX-A"-DTPA was removed by SEC on a Sephadex G-50 packed 1 x 10 cm column (Sigma), eluted with an isotonic phosphate-buffered saline (PBS) buffer. The average number of CHX-A"-DTPA on liposome was determined using standard yttrium arsenazo spectrophotometric method (*22*) which was found to be ~1750 ligands per liposome of ~100nm size.

To prepare DTPA functionalized liposomes, lipid, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-DTPA (Avanti Polar Lipids) was included instead of DMPE during the liposome preparation.

Mice, cell line, and Antibody

neu -N transgenic mice, at ages 6 to 8 wk, that overexpress rat HER-2/*neu* under the mouse mammary tumor virus promoter were maintained and obtained from Harlan. All experiments involving the use of mice were conducted with the approval of the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. The rat HER-2/*neu* --expressing mouse mammary tumor cell line NT2.5 was established from spontaneous mammary tumors in female *neu* -N mice (*4, 23*). The NT lines are grown in RPMI media containing 20% fetal bovine serum, 0.5% penicillin/streptomycin (Invitrogen), 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 0.02% gentamicin, and 0.2% insulin (Sigma) and maintained at 37°C in 5% CO_2.The hybridoma cell line for 7.16.4 was kindly provided by Dr. M. Greene (University of Pennsylvania). 7.16.4 collected from ascites of athymic mice was purified by a HiTrap protein G column (GE Healthcare Biosciences) using the Biologic LP purification system (Bio-Rad) and dialyzed into PBS using Centricon YM-10 filter units (Millipore).

Anti-HER2 Immunoliposomes

Immunoliposome was prepared by covalent conjugation of 7.16.4, a mouse anti-Her2/*neu* monoclonal antibody (MAb) to CHX-A″-DTPA liposomes by post insertion method as described elsewhere (*24*). As controls, non-targeted CHX-A″-DTPA liposomes were prepared identically except for the omission of MAb conjugation. 7.16.4

MAb and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(poly(ethylene glycol))2000] (DSPE-PEG-Maleimide lipid) (2 mol % of total lipids) was allowed to react with thiolated 7.16.4 MAb (thiolated using traut`s reagent (2-iminothiolane), Pierce Biotechnology) overnight at room temperature. 7.16.4 conjugated DSPE-PEG-Maleimide lipid (2 mol % of total lipid) in micellar form was later allowed to react with CHX-A″-DTPA liposomes for 1h at 55°C for insertion into liposome (post insertion method). The unbound antibody after post insertion method was removed by passing the liposomes through Sepharose 4B (Sigma) column. The liposome concentration was determined using phosphate assay (*25*). The antibody concentration was determined by Biorad protein assay. The antibody density turned out to be ~40 antibodies per liposome.

^{213}Bi Labeling

Bismuth-213 was generated from ^{225}Ac (10 day half life) (Institute for Transuranium Elements in Karlsruhe Germany). The ^{225}Ac is bound to an AGMP-50 cation exchange resin (200-400 mesh; Biorad) from which ^{213}Bi may be optimally eluted every 4-6 h (*26, 27*). The ^{213}Bi was eluted using 1.3ml of HI (0.1M HCl/NaI) to a 3M ammonium acetate (to decrease the pH to ~4) and ascorbic acid solution containing CHX-A″-DTPA -immunoliposomes. The binding reaction was carried out for 10 min at room temperature with occasional stirring. Upon quenching the reaction by the addition of 50μL 10mM EDTA, unlabeled ^{213}Bi was removed using a PD-10 size exclusion column (Sephadex G-25) (Biorad). The 7.16.4 antibody was labeled with ^{213}Bi as described previously (*5*). Briefly, 7.16.4 was first conjugated to *N*-[2-amino-3-(*p*-isothiocyanatophenyl)propyl]-*trans*-cyclohexane-1,2-diamine-*N,N',N',N'',N''*-pentaacetic acid (SCN-CHX-A''-DTPA). 7.16.4 conjugated to the chelate was incubated

with BiI_4^-/BiI_5^{2-} (at 10 mCi/mg) for 8 mins in a reaction buffer (pH 4.5) containing 3M ammonium acetate (Fisher Scientific, Pittsburgh, PA) and 150 mg/mL L-ascorbic acid (Sigma, St. Louis, MO) preheated to 37 °C.

Liposome Size Distribution Determination

Dynamic light scattering (DLS) of liposome suspensions was studied with a Zetasizer Nano ZS90 (Malvern Instruments), equipped with a 633 nm He–Ne laser (4mW) light source. To observe the size and external morphology of liposomes, a transmission electron microscope (Hitachi 7600) was used.

Flow cytometry, Immunoliposomes

To study the binding of 7.16.4 immunoliposomes to NT2.5 cell surface, the liposomes were prepared as explained above but with the inclusion of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(carboxyfluorescein)) (PE-CF) lipid as a fluorescent label. The BD FACScalibur cytometry system (BD Biosciences, San Diego, CA) equipped with 488nm laser source was used to analyze the cells.

***In-vitro* Bismuth Stability Measurements**

To experimentally test the stability of ^{213}Bi labeled liposome-CHX-A″-DTPA, the construct was added to 10% serum (Invitrogen-Gibco, Carlsbad, CA) and incubated over time at 37°C in 5% CO_2. After 7 h. the sample was quenched with 10mM EDTA and was run through a PD-10 column with phosphate buffered saline at pH 7.4 and the fractions were collected and counted in a gamma counter (CompuGamma Spectrometer, LKB-Wallac 1282). *In-vitro* stability in percent was calculated as the area under the curve corresponding to liposomal fraction elution divided by the total area under the elution curve times 100.

