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A Novel Nitroxide is an Effective Brain Redox Imaging Contrast Agent and *in vivo* Radioprotector

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Abstract

Individuals are exposed to ionizing radiation during medical procedures and nuclear disasters, and this exposure can be carcinogenic, toxic, and sometimes fatal. Drugs that protect individuals from the adverse effects of radiation may therefore be valuable countermeasures against the health risks of exposure. In the current study, the LD_{50/30} (the dose resulting in 50% of exposed mice surviving 30 days after exposure) was determined in control C3H mice and mice treated with the nitroxide radioprotectors Tempol, 3-CP, 16c, 22c, and 23c. The pharmacokinetics of 22c and 23c were measured with magnetic resonance imaging (MRI) in the brain, blood, submandibular salivary gland, liver, muscle, tongue, and myocardium. It was found 23c was the most effective radioprotector of the five studied: 23c increased the LD_{50/30} in mice from 7.9 ± 0.15 Gy (treated with saline) to 11.47 ± 0.13 Gy (an increase of 45%). Additionally, MRI-based pharmacokinetic studies revealed that 23c is an effective redox imaging agent in the mouse brain, and that 23c may allow functional imaging of the myocardium. The data in this report suggest that 23c is currently the most potent known nitroxide radioprotector, and that it may also be useful as a contrast agent for functional imaging.

Keywords

Radioprotection; Redox Imaging; Blood-Brain-Barrier Permeable Contrast Agents; Nitroxides; Magnetic Resonance Imaging

Introduction

Cancer imposes an immense burden on society. In the United States alone, the year 2008 saw an estimated 1.4 million new cancer cases [1]. During the same year, cancer-related decreases in workforce productivity resulted in an estimated cost of \$130 billion to the American public [2]. It is well established that cancer can be both caused by and treated with ionizing radiation, and that radiation is not only carcinogenic but also toxic to non-cancerous normal tissues [3–4]. For example, during cancer radiotherapy, controlled doses of radiation are administered to the tumor, but the inevitable exposure of normal tissues can promote carcinogenesis and a variety of toxicities including, mucositis, xerostomia, and fibrosis [3–4]. Normal tissue toxicity is undesirable because it causes patient discomfort, causes non-

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adherence to the treatment schedule [5–7], and places upper-limitations on the dose safely deliverable to the tumor [8–12]. Not only does radiation exposure occur during medical procedures, but also during a nuclear power plant meltdown or during the detonation of a nuclear weapon. In these cases, the entire body is often exposed, and whole-body exposure can lead to acute hematological, gastrointestinal, and central nervous system damage, cancer, and death [3, 13]. Thus, radiation exposure can occur in medical and non-medical scenarios, and can result in both carcinogenesis and normal tissue toxicity.

A promising strategy for reducing normal tissue toxicity during both therapeutic and involuntary scenarios involves pre-exposure administration of a drug that ameliorates the toxic effects of radiation; such drugs are termed radioprotectors. The current study presents *in vivo* radioprotection, toxicity, and pharmacokinetic data for three novel nitroxide radioprotectors: 16c, 22c, and 23c. The data in this report show that the nitroxide 23c is currently the most potent known nitroxide protector against lethal doses of radiation in mice.

For a radioprotector to be useful during cancer radiotherapy, it must exhibit at least three properties: it must protect non-cancerous cells from radiation induced lethality, it must provide little or no protection for cancer cells, and its toxicity must not preclude its use in humans. The nitroxide Tempol has been shown to exhibit these three properties. Preclinical studies in mice have shown that administration of non-lethal doses of Tempol decreases the severity of xerostomia (reduced saliva output) after salivary gland irradiation and increases the LD_{50/30} for whole-body irradiation by 25% [14–16]. Furthermore, Tempol does not alter the radiation-induced re-growth delay of SCCVII, RIF-1, and HT-29 tumors [15, 17], suggesting that Tempol selectively protects normal tissues from ionizing radiation. Thus, in addition to serving as a countermeasure against public radiation toxicity during a nuclear disaster, Tempol and perhaps other nitroxides may also serve as clinical radioprotectors.

