Clearance and Biodistribution of Liposomally Encapsulated Nitroxides: A Model for Targeted Delivery of Electron Paramagnetic Resonance Imaging Probes to Tumors

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ABSTRACT:

Electron paramagnetic resonance (EPR) imaging using nitroxides as molecular probes is potentially a powerful tool for the detection and physiological characterization of micrometastatic lesions. Encapsulating nitroxides in anti-HER2 immunoliposomes at high concentrations to take advantage of the “self-quenching” phenomenon of nitroxides allows generation of robust EPR signals in HER2-overexpressing breast tumor cells with minimal background from indifferent tissues or circulating liposomes. We investigated the *in vivo* pharmacological properties of nitroxides encapsulated in sterically stabilized liposomes designed for long circulation times. We show that circulation times of nitroxides can be extended from hours to days; this increases the proportion of lipo-

somes in circulation to enhance tumor targeting. Furthermore, nitroxides encapsulated in sterically stabilized anti-HER2 immunoliposomes can be delivered to HER2-overexpressing tumors at micromolar concentrations, which should be imageable by EPR. Lastly, after *in vivo* administration, liposomally encapsulated nitroxide signal also appears in the liver, spleen, and kidneys. Although these organs are spatially distinct and would not hinder tumor imaging in our model, understanding nitroxide signal retention in these organs is essential for further improvements in EPR imaging contrast between tumors and other tissues. These results lay the foundation to use liposomally delivered nitroxides and EPR imaging to visualize tumor cells *in vivo*.

Introduction

The presence of micrometastatic breast lesions (<2 mm) is correlated with poor clinical prognosis and decreased patient survival rate (Alix-Panabieres et al., 2007; Park et al., 2009). However, using current imaging methods to detect breast tumor micrometastases or monitor response to therapies remains a significant clinical challenge. Electron paramagnetic resonance (EPR) imaging is a magnetic resonance imaging modality that can image exogenous paramagnetic species, such as nitroxides, *in vivo*. If nitroxides can be selectively targeted to metastatic cells *in vivo*, EPR imaging becomes an attractive modality to image micrometastatic lesions. This is because nitroxides can be imaged with high contrast at micromolar concentrations and can furthermore be chemically tailored to report cellular

physiological information such as regional changes in pH (Halpern et al., 1989; Smirnov et al., 2004).

We have previously synthesized nitroxides that are suitable for cellular imaging: they are resistant to bio-reduction and are retained in cells for sufficiently long periods to permit imaging (Rosen et al., 2005; Kao et al., 2007). We have further demonstrated that these nitroxides can be encapsulated in liposomes at high concentrations (>100 mM) and delivered to cells through endocytosis of liposomes (Burks et al., 2009). Liposomes encapsulating high concentrations of nitroxides are especially attractive carriers because nitroxides exhibit the phenomenon of “self-quenching,” analogous to that of fluorophores, where spectral signals become greatly attenuated at high concentration. Therefore, intact liposomes in circulation are spectroscopically “dark” and thus contribute minimal background signal in imaging. After endocytosis by cells, liposomes are degraded, and the encapsulated nitroxides are released, and become greatly diluted in the intracellular volume. This dilution relieves self-quenching and restores the nitroxide spectral signal, making the cells appear “bright” and visible by EPR imaging. Moreover, this cell-activated, EPR signal-generating mechanism can be targeted to specific cells through the use of immunoliposomes. For example, we have demonstrated that anti-HER2 immunoliposomes can deliver high concentrations (~1 mM) of nitroxides selectively to HER2-overexpressing breast tumor

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ABBREVIATIONS: EPR, electron paramagnetic resonance; HER2, human epidermal growth factor receptor 2; Hc7, HER2-overexpressing breast tumor cell derived from MCF7 cells; PEG, polyethylene glycol; PEG-PE, ammonium 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[polyethylene glycol 2000]; nitroxide 1, dipotassium (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N,N*-diacetate; nitroxide 2, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl; DPBS, Dulbecco's phosphate-buffered saline; SNR, signal-to-noise ratio; ANOVA, analysis of variance.

cells in vitro (Burks et al., 2010b). With further improvements, it may be possible to generate high-contrast EPR images of these HER2-overexpressing cells in vivo.

