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Comparison of Two Nitroxide Labile Esters for Delivering Electron Paramagnetic Resonance Probes into Mouse Brain

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Abstract

In vivo quantitation of O₂ in brain has been hindered by a lack of suitable imaging modalities. Development of low-frequency electron paramagnetic resonance spectrometers that can detect free radicals in animals in real time makes it feasible to image paramagnetic oximetry probes such as nitroxides in brain tissue. We have shown that masking the carboxyl group of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **1**) as an esterase-labile acetoxymethyl ester yields 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **2**). Nitroxide **2** can cross the blood-brain barrier and is then hydrolyzed *in situ* by esterases to regenerate nitroxide **1**, which becomes entrapped in brain tissue. Seeking to improve the loading of nitroxides into brain, we synthesized the more lipophilic pentanoyloxymethyl ester, 3-pentanoyloxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **3**). We report that the higher lipophilicity of nitroxide **3** does not significantly increase its ability to generate EPR signals in the mouse brain. Therefore, irrespective of whether nitroxide **2** or **3** was injected, similar levels of nitroxide were entrapped in brain tissue. These findings suggest that nitroxides **2** and **3** perform comparably well as pro-imaging agents for measuring O₂ distribution in brain.

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INTRODUCTION

Molecular oxygen is central to normal brain physiology. For example, it is the terminal electron acceptor for critical enzymes in the metabolic pathways of the neurotransmitters dopamine, serotonin, and norepinephrine. Changes in brain-tissue O₂ levels can directly impact function. In light of the significance of O₂ in brain physiology, there has been inadequate development of non-invasive O₂ measurements, which is partly attributable to the difficulty of quantifying O₂ *in vivo*. O₂ has been determined in biological systems by invasive methods such as Clark-type electrodes,¹ while non-invasive blood oxygen level-dependent (BOLD) MR imaging is limited to *qualitative* estimates of tissue O₂ levels.^{2–5} Alternative minimally-invasive imaging modalities need to be developed for *quantifying* brain O₂ *in vivo* in real time. This could provide insights into drugs of abuse, and how they affect O₂ utilization, thus potentially altering brain function.

Molecular oxygen, with two unpaired electrons, is paramagnetic. The unpaired electron spins influence those of other paramagnetic molecules such as trityl and nitroxyl radicals.^{4,6} Interactions with O₂ broadens the electron paramagnetic resonance (EPR) spectral lines of these compounds, and changes in their EPR linewidths have been used to estimate O₂ concentrations in homogenous solutions.^{4,7–10} The development of low-frequency EPR spectroscopy using nitroxides and trityl radicals makes real-time estimation of local O₂ concentrations feasible.¹¹ Difficulty in transporting O₂-sensitive probes across the blood-brain barrier has been an obstacle to developing EPR imaging for quantitative measurement of O₂ in brain.

In an initial paper on this subject, we reported that 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **2**, in contrast to 3-methoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **4**, is hydrolyzed by neuronal cellular esterases, thereby resulting in high levels of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **1** entrapped in brain tissue⁶ (Fig. 1: Fig. 2). Expanding upon this earlier work, we prepared nitroxides **5** and **6**, both bearing 3-acetoxymethoxycarbonyl groups (Fig. 1); *in situ* esterase hydrolysis led to accumulation of the resulting carboxylates in brain tissue. Nevertheless, neither of these paramagnetic compounds afforded an improvement over nitroxide **2** as a pro-EPR imaging agent.¹² One of the limitations of these compounds was that they were not designed to enhance lipophilicity, but rather they were synthesized to test the importance of ionic charges on the retention of the nitroxide in brain tissue after esterase hydrolysis. Building on the results reported by Miyake et al.,¹² we recently synthesized a family of labile 3-alkanoyloxymethoxycarbonyl nitroxide esters whose increased lipophilicity could enhance loading of nitroxide **1** into brain tissue. In particular, 3-pentanoylmethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **3**; Fig. 1) had 35-fold higher lipophilicity than nitroxide **2**. *In vitro* experiments showed that cells incubated with nitroxide **3** achieved the greatest intracellular accumulation of nitroxide **1**.¹³ Therefore, nitroxide **3** is a promising candidate for *in vivo* tests.