Nitroxides exhibit an additional useful property besides radioprotection: they are paramagnetic and their pharmacokinetics can therefore be monitored indirectly with magnetic resonance imaging (MRI). Imaging of nitroxide pharmacokinetics has two potentially useful biomedical applications. First, nitroxide imaging allows quantification of the nitroxide radioprotector concentration in tissues. For example, a recent study found that in anesthetized mice, the peak concentrations of Tempol in SCCVII, HT-29, and KHT tumors were less than in non-cancerous tissues such as the brain, rectum, and salivary gland. The greater concentration of Tempol in the salivary gland may explain the above observation that Tempol protects mice from normal tissue damage such as xerostomia [15–16], but does not protect SCCVII, HT-29, or RIF-1 tumors from radiation-induced re-growth delay [15, 17]. The second application of nitroxide imaging is redox imaging [18]. Nitroxide-based redox imaging relies on the *in vivo* redox reactions that occur between nitroxides, reactive oxygen species, and intracellular antioxidants [18]. These redox reactions result in a net $1e^-$ reduction of the nitroxide into a diamagnetic and non-contrast enhancing hydroxylamine. During *in vivo* imaging experiments, the conversion of the nitroxide to the hydroxylamine can be measured on a T₁-weighted MRI scan, and the resulting signal loss can be modeled as an exponential decay. In preclinical cancer models, the decay rate constant positively correlates with tumor glutathione levels [19–20]. Furthermore, preclinical models of oxidative stress caused by ultraviolet and x-ray irradiation, hyperoxia, diabetes, asbestosis, and stroke show that the exponential decay rate of nitroxides increases or decreases after oxidative stress [21–29]. Together, these studies demonstrate that nitroxides provide an imaging based assay of tissue redox status.

Based on the initial findings on the radioprotective effects of Tempol [14, 17, 30], a systematic *in vitro* survey of approximately 90 different nitroxide-related compounds was initiated with the purpose of identifying additional nitroxide radioprotectors [31]. The study

identified three new nitroxides that are more effective radioprotectors than Tempol: 16c, 22c, and 23c. The current study builds upon the *in vitro* findings of the systematic nitroxide survey, and measures the *in vivo* radioprotection capability and toxicity of the three new nitroxides. Additionally, this study reports the *in vivo* pharmacodynamics of two of the nitroxides, 22c and 23c, in the salivary gland, kidney, brain, leg muscle, blood, tongue, and myocardium. Finally, the observation is made that 23c readily passes the blood brain barrier (BBB), and the current study investigates 23c as a BBB-permeable redox-sensitive MRI contrast agent.

Methods

Chemicals

4-(*N*-methyl piperidine)-2,2,5,5-tetramethylpyrroline-1-oxyl (23c), 4-(*N*-methyl pyrrolidine)-2,2,5,5-tetramethylpyrroline-1-oxyl (22c), and 4-dimethylamino-2,2,5,5-tetramethylpyrroline-1-oxyl (16c) were synthesized according to reference [31]. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) and 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-CP) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Cell Culture

Chinese hamster V79 cells were grown in F12 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Survival was assessed by the clonogenic assay. The plating efficiency ranged between 80–90%. Stock cultures of exponentially growing cells were trypsinized, rinsed, and plated (5×10^5 cells/dish) into a number of 100 mm Petri dishes and incubated 16 hr at 37°C prior to experimental protocols. Cells were exposed to different concentrations of hydrogen peroxide (H_2O_2) for 1 hr at 37°C in the presence or absence of Tempol (1000 μM) or 23c (250 and 1000 μM), which was added to the cells immediately before hydrogen peroxide treatment. For radiation studies, nitroxides (final concentration of 10mM) were added to exponentially growing cells in complete F12 medium at room temperature (RT) 10 min prior to X-irradiation. The time required for irradiation (at RT) was approximately 10 min. Immediately after X-ray treatment, cells were rinsed, trypsinized, counted, and plated for macroscopic colony formation. Under these conditions, none of the nitroxides alone exerted cytotoxicity. Each dose determination was plated in triplicate, and experiments were repeated a minimum of two times. Plates were incubated 7 days; colonies were then fixed with methanol/acetic acid (3:1) and stained with crystal violet. Colonies containing >50 cells were scored. Error bars shown in the figures represent S.E. of the mean, and are shown when larger than the symbol. For *in vitro* radiation survival studies, the radiation dose resulting in 10% survival was calculated separately for nitroxide-treated and non-nitroxide-treated control cells. The *in vitro* dose modifying factor (DMF) was calculated as the ratio of the 10% survival doses between the treated and untreated cells. Analogously, for the *in vitro* H_2O_2 studies, the protection factor (PF) was determined as the ratio of hydrogen peroxide concentrations resulting in 10% survival between nitroxide-treated and untreated cells.

