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Large anti-HER2/*neu* liposomes for potential targeted intraperitoneal therapy of micrometastatic cancer

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Abstract

Effective targeting and killing of intraperitoneally disseminated micrometastases remains a challenge.

Objective/Methods—In this work, we evaluated the potential of antibody-labeled PEGylated large liposomes as vehicles for direct intraperitoneal (i.p.) drug delivery with the aim to enhance the tumor-to-normal organ ratio and to improve the bioexposure of cancer cells to the delivered therapeutics while shifting the toxicities toward the spleen. These targeted liposomes are designed to combine: (1) specific targeting to and internalization by cancer cells mediated by liposome-conjugated tumor-specific antibodies, (2) slow clearance from the peritoneal cavity, and (3) shift of normal organ toxicities from the liver to the spleen due to their relatively large size.

Results—Conjugation of anti-HER2/*neu* antibodies to the surface of large (approximately 600 nm in diameter) PEGylated liposomes results in fast, specific binding of targeted liposomes to cancer cells *in vitro*, followed by considerable cellular internalization. *In vivo*, after i.p. administration, these liposomes exhibit fast, specific binding to i.p. cancerous tumors. Large liposomes are slowly cleared from the peritoneal cavity, and they exhibit increased uptake by the spleen relative to the liver, while targeted large liposomes demonstrate specific tumor uptake at early times. Although tissue and tumor uptake are greater for cationic liposomes, the tumor-to-liver and spleen-to-liver ratios are similar for both membrane compositions, suggesting a primary role for the liposome's size, compared to the liposome's surface charge.

Conclusions—The findings of this study suggest that large targeted liposomes administered i.p. could be a potent drug-delivery strategy for locoregional therapy of i.p. micrometastatic tumors.

Keywords

Large liposomes; targeted liposomes; trastuzumab; intraperitoneal therapy; ovarian cancer

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Micrometastatic dissemination in the peritoneal cavity remains a treatment challenge for patients with gastrointestinal and gynecological cancers. Effective elimination of micrometastatic tumors is of great importance, since it could delay the onset of cancer recurrence. A promising approach for the therapy of peritoneally disseminated cancer is the intraperitoneal (i.p.) administration of therapeutic agents. The rationale for i.p. administration is 2-fold. First, it is a direct way to access peritoneally disseminated disease. High concentrations of the therapeutic moiety can be achieved in the peritoneal cavity before its concentration reaches toxic levels in the dose-limiting organs (Buijs et al., 1998; Borchardt et al., 2003). Second, micrometastatic tumors in the peritoneal cavity do not have developed vasculature (Li et al., 2000). For such cases, i.p. administration of therapeutics may be the only route to reach i.p. micrometastases.

For naked, nontargeted drugs administered i.p., however, clearance from the peritoneal cavity would occur in relatively rapid rates, compared to therapeutics incorporated in nanometer-sized carriers, decreasing, therefore, the probability the therapeutic agents to encounter the tumor. In order to increase the peritoneal retention of therapeutics, their encapsulation in liposomes has been studied before (Ellens et al., 1981; Sadzuka et al., 2000). Liposomes are closed shell structures defined by a phospholipid bilayer membrane that encloses an aqueous compartment (Cullis and DeKruiff, 1979). The peritoneal concentration of chemotherapeutics packaged in liposomes after i.p. administration has been shown in humans to decrease by less than 10% during the first 48 hours (Verschraegen et al., 2003). Clinical trials on liposomally entrapped chemotherapeutic agents for i.p. therapy show encouraging results due to prolonged retention of liposomes in the peritoneal cavity (Delgado et al., 1989; Verschraegen et al., 2003). Similarly, for short-range radionuclide emitters, the i.p. administration of radioconstructs of nano- and micron size seems promising (Sofou, 2008).

To further improve the therapeutic index of delivered agents that act locally at the intracellular level (such as chemotherapeutics and short-range radionuclide emitters; Sofou, 2008), specific targeting and cell internalization of liposomes is required. This is achieved by the conjugation of multiple numbers of targeting antibodies per liposome, resulting in antibody-mediated internalization by the targeted cancer cells (Kirpotin et al., 1997; Sapra and Allen, 2003).

For liposomes with sizes of roughly 100 nm in diameter, the liver is the major site of normal organ uptake, followed by the spleen (Syrigos et al., 2003). In this study, the average size of liposomes for i.p. administration is increased with the aim to shift liposome uptake away from the liver toward the spleen (Liu et al., 1991), an organ that is often removed, sometimes laparoscopically, in patients with leukemia and lymphoma to improve therapeutic outcome (Heniford et al., 2001; Takemori et al., 1997). Although important to the defense of the body, in certain cases, the spleen could be compromised, since many of its functions can be assumed by the liver and other lymphoid tissues (Borley, 2005).

