Liposomal Formulations of Poorly Soluble Camptothecin

-Drug Retention and Biodistribution

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Abstract

Context: Camptothecin (CPT) represents a potent anticancer drug. Its therapeutic use however is impaired by both drug solubility, hydrolysis and protein interactions *in vivo*. Use of liposomes as drug formulation approach could overcome some of these challenges.

Objective: The objective of this study was to perform a mechanistic study of the incorporation and retention of the lipophilic parent CPT-compound in different liposome formulations using radiolabeled CPT and thus be able to identify promising CPT delivery systems. In this context we also wanted to establish an appropriate mouse tumor model, *in vivo* scintigraphic imaging and biodistribution methodology for testing the most promising formulation.

Materials and methods: CPT retention in various liposome formulations following incubation in buffer and serum was determined. The HT-29 mouse tumor model, ^{111}In-labeled liposomes as well as ^{3}H-labeled CPT were used to investigate the biodistribution of liposomes and drug.

Results and discussion: The ability of different liposome formulations to retain CPT in buffer was influenced by the lipid concentration and the drug:lipid ratio rather than lipid composition. The tested formulations were cleared from the blood in the following order:CPT-solution>CPT-liposomes>^{111}In-labeled liposomes, and liposomes mainly accumulated in liver.

Conclusion: Lipid composition did not influence CPT retention to the same extent as earlier observed in incorporation studies. The set up for the biodistribution study works well and is suited for future *in vivo* studies on CPT liposomes. The biodistribution study showed that liposomes circulated longer than free drug, but premature release of drug from liposomes occurred. Further studies to develop formulations with higher retention potential and prolonged circulation are desired.

1. Introduction

Camptothecin (CPT) and its analogues represent an important class of agents useful in the treatment of cancer. They act by stabilizing the covalent binding of the enzyme topoisomerase I to DNA and lead to reversible single-strand nicks (Burke *et al.*, 2000). Due to its mechanism CPT is S-phase specific, indicating that it is only toxic to cells that undergo DNA synthesis and is thus highly toxic to rapidly replicating cells, such as cancerous cells (Burke *et al.*, 2000, Garcia-Carbonero *et al.*, 2002). As a class, the CPTs have exhibited unique dynamics and reactivity *in vivo* with respect to both drug hydrolysis and blood protein interactions. These factors have impaired their pharmaceutical development and clinical implementation. The CPT molecule exists in equilibrium between a lactone form and a carboxylate form. This equilibration is pH-dependent and at pH values above 7 the carboxylate form is dominant. Unfortunately, the carboxylate form has only 10% pharmacological activity compared to the more lipophilic lactone form, and is also more toxic (Burke *et al.*, 1993). In blood and tissue, this equilibrium between the lactone-carboxylate forms can be greatly affected by the presence of human serum albumin (HSA). The carboxylate form binds tightly to HSA, displaying a 150-fold enhanced affinity for this serum protein compared to the lactone form. (Burke *et al.*, 2000). On the other hand, red blood cells/cell membranes stabilize the lactone form (Mi *et al.*, 1994).

Various drug delivery systems, including micelles (Kawano *et al.*, 2006, Watanabe *et al.*, 2006), liposomes (Sugarman *et al.*, 1996, Watanabe *et al.*, 2008), other nanoparticle drug formulations (Min *et al.*, 2008) and hydrogels (Berrada *et al.*, 2005) have been utilized to improve the lipophilic parent CPT- compound's solubility and lactone stability to bring it into the clinic. Liposomes are thus one advanced drug formulation approach that can be used to overcome some

of the challenges associated with CPT (Hatefi *et al.*, 2002). The lipids can dissolve the drug in its bilayer and the pH can be controlled inside the liposome in such a way that the equilibrium is forced towards the active lactone form. The lactone form also has been shown to be stabilized when harbored in the liposome bilayer (Burke *et al.*, 1992), the drug is protected from HSA, and the complexation with the carboxylate form is avoided (Emerson, 2000). In addition, the systemic environment should ideally only recognize the liposomes and not the free drug, and the pharmacokinetic profile of the drug will be determined by the physiochemical properties of the liposomes. A small selection of low-molecular weight anticancer drug compounds, which can via a pH-gradient or ion-gradient be loaded to and precipitate in the aqueous core of liposomes (active loading) (Li *et al.*, 1998), such as e.g. doxorubicin, have shown significantly enhanced accumulation within solid tumors upon entrapment in liposomes when administered intravenously (Gurung *et al.*, 2009, Li *et al.*, 1998). This is due to the so called "enhanced permeability and retention effect (EPR)" which takes place due to extravasation of small particulate drug carriers like liposomes in the leaky blood vessels in the tumors and a marginal expression of the lymphatic system compared to in normal tissue (Matsumura *et al.*, 1986). In contrast, effective tumor-targeting by liposomal carriers has not been achieved to the same extent for other cytostatics, such as drugs that cannot be actively loaded into liposomes and especially drugs of the class of poorly water soluble compounds. One hypothesis is that a premature loss of the anticancer compound from the liposome carrier is the reason for the decreased effectiveness. A central prerequisite for successful delivery of the anticancer drug, namely that the drug remains associated with the liposome carrier during transit in the blood stream and is only released upon arrival at the target site, may thus not have been sufficiently fulfilled with the liposome formulations of such drugs investigated to date.

Parental liposomal formulations have been shown to prolong the duration of CPT in systemic circulation as well as have the potential to direct more drug to the tumor, and hence decrease the systemic toxicity compared to free drug (Garcia-Carbonero *et al.*, 2002). Despite the fact that there is a number of liposomal CPT formulations described in literature it is still not quite clear if it is possible to design formulations, which retain the drug *in vivo* for sufficient periods of time in order to allow tumor targeting.