Imaging tumor cells in vivo requires several additional considerations. Of primary concern are clearance mechanisms that limit the efficacy of tumor targeting by liposomes. Liposomes must remain in circulation for a sufficient time to permit optimal tumor targeting and maximal delivery of EPR imaging agents. When liposomes are introduced into the circulation, opsonization promotes their rapid removal by the reticuloendothelial system (Liu and Liu, 1996; Yan et al., 2005). Liposomes can incorporate lipids that are conjugated to a high-molecular-mass (>1 kDa) hydrophilic polymer, such as polyethylene glycol (PEG), to form sterically stabilized liposomes. Such "PEGylated" liposomes differ from classical liposomes in their ability to evade clearance mechanisms and persist in the circulation for longer times (Woodle and Lasic, 1992).

The diameter of liposomes can drastically affect the rate of clearance from circulation. In general, clearance is faster for larger liposomes. An outer diameter of 100 nm is an optimal compromise for minimizing circulatory clearance while maximizing luminal capacity of the liposome (Harashima et al., 1994). With specific regard to in vivo tumor models, liposomes access tumors by extravasation into the interstitial space of the tumor. Increasing the liposomal diameter beyond 100 nm hinders this process and greatly reduces liposome distribution in the tumor volume (Charrois and Allen, 2003).

In this study, we demonstrate the feasibility of visualizing HER2-overexpressing tumors in vivo with EPR imaging using anti-HER2 immunoliposomes to selectively deliver nitroxides. We have characterized nitroxide-encapsulating, sterically stabilized anti-HER2 immunoliposomes with regard to their persistence in circulation, stability, and the ultimate biodistribution of encapsulated nitroxides. We demonstrate that nitroxides in sterically stabilized liposomes persist in circulation for days compared with hours with classical liposomes. Furthermore, these liposomes are highly stable in circulation and exhibit maximal self-quenching of encapsulated nitroxides; this minimizes background from the circulating liposomes. For tumor studies, we established xenograft tumors by inoculating mice with Hc7 cells, which are derived from the MCF7 breast tumor cell line and engineered to overexpress HER2 (for cellular characterization see Burks et al., 2010b). We demonstrate that through targeted delivery of immunoliposome-encapsulated nitroxides, Hc7 xenograft tumors can accumulate nitroxide at concentrations that should be sufficient to permit EPR imaging. Lastly, we have surveyed the biodistribution of nitroxides and measured the relative rates of nitroxide metabolism by the tumor and other tissues. This knowledge may guide the design and synthesis of novel nitroxides to create maximal image contrast in HER2-overexpressing tumors.

Materials and Methods

General Materials. Unless otherwise noted, all chemicals were obtained from commercial vendors and used without purification. Dipotassium (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N,N*-diacetate (nitroxide **1**) was synthesized as previously described (Rosen et al., 2005). 3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **2**; Sigma-Aldrich, St. Louis, MO) was converted to the corresponding potassium salt by the addition of KOH. Nitroxide molecular structures are shown in Fig. 1. Cell culture reagents and biochemicals were from Invitrogen (Carlsbad, CA) or HyClone (Thermo Fisher Scientific, Waltham, MA). Chromatography resins were from GE Healthcare Life Sciences (Piscataway, NJ).

Cell Culture. Hc7 cells were cultured at 37°C under humidified atmosphere containing 5% CO₂. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 µg/ml Hygromycin B.

FIG. 1. Molecular structures of nitroxides used in the study presented here.

EPR Spectroscopy. EPR spectra were recorded on an X-band EPR spectrometer (model E-109; Varian, Inc., Palo Alto, CA). Instrumental settings were as follows: microwave frequency, 9.55 GHz; microwave power, 20 mW; magnetic field center, 333.5 mT; time constant, 0.5 s; modulation frequency, 100 kHz; modulation amplitude, 0.05 to 0.2 mT. The magnetic field was scanned from 0.8 to 8 mT at a rate of 2.67 mT/min. Instrument control and data digitization were performed with EWIN32 (Scientific Software Solutions, Plymouth, MI). Reported spectral intensity is the intensity of the center peak in the nitroxide triplet spectrum.

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore. Female NIH Swiss or NOD SCID mice (6–10 weeks; Harlan, Indianapolis, IN) were used for experimentation. Mice were housed under positive pressure on an alternating 12-h light/dark cycle and given free access to food and water. SCID mice were previously ovariectomized by the vendor. Isoflurane inhalant was used for anesthesia (2.5% in O₂ for induction, 2.0% in O₂ for maintenance). Euthanasia was performed by asphyxiation with CO₂ followed by cervical dislocation.