In this study, liposomes composed of zwitterionic and cationic lipid membranes containing PEGylated lipids and anti-HER2/*neu* antibodies, conjugated at the free ends of PEG chains, were characterized for (1) size, (2) retention of encapsulated contents in challenging conditions, (3) *in vitro* cell binding and extent of internalization, and (3) tumor, liver, and spleen uptake in mice bearing i.p. disseminated ovarian carcinoma resembling micrometastatic disease. Ovarian cancer has the highest mortality among gynecological malignancies. Epithelial ovarian cancer, in the majority (70%) of patients, is first detected as a result of symptoms arising after the disease has spread outside of the pelvis and into the

peritoneal cavity. In such cases of advanced disease (FIGO stage III), the 5-year survival rate employing current treatment approaches is approximately 15–20%. These data suggest that a new treatment modality is needed for disseminated epithelial ovarian micrometastatic carcinoma.

Materials and methods

Reagents

The lipids, L- α -phosphatidylcholine (egg) (EPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (ammonium salt) (DPPE-PEG lipid), 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine (chloride salt) (cationic lipid), L- α -phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole) (egg) (NBD-labeled lipid), L- α -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (egg) (rhodamine-labeled lipid), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol) 2000] (ammonium salt) (DSPE-PEG-maleimide lipid), (purity >99%) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Cholesterol, phosphate-buffered saline (PBS), fluorexon (calcein), Sephadex G-50, diethylenetriaminepentaacetic acid (DTPA), 8-hydroxyquinoline (oxine), ascorbic acid, Triton X-100, and the control ascites fluid from murine myeloma were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Dithionite (sodium hydrosulfite, tech. ca. 85%) was obtained from Acros Organics (Morris Plains, New Jersey, USA). Traut's reagent (2-Iminoethiolane-HCl) was purchased from Pierce (Rockford, Illinois, USA). Indium-111 chloride was obtained from PerkinElmer Life Sciences, Inc. (Boston, Massachusetts, USA).

Liposome preparation

Mixtures of phosphatidyl choline (EPC), cholesterol (1:1 molar ratio), and PEG-labeled lipids (5.3 mol% of total lipid) in CHCl_3 were dried in a rotary evaporator (for cationic liposomes, cationic lipid was included in 10 mol% of total lipid). After the addition of the water phase that was intended to become encapsulated into liposomes, the lipid suspension was then annealed to 55°C for 2 hours (Castile and Taylor, 1999). To make liposomes, the lipid suspension was then taken through 21 cycles of extrusion (LiposoFast; Avestin, Ottawa, Ontario, Canada) through two stacked polycarbonate filters (800-nm filter pore diameter), and untrapped contents were removed by size-exclusion chromatography (SEC) in a Sephadex G-50 (Sigma-Aldrich) packed 1 × 10 cm column, eluted with a PBS isotonic buffer. For content retention measurements, dry lipids were resuspended in calcein solution (55 mM calcein in phosphate buffer, isosmolar to PBS; pH = 7.4).

For ^{111}In passive entrapment, dry lipids were resuspended in PBS containing chelated indium complexes (^{111}In -DTPA, 3.7–37 MBq per mL, depending on ^{111}In availability). For ^{111}In -encapsulating liposomes, 1 mM of DTPA (final concentration) was added to the liposome suspension 30 minutes prior to SEC. Also, a chemical method was followed to load ^{111}In into preformed liposomes. The loading protocol for indium is published elsewhere (Hwang et al., 1982). Briefly, to 1 mL of preformed liposomes, with the entrapped 2 mM of DTPA, 100 μL of InCl_3 in 3 mM of HCl and 100 μL of oxine in 1.8 (wt %) NaCl/20 mM sodium acetate (3 μL of 11 mM oxine in EtOH added to the acetate buffer; pH = 5.5) was added dropwise. After loading, untrapped ^{111}In was complexed with externally added 2 mM EDTA (ethylene diamine tetraacetic acid; final concentration) and was separated from the liposomal suspension by SEC in a Sephadex G-50 packed 1 × 10 cm column, eluted with phosphate buffer (pH = 7.4). Ascorbic acid (8 mmol/L) was coentrapped to minimize lipid oxidation due to radiation (Stensrud et al., 1999).

Retention of entrapped contents by liposomes

To study the retention of entrapped contents by liposomes, a fluorescent dye (calcein) was encapsulated at self-quenching concentrations. Liposome stability was determined over time at 37°C in PBS, in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) and in murine ascites fluid (Sigma-Aldrich). The fluorescence intensity of liposome suspensions was measured by a fluorescence microplate reader (Ex: 485 nm; Em: 538 nm). Destabilization of the liposomal membrane causes calcein leakage from liposomes, which is followed by the dilution of calcein into the surrounding solution and by relief of the fluorescence self-quenching effect, resulting in an increase of fluorescence intensity. To normalize and properly compare different samples, Triton X-100 (4.5% wt/wt) was added to achieve complete calcein release. To quantitate liposome stability, the fluorescence self-quenching efficiency, q , of liposome suspensions was compared over the period of 30 days ($q = I_{\max}/I$, where I is the measured fluorescence intensity before Triton X-100 addition and $I_{\max}$ the maximum fluorescence intensity after Triton X-100 addition).