Several patents (Burke, 1996, Perez-Soler *et al.*, 1998) are available and numerous studies (Sugarman *et al.*, 1996, Proulx *et al.*, 2001, Burke *et al.*, 1992, Saetern *et al.*, 2004b, Watanabe *et al.*, 2008, Eichhorn *et al.*, 2007, Clements *et al.*, 1996, Daoud *et al.*, 1995, Maitani *et al.*, 2008) reported on liposomal formulations of camptothecins, whereof the majority of studies is on liposomal CPT-formulations investigated water soluble CPT-derivatives such as topotecan (Yang *et al.*, 2012, Tardi *et al.*, 2000, Liu *et al.*, 2002, Subramanian *et al.*, 1995, Burke *et al.*, 1994, Zucker *et al.*, 2012, Drummond *et al.*, 2010, Dadashzadeh *et al.*, 2008), irinotecan (Chou *et al.*, 2003, Sadzuka, 2000, Sadzuka *et al.*, 1998, Sadzuka *et al.*, 1999, Sadzuka *et al.*, 1997, Drummond *et al.*, 2006, Zhang *et al.*, 2012, Hattori *et al.*, 2009), lurtotecan (MacKenzie *et al.*, 2004, Emerson *et al.*, 2000, Loos *et al.*, 2000, Desjardins *et al.*, 2001, Colbern *et al.*, 1998), SN-38 {Zhang, 2004 #1255;Atyabi, 2009 #1328;Sadzuka, 2005 #1329;Lei, 2004 #1330}, 9-nitro-CPT (Chen *et al.*, 2008, Chen *et al.*, 2006, Gilbert *et al.*, 2002, Koshkina *et al.*, 1999) and DB-67 (Bom *et al.*, 2001, Bom *et al.*, 2000). The focus on the present study is in contrast on poorly water soluble and lipophilic parent CPT-compound. Previously, several studies have been performed on incorporation of CPT in liposomes, however the experimental designs used were

different and none besides the one from Saetern and colleagues has reported a thorough study of many different liposome compositions. Despite of this the results from the mentioned studies could be summarized to relieve the following effect of different types of lipids: Inclusion of cationic lipids has shown to increase the incorporation of CPT (Eichhorn *et al.*, 2007, Saetern *et al.*, 2004b, Sugarman *et al.*, 1996), the same has been shown with anionic lipids even though not to the same extent (Burke *et al.*, 1993, Saetern *et al.*, 2004b, Sugarman *et al.*, 1996). Inclusion of fatty acids with increased degree of saturation as well as cholesterol has in most studies shown to decrease incorporation (Daoud *et al.*, 1995, Saetern *et al.*, 2004b, Sugarman *et al.*, 1996) but in some cases the opposite effect has been observed when fatty acids with increased degree of saturation has been included in liposome formulations (Burke *et al.*, 1993). The reason for the discrepancy between the results from Burke and colleagues and other studies is most probably that crystals of CPT might have interfered with the results due to choice of method. Lately, it also has been shown by Maitani and colleagues that inclusion of artificial lipids, dodecyloxy benzoic acids (DBs), could stabilize CPT in the bilayer. In addition, coating CPT with albumin has shown to have a positive effect on liposome incorporation of CPT.

A study of liposomal CPT is however not only of interest for this particular, drug but also for other similar poorly water soluble anti-cancer drugs. The development of high-throughput screening methods and combinatorial drug design during the 1990s and the move towards a more target-based approach to drug discovery have resulted in the identification of drug candidates with increasing lipophilicity and hence limited water solubility. It is thus of increasing importance to find ways to deliver these drug candidates to the target.

In an earlier study we performed *in vitro* characterization of CPT-containing liposomes (Saetern *et al.*, 2004b). In this study certain DOTAP-based liposome formulations were found to have a superior and sufficient incorporation capacity for CPT to reach therapeutically relevant concentrations (approximately 50 mg/ml) using physiological acceptable formulation characteristics. Later the EPC/DOTAP formulation was shown to not influence the cytotoxicity of the drug according to both the cytotoxicity profile and IC50 values (Saetern *et al.*, 2004a). However, this formulation, together with the other investigated formulations required further characterization, and stability studies of the drug retention was needed. A mechanistic study of the incorporation and retention of CPT in different liposome formulations has therefore been performed in this publication. The novelty is thus not the different liposome formulations tested but the systematic investigation of which factors affects the liposome formulation's ability to incorporate as well as to retain the drug, and thus be suitable as a drug delivery system for the lipophilic parent CPT-compound.

In this context another important area of interest is the behavior of CPT liposome formulations *in vivo*. Biodistribution studies are often performed to learn more about the liposome distribution in different organs and targets, as well as to monitor the circulation time and clearance from the body. Previous studies of lipid complexed CPT has shown a distribution preferentially to the gastrointestinal tract tissues, and later to the lung and liver, which all are organs that are common sites of metastasis (Sugarman *et al.*, 1996). A pharmacokinetics study in mice with CPT liposomes formulated with an artificial lipid and human serum albumin demonstrated prolonged circulation and an almost 10-fold increase in tumor accumulation after intravenous injection compared to injection of free CPT (Watanabe *et al.*, 2008). However, none of these studies have

employed scintigraphic imaging in their research. Scintigraphic imaging is a very valuable tool when it comes to biodistribution studies for the development of liposome-based drug formulations by enabling the non-invasive tracking of the liposome distribution in the body by providing an image of the whole body distribution of a liposome formulation over time after administration by various routes (Goins *et al.*, 2003, Phillips *et al.*, 2002). Scintigraphic imaging has been used for imaging the biodistribution of liposome formulations to lymph nodes, inflamed tissue, bone marrow and tumors (Sou *et al.*, 2010, Phillips *et al.*, 2002, Goins *et al.*, 1994, Awasthi *et al.*, 1998, Awasthi *et al.*, 2002). Different radionuclides with varying physical characteristics and half-lives can be associated with the liposomes. Three isotopes, indium-111 (^{111}In), gallium-67 (^{67}Ga) and technetium-99m (^{99m}Tc), have been shown to label liposomes with good *in vitro* and *in vivo* stability (Phillips *et al.*, 2002, ElBayoumi *et al.*, 2009, Phillips *et al.*, 2009, Ogihara *et al.*, 1986).

The objectives of this study were thus first to perform a mechanistic study to elucidate the influence of different factors on the liposome formulation's ability to retain the incorporated drug following contact with buffer and serum *in vitro* and compare this with the factors affecting incorporation. Second, *in vivo* scintigraphic imaging of tumor-bearing mice was developed and the biodistribution for the most promising CPT liposome formulation determined.

2. Materials and Methods

2.1. Materials

Egg phosphatidylcholine (EPC), 1,2-dioleyl-3-trimethylammonium-propane (DOTAP), 1,2-di-palmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG), 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC), 1,2-di-myristoyl-sn-glycero-3-phosphatidylglycerol (DMPG), 1,2-dioleyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleyl-sn-glycero-3-phosphatidylethanolamine (DOPE), 1,2-di-myristoyl-sn-glycero-3-phosphatidylethanolamine-N-diethylenetriaminepentaacetic acid (DMPE-DTPA), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-di-myristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and cholesterol (Chol) were purchased from Avanti Polar Lipids, Alabaster, AL and Camptothecin from TCI, Portland, OR. ^{3}H-CPT was purchased from Moravek Biochemicals and Radiochemicals, Brea, CA and $^{111}InCl_3$ from Nordion, Ottawa, Canada. Tissue solubilizing and liquid scintillation liquids, Solvable and UltimaGold, were bought from Perkin Elmer, Waltham, MA. Phospholipids C assay kit was purchased from Wako diagnostics, Richmond, VA. DC Protein Assay was bought from Bio-Rad Laboratories, Hercules, CA. Polyethylene glycol 400, propylene glycol and polysorbate 80 were purchased from Sigma Aldrich, St. Louis, MO. Fetal bovine serum (FBS) was purchased from Invitrogen, Grand Island, NY. SeparatorGel ACL was bought from Sooner Scientific, Garvin, OK and packed in spin columns from Bio-Rad, Hercules, CA. Sephadex G25 columns were bought from GE Healthcare, Piscataway, NJ. Isoflurane was purchased from VEDCO, Inc., St. Joseph, MO. The HT-29 cell line was bought from ATCC, Manassas, VA and the male Athymic Nude-Foxn1nu mice from Harlan Laboratories, Indianapolis, IN.

