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Multifunctional nanocarriers for mammographic quantification of tumor dosing and prognosis of breast cancer therapy

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ABSTRACT

Nanoscale therapeutic interventions are increasingly important elements in the portfolio of cancer therapeutics. The efficacy of nanotherapeutics is dictated, in part, by the access they have to tumors via the leaky tumor vasculature. Yet, the extent of tumor vessel leakiness in individual tumors varies widely resulting in a correspondingly wide tumor dosing and resulting range of responses to therapy. Here we report the design of a multifunctional nanocarrier that simultaneously encapsulates a chemotherapeutic and a contrast agent which enables a personalized nanotherapeutic approach for breast cancer therapy by permitting tracking of the nanocarrier distribution by mammography, a widely used imaging modality. Following systemic administration in a rat breast tumor model, imaging demonstrated a wide range of intratumoral deposition of the nanocarriers, indicating variable tumor vessel leakiness. Notably, specific tumors that exhibited high uptake of the nanocarrier as visualized by imaging were precisely the animals that responded best to the treatment as quantified by low tumor growth and prolonged survival.

1. Introduction

Nanocarriers [1,2] are excellent delivery vehicles for chemotherapeutic drugs, increasing delivery efficiency to the targeted tumor while reducing off-target delivery, thus increasing the overall therapeutic index [3]. For instance, liposomal anthracyclines [4] and nanoparticle albumin-bound paclitaxel [1] are examples of nanotherapeutics approved for clinical use. Furthermore, nanoscale control in fabrication allows for multifunctional carriers [5–7] that can (i) carry large payloads of therapeutic drugs or diagnostic imaging/contrast agents [8,9]; (ii) modulate pharmacokinetics and biodistribution when systemically administered to increase accumulation in tumor site, and (iii) enable presentation of targeting ligands to increase target tumor affinity and selectivity [10,11]. Here we demonstrate that incorporating a contrast agent along with anti-cancer therapeutic can potentially enable personalized cancer therapy that facilitates diagnosing, treating and monitoring of individual cancer treatment efficacy by a clinically relevant, breast cancer imaging modality, mammography [7,12–15].

The success of systemically delivered nanotherapeutics for solid tumors is critically dependent on the access that these agents have to tumors via the so-called tumor leaky vasculature. The tumor's complex microvasculature network [16] consists of immature blood microvessels with hypervascularization, abnormal vascular architecture, increased leakage through the vessel wall and lack of lymphatic drainage [17]. Nanoscale particles preferentially accumulate in solid tumors by passive convective transport through leaky endothelium (a process termed *extravasation*) [18–20]. Unlike small molecules, since the convective transport of these particles far outweighs the diffusive component, they do not return to the blood stream. To date however, no clinical tools exist to non-invasively determine whether the tumor blood vessels of an individual patient are permeable to a nanotherapeutic or not. For instance, the current clinical protocols for liposomal chemotherapy consist of a standard dose every 3–4 weeks [21]. No prior knowledge of tumor vessel status, especially leakiness, is taken into account for dose scheduling. However, it is well-known that the degree of tumor vasculature leakiness differs among same type tumors [22–24]. One recent example demonstrating the critical role that tumor vasculature plays in determining therapeutic outcomes comes from the work of Jain [25,26], where it was shown that the restructuring of tumor vasculature (a process termed 'normalization') leads to better chemotherapeutic outcomes. While antiangiogenic therapies focus on destroying tumor-related blood vessels

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compromising the efficiency of subsequent chemotherapy, optimal scheduling and dosing of these therapies can 'normalize' the abnormal tumor vasculature for better delivery of oxygen (eliminating hypoxia and its complications) and other drugs.

To address the need for non-invasive tracking of nanoscale chemotherapeutic dosing to individual tumors, a multifunctional liposomal nanocarrier was developed co-encapsulating an X-ray contrast agent (iodixanol) for imaging and a chemotherapeutic (doxorubicin) for treatment. In this work, the nanocarrier's extravasation in a rat breast tumor model was non-invasively quantified by mammographic imaging and used to predict the efficacy of the therapy as monitored by tumor growth and survival rates.

