In Vivo Tracking of Liposomes Using CT and MR Imaging

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ABSTRACT

There are limited methods that are currently available for longitudinal and non-invasive *in vivo* assessment of the transport kinetics of carrier-based therapeutics, such as those relying on liposomes. A viable strategy to non-invasively track liposomes *in vivo* is to load them with contrast-enhancing agents that are well retained within the carrier. The encapsulation of contrast agents such as iodine and gadolinium allows for the liposomes to be tracked by computed tomography (CT) and magnetic resonance (MR) imaging systems, respectively. This study evaluated the feasibility of using CT and MR imaging as a means to gain a quantitative assessment of the pathway and fate of liposomes *in vivo* in rabbits. The liposome formulation examined was composed of DPPC/Cholesterol/DSPE-PEG_{2000} in a 55/40/5 molar ratio and the vesicles had an average diameter of 80 nm. The commercially available agents iohexol (CT agent) and gadoteridol (MR agent) were encapsulated within the liposomes and found to be retained over a 15-day period *in vitro*. Following i.v. administration of the CT and MR agent-containing liposomes the relative signal intensities in both imaging modalities were monitored over a 7-day period. The signal intensities were then compared with the values obtained for the concentration of each agent present in blood as measured by HPLC and ICP-AES analyses. Overall, the values obtained for the amount of agent in blood using non-invasive imaging correlated well with that measured using the traditional analytical techniques (R^2=0.9). This pilot study demonstrated the potential of employing CT and MR imaging for longitudinal *in vivo* mapping of liposomes and other nano-sized carriers.

Keywords: liposome, pharmacokinetics, contrast agent, computed tomography, magnetic resonance imaging

1 INTRODUCTION

Limited methods are currently available for longitudinal and non-invasive assessment of the *in vivo* pathway of carrier-based therapeutics, such as those relying on liposomes. Radioisotope labeling has been used to track small molecules *in vivo* with positron emission tomography (PET) and single photon computer tomography (SPECT) [1-4]. However, due to the short half-life of these labeling radioisotopes they are not suitable as tracers for longitudinal tracking of long-circulating carriers. Optical probes have also been explored as potential labels for carrier-based technologies [5-7]; however, optical imaging is associated with limited tissue depth penetration and substantial light photon scattering. These limitations make optical imaging unsuitable for quantitative *in vivo* applications. A strategy to non-invasively track the carrier *in vivo* is to employ the same carrier loaded with contrast-enhancing agents such as iodine and gadolinium. If these agents are retained within the carrier they will allow for tracking using computed tomography (CT) and magnetic resonance (MR) imaging devices. If a correlation can be found between the change in signal intensities measured by CT and/or MR and the actual concentration of the liposomal agents then this method may be successfully adopted for the non-invasive quantification of the *in vivo* pharmacokinetics and biodistribution of liposomes. In this way, not only can the same animal be sampled over multiple time points, avoiding animal-to-animal variations, but the total number of animals required for each study can also be drastically reduced. This study is aimed to evaluate the feasibility of employing CT and MR to longitudinally and quantitatively track iohexol and gadoteridol-containing liposomes *in vivo*.

2 MATERIALS AND METHODS

2.1 Materials

The lipids employed for the liposome formulation: 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC, M.W. 734), Cholesterol (CH, M.W. 387) and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)2000] (PEG_{2000}DSPE, M.W. 2774) were purchased from Northern Lipids Inc. (Vancouver, British Columbia, Canada). Omnipaque® (300 mg/mL of iodine), the CT contrast agent containing iohexol (M.W. 821.14), an iodinated, water-soluble, non-ionic monomeric contrast medium, was purchased from Nycomed Imaging AS, Oslo, Norway. The MR contrast agent, ProHance® (78.6 mg/mL of gadolinium), contains gadoteridol (M.W. 558.7), a non-

ionic gadolinium complex of 10-(2-hydroxy-propyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid, was purchased from Bracco Diagnostics Inc. (Princeton, NJ, USA).