2.2 Liposome preparation

^{3}H-CPT-containing liposome formulations with different lipid compositions (see Table 1) were prepared by the film hydration method with both all lipids and drug in the film. The lipids and drug were dissolved in a mixture of chloroform and methanol (2:1 or 4:1 v/v). The dissolved lipids and drug were dried to form a thin lipid/drug film by rotary evaporation and desiccated overnight. The hydration medium was phosphate buffered saline (PBS) pH 6.0 and the temperature was kept above the phase transition temperature of the lipids during hydration (see Table 1).

To prepare small unilamellar vesicles (SUVs) the liposomes were bath sonicated using a G112SP1 Special Ultrasonic Cleaner (Laboratory Supplies Co., Hicksville, NY) until a mean diameter of about 30-35 nm was obtained. The sonicator used was developed for liposome production and is much more powerful than the conventional ultrasonic cleaners. The tank is running at 80 KHz and 80 watts. The size distributions of the liposomes were measured with a DynaPro laser light scattering instrument (Wyatt Technology Corp, Santa Barbara, CA). The rationale behind choosing bath sonication was out of safety reasons since this method allows working with closed containers to reduce the risk for the operator of contamination with cytostatic and/or radioactive material. The liposome formulations for the *in vivo* studies were also filtered through a Sephadex G25 column to remove any drug precipitate and free drug.

2.3 ^{111}In-labeling of liposomes

EPC/DOTAP liposomes with 1% DMPE-DTPA (formulation 10) were labeled with ^{111}In for the *in vivo* study to enable tracking of the liposomes after injection in the animal model. $^{111}InCl_3$ with the desired amount of radioactivity was diluted with citrate buffer pH 3 (0.1 M). The ^{111}In-citrate solution was then mixed 1:2 with the DMPE-DPTA containing liposome dispersion and

incubated at room temperature for 1 hr (Torchilin *et al.*, 2003). Unbound ^{111}In was separated from the liposomes on a Sephadex G25 gel column, and the ^{111}In activity of the liposome dispersion before and after separation was measured with a Wallac Wizard 3" 1480 Automatic gamma counter (Perkin Elmer Life Science, Turku, Finland). The labeling efficiency was calculated by dividing the activity in liposome fraction after separation by the total activity before separation. The labeling stability of the ^{111}In liposomes in buffer at room temperature was tested for up to 5 days by separation of unbound ^{111}In from the ^{111}In liposomes on a Sephadex G25 column after 24, 72 and 120 hrs. The serum challenge study was performed to test the stability of the ^{111}In-labelling in FBS at 37 °C. Free ^{111}In as well as protein bound ^{111}In were separated from the liposome bound ^{111}In using a SeparatorGel ACL spin column, after 6 and 24 hrs of incubation, followed by measurement of ^{111}In activity (Bao *et al.*, 2004, Chonn *et al.*, 1991).

2.4 CPT liposome retention studies

Two different approaches were used to investigate the CPT retention of the different liposome formulations. The less labour intensive and much faster ultracentrifugation approach was found appropriate for measuring the liposome formulations' retention ability during incubation in buffer only, whereas the more time consuming gel column approach was suitable to obtain the formulations' retention ability during serum incubation as well.

For the method using ultracentrifugation ^{3}H-CPT liposomes were diluted 1:2 with the same buffer that was used during the liposome production (PBS pH 6.0) and incubated at room temperature. Aliquots were withdrawn, diluted further 1:7 with PBS pH 6.0 and centrifuged at 200 000 g at 4 ºC for 1 hr (Optima^{TM} LE80K Ultracentrifuge with 50.2 Ti rotor, Beckman

Instrument, Fullerton, CA). Samples from the supernatants as well as the total liposome dispersion before centrifugation were prepared for the liquid scintillation counting and measured as described below in section 2.5. The percentages of ^{3}H activity associated with the liposome pellet compared to the total were calculated. Phospholipid C enzymatic colorimetric kit was used, with only small modifications from the method described earlier (Grohganz *et al.*, 2003), to assure that there were no lipids remaining in the supernatant after centrifugation. In brief, 25 µl of the samples were diluted with Triton X-100 solution (5%) up to 50 µl in a 96 well plate. Phospholipid C reagents (250 µl) were then added to each well and the plate well shaken. The plate was then incubated at 37 °C for 30 minutes before the absorbance was measured at λ=600 nm.

The pellets were also examined under long and short wavelength UV light to assure that there were no CPT crystals which would result in overestimation of retention ability for the formulations.

For the gel chromatography approach, ^{3}H-CPT liposomes were diluted 1:2 with FBS or PBS pH 6.0 and incubated at 37°C. The amount of ^{3}H activity associated with the liposomes at different time points was determined using SeparatorGel ACL spin column (Bao *et al.*, 2004, Chonn *et al.*, 1991). SeparatorGel ACL 4% (2.0 ml) was packed in a micro column by centrifugation at 1000 rpm for 1 min and equilibrated with 10 column volumes of PBS buffer, pH 6.0. An aliquot (100µl) of diluted ^{3}H-CPT liposomes was added to the column, the column centrifuged at 500 rpm for 1 min, and the first fraction collected in a tube. Then, 100 µl of PBS buffer, pH 6.0, was added and another centrifugation at 500 rpm for 1 min performed to collect the second fraction. This last step was repeated until a total of 20 fractions were collected. The ^{3}H activity in each fraction was counted as described below in section 2.5 and the percentage of ^{3}H activity

associated with the liposomes (fractions 2-6 based on phospholipid quantification) compared to the total was calculated. The protein concentration in each fraction was determined using DC Protein assay and the microplate assay protocol. In brief, 5 µl of the samples were mixed with 25 µl of working reagent A and 250 µl of reagent B in a micro titer plate and the plate shaken. After 15 min of incubation at room temperature, the absorbance was read at λ=650 nm Phospholipids were quantified using Phospholipid C enzymatic colorimetric kit as described previous in the section with only small modifications from the method reported earlier (Grohganz *et al.*, 2003).

