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In vivo EPR imaging of differential tumor targeting using *cis*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl

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Abstract

Purpose—EPR spectroscopy promises quantitative images of important physiologic markers of animal tumors and normal tissues, such as pO₂, pH, and thiol redox status. These parameters of tissue function are conveniently reported by tailored nitroxides. For defining tumor physiology, it is vital that nitroxides are selectively localized in tumors relative to normal tissue. Furthermore, these paramagnetic species should be specifically taken up by cells of the tumor, thereby reporting on both the site of tumor formation and the physiological status of the tissue. This study investigates the tumor localization of the novel nitroxide, *cis*-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl **3** relative to the corresponding di-acid **4**.

Methods—We obtained images of nitroxide **3** infused intravenously into C3H mice bearing 0.5-cm³ FSa fibrosarcoma on the leg, and compared these with images of similar tumors infused with nitroxide **4**.

Results—The ratio of spectral intensity from within the tumor-bearing region to that of normal tissue was higher in the mice injected with **3** relative to **4**.

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Conclusion—This establishes the possibility of tumor imaging with a nitroxide with intracellular distribution and provides the basis for EPR images of animal models to investigate the relationship between crucial aspects of tumor microenvironment and malignancy and its response to therapy.

Keywords

Nitroxides; Electron Paramagnetic Resonance Imaging

Introduction

Low-frequency electron paramagnetic resonance imaging (EPRI) is a magnetic imaging modality that can image the concentration of injected spin probes that report physiological aspects of tissue and tumor microenvironment (1). EPRI is a low-background magnetic imaging modality that is complementary to magnetic resonance imaging (MRI), which routinely affords exquisite anatomic definition of tissues. MRI produces a number of important images pertinent to tissue microenvironment and homeostasis. These include blood oxygen level-dependent (BOLD) MRI (2), which provides qualitative images of O₂ distribution in tissues and tumors (3–5), diffusion weighted MRI, which demonstrates vascular and neuronal orientation, (6), and dynamic contrast enhanced MRI, which images local tissue and tumor vascular volume and vascular permeability (7,8). In contrast, EPRI with well-designed spin probes, such as trityl radicals and nitroxides (9), can measure, in local microenvironments of tissues and tumors, quantitative parameters such as pO₂, (10–13), pH, (14,15), temperature (16), thiol concentration (17), and microviscosity (18), a measure of the ease of diffusion of small molecules, and redox status of cells, as estimated by the ease of disulfide reduction by GSH in the tissue (19). EPRI with certain nitroxides can also provide information about the perfusion and viability of tissues (20). Nitroxides have also been synthesized to target tissue compartments via functionalized lipophilic or hydrophilic moieties (21). For instance, isotopic-substituted nitroxides and EPRI (22,23) have resolved the paradoxical elevated pH reported using ³¹P MRI (24) with measurements of specifically extracellular pH showing expected acidosis in hypoxic tumors. The validity of some EPRI measurements has been demonstrated by comparison with other measurement techniques. For example, EPRI pO₂ measurements have been corroborated by spatial and quantitative correlation with well-established OxyLite probe measurements (25).

A number of years ago, we synthesized (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-yl-methyl)amine-*N,N*-diacetic acid diacetoxymethyl ester **1**, which was hydrolyzed by intracellular esterases in lymphocytes to (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-yl-methyl)amine-*N,N*-diacetate **2** (26) (Fig. 1). Concentration of intracellularly entrapped nitroxide **2** was sufficiently large to allow EPR imaging of cells at a resolution of 3 mm, which gave rise to the possibility of localizing labeled cells *in vivo* (27). Because the vasculature of tumors is both chaotic and permeable (28–30), we believed that a structural modification of nitroxide **1** could allow sufficiently high concentration of a nitroxide to be entrapped in the cells of a tumor, sufficient to permit the topography of the tumor to be mapped by EPRI.

Until recently, it was not clear what novel nitroxides might localize to tumors. A hint came from studies we reported five years ago. We found that the *trans* isomer of **3** crossed the blood-brain barrier of mice. After *in situ* hydrolysis, high local concentrations of the *trans* diacid **4** were entrapped in brain tissue (31). These findings suggested that a minor structural modification of the pyrrolidinyloxy ring might increase specificity of delivery to tumors. In a series of pilot experiments, however, we found that the *cis* isomer, rather than the *trans* isomer, was better suited for the tumor imaging experiments described in this paper.