The aim of the present study is to determine how the lipophilicity of the labile ester affects nitroxide loading into brain tissue. We compared the amount of EPR signal accumulated in the brain after injection with the EPR pro-imaging agents, nitroxides **2** and **3**. Irrespective of which compound is administered, the accumulated EPR signals measured in the head are sufficient for EPR imaging of O₂ in brain tissue. After injection of nitroxide **2** or **3**, the EPR signal in the mouse head declined at similar rates. Additionally, the percentage of nitroxide signal entrapped in brain tissue was similar after injection of either nitroxide **2** or **3**. Therefore, despite its increased lipophilicity, nitroxide **3** was comparable to nitroxide **2** as a pro-imaging agent. In the light of these results, the next logical step to improve oxygen measurement by EPR imaging is to prepare deuterium- and ¹⁵N-substituted nitroxide labile

esters. Such nitroxides are expected to have narrower EPR spectral peaks and concomitantly greater signal, both of which should improve the sensitivity of oxygen measurement.

MATERIALS AND METHODS

Chemicals

All chemical reagents and solvents were of ACS grade or better, were from commercial vendors and were used without further purification. 3-Acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **2** and 3-pentanoylmethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy **3** were synthesized as described in the literature.^{13,14}

Kinetics of response of nitroxides **2** and **3** in mouse head

Mice (C57 strain, weighing 16 – 20 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Animal housing, care, and experimental procedures were in accordance with the guidelines of the University of New Mexico Animal Care and Use Committee. Mice were maintained under appropriate lighting conditions for 4 days with free access to food and water. Anesthesia was induced with 4% isoflurane inhalant in N₂O/O₂ (7:3) and maintained with 1% isoflurane in N₂O/O₂ (7:3). Animal core temperature was maintained at 37°C with a heating pad. Nitroxides **2** and **3** were prepared as 50 mM solutions in phosphate buffered saline (PBS) from a 0.5 M stock in 95% ethanol. Nitroxides were injected at 0.4 mmol/kg body weight. For iv injections, Tygon microtubing (0.010-in ID, 0.030-in OD; Saint Gobain PPL Corp., Bridgewater, NJ) was cannulated into the left femoral vein for dose administration.

Anesthetized mice (n = 3) were placed in a custom-made head resonator in an L-band EPR imaging system (E540L, Bruker BioSpin, Billerica, MA), and the resonator was tuned. Nitroxide was then administered through ip or iv injection. Neither nitroxide caused obvious changes in animal behavior or any signs of acute toxicity. EPR spectra were recorded immediately after injections at 1-min intervals for up to 60 min. Each acquired spectrum measures the total EPR signal in the mouse head, and is the sum of the signals from the administered nitroxide and the hydrolytically regenerated nitroxide **1**. The following EPR acquisition parameters were used: microwave frequency, 1.12 GHz; microwave power, 18 mW; center field, 395 G; modulation frequency, 50 kHz; modulation amplitude, 1.0 G. Intensity values represent peak-to-peak amplitudes of the center spectral peak in the nitroxide triplet spectrum. Typically, between nitroxide injection and acquisition of the first EPR spectrum there was an interval of 30 – 60 sec during which instrument tuning and optimization occurred.

Pharmacokinetic analysis

The EPR signal of nitroxides **2** and **3** in the brain was determined in each individual animal. Analysis was performed on the individual EPR signal versus time data of each animal following ip or iv dosing. Pharmacokinetic parameters (maximal plasma concentration, C_{max}; area under the concentration-time curve, AUC_{0-t}; and terminal elimination half-life, t_{1/2}) were derived using a model-independent approach (non-compartmental analysis) with WinNonLin software (version 5.2; Pharsight, Cary, NC). Using the model-independent approach, the area under the concentration-time curve over the sampling period of 60 min (AUC_{0-t}) was determined using the linear trapezoidal rule. The area was extrapolated to infinity (AUC_{0-∞}) using the terminal elimination rate constant (*k*). The elimination rate constant, *k*, was determined from the slope of the terminal log-linear portion of the concentration-time curve. The elimination half-life (t_{1/2}) was calculated as ln2/*k*. Peak concentration (C_{max}) and the time of C_{max} (t_{max}) were determined directly from the

individual observed EPR signal versus time data. Estimated pharmacokinetic parameters are reported as mean value $\pm$ SD.

Effects of whole blood on EPR spectrum of nitroxides **2** and **3**

Mice were anesthetized following the protocol outlined above. At ~15 min prior to blood collection, mice were given ip injections of heparin (1 U/g body weight in PBS). Blood (~1 mL) was collected from the right atrium with a 27 G needle into tubes containing an additional 10 U of heparin, and was immediately used for measurements. Nitroxide **2** or **3** (10 μ M) was incubated in the whole blood at 37°C for 10 min. Samples were transferred to a flat cell quartz cuvette, and placed in the cavity of an EPR spectrometer (model E-109, Varian Associates, Palo Alto, CA). EPR spectra were recorded at room temperature. Reference EPR spectra of nitroxides **2** and **3** (10 μ M in PBS) were also acquired. Instrument settings were as follows: microwave frequency, 9.55 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; sweep time, 25 G/min; and response time, 0.5 s.