To determine the retention of ^{111}In by liposomes, DTPA was added for 30 minutes into a fraction of the liposome suspension, and after SEC, the γ -emissions of ^{111}In retained by liposomes were measured, using a Cobra γ -counter (Packard Cobra Gamma Counter; Packard Instrument Co., Inc., Meriden, Connecticut, USA). The energy window used for ^{111}In was 15–550 keV.

Liposome lamellarity

A dithionite assay was used to determine the lamellarity of the zwitterionic and cationic liposomes. Dithionite ion $\text{S}_2\text{O}_4^{2-}$ and the spontaneously produced $\text{SO}_2^{\cdot -}$ radical react with the NBD-labeled lipids of the outer membrane layer and produce nonfluorescent derivatives (McIntyre and Sleight, 1991). They diffuse very slowly through the bilayer and thus allow the quantitative distinction of the inner and outer layer lipids.

Liposome size-distribution determination

Dynamic light scattering (DLS) of liposome suspensions was studied with an N4 Plus autocorrelator (Beckman-Coulter, Brea, CA, USA), equipped with a 632.8-nm He-Ne laser light source. Scattering was detected at 15.7, 23.0, 30.2, and 62.6 degrees. Particle-size distributions at each angle were calculated from autocorrelation data analysis by CONTIN (Provencher, 1982). The average liposome size was calculated to be the y-intercept at the zero angle of the measured average particle-size values vs. $\sin^2(\theta)$ (Teraoka, 2002). All buffer solutions used were filtered with 0.22- μm filters just prior to liposome preparation. The collection times for the autocorrelation data were 1–4 minutes.

Liposome immunolabeling

The protocol to immunolabel liposomes has been previously described (Kirpotin et al., 1997). Briefly, trastuzumab (5–10 mg/mL, in PBS; pH = 8) was purified from Herceptin[®] (Genentech, South San Francisco, California, USA), using a Microcone[®] (Bedford, Massachusetts, USA) with a 30,000-MW cut-off for buffer exchange, following the manufacturer's instructions. Trastuzumab solution was then reacted with a 16-fold molar excess of Traut's reagent (2 mg/mL in PBS; pH = 8) for 1 hour at room temperature under a nitrogen atmosphere. Then, the antibody was purified by SEC in a 10-DG column (Bio-Rad, Hercules, California, USA) eluted with PBS (1 mM of EDTA; pH = 7.4). An average of five to seven sulfhydryl groups per antibody were determined from using the Ellman's assay and a protein assay (DC protein assay; Bio-Rad). Liposomes (7–15 mM of lipid), containing DSPE-PEG-maleimide lipid (1 mol% of total lipid), were then incubated with antibody solution (0.5–5 mg/mL, depending on the availability of antibody) overnight at room

temperature under N₂. After completion of the conjugation, excess maleimide groups were quenched with β -mercaptoethanol (3:1 mole ratio of mercaptoethanol to maleimide groups) for 30 minutes. Immunoliposomes were purified from unreacted antibody and β -mercaptoethanol by SEC in a 4B Sepharose (Sigma-Aldrich) packed 1 $\times$ 10 cm column, eluted with PBS. Liposome suspensions with conjugated antibodies (with and without mercaptoethanol treatment) were tested for binding efficacy, and no detectable difference in binding was measured. A protein assay was used to quantify the concentration of antibodies in the liposome suspension. Lipid concentration was determined by the fluorescence intensity of rhodamine-labeled lipids included in liposome membranes at fractions of 0.5–1 mol% of total lipid. The average number of antibodies per liposome was estimated based on the average liposome size, as measured by DLS, and assuming an average head-group area per lipid equal to 70 Å² for lipids in the fluid phase (Lasic, 1993; Kirpotin et al., 1997).

Cell line

Stock T-flask cultures of the human ovarian carcinoma cell line, SKOV3-NMP2 (Borchardt et al., 2003), were propagated at 37°C, in 5% CO₂ in RPMI-1640 media, supplemented with 10% FCS (Sigma-Aldrich), 100 units/mL of penicillin, and 100 μ g/mL of streptomycin. Cell concentration was determined by counting trypsinized cells with a hemocytometer. SKOV3-NMP2 was derived from the serial passage of the parental SKOV3 cell line in nude mice (Mujoo et al., 1996) and was used because of the higher probability to result in tumor growth in nude mice.

Cell binding and internalization of liposomes

To quantitate the cell binding and internalization of liposome-encapsulated contents, harvested SKOV3-NMP2 cells were washed twice with ice-cold media (RPMI –640/10% FBS), including 2% bovine serum albumin (BSA) (to avoid nonspecific and Fc-receptor binding), and then resuspended in cold media (as above) at a density of 10⁶ cells/mL. Radiolabeled liposomes (250 μ L of 1.8-mM lipid) were added to 3.5 mL of cell suspension, and two 200- μ L samples were immediately taken and processed, as described below. The cells were then placed in a humidified 37°C incubator with 5% CO₂, where they were periodically swirled and sampled at 0.5, 1, 2, 4, and 24 hours. The cells were washed three times with 2 mL of ice-cold PBS, and then, 1 mL of an acidic stripping buffer (50 mM of glycine and 150 mM of NaCl; pH = 2.8) was added for 10 minutes at room temperature to eliminate the charge-specific binding of membrane-bound conjugates and to remove the surface-bound immunoliposomes or antibodies. After centrifugation, the supernatant and pellet were counted to determine the percentages of membrane-bound and internalized counts.