2.5 Tissue, blood and liposome sample preparation for liquid scintillation counting

The samples were prepared for liquid scintillation counting by adding 1-1.5 ml of SOLVABLE according to sample weight. The samples were incubated at 55 – 60 °C with regular agitation until the samples were dissolved (1-5.5 hrs). Hydrogen peroxide (30%, 0.1 ml) was added with gentle agitation between additions to help reduce the amount of color present and thus reduce color quench in the final mixture. After incubation for 15 to 30 minutes at room temperature to complete the reaction, the vials were tightly capped and incubated at 55 – 60 °C for 1 hr. The samples were then allowed to cool to room temperature, ULTIMA Gold (10-15 ml) was added to the samples and the temperature and light adapted for 1 hour before counting in a Beckman LS 6500 Liquid scintillation counter (Beckman Instruments, Fullerton, CA).

2.6 Animal tumor model

The animal experiments were performed according to the National Institutes of Health Animal Use and Care Guidelines and were approved by the Institutional Animal Care Committee at University of Texas Health Science Center at San Antonio.

HT-29 cell line was cultured and maintained at 37 ºC in an incubator with 5% CO_2. When the cells were 80-90% confluent, they were collected and prepared into a single cell suspension in serum free media. Tumors were inoculated in male Athymic Nude-Foxn1nu mice at 3-4 weeks age (20 g) by subcutaneous injection of $1x10^7$ HT-29 human colon carcinoma cells in 0.2 ml serum free media into the left thigh area. The mice were anesthetized by inhalation with isoflurane (3% in 100% oxygen) using an anesthesia inhalation unit (Bickford, Wales Center, NY). Tumor dimensions were measured at day 3, 5, 7, 10, 11 and 12 after injection by measuring length (l), width (w) and height (h) using a digital caliper and the tumor volume calculated using the ellipsoid volume formula, $V= (lwh\pi)/6$ (Tomayko *et al.*, 1989).

2.7 Biodistribution studies

For the distribution study the animals were divided into three groups with 6 animals in each group. One group was injected with 90 µl of a solution (polyethylene glycol 400, propylene glycol and polysorbate 80 (40:58:2 volume ratios) containing ^{3}H-CPT (50 µCi/kg body weight) (Yang *et al.*, 1999). The second group was injected with 200µl EPC/DOTAP liposomes containing ^{3}H-CPT (150 µCi/kg body weight) (Wilson *et al.*, 2007). The third group was injected with 200µl ^{111}In-labeled EPC/DOTAP liposomes (250 µCi; 12.5 mCi/kg body weight). The total lipid dose was about 150 mg/kg body weight in the liposome formulations and the total CPT dose about 125 µg/kg body weight in all CPT containing formulations.

The pharmacokinetics of the different formulations were determined by collecting 10 µl blood samples from the tail vein at baseline (immediately after administration) and 1, 2, 4, 6, and 19 hrs after administration. The mice were anesthetized by inhalation with isoflurane (3% in 100% oxygen) during blood sampling. Twenty hours after administration the animals were euthanized,

cardiac puncture and exsanguination performed under deep isoflurane anesthesia and the following organs were collected from all the animals: heart, liver, spleen, lung, kidney, tumor, testicles. In addition, bone, brain, skin, muscles, intestine (with feces) and stomach were harvested for the mice injected with the ^{111}In-labeled liposomes. The tissue samples were weighed and the samples from the group injected with ^{111}In liposomes were measured for ^{111}In activity on a Wallac Wizard 3" 1480 Automatic gamma counter (Perkin Elmer Life Science, Turku, Finland). A standard of the ^{111}In liposomes formulation was also counted. The tissue samples from the mice injected with ^{3}H-CPT solution or ^{3}H-CPT liposomes were prepared for the liquid scintillation counting as described in section 2.5 and counted in a Beckman LS 6500 Liquid scintillation counter (Beckman Instruments, Fullerton, CA). All data are expressed as percentage of injected dose per organ and percent of injected dose per gram of the organ.

2.8 Scintigraphic imaging study

^{111}In has a penetrative gamma emission that allows for monitoring of the biodistribution of ^{111}In -labeled liposomes using gamma scintigraphy. Scintigraphic imaging was therefore used to investigate the liposome biodistribution at various time points after injection in the 6 animals injected with 200µl ^{111}In-labeled liposomes containing 250 µCi ^{111}In-activity and a total lipid dose of 150 mg/kg body weight. The mice were anesthetized by inhalation with isoflurane (3% in 100% oxygen) during imaging.

Single 1mm-pinhole collimator planar images and Single Photon Emission Computed Tomography (SPECT) images were acquired using a microSPECT scanner equipped with dual cadmium zinc telleruide (CZT) detectors (FLEX SPECT/CT/PET, Gamma Medica, Northridge, CA, USA).). A series of static planar anterior-posterior images were acquired at baseline, 1 hr,

2 hrs, 4 hrs, 6 hrs (5 min acquisitions) and 20 hrs (10 min acquisition). ^{111}In liposome sample (12 μCi) was placed in the field of view but outside the position of the mouse as a standard source during image acquisition. SPECT images (30 s per projection, 32 projections, 180º rotation, ROR 6.14 cm) were reconstructed using software with the FLEX unit. CT images (fly mode, 75 kVp, 0.25 mA, 256 projections) were also acquired and reconstructed using COBRA software supplied with FLEX unit. SPECT images were co-registered with CT images and displayed using VIVID software supplied by Gamma Medica (Northridge, CA, USA).

2.9 Statistical Methods

To identify significant differences between two sets of data the Student's t-test for comparison of two means was performed with Microsoft Excel 2010 (Richmond, WA). A significance level of $p < 0.05$ was used.

The first order exponential decay simulations to calculate clearance half-life and percentage cleared from circulation were performed using Origin Lab, version 7.5 (Northampton, MA).

3. Results and discussion

3.1 CPT retention within liposomes

A central prerequisite for successful delivery of the liposomal anticancer drug to the tumor is that the drug remains associated with the liposome carrier during transit in the blood stream and is then released upon arrival at the target site (Brandl, 2003). The factors influencing the ability of different liposome formulations to retain lipophilic CPT incorporated into the liposome bilayer upon contact with buffer and serum was therefore an important question to be answered in this study.