Immunoreactivity

The immunoreactive fraction of liposome-CHX-A″-DTPA-^{213}Bi(7.16.4) was measured by incubating (2 hrs on ice) a fixed amount of liposome-CHX-A″-DTPA-^{111}In(7.16.4) with increasing numbers of antigen-expressing cells (NT2.5) and then fitting the expression: $\frac{B}{T} = f \cdot (\frac{Ag}{Ag+K_D})$ to the cell-bound radioactivity (B) over the total activity (T) (*28*). The antigen concentration, Ag, was obtained as the product of cell concentration and antigen density (=1.5 x 10^5/Avogadro's number). The fitting software package SAAM II (University of Washington, Seattle, WA) was used to obtain fitted values of the immunoreactive fraction, f and the effective dissociation constant, K_D. Non-specific binding was evaluated using liposome-CHX-A″-DTPA-^{111}In.

Cell killing

The potency of tumor cell killing was measured using a colony formation assay. 1x10^4 cells (in 100μL media) plated on 96 well plate, in their exponential phase of growth were treated with serial dilutions of liposome-CHX-A″-DTPA-^{213}Bi(7.16.4) or liposome-CHX-A″-DTPA overnight. In vitro toxicity was evaluated by colony formation assay by plating ~1000 treated cells on separate (60mm) plates (in duplicate). After 10 days the colonies (>50 cells) formed were stained using crystal violet (1%) (Sigma) and counted. The cell-killing efficacy of each agent, in vitro, was taken as the slope of the survival curve (plot of surviving fraction against activity).

Maximum Tolerated Dose (MTD)

MTD was defined as the highest total activity that allows 100% survival of the mice with no significant (*i.e.*, <15%) body weight loss. Two injections of liposome-CHX-A″-DTPA-^{213}Bi were given to mice (five per group) on two consecutive days with

total administered activities of 29.6, 22.2, 20.4, 12.3, and 9.3 MBq (800, 600, 550, 350 and 250 μCi). All mice were euthanized, and major organs were collected and examined for histopathology.

The murine model of breast cancer metastases.

The murine model for rat HER-2/*neu* --expressing breast cancer metastasis has been described previously (*4*). Briefly, *neu* -N mice, ages 6 to 8 wk, were injected with 10^5 NT2.5 cells suspended in 100 μL cold PBS via the left cardiac ventricle (LCV) after anesthesia with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture. Establishment and progression of metastases in multiple organs, including bones, liver, and spleen was confirmed by histopathology. Necropsy was performed on every mouse to confirm successful LCV injection; mice that were not successfully injected could be identified by tumor confined to the chest wall. Unsuccessfully injected mice were excluded from the analysis.

Biodistribution/*In-vivo* Stability

The biodistribution of immunoliposome-CHX-A″-DTPA-^{213}Bi in tumor-bearing mice was obtained at 0.5, 1, 2 and 4h after injection (n=5 (0.5,1,2 h) or 4 (4h)). To enable identification and collection of metastatic tumor, LCV inoculation was performed 15 days before the biodistribution studies. This was the earliest time that we could reliably identify metastatic tumors. At each time, the mice were sacrificed and the blood, heart, lung, liver, kidney, spleen, intestine, stomach, femur and tumor were removed, weighed and counted directly in a gamma counter. Results were corrected for physical decay and presented as percentage of injected dose per gram (%ID/g). Stability, *in vivo*, was further evaluated in tumor-free mice by comparing the 4 hr biodistribution of

free ^{213}Bi to that of liposome-DTPA-^{213}Bi and liposome-CHX-A″-DTPA-^{213}Bi (n=3 in each group).

Dosimetry

Absorbed doses were calculated by fitting an exponential expression to each tissue time-activity curve obtained in the biodistribution study. The resulting total number of disintegrations was multiplied by the total alpha-particle or electron energy emitted per disintegration of ^{213}Bi (*29*) to give the alpha-particle or electron absorbed dose.

Targeted Therapy, *in-vivo*

Following LCV injection, radioimmunotherapy was started on the third day with two consecutive daily i.v. injections of (*a*) liposome-CHX-A″-DTPA-^{213}Bi-7.16.4 with a total administered activity of 19.2 MBq (520 μCi), $n = 9$; (*b*) liposome-CHX-A″-DTPA-^{213}Bi with a total administered activity of 19.2 MBq, $n = 8$; (*c*) unlabeled immunoliposome-CHX-A″-DTPA, $n = 5$; (*d*) no treatment control, $n = 8$; (e) ^{213}Bi-labeled 7.16.4 antibody. Macrophage depletion in mice was carried out by intravenous injection of liposome-DMDP (0.5-1 hour) prior to LCV injection. In LCV injected mice weight loss and survival time were monitored.

Histopathology

neu -N mice were treated with 22.2 MBq Liposome-CHX-A″-DTPA-^{213}Bi, 1.85 MBq greater than the MTD. Histopathology analysis was performed to determine the reason for dose-limiting toxicity. Major organs (e.g., heart, lung, liver, kidney, spleen and femur) were collected, fixed in formalin for 24h and later sectioned and stained with Hematoxylin and Eosin (H and E).

Statistical Analysis

The statistical significance of differences between two groups was analyzed with the *t* test and Kaplan-Meier survival analysis using MedCalc (MedCalc Software, Mariakerke, Belgium). Differences with *P* values of <0.05 were considered statistically significant.

RESULTS

Immunoliposome Characterization

Liposomes were prepared by the extrusion method with 100 nm filter pore sizes. Liposome size distribution was measured with DLS. The measured average liposome size was ~110 nm. Liposomes were also imaged by transmission electron microscopy using the negative staining method and the size was confirmed. Reactivity of the immunoliposomes against antigen-expressing cells was confirmed by a shift in cells exposed to fluorescently labeled 7.16.4-immunoliposomes compared to liposomes (Figure 1).