2.2 Preparation of Liposome Formulations

200 mmol/L of lipid mixture composed of DPPC, cholesterol and PEG_{2000}DSPE in 55:40:5 percent mole ratios, was dissolved in ethanol ($10\%_{vol}$). The resulting lipid-ethanol solution was hydrated at 70°C with Omnipaque® ($45\%_{vol}$) and Prohance® ($45\%_{vol}$). The liposome solution was then extruded [8, 9] at 70°C with a 10 mL Lipex™ Extruder (Northern Lipids Inc., Vancouver, British Columbia, Canada) with two stacked polycarbonate membranes of 0.2 μm pore size (Nucleopore® Track-Etch Membrane, Whatman Inc., Clifton, NJ, USA) for the first 5 passes and subsequently with two stacked polycarbonate membranes of 0.08 μm pore size for the next 5 passes.

2.3 Determination of Agent Loading

The un-encapsulated iohexol and gadoteridol were removed by membrane dialysis: one mL of the liposome sample was dialyzed against 250 mL of N-(2-Hydroxyethyl)Piperazine-N'(Ethanesulfonic Acid)(HEPES) buffer saline (HBS) in an 8000 molecular weight cut-off (MWCO) dialysis bag overnight. Using a 10-fold volume excess of ethanol, the liposomes were ruptured to allow for measurement of the concentration of encapsulated agents. The iodine concentration was determined using a UV assay with detection at a wavelength of 245 nm (Heλios γ, Spectronic Unicam, MA, USA). The gadolinium concentration was determined using an assay based on inductively coupled plasma atomic emission spectrometry (ICP-AES Optima 3000DV, Perkin Elmer, MA, USA).

2.4 Measurement of Liposome Size

The liposome diameter was measured by dynamic light scattering (DLS) using a DynaPro DLS (Protein Solutions, Charlottesville, VA, USA) at 25°C.

2.5 *In Vivo* CT and MR Imaging

All animal studies were performed under protocols approved by the Animal Care and Use Committee of the University Health Network. A New Zealand white rabbit (female, 2 kg) was first anaesthetized with an intramuscular injection of 40 mg/kg of ketamine and 5 mg/mL of xylazine and then with a 2% isoflurane vapor administered by inhalation. 10 mL of the contrast agent loaded liposome solution (200 mg/kg of iodine and 16 mg/kg of gadolinium) was injected into the marginal ear vein catheter as a bolus. Pre- and post-contrast injection images of the rabbit were acquired in CT (GE Discovery LS, General Electric Medical Systems, Milwaukee, WI, USA) and MR (GE Signa TwinSpeed MR scanner, General Electric Medical Systems, Milwaukee, WI, USA). 10, 60 minutes and 24, 48, 72, 96, 120 and 168 hours following the liposome sample injection, the rabbit was imaged in CT (120kV, 200mA, FOV = 22.0 x 22.0 cm, slice thickness = 1.25 mm and image matrix of 512 x 512). 30, 90 minutes and 24, 48, 72, 96, 120 and 168 hours post-injection, the rabbit was imaged in MR (3D FSPGR sequence with a TR of 9.8 ms, a TE of 4.3 ms, a flip angle of 15°, an FOV of 22.0 x 22.0 cm and an image matrix of 256 x 256). The relative signal intensities in MR and the mean attenuation values in Hounsfield units (HU) in CT were measured in the aorta with circular regions of interests of 4 mm^2. The cross-sectional images were exported from a review station (Merge eFilm, Milwaukee, WI, USA). The same window and level was used for the pre and post-contrast images.

2.6 *In Vivo* Pharmacokinetics

1.5mL of blood was collected from the ear vein of the same rabbit at the following time points: 5 minutes, 24, 48, 72, 96, 120 and 168 hours following liposome injection. The plasma was isolated by centrifugation of the blood samples at 3000 g for 10 minutes. Iodine and gadolinium were extracted from the plasma solution using 10% perchloric acid (5-fold volume excess). The resulting samples were analyzed using high pressure liquid chromatography (HPLC, PerkinElmer Series 200, C18 Xterra reverse-phase column, 5 μg/mL of p-aminobenzoic acid was used as the internal standard, the mobile phase was 90% composed of methanol and 10% composed of 100mM acetic acid buffer at a pH of 4.10, the flow rate was set to 0.9 mL/min and the UV detection was performed at a wavelength of 245 nm) to determine the iodine concentration, and ICP-AES to measure the gadolinium concentration.