Tumor inoculation

Tumor inoculation was prepared from a single cell suspension in serum-free media (Dulbecco's modified Eagle's medium; DMEM). Each 4–6-week-old female Balb/c nude mouse (Taconic, Germantown, New York, USA) received a 0.15–0.20 mL inoculum of 5 $\times$ 10⁶ cells administered by i.p. injection.

Mice were housed in filter-top cages and provided with sterile food and water. Animals were maintained according to the regulations of the Research Animal Resource Center (RARC) at Memorial Sloan-Kettering Cancer Center (MSKCC), and animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Excised organ quantitation

Mice were injected i.p. with liposomes (0.1–0.2 mL suspension, 1.4 μ mole total lipid) with entrapped ^{111}In (37 kBq average activity per inoculum) and were sacrificed at different time points postinjection by CO_2 intoxication for dissection. Whole-body clearance was measured with a dose calibrator (Model CRC-15R; Capintec, Ramsey, New Jersey, USA). Blood was collected via cardiac puncture. Organ and muscle tissues were washed in PBS and weighed. The samples were then counted for photons in a gamma-counter. Results were expressed for each organ as the percentage of total radioactivity injected divided by the organ mass (percent injected dose per gram; %ID/g). A biexponential curve of the form, $A_1 \cdot \exp(-t \cdot \ln(2)/T_1) - A_2 \cdot \exp(-t \cdot \ln(2)/T_2)$, was applied to fit the uptake and clearance phase of the blood kinetics, using a commercial software program (SigmaPlot; SPSS, Inc., Chicago, Illinois, USA).

In vivo imaging of liposomes

Due to the small size of mice used, sampling of their i.p. fluid to obtain quantitative information on the fraction of intact liposomes and to directly measure their clearance kinetics from the peritoneum was not possible. To obtain an estimate of liposome retention within the peritoneum, planar gamma-camera imaging was performed at various time points.

To image intact liposomes in the peritoneal cavity, mice were injected with nontargeted liposomes containing an encapsulated fluorophore (calcein) at self-quenching concentrations. Mice were anesthetized at 6 hours postinjection, using 100 mg/kg of ketamine and 10 mg/kg of xylazine, and were then sacrificed by cervical dislocation. The peritoneal cavity was exposed after removal of the abdominal skin and the peritoneal membrane, and fluorescent images of the peritoneal cavity were acquired before and after the addition of Triton X-100, which disrupts the liposomal membranes and causes relief of calcein self-quenching, if liposomes are still intact and containing their encapsulated contents. Mice were digitally imaged by using an ORCA CCD camera fitted with a macro-lens (Hamamatsu, Hamamatsu City, Japan) and MCID 5+ imaging software (Imaging Research, Hamilton, Ontario, Canada). Two mice were imaged per time point for each of the two liposome suspensions injected (i.e., zwitterionic, cationic). Fluorescent images were acquired from using an Illumatool Tunable Lighting System, with a 470 ± 20 nm exciter filter and a 525 ± 20 nm barrier filter (Lighttools, Encinitas, California, USA).

Results

Liposome size and lamellarity

The measured average liposome sizes for the zwitterionic composition of PEGylated liposomes was 646 ± 288 nm in diameter on the day of preparation and 657 ± 365 nm 30 days later. For the cationic composition, the corresponding values of PEGylated liposomes were 602 ± 385 and 678 ± 357 nm, respectively. Unilamellarity was verified by a 53 ± 1 (zwitterionic liposomes) and $52 \pm 1\%$ (cationic liposomes) decrease of the initial fluorescence of NBD-labeled lipids upon dithionite addition.

Retention of encapsulated contents

Incubation of liposomes in serum-supplemented media results, after the first 3 days, in a maximum decrease in calcein's self-quenching by $8 \pm 7\%$ for zwitterionic liposomes and $15 \pm 7\%$ for cationic liposomes (Table 1). Beyond this point and for 30 days, liposomes do not exhibit further content release. In ascites fluid, cationic liposomes retain contents to a greater extent, compared to zwitterionic liposomes (Table 2), and both compositions exhibit a significant retention of encapsulated contents during the first 3 days of incubation. After 30

days of incubation in ascites fluid, a more than 50% decrease in self-quenching was observed for both compositions.

In model conditions, in PBS at room temperature, the decay-corrected ^{111}In -encapsulated activity that was retained by the zwitterionic and cationic liposomes 3 days after preparation was >88 and >87% of the liposome initial activity (on the day of preparation) for the liposomes that were loaded with the passive and chemical method, respectively. The efficiency of ^{111}In entrapment by liposomes was 5–10 and 73–81% of the initial radioactivity from using the passive and chemical loading method, respectively.