Liposome Preparation. Classical, sterically stabilized immunoliposomes were prepared as described previously (Burks et al., 2009, 2010b). In brief, all liposomes were primarily composed of 1,2-distearoylphosphatidylcholine and cholesterol at a molar ratio of 3 distearoylphosphatidylcholine to 2 cholesterol. Sterically stabilized liposomes also incorporated 5 mol % ammonium 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[polyethylene glycol 2000] (PEG-PE). The formulation of anti-HER2 immunoliposomes was identical to sterically stabilized liposomes except for the inclusion of ammonium 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[maleimide-polyethylene glycol 2000] at 1 mol% to link trastuzumab Fab' fragments. All lipids were from Avanti Polar Lipids (Alabaster, AL). All liposomes were prepared to have a mean outer diameter of 100 nm and encapsulated 150 mM nitroxide **1**.

Clearance of Nitroxide-Containing Liposomes. NIH Swiss mice were placed in a restrainer (Braintree Scientific, Braintree, MA) and administered nitroxide **1** (25 nmol/g b.wt.) via tail vein injection. Nitroxide was encapsulated in classical liposomes or sterically stabilized liposomes, or it was used as free (unencapsulated) nitroxide in Dulbecco's phosphate-buffered saline (DPBS). Injection volumes were ~100 µl. At various times, blood (~10 µl) was collected into heparinized microhematocrit capillary tubes (Thermo Fisher Scientific) and then diluted into 1 ml of deionized water (resistivity = 18.3 MΩ-cm). Diluted blood samples were subjected to three freeze-thaw cycles to ensure lysis of liposomes. Samples were measured by EPR spectroscopy to determine nitroxide content. Each sample was also measured for plasma sodium content using a sodium-selective electrode (Thermo Fisher Scientific), and nitroxide content was normalized to plasma sodium content. To determine self-quenching of liposome-encapsulated nitroxides in circulation, a blood sample (~25 µl) was taken 24 h after injection with sterically stabilized liposomes and diluted into 450 µl DBPS. The same sample was immediately measured by EPR spectroscopy before and after the addition of Triton X-100 (0.5% v/v).

Establishing Hc7 Xenografts. At least 48 h before inoculation with Hc7 cells, biodegradable, time-release estrogen pellets (2.5 mg/90 days; Innovative Research of America, Sarasota, FL) were subcutaneously implanted into the shoulders of SCID mice. Hc7 cells were cultured to ~90% confluence in 150-cm² culture flasks. Cells were washed thrice with DPBS and then removed from the flask by incubation at 37°C in a solution containing 100 mM KCl, 50 mM sodium citrate (pH 7.0), and 0.5 mM disodium dihydrogen EDTA.

Collected cells were washed twice with DPBS and suspended in DPBS to a final concentration of 2×10^7 cells/ml. Approximately 2×10^6 cells were subcutaneously injected into each rear leg of a SCID mouse. Each week, tumor sites were inspected for palpable tumors, and volume was determined using radial measurements performed with calipers. Tumors were measured in two dimensions, length and width, and volume (V) was calculated by the formula $V = 4/3\pi r_1 r_2$, where r_1 is one half of the smaller of the two tumor dimensions and r_2 is one half of the larger dimension.

Biodistribution of Nitroxide-Loaded Immunoliposomes and Uptake by Hc7 Tumors. SCID mice bearing Hc7 xenograft tumors ($>200 \text{ mm}^3$) were administered anti-HER2 immunoliposomes containing 5 mol % PEG-PE and encapsulating nitroxide **1** by tail vein injection. Mice received immunoliposomes at 25 nmol nitroxide/g b.wt. At various times, mice were euthanized, placed on ice, and the vasculature was perfused with ice-cold DPBS to displace blood. DPBS was pumped through a 27-gauge butterfly needle inserted into the left ventricle. Part of the right atrium was excised to allow drainage. Animals were perfused until clear saline emerged from the right atrium (~ 5 min). Tumors were then excised and weighed. Each sample was homogenized in 3 ml DPBS and sonicated for 30 s. Tumor homogenates were measured by EPR spectroscopy for nitroxide content. Maximal tumor-associated nitroxide signal occurred 3 h after injection (see *Results*). Therefore, to assess biodistribution of nitroxides at that time point, other SCID mice were injected with immunoliposomes, and after 3 h, liver, spleen, kidney, brain, lung, and muscle were harvested, weighed, and measured for nitroxide content by the same protocol as that for the tumors.