^{213}Bi Labeling

The liposome-CHX-A″-DTPA radiolabeling efficiency with ^{213}Bi after 10 minute of reaction time at room temperature was 83% with a ^{213}Bi generator size of ~111-148 MBq. At a pH of 4-4.5 ^{213}Bi binds specifically to liposome-CHX-A″-DTPA constructs, otherwise at higher pH ^{213}Bi bound non-specifically to the phosphate head groups on the liposome surface. Decreasing the pH, protonates the head group and hence removes the non-specific binding sites (*12*). The radiochemical purity of the construct was ~99.8% as

assessed by instant thin layer chromatography. The radiochemical purity of ^{213}Bi labeled 7.16.4 antibody after purification was 98% and the immunoreactivity was between 80 and 85%.

Stability of ^{213}Bi labeled Liposomes, *ex vivo*

The ^{213}Bi-labeled immunoliposome-CHX-A″-DTPA construct was stable for 6 h with a stability of ~96%, since the half life is 45 min, the radiolabeled constructs only need to be stable for 6-7 h; the liposomes-DTPA construct was stable to ~85% over 6 h in *ex vivo* serum incubation assays.

Specific Activity of ^{213}Bi labeled Liposomes

A specific activity of 1 ^{213}Bi atom per 10 liposomes with liposome-DTPA and liposome-CHX-A″-DTPA constructs was obtained; 1 ^{213}Bi atom per ~1900 antibody molecules has been previously reported for antibodies (*5*) and was also achieved in the current study.

Immunoreactivity/Cytotoxicity

The immunoreactive fraction of liposome-CHX-A″-DTPA-^{213}Bi labeled with antibody (7.16.4) was 28 ± 2% and liposome-CHX-A″-DTPA-^{213}Bi without antibody that non-specifically adhered to the cell surface gave 3.2 ± 0.4%; the corresponding fitted values for the K_D parameter were 5 ± 3 nM and 9 ± 14 nM, respectively (Figure 2).

Cell killing experiments were performed with CHX-A″-DTPA chelated liposome or immunoliposome ^{213}Bi constructs. Immunoliposomes were more potent at killing cells than liposomes alone but less potent than ^{213}Bi-conjugated 7.16.4 antibody (Figure 3). The concentration required to yield 37% cell survival was 0.1, 0.3 and 0.5 MBq/mL (3, 8,

and 14 μCi/mL), for antibody, targeted immunoliposomes, and non-targeted liposomes, respectively.

Organ Biodistribution

The biodistribution of liposome-CHX-A″-DTPA-^{213}Bi(7.16.4) in tumor-bearing mice is shown on Figure 4A. At 0.5 h, the construct had already reached its maximum concentration in the blood, heart, lungs, femur and tumor with mean and standard deviation values of 15 ± 2, 1.8 ± 0.2, 4.0 ± 0.4, 0.7 ± 0.1 and 3 ± 2 %ID/g, respectively. The liver, spleen, kidneys and intestines reached a maximum 1 h after injection with mean and standard deviation values of 14 ± 2, 63 ± 15, 9 ± 2, 0.9 ± 0.4 and 36 ± 13 %ID/g, respectively. The construct cleared from the blood with a half-life of 1.1 ± 0.4 h. After a drop from the 1 hr maximum, construct accumulation in the spleen continued to increase over the 4-hr period. At 4 h after injection, the organ biodistribution of liposome-DTPA-^{213}Bi and free ^{213}Bi was compared with liposome-CHX-A″-DTPA-^{213}Bi. Liposome-DTPA-^{213}Bi and free ^{213}Bi showed a greater than 70% ID/g in the kidneys whereas less than 5% ID/g was seen in the liposome-CHX-A″-DTPA-^{213}Bi injected group, suggesting that the construct was very stable *in-vivo*. Free bismuth is known to accumulate in kidneys (*30*), therefore, similar to the observation made in the case of antibody-based chelated constructs (*31-33*), CHX-A″-DTPA is needed for the retention, *in vivo*, of ^{213}Bi on to the liposome surface. The 4 h stability results, *in vivo*, are shown on Fig. 4B. The high spleen uptake seen for both the antibody-conjugated and unconjugated liposome-CHX-A″-DTPA-^{213}Bi, (Fig. 4A and 4B, respectively) is consistent with the known splenic uptake of PEGylated liposomes (*34*).

Dosimetry

Mean absorbed doses are listed on Table 1. The spleen, liver and intestine received the three largest absorbed doses. The stomach, femur, and heart received the three lowest absorbed doses. The total absorbed dose to tumor was 2.1 ± 0.7 Gy; the total blood absorbed dose was 9 ± 6 Gy. The high level of uncertainty in the absorbed dose estimates, with standard deviation (SD) values ranging from 31 (tumor) to 73% (stomach) of the mean values, respectively, reflect the uncertrainty in the fitted parameter values.

Histopathology

Histopathology of liposome-CHX-A″-DTPA-^{213}Bi treated *neu* -N mice demonstrated marrow suppression as a probable reason for MTD limitation. Slides of the heart, lung, liver and kidney clearly show bacterial colonies (septicemia) due to opportunistic infection. Femur slide shows depletion of red marrow cells and spleen slide shows depletion of haematopoietic cells suggesting immune system suppression (Figure 5).

Targeted Therapy, *in-vivo*

In the metastatic LCV animal model, the median survival of untreated and cold liposome-CHX-A″-DTPA-7.16.4 controls was 29 and 27 days, respectively. Liposome-CHX-A″-DTPA-^{213}Bi-7.16.4 treated mice had a median survival time of 38 days (p = 0.0002 relative to untreated controls). This was not significantly different from the 39 day median survival obtained with ^{213}Bi-labeled 7.16.4 antibody (p = 0.5). The group treated with liposome-CHX-A″-DTPA-^{213}Bi with no antibody had a median survival time of 34 days (p = 0.013) compared to control (Figure 6); the 4 day gain in median survival of immunoliposome- vs liposome-treated mice was not statistically significant (p =0.08).