Accumulation of EPR signal in mouse brain after injection of nitroxides **2** and **3**

Mice (C57 strain, weighing 16 – 20 g) were anesthetized and either nitroxide **2** or **3** was injected intravenously at a dose of 0.4 mmol/kg body weight using the protocol outlined above. EPR spectra of the mouse head were recorded at 1-min intervals immediately after injection. At either 10 or 20 min post-injection, the mouse was removed from the cavity for perfusion ($n = 3$ for each time point). The mouse was placed on ice and a 25 G butterfly needle was inserted into the left ventricle and part of the right atrium was excised to allow the blood to drain. PBS (2.0 – 2.5 mL/min; 4 °C) was injected with an infusion pump through the butterfly needle until clear saline emerged from the right atrium. The animal was returned to the spectrometer, and post-perfusion EPR spectra were recorded. The perfusion procedure took approximately 8 min on average. Instrument settings were: microwave frequency, 1.12 GHz; microwave power, 18 mW; center field, 395 G; modulation frequency, 50 kHz; and modulation amplitude, 1 G.

Hydrolytic stability of nitroxides **2** and **3** in serum

For each nitroxide, a 100 μ M solution in fetal bovine serum (FBS) was made by rapidly vortexing 25 μ L of 40 mM nitroxide in DMSO into 10 mL of FBS; the solution was maintained at 25 °C. At various times after mixing, 0.5-mL aliquots of the solution were transferred to 1.5-mL conical microcentrifuge tubes. Each tube then received 0.75 mL of ethyl acetate (EtOAc) and was shaken vigorously for 15 sec to extract unhydrolyzed ester. The tubes were centrifuged at $20,937 \times g$ for 2 min at 4 °C. From each centrifuged sample, 0.5 mL of the EtOAc phase was transferred to a new tube and reduced to dryness in a centrifugal vacuum concentrator (SpeedVac SVC100H; Savant Instrument, Inc., Farmingham, NY). Each dried sample was dissolved in 0.4 mL of water-DMSO mixture (9:1, v/v), and the solution was transferred into a quartz cuvette for quantitation of intact (EtOAc-extractable) nitroxide **2** or **3** by EPR spectroscopy (model E-109). Spectrometer settings were identical to those listed in the previous paragraph. The EPR spectral peak intensity of each sample was plotted against time and fitted to a single-exponential decay. Nonlinear least-squares curve fitting was performed with Origin software (OriginPro 8; OriginLab Corp., Northampton, MA). The exponential time constant, $t_{1/e}$, was converted to the half-life: $t_{1/2} = \ln 2 \times t_{1/e}$. The half-lives of the two nitroxides were $t_{1/2} = 34 \pm 6$ min for nitroxide **2** and $t_{1/2} = 49 \pm 7$ min for nitroxide **3** ($p = 0.039$). The data and fitted curves are presented as Supplementary Material.

Calibration of EPR spectrometer response

To ensure consistency in measurement, the EPR spectrometer is calibrated daily before experiments. Because the spectrometer detects the total number of unpaired electrons within the resonant cavity, a standard sample of a stable free radical is used for calibration. The free radical standard is DPPH (2,2-diphenyl-1-picrylhydrazyl), and is supplied by Bruker BioSpin, the manufacturer of the EPR spectrometer. Reproducible recording of spectral parameters (e.g. EPR signal intensity, line width, etc.) for the DPPH standard confirms proper functioning of EPR spectrometer.

Statistical analysis

Data are expressed as mean values $\pm$ SD. A Student's unpaired t-test was used to assess statistical differences.

RESULTS AND DISCUSSION

Previously, we found nitroxide labile ester **2** to be a viable EPR pro-imaging agent capable of delivering sufficiently high concentrations of nitroxide to brain tissue to permit EPR imaging.^{6,12} In continuing to improve nitroxide transport across the blood-brain barrier, we have recently synthesized a family of lipophilic nitroxide labile esters, and have identified nitroxide **3** as a potentially superior pro-imaging agent.¹³ Here we compare the abilities of nitroxide **2** and nitroxide **3** to cross the blood-brain barrier after injection in mice and lead to the accumulation of EPR signal in brain tissue.