Reduction of Nitroxides by Tissue Homogenates. NIH Swiss mice were euthanized, and kidney, liver, and spleen were harvested. Tissues were washed with DPBS and maintained on ice. Tissue samples were weighed and then homogenized in DPBS (0.210 mg tissue/ml DPBS). Homogenates were pulse-sonicated for 10 s at 20 W (Sonifier 450; Branson Ultrasonics Corporation, Danbury, CT). At time 0, 5 μl of a stock solution of nitroxide **2** (40 mM in water) was added to 400 μl of the freshly prepared homogenate (final nitroxide concentration of 500 μM). The mixture was immediately transferred to an EPR cuvette that had previously been purged with N_2 and sealed. Nitroxide reduction proceeded at room temperature (23°C), and at various times, EPR spectra were acquired. To measure nitroxide reduction by Hc7 tumors, Hc7 cells were used in place of dissected tumors because tumors sometimes showed necrotic interiors, which would have given less consistent bio-reduction. Cultured Hc7 cells were detached from culture flasks by treatment with trypsin and centrifuged (805g). The pellet was weighed and suspended in DPBS at the same concentration as harvested tissues. Cells were lysed by sonication for 60 s in a bath sonicator (80 W power; Laboratory Supplies Company, Hicksville, NY), and nitroxide reduction was measured following the same procedure as that used for other tissues. Each time course was measured in duplicate, and plotted points are averaged measurements.

Data Analyses. OriginPro 8 (OriginLab Corp., Northampton, MA) and CorelDraw X4 (Corel Corporation, Ottawa, ON, Canada) were used for data analyses and presentation. Mean values are plotted and error bars represent S.D. One-way analysis of variance (ANOVA) was used to determine significance among data sets. Differences above the 95% confidence interval ($p < 0.05$) were considered significant. Bonferroni tests were used for post hoc analyses. Exponential time courses were fit with first-order exponential functions. For nitroxide reduction measurements, irregular measurement intervals in the repeated measurements were regularized by interpolation after fitting a second-degree polynomial function to the three nearest points. The initial value (at time 0) of each time course was extrapolated by linear least-squares analysis performed on the first three data points. Data points in each curve were then normalized to the extrapolated initial value.

Results

Circulatory Clearance of Nitroxide-Containing Liposomes. To assess the improvement in circulatory retention of sterically stabilized liposomes, mice were given classical liposomes (lacking PEG-PE) or sterically stabilized liposomes (containing 5 mol % PEG-PE) that both encapsulated 150 mM nitroxide **1**. At various times, blood samples were drawn from the mice, and intact liposomes in the blood were lysed by repeated freeze-thaw cycles; nitroxide content in each sample

was then measured. Clearance results are summarized in Fig. 2. The clearance time course of classical liposomes is best fit by a first-order exponential decay function having a time constant ($\tau = t_{1/e}$) of 6.9 ± 1.1 h, which is equivalent to a half-life of $t_{1/2} = 4.8 \pm 0.8$ h ($t_{1/2} = \ln 2 \times \tau$). Clearance is more than 95% complete after 3τ , or ~ 20 h for classical liposomes. However, sterically stabilized liposomes remained in circulation 2.3-fold longer ($\tau = 16.0 \pm 3.2$ h, or $t_{1/2} = 11.1 \pm 2.3$ h). Thus, by incorporating PEG-PE into the liposomal formulation, even after 36 h, greater than 10% of the injected nitroxide signal is still present in the circulation.

Although background EPR signal in the circulation can be minimized by encapsulating quenched concentrations of nitroxides in liposomes, any nitroxide that has leaked from liposomes would become dequenched and thus contribute unwanted background signal in the circulation. We expected signal from leaked nitroxides to be minimal because renal filtration should efficiently remove small ionic solutes such as nitroxide **1** from circulation. Indeed, when mice were injected with free nitroxide **1** (not encapsulated in liposomes), clearance was very rapid ($\tau = 2.88 \pm 0.34$ min, $t_{1/2} = 2.00 \pm 0.24$ min). Therefore, extraliposomal nitroxide is unlikely to contribute significant background signal in the circulation.