Cell binding and internalization of large PEGylated anti-HER2/*neu* liposomes

The conjugation reaction resulted in 65–90 antibodies per zwitterionic large liposome and 90–145 antibodies per cationic large liposome. Flow cytometry, using membrane-labeled liposomes, confirmed that the specific activity toward the HER2/*neu* receptor is conserved by antibodies conjugated to large PEGylated liposomes, with cationic targeted liposomes exhibiting a greater fluorescence shift, compared to zwitterionic targeted liposomes (see Figure S1 in Supplemental section). Not surprisingly (Miller et al., 1998), cationic nontargeted PEGylated liposomes exhibit nonspecific binding with and without the blocking of the HER2/*neu* cell receptors, suggesting electrostatic attraction to the overall negatively charged cell-plasma membrane (see Figure S1 in Supplemental section) (Devaux, 1992). A leakage of entrapped radioactive (or fluorescent) contents due to conjugation was not detected (data not shown).

Incubation of cells with anti-HER2/*neu* liposomes encapsulating ^{111}In -DTPA results in the increase of the total cell-associated radioactivity, over time, of incubation. At 4 hours of incubation, zwitterionic anti-HER2/*neu* liposomes (Figure 1A, filled circles) result in a 16-fold greater cell-associated radioactivity than zwitterionic nontargeted liposomes (Figure 1A, filled triangles). Similarly, targeted cationic liposomes result in a 53-fold increase of cell-associated radioactivity (Figure 1B). After 24 hours of incubation, 1.2% of the total zwitterionic and 13.3% of the total cationic anti-HER2/*neu* liposome radioactivity is associated with cells (data not shown). When nontargeted liposomes are evaluated, only low cell-associated radioactivities are observed, compared to the corresponding targeted liposomes (Figure 1A and 1B, filled triangles): Only 0.3% of total zwitterionic and 0.9% of total cationic nontargeted liposome activity is cell associated. The increase in nonspecific association with cells of nontargeted cationic liposomes, as shown from using flow cytometry, is not confirmed in these studies, where the liposome contents are labeled. Content release during electrostatic binding of cationic nontargeted liposomes to cancer cells could explain this difference.

Zwitterionic anti-HER2/*neu* liposomes exhibit internalization up to 50% of cell-associated radioactivity within the first 4 hours of incubation. The total cell-associated activity continues to rise at 24 hours to the value of 439 ± 48 cpm, while internalization of targeted liposomes is retained unaltered at 161 ± 27 cpm. These findings suggest that internalization and replacement on the cell membrane of the HER2/*neu* target receptor seems to occur only in the first few hours of incubation.

Cationic anti-HER2/*neu* liposomes exhibit internalized radioactivity approximately 67% over the first 4 hours of incubation. Total cell-associated radioactivity continuously increases during the 24 hours of incubation. The total cell-associated activity at 24 hours is $5,299 \pm 35$ cpm, and internalization of targeted liposomes reaches the value of $2,731 \pm 90$ cpm.

Pharmacokinetics of large liposomes in tumor-free mice

In tumor-free mice, at early time points (1–4 hours) upon i.p. administration of liposomes encapsulating ^{111}In -DTPA, blood sampling shows a significant uptake, ranging from 6.3 ± 4.2 to $8.2 \pm 7.5\%$ ID/g that may reflect a fraction of rapidly disrupted liposomes after i.p. injection, or smaller size liposomes that escape the peritoneal cavity through lymphatic drainage (Tables S1 and S2 and Figure S2A and S2B in Supplemental section). Blood uptake and elimination kinetics of large zwitterionic liposomes is described by a biexponential curve with mean half-lives of 0.51 ± 0.29 and 5.79 ± 0.24 hours, respectively. The corresponding values for large cationic liposomes are 0.68 ± 0.84 and 2.74 ± 0.71 hours.

The whole-body clearance is low for both liposome compositions encapsulating ^{111}In -DTPA. Twenty-four hours postadministration, at least 73% of the initial whole-body activity is retained. On the contrary, rapid whole-body clearance (that occurs through the kidneys) is observed after i.p. administration of the free ^{111}In -DTPA (Table S3 and Figure S2C in Supplemental section).

A shift of normal organ accumulation from the liver—common localization site for smaller liposomes (Syrigos et al., 2003)—to the spleen is observed for large PEGylated liposomes administered i.p. The spleen-to-liver ratios of %ID/g for zwitterionic nontargeted liposomes on tumor-free mice vary over time as follows: 6.9 ± 0.8 , 21.4 ± 0.7 , 33.5 ± 1.8 and 13.2 ± 1.1 at 1, 4, 8, and 24 hours post-i.p. administration, respectively. The spleen-to-liver ratios for cationic nontargeted liposomes on tumor-free mice are 4.5 ± 0.8 , 13.1 ± 0.9 , 11.9 ± 0.2 and 16.3 ± 0.6 at 1, 4, 8, and 24 hours post-i.p. administration, respectively.

Fluorescence imaging of intact liposomes in the peritoneal cavity

Planar gamma-camera imaging of mice during the first 24 hours after i.p. administration of liposomes suggests a slow clearance of liposomes from the peritoneum (see Figure S3 in Supplemental section).