3.1.1 CPT retention within liposomes in buffer

In our earlier studies we investigated the CPT incorporation capacity for different liposome formulations (Saetern *et al.*, 2004b). These studies revealed that the cationic EPC/DOTAP containing liposomes had a superior CPT incorporation capacity compared with the other formulations, whereas the formulations that contained DMPC or cholesterol resulted in stiffening of the bilayer and showed the lowest incorporation (Saetern *et al.*, 2004b). The investigated formulations needed to be further characterized to determine drug retention after incorporation. Formulations 1-8 in Table 1 were therefore prepared with a total lipid concentration of 12.5 mg/ml and a CPT concentration of 16 µg/100 mg lipid. Their ability to retain CPT over time during incubation in buffer was investigated by separation of liposomes from the free drug by ultracentrifugation. The results are displayed in Figure 1. All the investigated formulations showed, under the chosen conditions, a rapid drug release upon dilution in the range between 70 and 85%, whereas no significant further release occurred thereafter. Only minor, non-significant ($p>0.064$) variations in drug retention were seen between the different lipid compositions. However, even though the drug retention for the different liposome formulations was not statistically different, the formulation containing DMPC (formulation 7) had slightly higher drug retention and the formulation containing EPC/DOTAP/DOPE (formulation 5) had slightly lower retention compared with the control (formulation 1). The difference in retention between formulation 5 and formulation 7 is also significant ($p<0.05$). One explanation for the improved drug retention ability for formulation 7 is that the more rigid DMPC bilayer might be better in keeping the incorporated drug inside the phospholipid bilayer compared to the more fluid DOPE containing lipid bilayer (Brandl, 2001). This is an indication that the lipid composition, in contrast to what was observed for the incorporation study, has minimal influence on the retention

ability at the chosen test conditions and the most promising formulation from our incorporation studies (Saetern *et al.*, 2004b), containing EPC/DOTAP (formulation 3), appears to be equal to the other formulations when it comes to keeping the incorporated drug within the liposome.

Since the lipid composition did not seem to have a pronounced effect on the drug retention in the tested liposome formulations we chose to further investigate the EPC/DOTAP formulation (formulation 3), which earlier had shown the highest drug load capacity. Here we wanted to determine the influence of lipid concentration and/or drug concentration on drug retention ability. We first kept the total lipid concentration constant while increasing the drug concentration to 100 µg/100 mg lipid, then increased the total lipid concentration to 100 mg/ml while keeping the drug:lipid ratio at 100 µg/100 mg lipid. Retention experiments were performed as described above and the results given in Figure 2 showing a large and significant ($p<0.05$) increase in CPT retention when increasing the drug:lipid ratio to 100 µg/100 mg lipid and an even larger increase when the total lipid concentration was increased to 100 mg/ml.

Thus, it seems that CPT retention within the liposomes is a question of establishing equilibrium between the hydrophobic lipid phase and the hydrophilic buffer phase. There were no significant changes in CPT retention for any of the liposome formulations as a function of incubation time ($p>0.21$).

Further the drug retention of the EPC/DOTAP-containing liposome formulations e.g. formulations 9-11 in Table 1 at high lipid and high CPT concentration (100 µg CPT/100 mg lipid and 100 mg lipid/ml) were measured. The EPC formulation (formulation 3) was included as a control, DMPE-DPTA (formulation 9 and 10) was added for the purpose of labeling the

liposomes with ^{111}In, and DPPE (formulation 11) was added to elucidate if any effect of the presence of DMPE-DTPA is due to the anchor in the bilayer or to the chelators on the surface. The retention of CPT was found to be 86%, 88.5% and 92% for formulation 9, 10 and 11 respectively. Compared to the control (formulation 3) that showed CPT retention of 86% these additives did not seem to influence the drug retention of the formulations in buffer to a high extent. Only the addition of DPPE gave a slightly yet significant ($p<0.05$) higher retention of CPT compared to the control.

3.1.2 CPT retention within liposomes after incubation in serum

Since the most promising formulation from our incorporation studies, containing EPC/DOTAP (formulation 3), appears to be able to retain the drug to the same extent as the other formulations, the serum stability of this formulation was further investigated. A control experiment was performed by incubating the formulation in buffer instead of serum. Spin columns used to separate the free drug from liposome bound drug were able to effectively separate liposomes, proteins and free drug to ensure reliable results.

For the samples incubated in buffer there was only a small decrease in retention over time up to 24 hrs. After 24 hrs, 40.3% of the drug was still associated with the liposomes. For the samples incubated in serum there was no difference in retention immediately after dilution with serum compared to the situation in buffer. However, after 4 hrs of incubation in serum 10.3% of the drug was still associated with the liposomes meaning that some drug had been released. The reason for this might be partly due to the affinity of the CPT carboxylate form for blood proteins. This could push the equilibrium in the favor of the more soluble carboxylate form showing lower

affinity to the phospholipid bilayer compared to the more lipophilic lactone form (Mi *et al.*, 1994).

3.2. Biodistribution and imaging studies

The second part of this study was the establishment of an appropriate mouse tumor model and in *vivo* scintigraphic imaging protocol, as well as determination of the biodistribution of the most promising formulation. In an earlier *in vitro* study a dose-dependent cytotoxicity was seen upon incubation of the EPC/DOTAP formulation with HT-29 and SW-480 cell lines. However the cytotoxic effect was observed to be higher in the cell line HT-29, where the p53 proto-oncogene is mutated (Saetern *et al.*, 2004a). This strongly indicates that the effect is at least partly independent of intact p53, which is in accordance with previous studies, where a p53-independent apoptosis and even selectivity of CPT towards tumors with p53 mutants has been reported (Stella *et al.*, 1997, Sugarman *et al.*, 1996). HT-29 cell line was therefore chosen as an appropriate cell line for the *in vivo* mouse tumor model.

3.2.1 Mouse tumor model

Male Athymic Nude-Foxn1nu mice were injected subcutaneously with the HT-29 cell line. Nineteen out of 20 of the injected mice developed a tumor, resulting in a 95% tumor take rate. The mouse without a tumor was excluded from the biodistribution study. At day 12 post injection the tumor volumes were between 100 and 170 mm^3 and the experiments were initiated.

3.2.2 ^{111}In-labeled liposomes

With the intention to track the liposomes *in vivo* over time by scintigraphic imaging the liposome formulation was labeled with the gamma emitter ^{111}In. Immediately after the labeling process 97% ± 0.7% of the added ^{111}In was bound to the DTPA on the liposome surface. The labeling stability in buffer over time was found to be satisfactory with 98% ± 1.4%, 97% ± 0.7% and 93% ± 0.7% of the ^{111}In still bound to the liposomes after 24, 72 and 120 hrs incubation at room temperature, respectively, which gives the opportunity to store the liposome formulation for some time after labeling before starting the animal experiments. The results from the serum challenge study at different time points was found to be 94.5% ± 0.7% and 94% ± 2.5% after 6 hrs and 24 hrs incubation, respectively. The labeling was thus stable in serum during the duration of the biodistribution studies and suggested the ^{111}In-activity observed in the images could be attributed to ^{111}In-labeled liposomes and not free ^{111}In. In summary, the ^{111}In-labeled liposomes were thus found suitable for further *in vivo* studies.