Herein we report that the EPR imaging showed nitroxide **3** to be distributed at high concentration within the tumor. In contrast, nitroxide **4**, which is predominately dianionic at physiologic pH, is poorly taken up into the intracellular compartment of the tumor. In the present study, we did not analyze the EPR spectra of the nitroxides. Rather, spectral amplitudes were imaged to establish the distribution of the nitroxides *in vivo*. This is an essential first step towards spectroscopic imaging of tumor microenvironments. With tumor targeting established, spectroscopic images can then be the basis for investigating the physiological status of tumors and normal tissues in the native state. The ability to image the tumor by *in vivo* EPR imaging can provide a quantifiable measurement of the heterogeneous environment of the tumor cell.

Methods

Nitroxides and Trityl Radical

cis-3,4-Di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **3** and *cis*-3,4-dicarboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **4** were synthesized as previously published (32). The structure of each compound is presented in Fig. 1. Narrow-lined trityl radical (OX063) was a gift from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Mice and Tumor Preparation

FSa methylcholanthrene-induced fibrosarcoma cells were provided as a kind gift from Kathy Mason of the MD Anderson Hospital and Tumor Institute, Houston TX. Cells were grown in the legs of 6–8 week old C3H mice. The tumors were surgically excised, minced, treated with trypsin and stirred to a consistent brie, strained, and centrifuged to a pellet. The dissociated tumor cells were resuspended in MEM and counted. One million cells in ~100 μ L of medium were injected intramuscularly into the gastrocnemius of the legs of C3H/HeN:Hsd female mice (Harlan Sprague Dawley, Indianapolis, IN). Tumors were grown to a diameter of ~10 mm in 10 – 14 days. Animals were anesthetized with 1 – 2 % isoflurane mixed with medical grade air. The fraction of isoflurane was adjusted based on the respiration amplitude and frequency, which were continuously monitored during image acquisition by direct observation and by monitoring the power reflected from the imager resonator. In addition to anesthesia, the leg bearing the tumor and the tumor were immobilized using vinyl polysiloxane (VPS) dental mold material (GC America Inc., Alsip, IL). This provided soft, rubbery but consistent immobilization to minimize animal motion during imaging. Body temperature was measured throughout imaging with a Physitemp thermocouple probe (Physitemp, Clifton, NJ) coupled to the mouse torso and maintained in the range of 36–38 °C using adjustable opposed heat lamps. Following imaging, skin

temperature at the installed tumor was measured. This temperature was in the range of 33–35 °C. In the tail vein of a mouse, 1 mL of a 4 mM solution (0.13 mmol/kg) of either nitroxide **3** or **4** in a 5:95 (v/v) mixture of DMSO and phosphate-buffered saline was injected as bolus over ~2 min and then continuously infused throughout imaging. Accumulation of nitroxide in the nearby animal bladder was minimized by the use of a double lumen catheter and a Harvard 22 syringe pump (Harvard Apparatus, Holliston, MA) to flush the bladder at 15 mL/hr (33). Total imaging time was 10 min.

Pharmacokinetic measurements for nitroxides **3** and **4** were conducted in the same manner as the imaging experiments. However, following the bolus injection there was no continuous infusion and EPR spectroscopy was performed, rather than imaging. During the bolus injection the EPR signal height (peak-to-peak amplitude of the EPR spectrum) increased accordingly. Signal height at time, $t = 0$, was recorded just before the bolus injection and represents the amplitude of the background noise present. Immediately following the bolus injection a maximum signal height was reached and the signal decay was recorded for ~ 60 min. A pharmacokinetic analysis was performed using a model independent approach (non-compartmental analysis) as well as modeling the signal versus time data by fitting to a one compartment open linear model with first order input using Pharsight Phoenix version 3.2 (Pharsight, Carey, NC). Non-compartmental parameters determined included the time of the maximum observed signal ($t_{\max}$). Model dependent parameters estimated were local signal elimination rate constant (k) and the elimination half-life ($t_{1/2}$).

All animal experiments were performed according to the U.S. Public Health Service “Policy on Humane Care and Use of Laboratory Animals” and the protocols were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP No 69681). The University of Chicago Animal Resources Center is an AALAC approved animal care facility.