Cell Irradiation

Cells were irradiated at RT with a X-RAD 320 x-ray unit (Precision X-Ray, North Branford, CT) using 2.0 mm Al filtration (300 KVp) at a dose rate of 2.4 Gy/min. Full electron equilibrium was ensured for all irradiations.

Animal Studies: Radioprotection Studies

Female C3H_{He}CrMTV- (abbreviated C3H) mice, bred in the National Cancer Institute Animal Production Area (Frederick, MD), were used for this study. The mice were 7–9 weeks of age at the time of experimentation and weighed between 20–30 grams. All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Nitroxides were injected (i.p.) 5 min before whole body radiation (X-RAD 320 x-ray unit (Precision X-Ray, North Branford, CT) using 2.0 mm Al filtration (300 KVp) at a dose rate of 2.4 Gy/min) over a radiation dose range of 6 – 12.5 Gy. Control animals received i.p. injections of 1X phosphate buffered saline (PBS) 5 min prior to radiation treatment. Mice were placed in a specially designed jig to allow the delivery of total body irradiation without the use of anesthetics. Each radiation dose group consisted of 10 mice and studies for selected nitroxides were repeated at least twice. Following radiation treatment the mice were observed daily to 30 days post-radiation at which time survival was recorded. Animals were euthanized when humane endpoints were reached before death. The *in vivo* DMF was determined by taking the ratio of the LD_{50/30} of mice treated with nitroxide to the LD_{50/30} of control mice treated with PBS. Thus, a DMF equal to one suggests no radioprotective effect, and a DMF greater than one suggests a radioprotective effect.

For the maximum tolerable dose (MTD) studies, each nitroxide was tested separately for acute toxicity. Nitroxide solutions were prepared at pH 7.5, and injected intraperitoneally into 1–3 mice at an initial dose of 250 mg/kg. At 10 minutes after injection, the mouse was assessed for signs of seizure, which included shaking, face rubbing, and salivating. If the intensity of the seizure was increasing 10 minutes after injection, the dose (250 mg/kg) was determined to exceed the maximum tolerable dose. Next, separate mice were injected with a modified dose: if the initial 250 mg/kg dose was greater than the MTD, the dose was decreased by 25–50 mg/kg, and if the initial dose was less than the MTD, the dose was increased by 50 mg/kg. These experiments were repeated until the MTD was determined for each nitroxide. For the MTD studies, approximately 5–10 mice were used per nitroxide.

Animal Studies: Imaging

Female C3H mice were obtained from the Frederick Cancer Research Center, Animal Production (Frederick, MD, USA). Mice were housed in a climate controlled circadian rhythm adjusted room, and were allowed access to food and water *ad libitum*. The body weight of the mice at the time of imaging was 22–29 grams, and the age at the time of imaging was 11–20 weeks. All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the National Cancer Institute Animal Care and Use Committee.

Mice were anesthetized with a mixture of isoflurane (4% to induce, 1–2% to maintain) and medical air (750 mL/min). A catheter was made by breaking the tip off of a 30½ gauge needle (Becton Dickinson and Company, Franklin Lakes, NJ) and inserting it into Tygon tubing (inner diameter (id): 0.01 inches, Norton Performance Plastics, Akron, Ohio.) This catheter was placed in a tail vein and connected to a syringe containing 22c or 23c (3μL/g body weight of 150mM solution), with an injection volume of 60–90 μL, depending on the weight of the mouse. The mouse was then placed inside the MRI coil in a prone position, and lightly taped to the cradle once at the head, once just above the hind legs, and once on the tail. The syringe was placed outside of the scanner so that it could be accessed during imaging. The surface body temperature was maintained between 35°C and 36°C, and the breathing rate was maintained between 60–90 breaths per min.