Intact liposomes were directly detected in the peritoneal cavity 6 hours after i.p. injection of zwitterionic liposomes encapsulating self-quenching concentrations of calcein. Figure 2A and 2B show the fluorescence emitted by liposome contents before and after the addition of Triton-X 100, respectively. After the addition of the lipid solubilizer, a dramatic increase of fluorescence in the peritoneal fluid was observed, indicating that 6 hours after i.p. administration, there are still intact liposomes in the peritoneal fluid retaining their encapsulated contents (Figure S4 in Supplemental section shows the same behavior by cationic liposomes). Forty-eight hours postinjection, intact zwitterionic and cationic liposomes were observed only in the mesentery, as verified by the enhancement of fluorescence intensity after Triton-X 100 addition (data not shown).

Biodistributions of large liposomes in tumor-bearing mice

Biodistributions of anti-HER2/*neu* and nontargeted liposomes were determined in tumor-bearing mice. Fourteen days after inoculation, a tumor pattern is formed, consisting of nodules on the ventral side of the spleen. Smaller nodules (~1 mm in diameter) are frequently observed within the mesentery. At 14 days postinoculation, the mean weight of observed tumor nodules was 0.269 ± 0.242 g ($n = 30$; total number of animals). Biodistributions in tumor-bearing mice were evaluated 4 and 24 hours after i.p. administration of liposomes.

Targeted zwitterionic and cationic liposomes

Four hours after administration, anti-HER2/*neu* zwitterionic and cationic liposomes exhibited comparable tumor uptake: 17.6 ± 3.7 and $21.3 \pm 19.5\%$ ID/g, respectively (open

bars in Figures 3A and 4A, respectively). After 24 hours, the tumor accumulation of targeted zwitterionic liposomes was $12.0 \pm 4.0\%$ ID/g (shaded bars, Figure 3A) and of targeted cationic liposomes was $21.7 \pm 14.3\%$ ID/g (shaded bars, Figure 4A).

For both compositions of targeted liposomes, the liver uptake increased with time. For targeted zwitterionic liposomes, the liver uptake increased from $9.1 \pm 0.4\%$ ID/g at 4 hours (open bars, Figure 3A) to $14.1 \pm 2.2\%$ ID/g at 24 hours (shaded bars, Figure 3A). For targeted cationic liposomes, the liver uptake increased from $12.9 \pm 11.3\%$ ID/g (open bars, Figure 4A) at 4 hours to $21.4 \pm 7.8\%$ ID/g at 24 hours (shaded bars, Figure 4A).

For both compositions of targeted liposomes, the spleen uptake increased with time. For targeted zwitterionic liposomes, the spleen uptake increased from $61.9 \pm 22.7\%$ ID/g at 4 hours (open bars, Figure 3A) to $83.3 \pm 19.7\%$ ID/g at 24 hours (shaded bars, Figure 3A). For targeted cationic liposomes, the spleen uptake at 4 hours was $115.5 \pm 135.9\%$ ID/g (open bars, Figure 4A) and at 24 hours was $148.7 \pm 67.2\%$ ID/g (shaded bars, Figure 4A).

Kidney uptake was insignificant. Blood uptake for all types of liposomes was between 2.7 and 8.0% ID/g at 4 hours, decreasing to values below 0.8% ID/g at 24 hours.

Nontargeted zwitterionic and cationic liposomes

Nontargeted cationic liposomes exhibit greater tumor uptake than nontargeted zwitterionic liposomes: 4 hours after administration, nontargeted zwitterionic and cationic liposomes exhibited tumor uptake of 2.7 ± 0.7 and $7.1 \pm 2.5\%$ ID/g, respectively (open bars in Figures 3B and 4B). After 24 hours, the tumor accumulation of nontargeted zwitterionic liposomes increased to $8.7 \pm 6.1\%$ ID/g (shaded bars, Figure 3B), and the tumor uptake of nontargeted cationic liposomes increased to $18.2 \pm 13.0\%$ ID/g (shaded bars, Figure 4B).

For both compositions of nontargeted liposomes, the liver uptake increased at the later time point. Liver uptake was higher for nontargeted cationic liposomes, compared to nontargeted zwitterionic liposomes, at both time points. For both compositions of nontargeted liposomes, the spleen uptake also increased at the later time point. Spleen uptake was greater for nontargeted cationic liposomes, compared to nontargeted zwitterionic liposomes, at both time points. The spleen and liver uptake of nontargeted zwitterionic liposomes was not significantly different in mice with and without tumor. The biodistributions of nontargeted cationic liposomes were also similar in tumor-bearing and -free mice, except for the spleen uptake, which was significantly higher in the latter case.

Tumor-to-liver ratios

The tumor-to-liver ratio of %ID/g values was similar between anti-HER2/*neu* zwitterionic and cationic liposomes and decreased at the later time point for both types of liposomes (see Table S4 in Supplemental section). In particular, 4 hours after administration, anti-HER2/*neu* zwitterionic and cationic liposomes exhibited comparable tumor-to-liver ratios: 1.9 ± 0.2 and 1.7 ± 1.3 , respectively. After 24 hours, the tumor-to-liver ratio of targeted zwitterionic liposomes decreased to 0.8 ± 0.3 and of targeted cationic liposomes decreased to 1.0 ± 0.8 .