3 RESULTS

The physico-chemical properties of the iohexol and gadoteridol containing liposome formulation used in this study have been characterized previously [10, 11]. In brief, the iohexol and gadoteridol loading efficiency into the liposome was found to be approximately 20% (Table 1). The average liposome size was measured to be approximately 74.4 ± 3.3 nm. Following a 15-day incubation period at 37°C, there was 9.1 ± 2.5 % and 7.5 ± 1.4 % release of the total encapsulated iodine and gadolinium, respectively. The liposome size remained constant throughout the incubation period.

Liposome diameter (nm)	Iohexol loading efficiency (%)	Gadoteridol loading efficiency (%)
74.4 ± 3.3	19.6 ± 2.8	18.6 ± 4.4

Table 1: Size and loading characteristics of the agent-modified liposome formulation [10, 11].

In this study, an imaging-based assessment of the *in vivo* stability (Figures 1) of the contrast agent modified liposome was conducted in a rabbit model. Visual contrast enhancement and measurable signal increases produced by the presence of the contrast agent carrying liposomes was induced in various organ systems (i.e. heart and blood vessels, liver, spleen, kidney and intestines) in both CT and MR over a 7-day period. Following the extraction of each agent from rabbit plasma, it was determined that 17.7% of the injected iohexol (95.9 μg/mL of iodine) and 17.3% of the injected gadoteridol (7.9 μg/mL of gadolinium) still circulated in the bloodstream 7 days post-injection. The plasma circulation half-life of the present liposome formulation in rabbits was found to be approximately 45 hours (unpublished data).

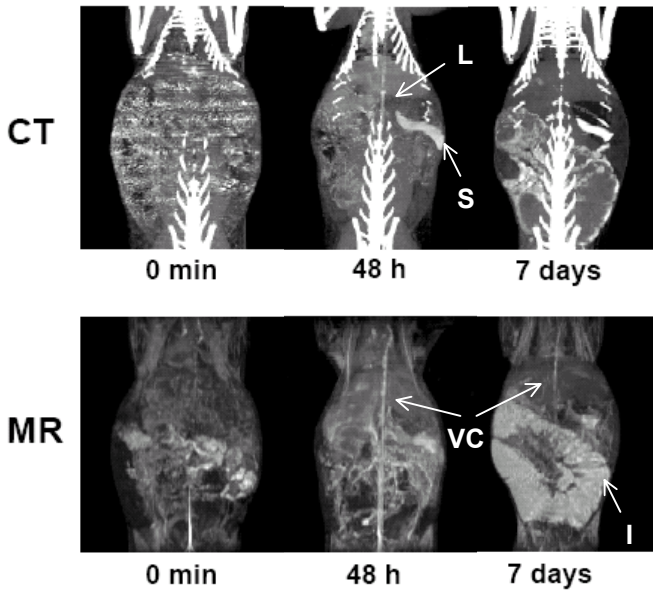

Figure 1: 3D maximum intensity projection images (anterior view) of the rabbit in CT (120 kV, 200 mA) and MR (3D FSPGR, TR/TE=9.8/4.3) before the injection of the contrast agent modified liposomes (0 minutes), 48 hours post-injection and 7 days (168 hours) post-injection. The parallel visual enhancement seen in both CT and MR obtained in the vena cava (VC), liver (L), spleen (S) and intestines (I) represents the liposome distribution over a 7-day period. The spine and part of the ribs of the rabbit have been masked in the CT image set for improved soft tissue visualization.

Correlations were established between the iodine and gadolinium concentrations found in the rabbit plasma and the signal enhancement obtained in the rabbit aorta in CT and MR, respectively, using circular regions of interest over 6 time points (i.e. 10 minutes, 24, 48, 72, 120 and 168 hours post-injection). Fairly linear relationships (R^2=0.9) were observed between both the iodine concentration and relative HU increase in CT, and the gadolinium concentration and relative signal intensity increase in MR (Figure 2).