Although nitroxides leaked from circulating liposomes are cleared rapidly, a potential problem arises if liposomes lose sufficient nitroxide so that the spectroscopic signal of the nitroxides that remain entrapped becomes incompletely quenched. In this case, the circulating liposome itself would become the source of unwanted background signal. Analysis of blood collected 24 h after injection with sterically stabilized liposomes demonstrates that they are exceptionally stable in vivo (Fig. 3). Blood samples were measured by EPR spectroscopy before and after liposomal lysis. The robust postlysis spectrum shows ~ 50 -fold greater amplitude than the prelysis spectrum; this leads to three significant inferences. First, the robust postlysis spectrum shows that a significant number of liposomes remain in circulation after 24 h. Second, the minimum prelysis spectrum demonstrates that circulating liposomes have retained enough nitroxide to exhibit essentially com-

FIG. 2. Clearance of free and liposomally encapsulated nitroxides. Circulatory clearance of nitroxide **1** was measured after intravenous injection in mice ($n = 6$). Injections were of nitroxide **1** encapsulated in sterically stabilized liposomes ($\square$; solid curve is single-exponential fit), encapsulated in classical liposomes ($\circ$; dashed curve), or free in solution ($\triangle$; dotted curve). Addition of 5 mol% PEG-PE to form sterically stabilized liposomes increases the exponential lifetime ($t_{1/e}$) of nitroxides in circulation from ~ 7 to 16 h. Unencapsulated nitroxide is cleared from circulation with an exponential lifetime of ~ 3 min. Where not shown, error bars are smaller than symbols.

FIG. 3. Liposomally encapsulated nitroxides exhibit self-quenching after 24 h in circulation. Blood was drawn from mice 24 h after intravenous injection of sterically stabilized liposomes encapsulating 150 mM nitroxide **1**. The prelysis EPR spectrum (red trace) shows minimal nitroxide signal. However, after liposomes in the same blood sample were lysed and the EPR spectrum was recorded again (black trace), robust nitroxide signal was observed. This verifies the presence of intact liposomes that still encapsulate high concentrations of nitroxide as well as the absence of leaked nitroxides in circulation.

plete quenching of the EPR signal. Third, the prelysis spectrum confirms what is shown in Fig. 2: that if any nitroxide did escape the liposome, it was efficiently removed from the circulation and did not contribute a background signal.

Before *in vivo* tumor targeting experiments can be performed, Hc7 xenograft tumors must be generated. Hc7 cells were subcutaneously injected into both flanks of SCID mice ($n = 5$), and cell proliferation was stimulated with estrogen. Tumors were established at all injection sites ($n = 10$). The tumors became palpable within 1 week and reached an average volume of 300 mm³ in <2.5 weeks (Fig. 4A). At 5 weeks after implantation, the average tumor volume was almost 2000 mm³ (representative mouse shown in Fig. 4B).

EPR imaging of Hc7 tumors should be performed when accumulated nitroxide signal in the tumors is maximal. To investigate this, Hc7-tumor-bearing mice were administered anti-HER2 immunoliposomes encapsulating 150 mM nitroxide **1**. At various times, mice were euthanized and the vasculature was perfused with DPBS to remove circulating liposomes. Tumors ($n = 5$ –8) were excised at each time point, rapidly homogenized, and the tissue homogenate was immediately measured by EPR spectroscopy to determine tumor-associated nitroxide content. As shown in Fig. 5, tumors accumulated maximal nitroxide (~ 5 nmol/g) by 3 h postinjection. The measured EPR signal persisted until 6 h, and then declined. By 9 h postinjection, nitroxide signal decayed to approximately 35% of the maximum measured at 3 h (ANOVA $F_{4,26} = 7.30$).

FIG. 5. Tumor-associated nitroxide EPR signal. SCID mice bearing Hc7 tumors ($n = 5$ –8) were intravenously injected with anti-HER2 immunoliposomes containing 5 mol% PEG and encapsulating nitroxide **1**, and nitroxide signal accumulation in the tumors was monitored. Maximal signal is reached by 3 h, persists through 6 h, and declines afterward. *, statistical significance. Groups marked by identical symbols are statistically similar to one and other and are different from groups marked by other symbols. ANOVA $F_{4,26} = 7.30$.