DISCUSSION

We have developed liposome-CHX-A″-DTPA-^{213}Bi constructs and demonstrated their stability and therapeutic efficacy, *in vivo*. Surface chelation yielded a maximum efficiency of 83% of the initial radioactivity, this efficiency translates into 0.1 ^{213}Bi atoms per liposome. In this highly aggressive metastatic breast carcinoma model (*4*), immunoliposomal delivery of the α-particle emitter, ^{213}Bi, led to a significant increase in median survival over untreated controls.

The use of DTPA is the most commonly used chelating agent but that has been found to be unsuitable for bismuth (^{213}Bi) conjugation as ^{213}Bi-DTPA complex is unstable in-vivo. The use of CHX-A″-DTPA for bismuth conjugation results in a complex that has been proven to be extremely stable (*32, 33*) *in vitro* and *in vivo*. By surface binding ^{213}Bi onto immunoliposomes via CHX-A″-DTPA a number of critical advantages are obtained: 1. The chemistry is simpler and faster because, in contrast to ^{213}Bi conjugation of antibodies, only a single purification and buffer exchange step is needed. 2. The number of ^{213}Bi atoms per carrier is substantially increased over that achievable with antibodies. 3. the liposomal platform makes possible surface coating of liposomes with antibodies against more than one target antigen, thereby reducing the possibility of treatment failure due to escape of a sub-population of low antigen-expressing tumor cells. Also, receptor-mediated endocytosis of immunoliposomes has been previously reported (*35, 36*). Endocytosis of such radio-labeled immunoliposomes would deliver a much greater effective number of radioactive atoms to a tumor cell than would be possible with radiolabeled antibodies, given currently achievable specific activities and typical cell surface antigen densities.

The increase in survival using the higher specific activity immunoliposomal construct was not better than that obtained by antibody-mediated delivery of ^{213}Bi. The higher specific activity is counterbalanced by the low, 28% immunoreactive fraction of the immunoliposome-CHX-A″-DTPA-^{213}Bi construct compared with the 80-85% achievable with 7.16.4 antibody. This is reflected in the 3-fold higher activity concentration required to yield 37% cell survival in the cytotoxicity assay. The MTD of the liposomal constructs was about 4.5 times greater than the 4.44 MBq (120 μCi) value reported for ^{213}Bi-7.16.4 (*5*). This may be explained by the larger size and, therefore, reduced mobility of liposomes compared to antibodies. The immunoreactive and cell killing experiments would favor antibody-mediated ^{213}Bi delivery. The comparable efficacy, *in vivo*, of the ^{213}Bi-labeled immunoliposomal construct compared to the ^{213}Bi-labeled antibody, may be explained by a combination of the greater MTD of the labeled immunoliposomes and the high specific activity of the fraction that is reactive. The larger size of liposomes provides a number of disadvantages as a target vehicle, in this study mice had to be macrophage-depleted prior to treatment suggesting that the constructs were recognized by the RES system and that additional liposomal engineering is needed to reduce such uptake. Possible modifications would include conjugating F_{ab} antibody fragments, instead of intact antibody, to reduce F_c-mediated recognition of the constructs and also the use of longer PEG chains to assure that the CHX-A”-DTPA-Bi-213 groups do not extend beyond the RES-repelling PEG brush border.

The estimated absorbed doses resulting from the near-MTD administered activity level are generally higher than corresponding values previously reported for antibody-mediated delivery of ^{213}Bi (*5*). In the antibody targeting studies, blood received the

greatest absorbed dose at the MTD administered activity level. In this study, the spleen and intestine received higher absorbed doses than the blood but the blood alpha-particle absorbed dose of 9 ± 6 Gy is within the 6.2 Gy value reported previously. In contrast, the tumor absorbed dose of 1.9 ± 0.6 Gy is substantially lower than the 4.43 Gy reported for micrometastases. The assumption of a uniform alpha-particle energy distribution within tumor and normal tissue for both constructs is known to not accurately represent the true distribution; the latter is essential in predicting the likely biologic effects of alpha-emitters. More detailed, microscopic distributions of alpha-emissions in each organ and in the tumor are required to explain these discrepancies.

In conclusion, high-specific activity ^{213}Bi conjugated immunoliposome constructs were produced that were stable *in-vitro* and *in-vivo*. Specific cell killing, *in vitro*, and a significant increase in median survival over untreated mice was demonstrated. The median survival was similar to that obtained for antibody-mediated targeting of ^{213}Bi in the same highly aggressive metastatic breast cancer model. Immunoliposomal delivery of alpha-emitters to target rapidly accessible metastatic cancer merits further consideration.

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FIGURE Legends

Figure 1: A) Schematic illustration of immunoliposome-CHX-A″-DTPA, B) TEM image of liposomes and C) Histogramic representation of flow cytometric analysis showing positive shift in fluorescence intensity of (i) cell-bound fluorescent immunoliposomes indicating specific binding with respect to (ii) cell-bound fluorescent liposome without the specific antibody and (iii) untreated cells.

Figure 2: The immunoreactive fraction of liposome-CHX-A″-DTPA with (specific, closed circles) and without 7.16.4 antibody (non-specific, open circles) was obtained by plotting the bound to total ratio against antigen concentration and fitting a receptor-ligand binding expression that included immunoreactive fraction. The fits are shown as solid or dotted lines. The immunoreactive fraction of liposome-CHX-A″-DTPA with and without antibody was 28 ± 2% and 3.2 ± 0.4%, respectively

Figure 3: *In-vitro* cell kill assay comparison of antibody (7.16.4) with liposome-CHX-A″-DTPA and immunoliposome-CHX-A″-DTPA. D_0 is about 0.12 MBq/mL for antibody, ~0.52 MBq/mL for non-targeted liposome and the targeted immunoliposome of about 0.3 MBq/mL.