EPR Spectroscopy and Imaging

Animal imaging and spectroscopy were performed with a radiofrequency (RF) EPR imaging spectrometer with an air core magnet. At 249.7 MHz operating frequency the magnetic field was 8.95 mT. Zeeman field modulation was accomplished with oversized Helmholtz coils operating at 4.98 MHz with a peak-to-peak amplitude of 0.10 mT. The resonator was a loop-gap resonator with a single loop and single gap, fabricated from ABS plastic and plated with ~12 μm of copper flashed with gold. With the mouse leg immobilized in a VPS cast and installed in the resonator, the resonator quality factor was $Q \approx 120$, and the resonator efficiency, $B_1 / \sqrt{W}$, was ~1.4. We operated with an RF power of 10 mW at the resonator. Acquisition of the fixed stepped gradient angle scans involved the acquisition of 256 data points every 0.001 s with the same lock-in time constant, $\tau_c = 0.001$ s. A single-pole 12 dB per octave lock-in filter was used. Four scans were averaged for each projection. The full spatial image of the nitroxide required 66 projections at different angles equally covering 2π solid angle. Three or four and in one case six 10-min images were obtained approximately every 20 min for each tumor. The gradient magnitude was 50 mT/m. With a spatial field-of-view of 3 cm, and the nitroxide splitting of approximately 1.5 mT, this gradient excludes additional spectral lines in the field-of-view. Images were reconstructed from the projections

using a filtered back-projection. Projections were filtered using a simple cutoff of the image response frequency to half of the Nyquist sampling limit. Images were acquired in 12 min, of which approximately 3 min was overhead due to pauses to allow fields to settle. Image resolution was approximately 3 mm based on the acquisition of images under similar conditions from 4.7-mm glass tubes loaded with nitroxide **3**. Pure spectral acquisitions were made under conditions that mimicked those under which the images were obtained. As a control, the EPR spectrum of each nitroxide (1 mM) in a phosphate-buffered saline was acquired. Although the same resonator was used as that for imaging, these samples produced slightly different acquisition conditions ($Q = 94$). Power was adjusted for equivalent B_1 and the same modulation amplitude was used so that this control spectrum reflects that used for imaging. The image was obtained using the central manifold of the 3-line EPR spectrum. Reported numerical values are mean $\pm$ standard deviation.

Tumor Localization in the EPR Image with Registered MRI

Because EPRI is not an anatomic imaging modality, it does not permit unambiguous assignment of EPR spectral signal specifically to tumor tissue. Therefore, another, more anatomically accurate image must be registered with the EPR image to distinguish voxels in tumor tissue from those in normal tissue. Anatomy from T_2 -weighted MR images were registered with the EPR images to permit voxel-by-voxel registered assessments of normal and tumor tissue physiology. Immobilization of the leg bearing the tumor, placement of fiducials for registration of the MR and EPR images, and the registration process itself have been described in detail (34,35). In the VPS cast, molded holes tightly accommodated two or three borosilicate capillaries that were 1 mm in ID and 3 – 4 cm in length. These tubes contained either 3 mM narrow-lined trityl radical, OX063, (for EPRI) or water (for MRI) to serve as fiducials for registration of EPRI with MRI.

T_2 -weighted MRI Imaging and Tumor Identification

Rapid Acquisition with Refocused Echoes (RARE) T_2 -weighted images were acquired at 4.7 T on an Omega Bruker/GE imager (Billerica, MA) with the following parameters: TR = 4000 ms, effective TE = 27 ms, FOV = 3.0 cm, matrix size = 256×256 , slice thickness = 0.75 mm, NEX = 2, rare factor = 4. MRI using the water fiducials was registered with the EPR image acquired with the OX063 fiducials. Initial registration estimate was done based on alignment of surfaces of the external fiducials. Then a manual fine-matching of the tumor EPRI and MRI leg/tumor surfaces was performed, going through several slices within the tumor volume in three directions. Compared to normal tissue, tumor was identified as that which had high T_2 contrast signal, was not of the morphology of normal muscle groups, bone or joint, and approximately conformed to the tumor caliper dimensions measured before the image was obtained. These criteria are commonly used in clinical MRI registered with CT scans to determine tumor contours for radiation therapy treatment planning (36).

Results

Shown in Fig. 2 is the 250 MHz EPR spectrum of an air-equilibrated aqueous solution of nitroxide **3**, acquired with sufficiently high field modulation to suppress the hydrogen super hyperfine splitting. Because the spectrum was obtained at a Zeeman field of 8.9 mT, which

is comparable to the nitrogen splitting, the spectral lines are not equally spaced, owing to Breit-Rabi effect (37). Extrapolated to high field, the equally spaced nitrogen manifold splitting is 1.625 mT.

The pharmacokinetic parameters, determined as described in the methods section, are shown in Table 1. The average signal elimination half-life was 21.4 ± 2.0 min for nitroxide **4** and 29.7 ± 3.6 min for nitroxide **3**. Both compounds were detectable for up to one hr after injection in all animals (Fig. 3). The fit used to determine the pharmacokinetics for each nitroxide is shown as the solid line in the plots in Fig. 3.