Nontargeted liposomes exhibited lower tumor-to-liver ratios at the earlier time point, compared to anti-HER2/*neu* liposomes. However, at the later time point, the values increased and were similar to the ratios obtained with targeted liposomes.

Spleen-to-liver ratios

The spleen-to-liver ratio of %ID/g values decreased with time for both compositions of targeted liposomes (see Table S4 in Supplemental section). Cationic and zwitterionic targeted liposomes exhibited comparable ratios. In particular, 4 hours after administration, anti-HER2/*neu* zwitterionic and cationic liposomes exhibited spleen-to-liver ratios equal to 6.8 ± 0.4 and 9.0 ± 1.5 , respectively. After 24 hours, the spleen-to-liver ratio of targeted zwitterionic liposomes decreased to 5.9 ± 0.3 and of targeted cationic liposomes decreased to 6.9 ± 0.6 .

For nontargeted liposomes, the spleen-to-liver ratio increased with time, and nontargeted liposomes exhibited similar values independent of membrane charge (see Table S4 in Supplemental section).

Summary and discussion

I.p. micrometastatic dissemination is common in gastrointestinal and gynecological cancers, but still presents a treatment challenge. In such cases of a vascular disease, i.p. administration of therapeutics is a promising strategy for drug delivery. This study evaluated the potential of engineered liposomes labeled with the tumor-targeting internalizing antibodies as drug-delivery carriers, with the aim to increase the bioexposure of cancer cells comprising the tumor to the administered therapeutics, to increase the tumor-to-normal organ ratios, and to shift the toxicities toward less-essential normal organs, such as the spleen.

In this study, large PEGylated liposomes were designed and labeled with internalizing tumor-targeting antiHER2/*neu* antibodies conjugated to the free ends of grafted PEG-chains. These liposomes rapidly, extensively target i.p. tumors in an ovarian carcinoma animal model resembling i.p. disseminated micrometastases. Targeted large liposomes become internalized, to a great extent, by HER2/*neu*-expressing cancer cells *in vitro*, and, although not evaluated, it is expected that the observed specific tumor uptake *in vivo* should be followed by the cellular internalization of these liposomes. Large liposomes effectively shift the major normal organ uptake from the liver to the spleen. Reported spleen-to-liver ratios of %ID/g values for small liposomes following i.p. administration range from 0.7 ± 0.3 to 1.3 ± 0.6 from 1 to 192 hours postadministration (Syrigos et al., 2003), whereas the large liposomes in this study exhibited spleen-to-liver ratios of %ID/g values that ranged from 5.9 ± 0.3 to 9.6 ± 0.8 during the first 24 hours postadministration.

Liposomes are intact in the peritoneal cavity of mice for several hours after i.p. administration, therefore, increasing the probability for extensive interactions with the peritoneal tumors. Alternatively, due to their slow clearance from the peritoneum, these liposomes (not necessarily in the targeted form) could be used as controlled-release depots of delivered therapeutics.

For delivery vehicles, it is well established that the carrier's size, charge, and surface modification are the major factors affecting their interactions with the biological milieu (Sofou, 2007). In these studies, tumor and normal organ uptake values are generally greater for cationic than for zwitterionic liposomes. Interestingly, however, both the targeted zwitterionic and cationic liposome compositions exhibit similar tumor-to-liver and spleen-to-liver ratios. These findings, therefore, suggest that for such large sizes of PEGylated liposomes, the liposome's surface charge may play a secondary role, compared to the liposome's size. The observed similarity should not be entirely the result of liposome PEGylation on screening the liposomes' electrostatic interactions with the biological milieu, since, on relatively smaller liposomes (with diameters approximately of 150 nm),

PEGylation does not entirely screen the liposomal surface potential, therefore resulting in significantly different biodistributions and relative tissue ratios (Campbell et al., 2002).

in vitro, the cationic anti-HER2/*neu* liposome composition exhibits excessive binding to HER2/*neu*-expressing cancer cells, compared to the zwitterionic liposome analog. In addition to a potential electrostatic attraction of cationic liposomes with the cell's locally negatively charged surface (Devaux, 1992), the consistently higher surface density of conjugated antibodies on cationic, compared to zwitterionic, liposomes—obtained in our preparations—could explain this result (Allen et al., 1995). In our studies, the surface grafting density of intact antibodies on liposomes with a 600-nm average diameter was estimated to be approximately one antibody per 12,000–17,000 nm² for zwitterionic liposomes and one antibody per 8,000–12,000 nm² for cationic liposomes.