Figure 4: A. Organ biodistribution at 0.5, 1, 2 and 4 h after intravenous injection of liposome-CHX-A″-DTPA-^{213}Bi(7.16.4). The tumor kinetics shown are for tumors visible 15 days after left cardiac ventricle injection of tumor cells. B. The 4 hr biodistribution of liposome-CHX-A″-DTPA-^{213}Bi, liposome-DTPA-^{213}Bi and ^{213}Bi alone shows

accumulation of free and liposome-DTPA-^{213}Bi in the kidneys while the construct using CHX-A″-DTPA has a high splenic localization consistent with that observed for liposomes.

Figure 5: Histopathology staining slides showing (A) bacterial colonies in liver (arrows), (B) bone marrow depeletion in the femur (arrows), (C) depletion of haematopoietic cells in the spleen (arrows), (D) bacterial colonies in the lung (arrows), (E) bacterial colonies in the kidney glomerular capillaries (arrow) and (F) multifocal bacterial colonies in capillaries in the heart (arrow) of *neu* -N mice following treatment with liposome-CHX-A″-DTPA-^{213}Bi at dose greater than MTD.

Figure 6: Survival probability plot (Kaplan Meier survival curve) for metastatic mouse model in *neu*-N transgenic mice with treatment groups that include 19.2 MBq of *neu* targeted liposome-CHX-A″-DTPA-^{213}Bi (n=9), 19.2 MBq of non-targeted liposome-CHX-A″-DTPA-^{213}Bi (n=8), unlabeled targeted liposome-CHX-A″-DTPA (cold) (n=5) and no treatment control group (n=8) and 4.44 MBq ^{213}Bi-labeled 7.16.4 antibody (n=9).

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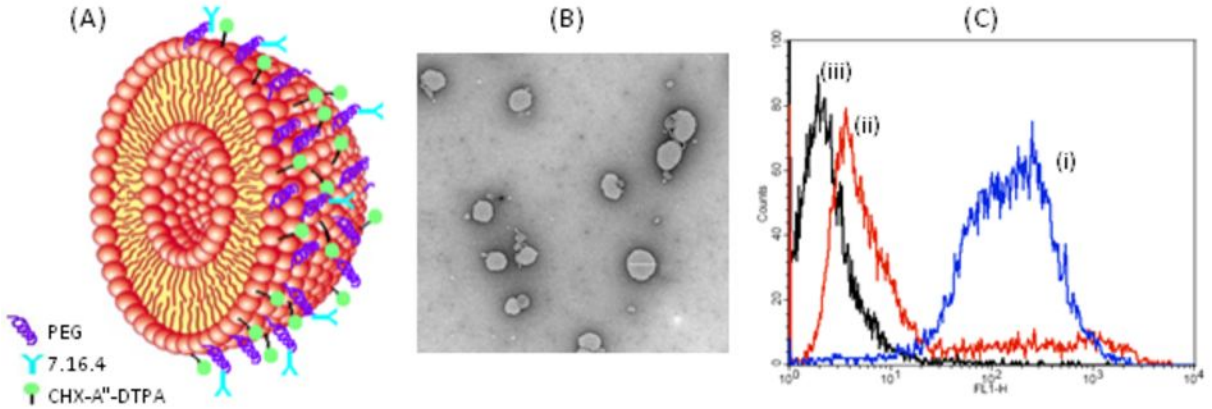
1

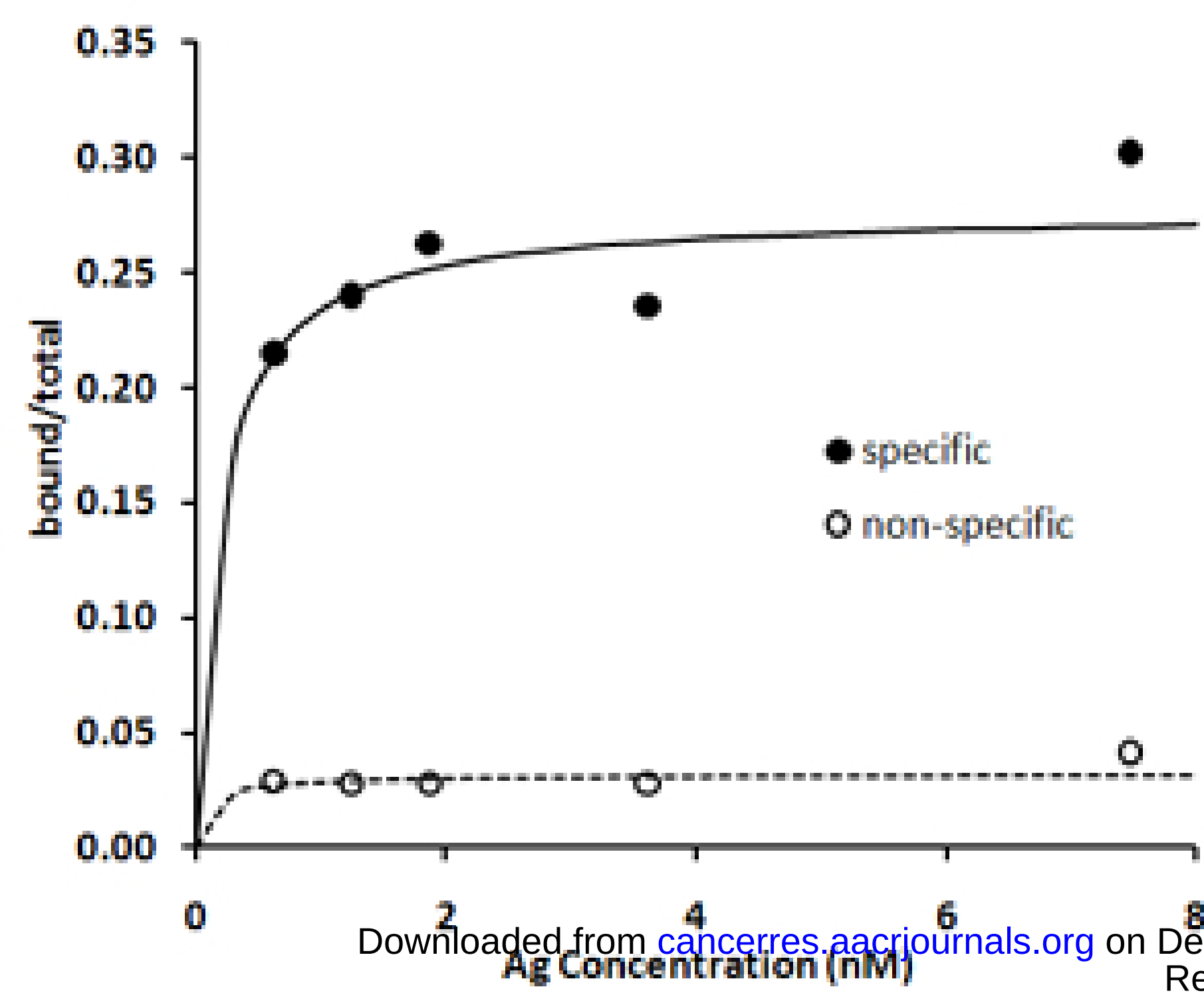
Table 1. Normal tissue and tumor absorbed doses ± SD (Gy) from 19.2 MBq (520 μCi) liposome-CHX-A"-DTPA-^{213}Bi(7.16.4)