Knowing that Hc7 tumors reach peak EPR signal intensities by 3 h, we measured the distribution of nitroxides in other tissues at that time and determined the nitroxide content associated with other tissues. Measurable nitroxide accumulated in tumor tissue at a concentration of ~ 5 nmol/g at 3 h postinjection (Fig. 6). Organs that are associated with clearance of nitroxides and liposomes also accumulate measurable nitroxide content: ~ 10 nmol/g in the kidneys, ~ 2.5 nmol/g in the liver, and ~ 30 nmol/g in the spleen. On the basis of the weight of each organ, the fraction of the total injected dose of nitroxides that appears in each tissue is $\sim 2.0 \times 10^{-3}\%$ in the tumors, $\sim 3.5 \times 10^{-3}\%$ in the kidneys, $\sim 1.5 \times 10^{-3}\%$ in the liver, and $\sim 3.0 \times 10^{-3}\%$ in the spleen. Other tissues that would not be expected to interact with liposomes (e.g., brain, muscle, and lungs) did not have any measurable nitroxide content.

Lastly, because nitroxides are susceptible to metabolism in tissue, the observed nitroxide signal in any tissue depends on liposomal delivery to and nitroxide metabolism by that tissue. Therefore, we measured the relative rates of nitroxide signal decay in each tissue. Freshly homogenized tissue was mixed with nitroxide and sealed in a cuvette under inert atmosphere; EPR signal intensity was monitored

FIG. 4. Hc7 xenograft model. SCID mice ($n = 5$) were subcutaneously inoculated with a suspension of Hc7 cells, and tumor growth was stimulated with estrogen. A, the tumor growth curve: smooth curve, least-squared fit of the data to a single-exponential function; dashed line, time at which the average tumor volume reached 300 mm³. Where not shown, error bars are smaller than symbols. B, representative mouse 5 weeks after inoculation: arrows, tumors on each side of the mouse.

FIG. 6. Biodistribution of nitroxides 3 h after injection of nitroxide 1 encapsulated in anti-HER2 immunoliposomes. SCID mice bearing Hc7 tumors ($n = 4$) were intravenously injected with immunoliposomes encapsulating 150 mM nitroxide 1. After 3 h, mice were euthanized and the vasculature was perfused with saline. Tissues were harvested, homogenized, and analyzed for nitroxide content. Tumor, kidney, spleen, and liver tissue accumulated nitroxide to varying extents, whereas brain, muscle, and lung had no detectable nitroxide (indicated by n.d.). * and †, statistical significance. Groups marked by identical symbols are statistically similar to one and other and are different from groups marked by other symbols. ANOVA $F_{6,28} = 16.13$.

over 3 h (Fig. 7). It was not surprising to note that the nitroxide signal disappeared most rapidly in the liver and kidney homogenates. After 3 h, only 5 and 15% of the original nitroxide signal was detectable in the liver and kidney homogenates, respectively. In contrast, at the same time point, 50% of the nitroxide signal was still detectable in the spleen sample, and ~65% was measured in the Hc7 homogenate.

Discussion

We have demonstrated that administering sterically stabilized anti-HER2 immunoliposomes encapsulating quenched concentrations of nitroxide 1 to mice bearing Hc7 tumors results in accumulation of nitroxide in the tumors. We have shown that encapsulation in sterically stabilized liposomes enables nitroxides to remain in the circulation for days. Furthermore, these liposomes are sufficiently biostable to maintain essentially maximal quenching of the EPR signal of encapsulated nitroxides. Such stability is important for minimizing background signal in the circulation to make high-contrast imaging possible. We show that the nitroxide signal in Hc7 tumors was maximal after 3 h, which allows imaging studies to be conducted shortly after nitroxide administration. At that time point, tissues that were not expected to interact with the immunoliposomes (e.g., brain, lung, and muscle) showed no measurable nitroxide signal. It was not surprising to note that organs that mediate clearance of nitroxides and liposomes (liver, kidney, and spleen) did accumulate nitroxide signal. Importantly, the total amount of nitroxide detected in tissues by 3 h postinjection (<1% total nitroxide dose) is only a small fraction of what has been cleared from the circulation at that time (~20% total dose). The fact that these nitroxides are exceptionally resistant to bioreduction in the blood/plasma (Burks et al., 2009; Miyake et al., 2010) and are further protected from metabolic degradation by being encapsulated in liposomes implies that most cleared nitroxides are subjected to one of two fates: metabolic degradation by the liver and kidney (Fig. 7) or possibly excretion by the kidneys into the urine.