In the first series of experiments, nitroxide **2** and nitroxide **3** were introduced into mice by either ip or iv administration (Fig. 3). Pharmacokinetic parameters derived from non-compartmental analysis (NCA) are presented in Table 1. No significant difference between the parameters for nitroxides **2** and **3** was observed with either route of administration. Kinetic profiles of response for nitroxide **2** and nitroxide **3** in the mouse head after ip injection are shown in Fig. 3, panel A ($n = 3$). Two observations are worth noting. First, after ip administration of nitroxide **2** or **3**, growth of EPR spectral signal in the mouse head was comparable, reaching similar maxima after 5.67 ± 0.58 min and 8.00 ± 4.36 min, respectively, even though nitroxide **3** is more lipophilic than nitroxide **2** by 35-fold.¹³ Second, clearance of nitroxides **2** and **3** was similar—the corresponding elimination half-lives were 24.1 ± 3.3 min and 20.4 ± 1.5 min, respectively ($p = 0.15$). After iv administration of nitroxide **2** or **3**, the maximal EPR signal measured in the mouse head was essentially identical, and was approximately twice that attained after ip injection (Fig. 3, panel B). The elimination half-life was $t_{1/2} = 17.8 \pm 0.6$ min for nitroxide **2** and $t_{1/2} = 22.8 \pm 7.4$ min for nitroxide **3** ($p = 0.31$). As the values in Table 1 indicate, brain exposure for nitroxide **2** as measured by EPR signal was higher for iv relative to ip administration: $C_{\max}$ was 876 ± 121 and 362 ± 95 for ip and iv injection, respectively ($p = 0.00447$). Similarly the $AUC_{0-\infty}$ for nitroxide **2** was greater following iv administration at $19,900 \pm 3,000$ versus $12,400 \pm 3,100$ for ip administration ($p = 0.0399$). Interestingly, a difference in half-life was also observed with $t_{1/2} = 17.8$ min following iv administration and a $t_{1/2} = 24.1$ min for ip administration ($p = 0.00078$). This difference in half-life is possibly due to delayed absorption from the peritoneum. As judged by AUC, brain exposure to nitroxide **3** was not statistically different for ip and iv administration. However differences in $C_{\max}$ and $t_{\max}$ were noted. The $C_{\max}$ for nitroxide **3** was 866 ± 132 and 427 ± 89 for iv and ip administration, respectively ($p = 0.00879$). Not surprisingly, $t_{\max}$ was less than 1 min following iv administration, whereas following ip administration $t_{\max}$ was 8.00 ± 0.36 min ($p = 0.0446$).

Nitroxide **3**, being more lipophilic than nitroxide **2**, could bind more avidly to serum proteins and thus potentially give rise to a spectroscopic artifact. This is because binding of nitroxides to proteins restricts the motion of the free radical and causes broadening of the EPR spectral lines, with corresponding reduction of spectral peak height.¹⁵ Since EPR spectral peak height was used as an index of nitroxide concentration in the mouse head, any nitroxide spectral line broadening would underestimate the actual nitroxide concentration in the head. To test this possibility, we measured the EPR spectral peak height of nitroxides **2** and **3**, each at 10 μ M in both PBS and whole blood. For nitroxides **2** and **3**, the ratio of the peak height in blood to that in PBS measured 0.907 and 0.870, respectively. That is, relative to PBS, the EPR spectral peak height in whole blood was reduced by 9.3% for nitroxide **2** and by 13% for nitroxide **3** ($n = 2$). In light of the fact that the two nitroxides do not differ in their susceptibility to bioreduction,¹³ these findings suggest that the greater lipophilicity of nitroxide **3** only slightly increased its binding to serum proteins. The minor difference in binding to blood components between nitroxides **2** and **3** constitutes a negligible perturbation in the present experiments.