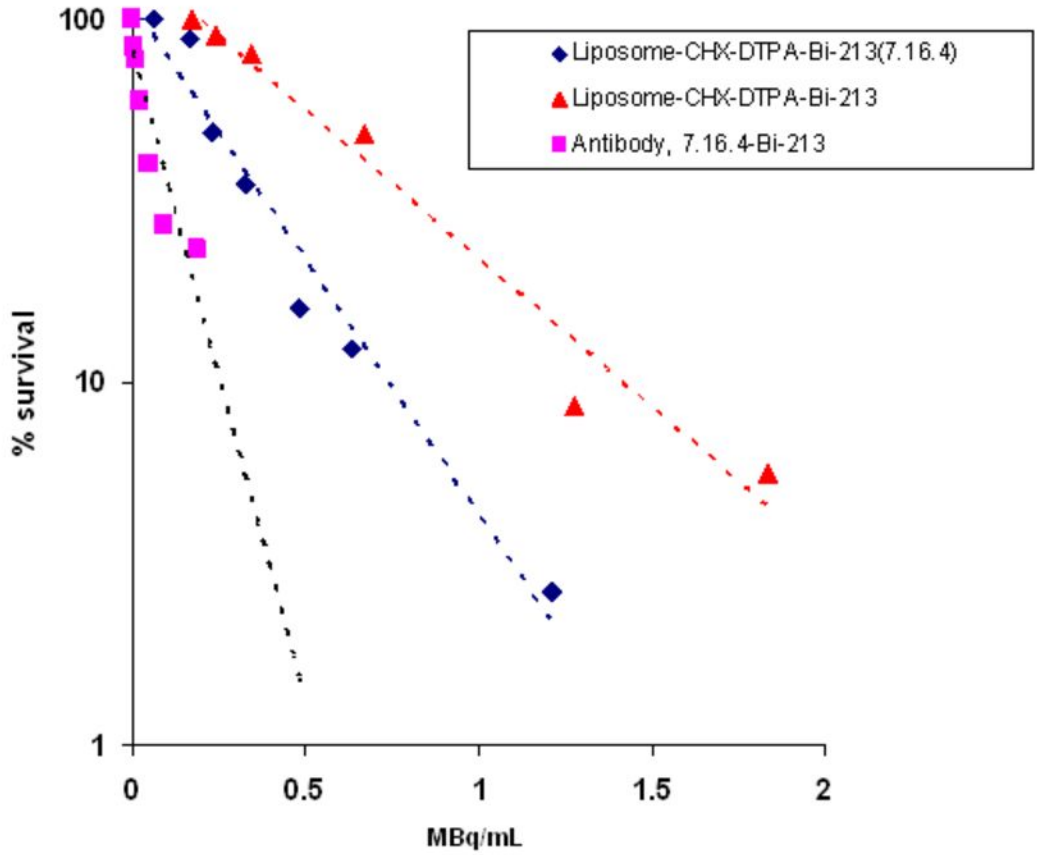
	Alpha-particle	Electron
Spleen	28 ± 16	2.2 ± 1.3
Intestine	11 ± 9	0.9 ± 0.7
Liver	9 ± 6	0.7 ± 0.5
Blood	9 ± 5	0.7 ± 0.4
Kidneys	6 ± 2	0.5 ± 0.2
Lungs	3 ± 1	0.2 ± 0.1
Tumor*	1.9 ± 0.6	0.15 ± 0.05
Heart	1.2 ± 0.6	0.10 ± 0.05
Femur	0.5 ± 0.3	0.04 ± 0.02
Stomach	0.4 ± 0.3	0.04 ± 0.03