3.2.3 Biodistribution studies

Biodistribution studies with the ^{3}H-CPT solution and EPC/DOTAP liposome formulations were performed to track the retention of both CPT in solution and liposome incorporated CPT as well as the liposomes themselves. The animals were divided into three groups of six animals where one group was injected with ^{111}In-labeled liposomes, one group with ^{3}H-CPT liposomes and one control group with ^{3}H-CPT solution.

The sample preparation before counting of the ^{3}H-CPT containing tissue, blood, and liposome samples appeared to work satisfactory, and is thus a good method for this study as well as future

in vitro and *in vivo* studies with CPT formulations and other formulations with ^{3}H-labeled anticancer drug expected to be active against colon cancer.

Blood samples were collected from all animals immediately as well as 1, 2, 4, 6 and 19 hrs after injection. The blood concentration curves are displayed in Figure 3 and the clearance half-lives from first order exponential decay simulations were found to be 0.5 hr, 0.6 hr and 2.1 hrs for the CPT solution, CPT liposomes and the ^{111}In-labeled liposomes, respectively. Drug in solution is thus cleared from circulation faster than the drug in the liposome formulation which is according to what has been reported earlier for other drugs (Forssen *et al.*, 1994). The liposomal CPT has a shorter clearance half-life than the ^{111}In-labeled liposomes, however, only 76.5% of the liposomal CPT was cleared from circulation compared to 96.4% for the ^{111}In-labeled liposomes. This might indicate that CPT is partly prematurely released from the liposomes, which also is in agreement with the liposomes' drug retention ability upon incubation in serum.

The results from the biodistribution study at 20 hours post-injection are given in Table 2 and show that the formulations are cleared from the blood in the ascending order CPT solution > CPT liposomes > ^{111}In-labeled liposomes. However, liposomal CPT stays in circulation longer than what would be anticipated from the clearance half-life, and based on the first order exponential decay simulation 23.5% of liposomal CPT had permanent retention. This could be due to release of drug and subsequent protein binding of the free drug, since CPT carboxylate is known to have a high affinity for plasma proteins (Kruszewski *et al.*, 2002), and thus a prolonged circulation time. It thus appears that all the CPT does not stay associated with the liposomes throughout the experiment. The premature loss of drug is however as already pointed

out a well-known problem for lipophilic drugs (Fahr *et al.*, 2006). When looking at the percentage of the total dose found in the tumor 20 hrs after injection, the level is significantly higher for both liposome formulations compared to the CPT solution. This is also what was expected due to the longer circulation time and EPR effect. On the other hand, there is no significant difference between the two liposome formulations. Taking into account that the ^{111}In-labeled liposomes had a longer circulation time compared to the CPT liposomes this is a bit surprising, but the amounts found in the tumors are small so it is difficult to draw clear conclusions. What also can be seen is that the ^{111}In-labelled liposome formulation accumulates in the lungs to a higher extent, compared to the others. Studies of lipid complexed CPT has also earlier shown a distribution to the lung (Sugarman *et al.*, 1996). DOTAP/cholesterol-based lipoplexes have furthermore shown to be successfully delivering plasmid DNA *in vivo* in particular to the lungs (Senmaru *et al.*, 1998).

3.2.4 Scintigraphic imaging study

To track the liposomes *in vivo* before sacrificing the animals, scintigraphic imaging of the animals injected with ^{111}In-labeled liposomes was performed. Planar images, with mice outlines, acquired of a tumor-bearing mouse over time after injection of ^{111}In-labeled liposomes are shown in Figure 4 and a SPECT/CT image acquired 2 hrs after injection is shown in Figure 5. At baseline some of the activity can still be found in the blood pool, represented by the heart, but at 2 hrs after injection, only the liver is evident in the image. It thus appears the liposomes are quickly taken up by the liver. In the image acquired at 20 hrs post-injection, the liver still has the highest activity which corresponds to the results from the biodistribution study given in Table 2, where the liver counts accounts for about 30% of the injected dose. The intestine (with feces),

the organ showing the second highest activity, can also be seen in the image acquired after 20 hrs.

In vivo labeling stability of ^{111}In is a point to consider when discussing the liposomes biodistribution. However, other studies with liposomes labeled with ^{111}In in the same way as in our study, has shown that the radiolabeling was very stable in serum *in vivo* (Goins *et al.*, 2003, Mougin-Degraef *et al.*, 2007). Radiolabeled phospholipids released from degraded liposomes have also been shown to stay in the liver, and will thus not be present in circulation. ^{111}In is further usually cleared from the body by hepatobiliary path and the intestine activity could most probably be post processing from liver (Goins *et al.*, 2003, Mougin-Degraef *et al.*, 2007).

The reason for the fast clearance of liposomes to the liver is most probably due to the presence of the positively charged DOTAP in the liposome formulation, which could lead to recognition by the macrophages (Kelly *et al.*, 2011). Our theory was that the presence of DTPA on the surface would shield the charge from the blood compartment. But unfortunately DTPA is obviously not able to shield the positive charge on the liposome surface to a satisfactory level. When the target is macrophages and/or the liver, our formulation could be a very promising formulation, but when the goal is for the liposomes to circulate for longer time in the blood, changes are needed. Polyethylene glycol (PEG) is often added to liposome formulations to increase the circulation time *in vivo*. However it appears from preliminary studies (results not included in this paper) that the addition of low concentrations of 1,2-distearyl-sn-glycero-3-phosphatidylethanolamine (DSPE)-PEG 2000 influence the drug incorporation and retention. Further research on what influences a formulation's capacity to incorporate and retain the drug is clearly needed and is under way in our research group.

4. Conclusions and future perspectives

The ability of the liposome formulations to retain the lipophilic CPT in the bilayer was shown to be more dependent on the lipid concentration and the drug:lipid ratio rather than the lipid composition, unlike what was observed during incorporation studies. No significant variations in drug retention were seen between the different liposome compositions tested.

The present tumor model, ^{111}In-labeling of the liposomes as well as the preparation and analysis of the ^{3}H-CPT containing tissue and blood samples were shown to work satisfactorily and is thus a good methodology for future *in vivo* studies on both CPT formulations as well as other formulations with ^{3}H-labeled anticancer drug expected to be active against colon cancer.

The EPC/DOTAP liposomes, showing the highest incorporation in earlier studies, seem to release some of the drug prematurely and they accumulate in the liver shortly after injection. Unless the liver is the therapeutic target, further studies to develop the formulation towards higher CPT retention as well as increased circulation time are necessary. The addition of PEGylated lipids to the formulation needs to be further investigated both in terms of its influence on incorporation capacity and retention ability, and eventually *in vivo* studies to assess tumor accumulation.