Imaging Parameters

Images were acquired on a 3T Phillips clinical scanner with a custom-built small animal receive-only saddle-shaped coil (diameter: 3.8 cm, length 7.0 cm.) After localizing scans, a multi-slice T_2 weighted turbo spin echo (TSE) ($TR = 4s$, $TE = 30ms$, $\alpha=90^\circ$, $NEX=1$, $FOV = 8 \times 3.96$ cm, slice thickness = 1 mm, number of slices = 22) was acquired to aid in identification of tissue boundaries. Then, a 3D spoiled fast field echo (FFE) ($TR = 8.5$ ms, $TE = 2.302$ ms (fat and water in phase), $\alpha=3^\circ$, $NEX = 3$, $FOV = 8 \times 3.4$ cm, slice thickness = 1mm, number of slices = 22) was acquired for T_1 map calculation (described below in “Tissue concentration calculation”). A flip angle map was acquired using the signal ratio between T_1 -weighted images corresponding to two separate repetition times ($T_{r,low} = 20$ ms, $T_{r,high} = 120$ ms, $T_e = 4.6$ ms (fat/water in phase), $NEX = 2$). The flip angle map was automatically calculated from the raw data by the Philips scanner software, and the output was a map of the percentage of the nominal flip angle. A 3D spoiled fast field echo (FFE) was used for the T_1 -weighted dynamic scans ($\alpha=19^\circ$, $NEX = 1$, time per scan = 20 seconds, number of dynamic scans was 60). After 2 min of baseline imaging, the nitroxide was manually injected starting at the beginning of the seventh image. Imaging resumed for 18 min in the case of Tempol and 18 or 38 min in the case of 3-CP. Once the scanning was complete, the animal was allowed to recover from anesthesia, and was then returned to its cage.

Nitroxide concentration calculation

The concentration of 22c and 23c were calculated using Matlab (The MathWorks, Inc., Natick, MA, USA). Calculations of nitroxide concentration were performed on a voxel-by-voxel basis, and were made based on the longitudinal relaxivity (r_1) of the nitroxides, a flip angle (α) map, a pre-injection T_1 ($T_{1,0}$) map, and dynamic (serial) T_1 -weighted fast field echo images (S). The longitudinal relaxivity of 22c and 23c were determined to be equal in blood, with a value of 0.22 ± 0.03 $\text{mM}^{-1}\text{s}^{-1}$. The procedure for calculating the r_1 of nitroxides in blood was described in a previous study [32]. The flip angle map was generated as described in the “imaging parameters” methods section. Calculation of the dynamic nitroxide concentration requires the fast field echo signal equation, which is:

$$S(T_{1,t}, \alpha, T_R) = A \sin(\alpha) \left(\frac{1 - e^{-\frac{T_R}{T_{1,t}}}}{1 - \cos(\alpha)e^{-\frac{T_R}{T_{1,t}}}} \right), \quad (1)$$

where S is the signal amplitude, T_R is the repetition time of the pulse sequence, A is a dimensionless constant of proportionality, $T_{1,t}$ is the longitudinal relaxation time at time “t”, and α is the flip angle. In the subsequent calculations, α refers to the actual flip angle given by the flip angle maps, not the nominal flip angle that was entered as an imaging parameter. Usually the actual flip angle “ α ” was equal to the nominal flip angle $\pm 20\%$.

The nitroxide concentration calculation took place in three steps: calculation of a pre-injection T_1 ($T_{1,0}$) map, calculation of a map of the “A” constant in Equation 1, and finally calculation of the dynamic concentration maps ($[N]_t$). The analytical expressions used to make each of these three calculations are given below in Equations 2, 3, and 5, respectively. The first step was to calculate the pre-injection $T_{1,0}$ images, and this was achieved by taking the ratio of two T_1 weighted fast field echo images: one with $\alpha_l = 3^\circ$ (with corresponding signal intensity $S_{l,0}$) and one with $\alpha_h = 19^\circ$ (with corresponding signal intensity $S_{h,0}$). The “0” subscripts indicate that the images were taken prior to nitroxide injection, and the subscripts “l” and “h” designate a low and high flip angle, respectively. Dividing Equation 1 for the α_h case by the α_l case and rearranging for $T_{1,0}$ yields the equation

$$T_{1,0} = -T_r \left[\ln \left[\frac{\left[\frac{S_{h,0} \tan a_l}{S_{l,0} \sin a_h} - \frac{1}{\cos a_l} \right]}{\frac{S_{h,0} \tan a_l}{S_{l,0} \tan a_h} - 1} \right] \right]^{-1}. \quad (2)$$