2. Methods

2.1. Fabrication of the multifunctional nanocarrier

A highly concentrated iodine solution (550 mg/mL iodine) was prepared by dissolving iodixanol powder (lyophilized from Visipaque 320, GE Healthcare, Milwaukee, WI) in a 250 mM ammonium sulfate solution under stirring and heating at 70 °C. The phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2 disteroyl-*sn*-glycero-phosphoethanolamine poly(ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) were used as the lipid matrix for the liposomes (Genzyme Pharmaceuticals, Cambridge, MA). A lipid composition of DPPC, cholesterol and DSPE-PEG$_{2000}$ in the molar ratio of 55:40:5 was used. The lipids were dissolved in ethanol and hydrated with the iodine solution at 70 °C followed by sequential extrusion in a Lipex Biomembranes Extruder (Northern Lipids, Vancouver, Canada), to size the liposomes to 100 nm. Free, unencapsulated ammonium sulfate (and iodixanol) was replaced by a saline solution (400 mM NaCl at pH ~ 7) using a 1-day dialysis with a 100 kDa MWCO dialysis tubing to establish an ammonium sulfate gradient for remote loading of doxorubicin (DXR). Briefly, liposomes and DXR were mixed at a ratio of 0.1 mg DXR per 1 mg of DPPC in the liposomes. The liposome–DXR suspension was heated at 35 °C for 25 min. The liposomes were then left overnight at room temperature and then dialyzed twice in 100 kDa MWCO membrane against 400 mM NaCl to remove unencapsulated DXR. Following concentration via diafiltration using MicroKros modules (Spectrum Laboratories, California) with a 50 nm cutoff pore size, the size of the liposomes was determined by dynamic light scattering (90 Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY). Prior to administration, the final iodine and DXR levels were quantified through spectrophotometry at 245 and 480 nm, respectively.

2.2. Mammary adenocarcinoma cell culture

The 13762 MAT B III cells (American Type Culture Collection), a rat mammary adenocarcinoma cell line, was maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin under conditions of 5% CO_2 and 95% humidity at 37 °C.

2.3. In vitro cytotoxicity experiment

Cytotoxicity studies were performed by seeding the 13762 MAT BIII cells at a density of 10^5 cells/well in 6-well plate 24 h before incubation with the formulations. Prior to incubation, cells were washed three times with fresh medium and then incubated with the treatment for 180 min at a concentration of 150 μM doxorubicin per well. The treatments consisted of doxorubicin, doxorubicin loaded liposomes, doxorubicin/iodine co-loaded liposomes, and blank liposomes. Doxorubicin loaded liposomes were prepared following established methods [27]. After treatment application, the cells were washed three times with fresh medium and then incubated for 48 h at 37 °C and 5% CO_2 in a humidified environment. The number of viable cells was determined using a formazan-based cell counting assay (CCK-8). Untreated cells served as live controls for normalization of the data.

2.4. Animal model

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Georgia Institute of Technology. For the tumor model, the 13762 MAT B III cell line was used. Before inoculation, the cells were grown in 90% McCoy's 5A medium and 10% fetal bovine serum. A 0.2 mL aliquot containing 10^6 cancer cells was subcutaneously injected into the right flank of female Fisher rats with ages of 8–9 weeks (Harlan, Indianapolis, IN). Caliper measurements were used to estimate tumor size and the tumor volume was calculated as: $V_{\text{tumor}} = (d_1^2 \times d_2)/2$, where d_1 and d_2 are the minimum and maximum diameters.