In Figure 2, the relative HU (ΔHU_{rel}) was calculated as a function of the HU value found at the same anatomic location prior to the injection of the liposome sample (ΔHU_0) as described in equation (1):

$$\Delta HU_{rel} = \frac{(\Delta HU_{rel} - \Delta HU_0)}{\Delta HU_0} \quad (1)$$

Similarly, the relative MR signal intensity (ΔSI_{rel}) was calculated as a function of the pre-injection signal intensity value (ΔSI_0) as described in equation (2):

$$\Delta SI_{rel} = \frac{(\Delta SI_{rel} - \Delta SI_0)}{\Delta SI_0} \quad (2)$$

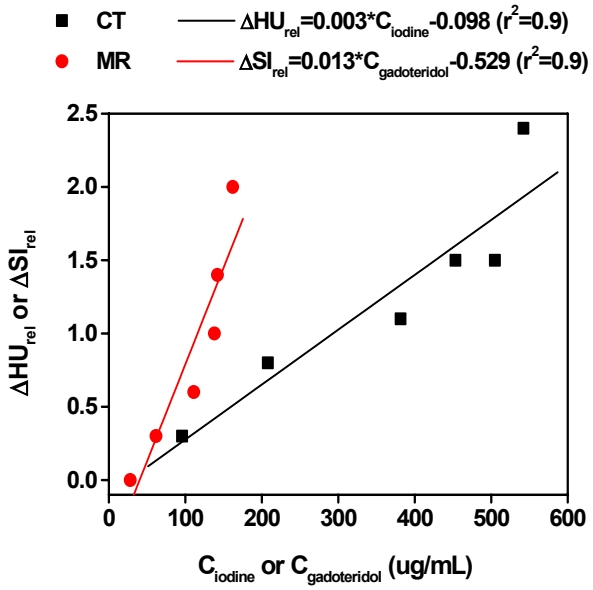

Figure 2: The relative signal differences measured in the rabbit aorta using CT and MR versus the concentration of iodine and gadoteridol, repectively, in blood as measured using HPLC and ICP-AES assays.

4 DISCUSSION

Traditional pharmacokinetics and biodistribution studies involve invasive plasma and tissue sampling. The invasive nature of these procedures can change the biological system under observation resulting in unreliable data collection. In small animal models such as mice and rats, the animals need to be sacrificed in order to allow for sufficient amounts of plasma and tissues to be sampled for analysis. As a consequence, multiple animals are needed for longitudinal studies involving sampling at multiple time points. This may result in greater data variability due to animal-to-animal heterogeneity. These limitations

associated with traditional pharmacokinetics and biodistribution studies can be either minimized or avoided through the use of non-invasive imaging systems in conjunction with appropriate labeling techniques. Imaging-based measurements, when successfully correlated with tissue concentrations, can be used to collect meaningful data on the same animal over multiple time points, and therefore not only avoid animal-to-animal variations, but also reduce the total number of animals required for each study. In addition, the same medical imaging assays can be employed in both preclinical and clinical settings due to their non-invasive nature. Furthermore, an imaging-based tool could, in theory, allow for real-time tracking over the entire course of a study, providing information on the complete *in vivo* pathway of the system under investigation. In practice, although imaging would allow for more time points to be sampled, the frequency of sampling greatly depends on the ability of the animal to remain immobilized.

This pilot study has demonstrated the feasibility of employing non-invasive imaging-based tools to visually track and quantify the concentrations of contrast agent containing liposome carriers *in vivo*. A linear concentration prediction range was found in both CT and MR with correlation coefficients of 0.9. Specifically, a clinical CT system operating at 120 kV and 200 mA was able to detect plasma iodine concentrations ranging from 100 μg/mL to 500 μg/mL in a 2 kg rabbit, while a clinical MR system (3D FSPGR, TR/TE=9.8/4.3) was able to measure plasma gadolinium concentrations ranging from 20 μg/mL to 150 μg/mL in the same rabbit (Figure 2).

These results indicate the potential of employing a multimodality imaging approach to map the distribution of liposome carriers *in vivo*. The successful refinement of this imaging tool may facilitate delivery carrier design and optimization in both the preclinical and clinical settings.

ACKNOWLEDGEMENTS

J. Zheng is grateful for the CIHR Training Grant Fellowship (EIRR21st) and the University of Toronto Mitchell Scholarship. C. Allen and D. Jaffray thank CIHR for an Operating Grant.

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