I.p. administration of chemotherapeutics to address peritoneal carcinomatosis has been evaluated in several studies (Rossi et al., 2003). The efficacy of this approach could be further improved by the encapsulation of chemotherapeutics in large targeted PEGylated liposomes to extend their i.p. residence time and increase tumor uptake. Gene therapy of disseminated i.p. cancer, mediated by these large cationic liposomes following i.p. administration, may potentially also address some of the toxicity issues arising from the enhanced liver localization of small cationic lipoplexes (Zhang et al., 2005; Mangala et al., 2009; Namiki et al., 1998). In addition, clinical studies on different therapeutic modalities, such as radionuclides for internal radiotherapy, have demonstrated that i.p.-administered radiotherapy could be advantageous in cases of small-volume disease (Epenetos et al., 1987; Crippa et al., 1995; Meredith et al., 1996). In the particular case of liposomes for internal radiotherapy, toxicities to normal organs could be minimized by choosing radionuclides with half-lives shorter than the characteristic times required by liposomes to reach toxic levels in the spleen or the liver. Since the enclosed aqueous compartment of liposomes can be used to entrap up to 10³–10⁶ water-soluble molecules, the loading extent of chemotherapeutics and the radiolabeling efficiency of antibody-labeled liposomes should not present a constraint (Drummond et al., 1999; Sofou, 2008).

Conclusions

Large PEGylated targeted liposomes are slowly cleared from the peritoneal cavity, demonstrate specific tumor uptake, and exhibit a significant shift of normal organ uptake from the liver to the spleen. Although the absolute tissue and tumor uptake is greater for cationic liposomes, the tumor-to-liver and spleen-to-liver ratios are similar for both membrane compositions, suggesting a general applicability of these liposomes for various types of therapeutics (e.g., chemotherapeutics, oligonucleotides, and radionuclides). The findings of this study suggest that i.p. administration of therapeutics encapsulated in large PEGylated targeted liposomes could be a potent drug-delivery strategy for the locoregional therapy of i.p. micrometastatic tumors.

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Figure 1. Cell binding and internalization of large PEGylated liposomes. (A) zwitterionic liposomes; (B) cationic liposomes. (●) total cell-associated activity of targeted liposomes; (○) cell-internalized activity of targeted liposomes; (▼) total cell-associated activity of nontargeted liposomes. The error bars correspond to standard deviations of repeated measurements. Lines are guides to the eye.

Figure 2.

Fluorescence imaging of intact large PEGylated liposomes in the peritoneal cavity. Detection of intact liposomes in the peritoneum came from using fluorescence imaging of i.p.-administered calcein-containing zwitterionic liposomes. Mice were sacrificed 6 hours after the administration of zwitterionic liposomes with entrapped self-quenching concentrations of calcein. The addition of Triton-X 100 disrupts the intact liposomes, calcein is released from the liposomes, and the fluorescence signal is increased due to relief of self-quenching. (A) Fluorescence image of the peritoneal cavity before addition of Triton-X 100; (B) after detergent addition.

Figure 3.

%ID/g for i.p.-administered large PEGylated zwitterionic liposomes encapsulating ^{111}In -DTPA. Female athymic nude mice bearing 14-day-old i.p. xenographs of SKOV3 cells were sacrificed at 4 (open bars) and 24 hours (shaded bars) after the administration of targeted liposomes (A) and nontargeted liposomes (B) ($n = 3-5$). Error bars correspond to standard deviations.

Figure 4. %ID/g for i.p.-administered large PEGylated cationic liposomes encapsulating ^{111}In -DTPA. Female athymic nude mice bearing 14-day-old i.p. xenographs of SKOV3 cells were sacrificed at 4 (open bars) and 24 hours (shaded bars) after the administration of targeted liposomes (A) and nontargeted liposomes (B) ($n = 3-5$). Error bars correspond to standard deviations.

Table 1

Fractional fluorescence self-quenching decrease $\Delta q/q$ due to calcein release from PEGylated liposomes incubated in serum supplemented media at 37°C for 30 days.

Time(days)	Large zwitterionic liposomes($\Delta q/q \times 100^a$)	Large cationic liposomes($\Delta q/q \times 100^a$)
1	0 ± 6	0 ± 6
2	6 ± 6	2 ± 5
3	8 ± 7	15 ± 7
14	0 ± 6	5 ± 6
30	-9 ± 6	-5 ± 6

^a $\Delta q(t)/q = (q_{day_1} - q_{day_t}) / q_{day_1}$, where q is the self-quenching ratio of encapsulated calcein. The uncertainties correspond to standard errors of repeated measurements.

Table 2

Fractional fluorescence self-quenching decrease $\Delta q/q$ due to calcein release from PEGylated liposomes incubated in ascites fluid at 37°C for 30 days.

Time(days)	Zwitterionic liposomes	Cationic liposomes
	$\Delta q/q \times 100^a$	
1	0 ± 9	0 ± 12
2	0 ± 9	-9 ± 10
3	0 ± 8	-4 ± 11
10	19 ± 8	1 ± 10
15	21 ± 7	0 ± 9
20	47 ± 7	26 ± 9
30	69 ± 7	52 ± 9

^a $\Delta q(t)/q = (q_{day_1} - q_{day_t}) / q_{day_1}$, where q is the self-quenching ratio of encapsulated calcein. The uncertainties correspond to standard errors of repeated measurements.