The relatively long half-lives for both nitroxides enabled us to perform EPR imaging after introducing nitroxide **3** ($n = 4$) or nitroxide **4** ($n = 4$) into mice. Typical slices from 3D EPRI images of a leg bearing a tumor after introducing nitroxide **3** or nitroxide **4** into the tail vein of a mouse and the corresponding T_2 weighted MRI anatomic images are shown in Fig. 4. Tumor localization was achieved by using high T_2 contrast from T_2 -weighted MRI as described in the Methods section. The tumor contours for each imaged animal are shown in Fig. 4. These data clearly show that the tumor uptake of nitroxide **3** is higher than nitroxide **4**. It can be seen that in the case of nitroxide **4**, the EPR image shows less uniformity and intensity in the tumor. This finding is consistent with what has been observed with FSa tumors imaged with the highly charged trityl radical, used for EPR oximetry (2).

The ratio of the nitroxide signal amplitude in image voxels inside the tumor volume to the amplitude from the voxels outside the tumor volume was evaluated for each of the four tumors. Over the course of the images, despite the variation in overall amplitude, the ratio of the tumor tissue intensity to the normal tissue intensity remained constant. The standard deviation of the tumor to normal tissue ratio for nitroxide **4** ranged from 2% to 9% for the four tumors. For nitroxide **3**, the standard deviation of the tumor to normal tissue ratio ranged from 15% to 30% among the tumors. The key measure of the selective uptake of nitroxide **3** relative to nitroxide **4** is the increased tumor to normal tissue ratio of their intensity in the EPR images obtained in this study. Overall, the signal amplitude ratio computed for tumor tissue to normal tissue was 2.2 ± 0.5 for nitroxide **3**, and 1.7 ± 0.2 for nitroxide **4** ($p = 0.1812$).

The differences in the tumor tissue to normal tissue amplitude ratio for nitroxides **3** and **4** were also compared by looking at smaller voxel clusters within each tumor. These clusters consisted of separate groups of $4 \times 4 \times 4$ voxels. The cluster dimensions represent a volume of $(2.7 \text{ mm})^3$, which corresponds to the resolution of these images as previously determined (38). This defines independent portions of the tumor. These clusters were counted as such if they contained at least 8 voxels within the tumor boundary. For each of these clusters, the average signal intensity was calculated and then normalized by the average signal intensity found outside of the tumor boundary and within the leg boundary, as determined by the registered anatomical MRI. This resulted in a total of 184 clusters for nitroxide **3** and 160 clusters for nitroxide **4** from all eight animals examined. The mean tumor tissue to normal tissue amplitude ratio for these clusters was 1.92 ± 0.11 for nitroxide **3**, and 1.74 ± 0.05 for nitroxide **4** ($p = 0.07$).

Discussion and Conclusions

The purpose of this study was to establish what structural features of a nitroxide-containing an acetoxymethoxycarbonyl group are important in designing EPR tumor imaging agents. Previously, we prepared both the *cis* and *trans* isomers of nitroxide **3** (39). We found that the *trans* isomer was able to cross the blood-brain barrier where, after *in situ* hydrolysis to the corresponding *trans*-dicarboxylate (the *trans* analogue of nitroxide **4**), it was entrapped in brain tissue (31,40). In preliminary studies, we found that the *trans* isomer of nitroxide **3** is much less soluble in aqueous medium than nitroxide **3** (the *cis* isomer). Indeed, the lower aqueous solubility of the *trans* isomer precluded its use in tumor imaging. Therefore, the *cis* isomer, nitroxide **3**, and the corresponding free-acid form, nitroxide **4**, were used in the studies reported here. The di-AM ester (nitroxide **3**) is essentially a pro-drug form of nitroxide **4**. Being neutral and relatively lipophilic, AM esters readily pass through the cell membrane. Once inside, abundant cellular esterases hydrolyze the AM esters to unmask carboxyl groups, which are anionic at physiologic pH, thus enabling the hydrolyzed form of the nitroxide to be trapped intracellularly (26,41). We have demonstrated that it is the AM ester function and not lipophilicity per se that enables intracellular accumulation of the nitroxide imaging agent (42). Therefore, nitroxide AM esters represent an attractive approach to load nitroxides into tumor tissue.

In this study, we first conducted pharmacokinetic measurements on nitroxides **3** and **4**. Nitroxide **3** has two acetoxymethyl (AM) ester groups at positions 3 and 4, whereas the corresponding positions in nitroxide **4** bear carboxyl groups, which are predominately anionic at physiologic pH (Fig. 1). We found that the half-lives of nitroxides **3** and **4** in FSa tumors were sufficiently long to enable EPR imaging (Fig. 3, Table 1).