2.5. X-ray imaging and treatment

Once the appropriate tumor sizes were established (day 7 after tumor inoculation, volume ~ 500 mm^3), the animals were used in the imaging studies. The animals were imaged using a clinical digital mammography system (Senographe 2000D, GE Healthcare, Milwaukee, WI). To maximize the number of photons with energies above the K-edge of iodine (approx. 33.2 keV) [28], the imaging studies were performed with a 49 kVp, 63 mAs X-ray spectrum, using a rhodium target and a 25 μm thick rhodium filter with an added 0.254 mm thick copper filter. The resultant X-ray spectrum was estimated using the XSPECT simulation program developed at Henry Ford Health Systems (Detroit, MI) based on semi-empirical models. To compute the radiation dose to the animals during X-ray imaging, a previously validated Monte Carlo simulation for dosimetry studies [29] was modified to include a simplified version of the animal geometry. In the simulation, the animals were represented as a cylinder of water with a length of 10 cm and a diameter of 4 cm. To estimate the dose to the cylinder from the X-ray spectrum used in the imaging studies, the Monte Carlo simulation was performed repeatedly with monochromatic X-rays with energies from 20 keV to 49 keV in 0.5 keV steps. To achieve the necessary statistical accuracy, one million photons per energy level were simulated. The monochromatic results were combined with the X-ray spectrum obtained with the XSPECT simulation program using the method described by Boone [30].

At day 7 after tumor inoculation, a group of animals ($n = 8$) was imaged before ($t = 0$) and at defined time points after IV injection ($t = 2$ and 30 min, 24 h, 72 h) of the nanocarrier. A control group of animals ($n = 8$) was imaged at the same time points upon injection of 0.5 mL of saline. The tumor growth of each animal was monitored every day using caliper measurements. In the end of the study, the animals were euthanized using a CO_2 chamber.

2.6. Intratumoral levels of doxorubicin

A different group of animals ($n = 8$) was imaged before ($t = 0$) and at defined time points after IV injection ($t = 24$ h and 72 h) of the nanocarrier. Immediately after the last imaging session, animals were anesthetized with an intraperetoneal injection of 50 and 10 mg/kg of ketamine and xylazine respectively and transcardially perfused with heparinized (1 unit/mL) phosphate buffered saline (PBS). The tumors were retrieved, washed with PBS and blotted dry. Tumors were weighed and DXR was extracted following methods described elsewhere [31]. Briefly, the tumors were homogenized in distilled, deionized water (20% wt/vol) using a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates (200 μL) were mixed with 100 μL of 10% Triton X-100, 200 μL of water and 1500 μL of acidified isopropanol (0.75 N HCl). Mixtures were stored overnight at −20 °C to extract the drug and then warmed at room temperature and vortexed for 5 min. The samples were then centrifuged at 15 000 g for 20 min. Fluoresence of suparnatants was analyzed to determine DXR content ($\lambda_{ex} = 485$, $\lambda_{em} = 590$). Tumor samples from an animal treated with a saline injection were used to correct for background fluorescence.

2.7. Survival study

At day 7 after tumor inoculation, a different group of animals ($n = 8$) was imaged before and after administration ($t = 2$ min, 24 h, 72 h) of the nanocarrier using the mammography system. At day 14 after tumor inoculation, the animals were injected again with the same dose of the nanocarrier and imaged at the same time points. Tumor growth was allowed to progress until the animal showed signs of morbidity, at which point, the animals were euthanized using a CO_2 chamber. Time of death was determined to be the following day. The survival time of a control group ($n = 10$) was also determined.

2.8. Image analysis

The sequential image acquisitions provided the dynamics of the nanocarrier's intratumoral accumulation over time. The grey levels in raw data (DICOM format) were measured using ImageJ software (NIH, Bethesda, MD). An ellipsoid region of interest was used for the measurements surrounding the entire tumor lesion. Since mammography is not a tomographic but a summation procedure, the observed tumor enhancement represents the summation of the absolute enhancement due to the contrast agent and the enhancement of overlying tissue structures. To normalize with respect to the overlying tissues, we computed a relative enhancement by subtracting the pre-contrast enhancement value from the post-contrast enhancement value.

For display purposes only, the histograms of the radiographic images were matched using ImageJ. The quality of these images was improved by sharpening the images using the 'unsharp mask' function in ImageJ. This simple sharpening operator enhances edges and other high frequency components in an image by subtracting a blurred copy of the image (created by Gaussian blurring) and rescaling the image to obtain the same contrast of large (low-frequency) structures as in the input image [32]. The mask weight (intensity of sharpening) was set at 0.7 and the Gaussian blur had a radius of 15 pixels. This processing was performed for display of the images only whereas quantitative analysis was performed with the original, unprocessed images.