In the studies presented in Fig. 3, the measured quantity is the total EPR signal in the mouse head, and represents the signals from the administered labile ester (i.e., nitroxide **2** or **3**) and the hydrolytically regenerated nitroxide **1**, which notably, are equally sensitive as O₂ sensors (see Supplementary Material). However, these studies cannot differentiate between a nitroxide molecule that is retained in the vasculature and one that has crossed the blood-brain barrier and become trapped in brain tissue. Therefore, we conducted experiments designed to measure the distribution of nitroxide in these two compartments. In a typical series of experiments, a group of mice received an iv injection of either nitroxide **2** or nitroxide **3**. EPR spectra were continually recorded from the head of each mouse, and at the 10 min or 20 min time points, blood was completely displaced from the vasculature by saline perfusion, and EPR spectra were again acquired from the head region ($n = 3$ animals at each time point). The EPR spectral signal measured after perfusion reveals the amount of nitroxide that became entrapped in brain tissue (Fig. 4). For both nitroxides **2** and **3**, the values representing the pre-perfusion signal were corrected for the EPR line-broadening effect of blood (see above). At 10 min after iv administration, as a percentage of the pre-perfusion EPR signal, the post-perfusion signal averaged $48 \pm 8\%$ and $66 \pm 9\%$ for nitroxides **2** and **3**, respectively ($p = 0.06$). At 20 min after iv administration, the corresponding values were $47 \pm 9\%$ and $62 \pm 11\%$, respectively ($p = 0.13$). Thus, for the purpose of generating EPR signal in the brain, nitroxide **3** is not a significant improvement over nitroxide **2**.

In view of the above findings, it is interesting to note that nitroxide **3** has higher hydrolytic stability than nitroxide **2**; the half-lives in serum are $t_{1/2} = 49 \pm 7$ min for nitroxide **3** and $t_{1/2} = 34 \pm 6$ min for nitroxide **2** ($p = 0.039$; see Materials and Methods). Despite its higher stability and lipophilicity, nitroxide **3** is not superior to nitroxide **2** for delivering nitroxide probes to brain tissue. The observed accumulation and persistence of EPR signals after injection of nitroxides **2** and **3** may be influenced by the physiology of the BBB, which is a physiologically complex barrier rather than just a physical barrier. Notably, several transport mechanisms exist in the BBB that result in efflux of xenobiotics from brain tissue. Transport of nitroxides may account for differences in the kinetics of response, and ultimate accumulation of each nitroxide in the brain. For example, the multi-drug resistant transporter, *P*-Glycoprotein (Pgp) is expressed on endothelial cells of the brain microvasculature. Pgp is capable of transporting a multitude of lipophilic substrates across the BBB back into the vasculature¹⁶ and could transport nitroxide-esters **2** and **3** at different rates. Additionally, after hydrolysis of either nitroxide ester in the brain, organic anionic transporters in the BBB could extrude the hydrolysis product, nitroxide **1**¹⁷ and thus cause lower levels of total nitroxide to be accumulated. In balance, nitroxide **3**, despite its superior

performance in *in vitro* cellular experiments, is not superior to nitroxide **2** as a pro-imaging agent *in vivo*. When administered in mice, both nitroxides **2** and **3** enable sufficient loading in brain to permit quantitative imaging of O₂ *in vivo*.¹⁸

Our long-term objective is to map changes in O₂ concentration in brain using EPR imaging. Before we can reach this goal, a pro-imaging nitroxide must be synthesized that delivers sufficiently high concentrations of nitroxide to be detected by EPR imaging. In the present study, we compared the ability of two pro-imaging compounds, nitroxides **2** and **3**, to deliver nitroxide into brain tissue. Despite the 35-fold increase in lipophilicity exhibited by nitroxide **3** as compared to nitroxide **2**, the data presented here suggest that nitroxide **3** is not more efficacious than nitroxide **2** as a pro-imaging agent. Current efforts are aimed at synthesizing deuterium- and ¹⁵N-substituted nitroxides, which are expected to have reduced EPR spectral line widths and concomitantly greater signal, both of which should improve the sensitivity of oxygen measurement. The isotopically substituted nitroxides can then be administered as labile esters *in vivo* for oxygen measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Pharmacokinetic parameters of nitroxides 2 and 3

Group	$C_{\max}$ (EPR signal*)	$t_{\max}$ (min)	$AUC_{0-\infty}$ ($10^3 \cdot \text{EPR signal} \cdot \text{min}$)	$t_{1/2}$ (min)
IP				
Nitroxide 2	362 ± 95	5.67 ± 0.58	12.4 ± 3.1	24.1 ± 3.3
Nitroxide 3	427 ± 89	8.00 ± 4.36	13.0 ± 1.4	20.4 ± 1.5
IV				
Nitroxide 2	876 ± 121	1.33 ± 0.58	19.9 ± 3.0	17.8 ± 0.6
Nitroxide 3	866 ± 132	0.667 ± 0.577	14.4 ± 2.7	22.8 ± 7.4

Mean ± SD (n = 3 animals per group)

* EPR signal is in arbitrary units that are a function of spectrometer settings