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Declaration of interest

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Table 1: Lipid composition (mol%) of the different CPT-liposome formulations

Lipids	T_m[a]	Formulation #										
	(°C)	1	2	3	4	5	6	7	8	9	10	11
DLPC	-1								100			
DMPC	23							100				
DPPG	41						10					
Chol	-		50									
EPC	-15 to -17[b]	100	50	80		40	90			79.75	79.5	79.5
DOPC	-20				40							
DOPE	-16				40	40						
DOTAP	0[c]			20	20	20				19.75	19.5	19.5
DMPE-DTPA	50[d]									0.5	1	
DPPE	63											1

[a] from Analytical Data from Avanti Polar Lipids if not stated otherwise
[b] from (Bordi *et al.*, 2006)
[c] from (Ogiso *et al.*, 1996)
[d] T_m for DMPC that is the bilayer forming part of the lipid

Bordi, F., Cametti, C., Sennato, S. & Diociaiuti, M. 2006. Direct evidence of multicompartment aggregates in polyelectrolyte-charged liposome complexes. *Biophys. J.*, 91: 1513-20.

Ogiso, T., Niinaka, N. & Iwaki, M. 1996. Mechanism for enhancement effect of lipid disperse system on percutaneous absorption. *J. Pharm. Sci.*, 85: 57-64.

Table 2: The biodistribution to selected organs 20 hrs after administration of the three formulations displayed in % of injected dose per organ (%ID) and as % injected dose per gram (%ID/g).

Organ	^{3}HCPTsol		^{3}HCPTlipo		^{111}Inlipo	
	Mean ± SD (%ID)	Mean ± SD (%ID/g)	Mean ± SD (%ID)	Mean ± SD (%ID/g)	Mean ± SD (%ID)	Mean ± SD (%ID/g)
Heart	0.01 ±0.00	0.05±0.02	0.02±0.00	0.17±0.04	0.07±0.01	0.62±0.06
Liver	0.16 ±0.04	0.17±0.06	0.51±0.10	0.48±0.12	29.9±8.33	25.94±6.99
Spleen	0.01 ±0.00	0.11±0.05	0.02±0.01	0.31±0.10	0.47±0.13	5.41±2.00
Lung	0.01 ±0.00	0.08±0.04	0.03±0.01	0.20±0.08	0.26±0.03	1.32±0.35
Kidney	0.04 ±0.01	0.12±0.03	0.10±0.02	0.27±0.06	1.21±0.12	3.44±0.30
Tumor	0.04 ±0.01	0.15±0.04	0.09±0.03	0.33±0.08	0.11±0.03	0.42±0.15
Blood	1.59 ±0.47	0.15±0.05	5.60±2.64	0.53±0.25	4.51±0.97	0.39±0.08
Testis	0.03 ±0.01	0.15±0.07	0.05±0.01	0.28±0.04	0.05±0.01	0.28±0.07
Total	1.88± 0.61		6.40±2.32		37.8±6.86	
Bone	-	-	-	-	2.70±0.45	1.34±0.26
Brain	-	-	-	-	0.03±0.02	0.10±0.05
Skin	-	-	-	-	1.46±0.23	0.56±0.10
Muscle	-	-	-	-	0.06±0.02	0.31±0.10
Intestine	-	-	-	-	23.2±1.54	2.86±0.11
Stomach	-	-	-	-	1.37±0.80	2.03±1.23
Total					65.4±7.94	

Figure legends:

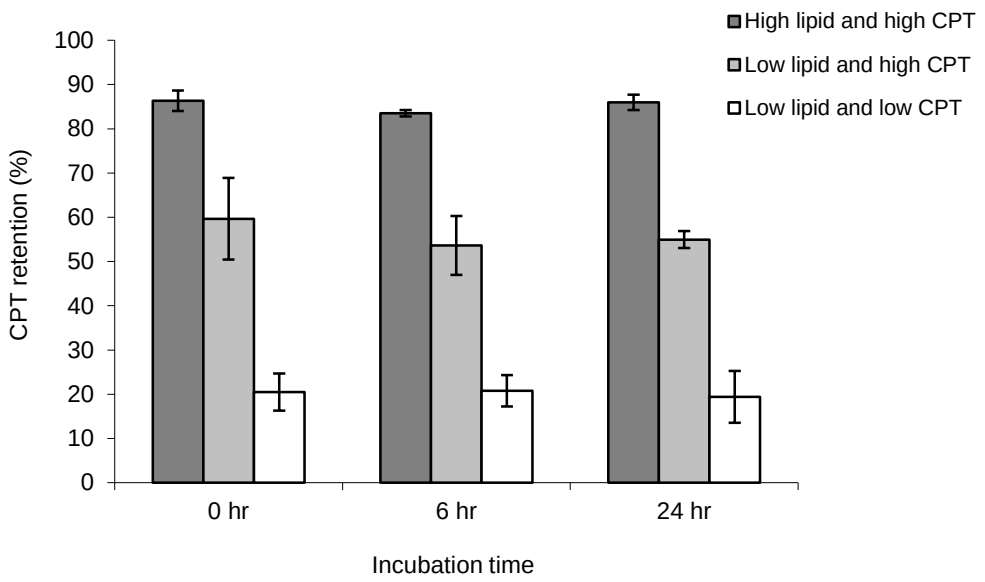
Figure 1: CPT Retention in liposomes incubated in PBS buffer pH 6.0 over time for different liposome formulations (n=3).

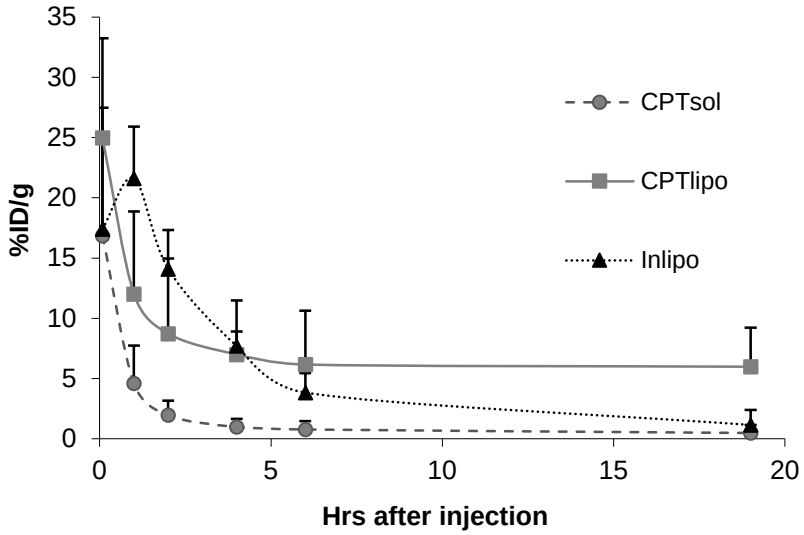
Figure 2: The effect of different lipid (DOTAP/EPC (2:8)) and drug concentrations on retention of CPT with the liposomes in PBS buffer pH 6.0 over time (n=3).