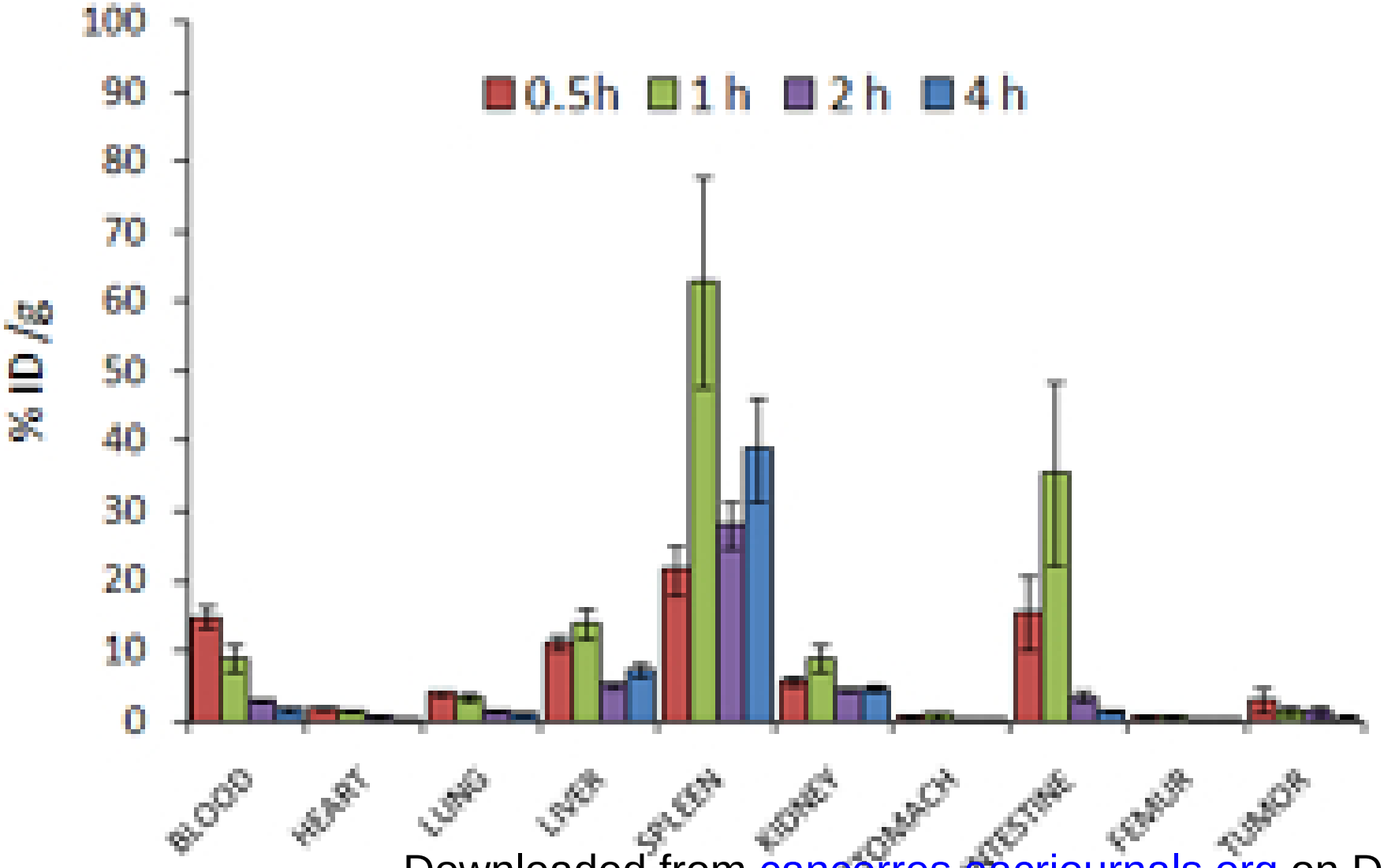
*Tumor micrometastasis identified 15 days after LCV injection.