The biodistribution of nitroxides almost completely agrees with previous biodistribution measurements of ^{67}Ga encapsulated in anti-HER2 immunoliposomes (Kirpotin et al., 2006). However, the quantities of tumor-associated nitroxide and ^{67}Ga are different with respect to the amount in the liver and kidneys. Compared with nitroxides, ^{67}Ga accumulated more in the liver but less in the kidney. This result is of interest because signal intensity differences between tissues facilitate differentiation of those tissues in imaging. Tumor-associated nitroxide signal is statistically similar to that associated with liver and kidneys, whereas tumor-associated ^{67}Ga is statistically different from that in the liver and kidneys. It is convenient that in the current Hc7 model, the tumors are spatially distinct from the liver and kidneys, which enables the different tissues to be readily discerned in images.

In the liver, which is highly metabolically active, nitroxides are reduced to the corresponding hydroxylamines, which do not produce EPR signal (Rosen and Rauckman, 1977; Kocherginsky and Swartz, 1995). Although the nitroxides used in this study were specifically designed to resist bioreduction (Keana et al., 1987; Halpern et al., 1996), bioreduction can still occur. This could account for relatively less nitroxide being found in the liver compared with ^{67}Ga , the clearance of which from the liver occurs over days to months (Dudley et al., 1949). The relationship between molecular structure and susceptibility to reduction has been extensively studied in nitroxides (Keana et al., 1987). If nitroxides could be designed to increase reduction in the liver without a corresponding change in tumor tissue, then EPR signal from liver can be markedly reduced. This would enable tumor and liver to be distinguished more readily on the basis of signal intensity.

Nitroxides and ^{67}Ga also accumulate differently in the kidneys. Administration through immunoliposomes results in relatively more nitroxide than ^{67}Ga in the kidneys. One potential explanation is partial leakage of nitroxides from circulating liposomes, which previous studies have suggested is a possibility (Burks et al., 2009). Such leaked nitroxides, being small anions, would be filtered by, and thus

FIG. 7. Decay of nitroxide signal in different tissues. Nitroxide 2 was added to freshly prepared tissue homogenates under inert atmosphere, and EPR signal intensity was monitored over 180 min. Nitroxide signal disappeared relatively quickly from the liver and kidney samples. Only 5 and 15% of the original nitroxide signal was detectable in the liver and kidney sample, respectively, after 180 min. Signal was more persistent in the spleen and Hc7 samples, which retained 50 and 65%, respectively, of the original nitroxide signal after 180 min. Measurements were repeated twice in random order, and each point is the average of two measurements.

appear in, the kidneys. A potential solution would be to modify the nitroxide molecular structure to improve entrapment within liposomes. The most straightforward approach would be to increase the number of ionic groups on the nitroxide.

The amount of nitroxide observed in tumors after 3 h is ~ 5 nmol/g, which corresponds to a concentration of ~ 5 μ M. Our preliminary imaging studies of Hc7 tumors and previous studies (Burks et al., 2009, 2010b) suggest this concentration should be just visible in reconstructed images, which have the benefit of signal averaging to increase signal-to-noise ratio (SNR). Several recent advances in EPR imaging technology should markedly improve the ease of tumor imaging with nitroxides delivered through immunoliposomes. First, the intrinsic SNR of nitroxides can be improved by more than 7-fold through systematic ^{15}N and ^2H isotopic substitution in the nitroxides (Burks et al., 2010a). In addition, the development of rapid-scan continuous-wave acquisition permits megagauss-per-minute scanning by the imaging spectrometer—an improvement of 5 orders of magnitude over current scanning speeds. This should afford markedly improved SNR through signal averaging (Quine et al., 2010; Tseitlin et al., 2010).

In total, our results lay the groundwork to use sterically stabilized immunoliposomes to deliver nitroxides to HER2-overexpressing breast tumors in vivo. By characterizing the pharmacological properties of nitroxides in liposomes, we have demonstrated that only modest improvements are required to enable high-contrast EPR images of Hc7 tumors in vivo. These studies are currently underway in our laboratory.

Authorship Contributions

Participated in research design: Burks, Rosen, and Kao.

Conducted experiments: Burks, Legenzov, and Kao.

Contributed new reagents or analytic tools: Rosen.

Performed data analysis: Burks and Kao.

Wrote or contributed to the writing of the manuscript: Burks, Rosen, and Kao.

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