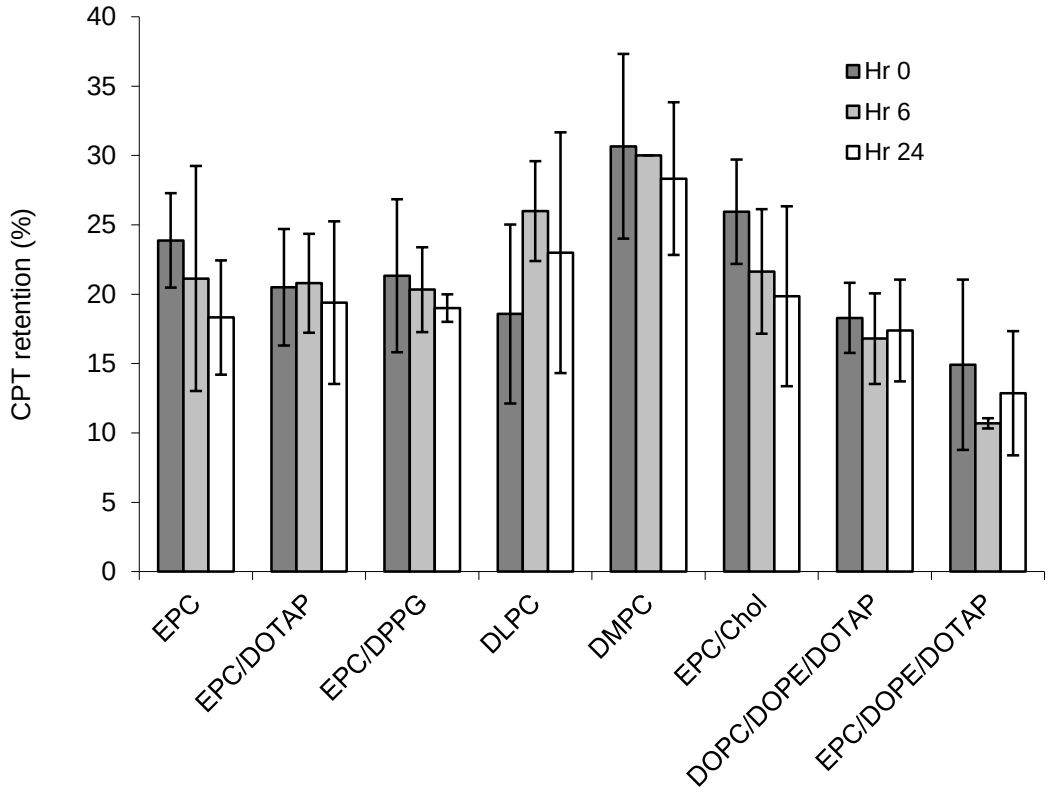
Figure 3: The blood concentration curves after intravenous injection with ^{111}In-labeled liposomes, ^{3}H-labeled CPT liposomes and ^{3}H-labeled CPT solution respectively.

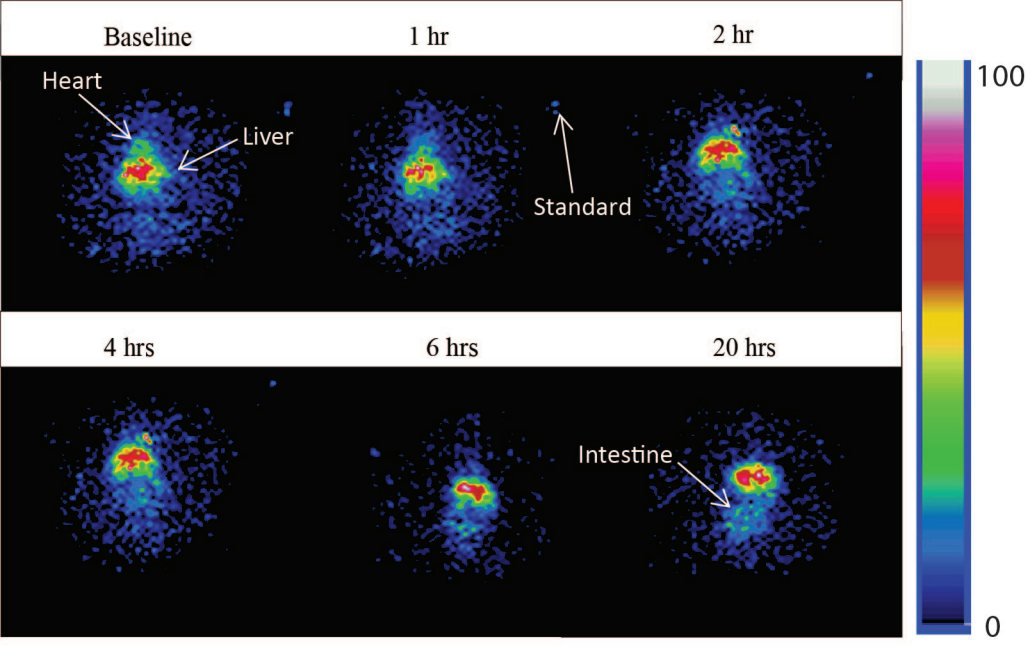
Figure 4: Planar images of a tumor-bearing mouse at baseline and 1, 2, 4, 6 and 20 hrs after injection of ^{111}In-labeled liposomes

Figure 5: SPECT/CT image of a tumor-bearing mouse acquired 2 hr after injection of ^{111}In labelled liposomes. SPECT image shows radioactive liposomes are predominately taken up by the liver (green) and co-registered with CT image windowed for bone (white).


High lipid and high CPT
Low lipid and high CPT
Low lipid and low CPT
CPT retention (%)
100
90
80
70
60
50
40
30
20
10
0
0 hr
6 hr
24 hr
Incubation time


35
30
25
20
15
10
5
0
%ID/g
0
5
10
15
20
Hrs after injection
CPTsol
CPTlipo
Inlipo


CPT retention (%)
40
35
30
25
20
15
10
5
0
Hr 0
Hr 6
Hr 24
EPC
EPC/DOTAP
EPC/DPPG
DLPC
DMPC
EPC/Chol
DOPC/DOPE/DOTAP
EPC/DOPE/DOTAP


Baseline
1 hr
2 hr
Heart
Liver
Standard
4 hrs
6 hrs
20 hrs
Intestine
100
0