The important finding from this study is seen in Fig. 4. This shows two representative slices of 3D EPR images from tumors obtained from each of the tumor-bearing mice that have received either nitroxide **3** or nitroxide **4**. This robust and persistent nitroxide signal has allowed the imaging of the C3H mouse bearing an FSa fibrosarcoma in approximately 9 min. Interestingly, both nitroxides accumulate in the tumor; however, compared to nitroxide **4**, nitroxide **3** preferentially accumulates in the tumor region, as judged by the spectral intensity images. This result approaches significance, indicated by $p = 0.07$. During imaging, the nitroxide imaging agent was continuously infused intravenously. An experiment in which an initial bolus injection of the nitroxide imaging agent is given while temporally subsequent images are acquired would be an ideal method to observe and compare how well nitroxides **3** and **4** are retained in tumor and/or normal tissue over time. However, with current EPRI it is necessary to use the continuous infusion methodology to ensure acceptable image quality. Future studies will focus on using AM ester forms of nitroxides with longer intracellular retention times, which may allow an initial bolus injection methodology to be used in order to observe the kinetics of interest.

The differential localization of nitroxides **3** and **4** is likely attributable to chemical differences that lead to different behavior *in vivo*. Nitroxides **3** and **4** can both extravasate from the tumor vasculature into the tumor tissue. But whereas dianionic nitroxide **4** is confined to the extracellular fluid, nitroxide **3** can enter the tumor cells and be converted

enzymatically into the dianionic form, which becomes entrapped within tumor cells. At physiological temperature, organic anion transporters efficiently extrude xenobiotic anionic species from cells. We have shown that nitroxides bearing two ionic groups have an intracellular half-life of ~20 min at 37 °C (26). Therefore, the concentration of intracellularly entrapped nitroxide depends on the balance between loading and extrusion. The extrusion kinetics depend on the number of ionic groups in the molecule, thus suggesting that nitroxide loading and retention in tumor cells can likely be improved by using AM ester forms of nitroxides bearing more than two ionic groups.

Previously, we described the EPR imaging of cells that had accumulated a carboxylic nitroxide through incubation with the corresponding esterase-labile acetoxymethyl (AM) ester (27). This immediately suggested the possibility of EPR imaging tumors in real time *in vivo* by loading tumors with a nitroxide AM ester. Preliminary studies suggested the di-AM ester nitroxide **3** as a candidate for *in vivo* imaging. The finding that application of nitroxide **3** gave EPR signal primarily within the tumor tissue (Fig. 4a and 4b), whereas for nitroxide **4**, the signal was distributed mainly in regions surrounding the tumor (Fig. 4c and 4d) is most encouraging. Future studies will be designed to access the intracellular and extracellular compartments of tumors to probe their physiological characteristics.

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Fig. 1.
Chemical structures of the nitroxides described herein.

Fig. 2.

250 MHz EPR spectrum of an aqueous solution of nitroxide **3**. The nitrogen manifold splittings are: low field line-central line: 1.448 mT; central line-high field line: 1.738 mT. The ~0.3 mT difference is characteristic of the nitroxide EPR spectrum at 250 MHz due to Breit-Rabi oscillations, the breakdown of the high field approximation.

Fig. 3. Pharmacokinetic plots in a FSa tumor-bearing leg of a C3H mouse showing compound elimination after iv bolus injection of (a) nitroxide **4** and (b) nitroxide **3**. Observed signal heights (data points) and the resulting fit (solid line) used to determine the pharmacokinetic parameters are shown.

Fig. 4.

Typical slices from 3D spatial EPR images of C3H mouse legs bearing 9 mm FSa fibrosarcomas after iv administration of either nitroxide **3** or **4** (top images), as well as the corresponding T_2 weighted MRI images used for anatomic information (bottom images). The tumor contour is obtained from the high T_2 contrast in the T_2 weighted MRI as described in the text. The leg contour is also from the MRI. EPR images were obtained 9 min after introducing nitroxide **3** or **4** into the tail vein of the mouse. The EPR image slices shown in (a) and (b) are obtained using nitroxide **3** and those shown in (c) and (d) are obtained using nitroxide **4**.

Table 1

Pharmacokinetic data for both nitroxides **3** and **4** showing elimination half-life ($t_{1/2}$), local signal elimination rate constant (k), and the time of maximum observed signal ($t_{\max}$).

Compound	$t_{1/2}$ (min)	k (min^{-1})	$t_{\max}$ (min)
Nitroxide 3	29.7 ± 3.6	0.0236 ± 0.0030	8.4 ± 5.2
Nitroxide 4	21.4 ± 2.0	0.0326 ± 0.0029	7.6 ± 3.0