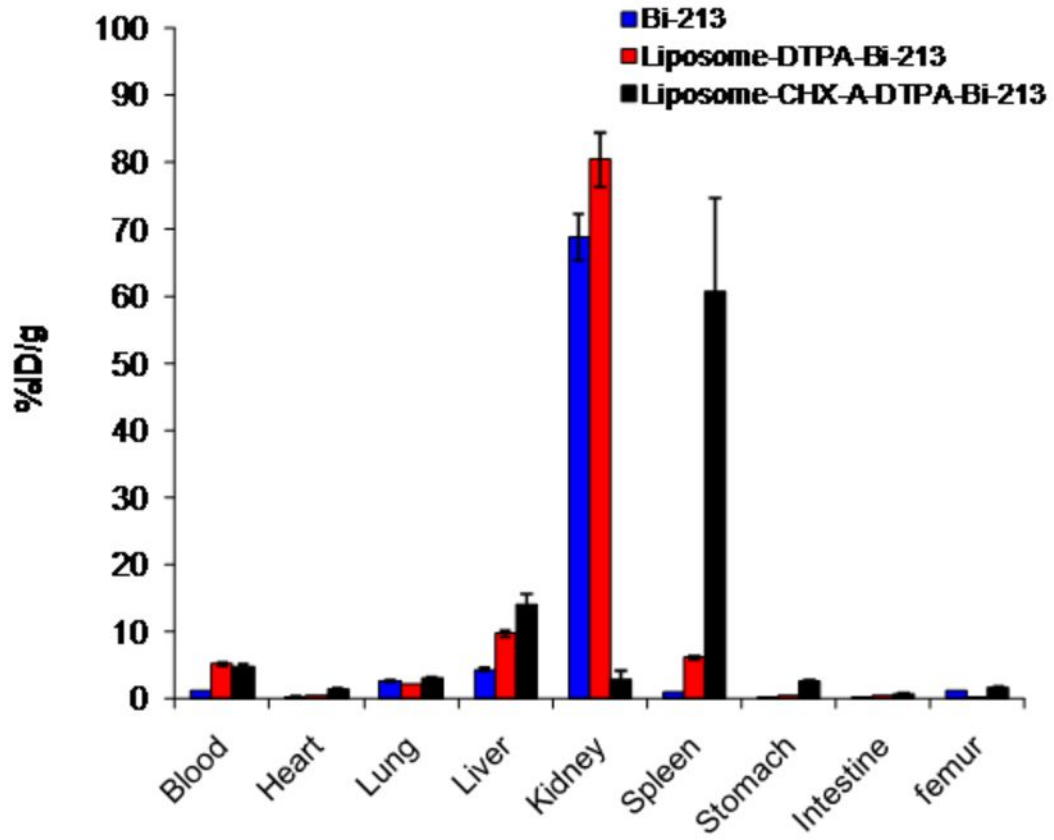
2

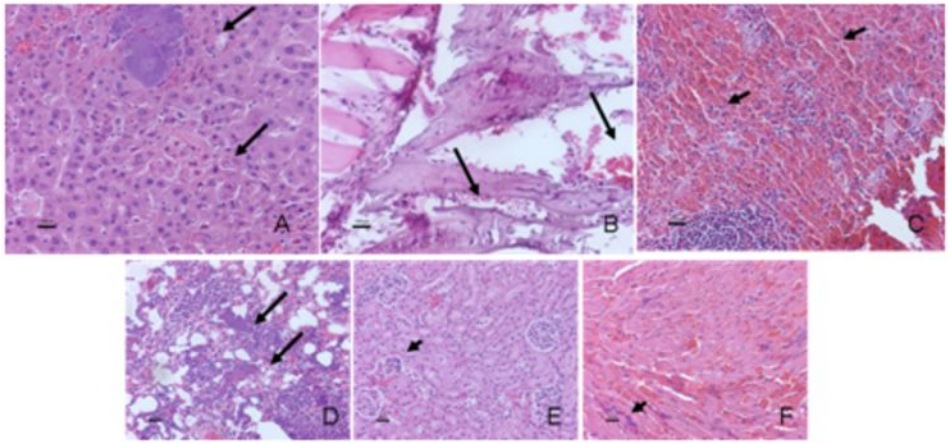


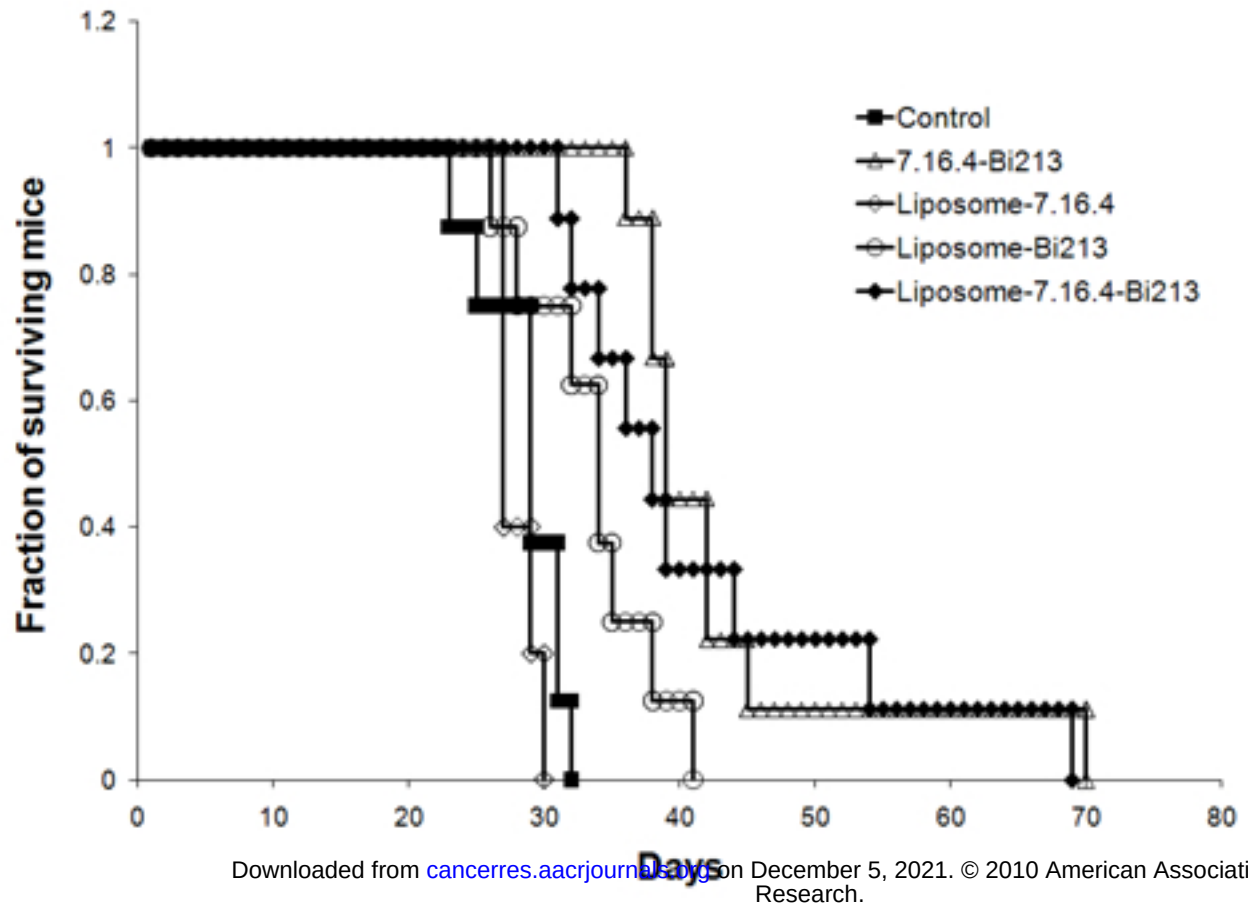












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