Equation 2 was used to calculate a pre-injection $T_{1,0}$ map for each imaging slice. After calculation of the $T_{1,0}$ maps, maps of the “A” constant from Equation 1 were calculated. Rearrangement of Equation 1 gives

$$A = \frac{S_{h,0}(1 - \cos(a_h)\exp(-T_r/T_{1,0}))}{\sin a_h(1 - \exp(-T_r/T_{1,0}))}. \quad (3)$$

The constant of proportionality “A” was assumed not to change throughout the course of the dynamic imaging experiment. Finally, with the $T_{1,0}$ and A maps known, the nitroxide concentration maps were calculated. This calculation was performed based on the longitudinal relativity equation, which relates $T_{1,t}$ to the longitudinal relaxivity of the nitroxide and the concentration of the nitroxide at a given time ($[N]_t$):

$$r_1[N]_t = \frac{1}{T_{1,t}} - \frac{1}{T_{1,0}}. \quad (4)$$

Solving for $T_{1,t}$ in equation 4, substituting the resulting expression for $T_{1,t}$ into Equation 1, and solving for $[N]$ gives

$$[N]_t = -\frac{1}{r_1} \left[\frac{1}{T_r} \ln \left[\frac{\frac{S_{h,t}}{A \sin a_h} - 1}{\frac{S_{h,t}}{A \tan a_h} - 1} \right] + \frac{1}{T_{1,0}} \right], \quad (5)$$

which was the expression used to solve for the dynamic nitroxide concentration ($[N]_t$). The nitroxide concentrations presented in the results section of the current manuscript were calculated for each tissue of interest by taking averages within a region of interest (ROI) in the concentration images generated by equation 5. In the case of the blood measurements, the ROIs were drawn in the ventricle, excluding the myocardium.

Decay rate calculation

The exponential decay rate of the nitroxide was calculated by least-squares-fitting the average nitroxide concentration within a tissue with a three-parameter exponential decay function using Matlab. The decay rate calculation is described in more detail in reference [32].

Determination of the total nitroxide concentration in the mouse brain

The total (oxidized plus reduced) nitroxide concentration was determined in the brain for three time-points after injection: 1 min, 4 min, and 20 min. The experiments were performed by injecting 40 mg/kg 23c into the tail vein of anesthetized mice, and then sacrificing the mouse by cervical dislocation at the appropriate time-point. After the mouse was euthanized, the entire brain of the mouse was harvested, weighed, and snap frozen until all samples were ready. Brain samples were assumed to exhibit a density of 1 g/mL, and were mixed with PBS (Cellgro, Mediatech, Inc., Manassas VA) equal to 3 times their volume (i.e. the brain samples were diluted four-fold.) After homogenization of the brain/PBS samples, 150–200

μL aliquots were mixed with potassium ferricyanide (Sigma Chemical Company, St. Louis, Mo, 63178) (2 mM final concentration) in order to oxidize all remaining hydroxylamine into the nitroxide. Aliquots were placed in gas-permeable (Zeus, Inc., Orangeburg, SC) tubing and the central peak height of each 23c diluted sample was measured with a 9.36 GHz X-Band electron paramagnetic resonance (EPR) spectrometer. Subsequent to measuring the peak heights of each sample, standard samples of known [23c] were measured in the 0.005 mM – 0.37 mM range. The relationship between peak height and nitroxide concentration was linear in this concentration region. Based on the peak-height standard curve, the concentration of nitroxide in the intact brain was calculated after correcting for the four-fold dilution that was made during homogenization. Care was taken to ensure that the samples and standards occupied the entire sensitive region of the EPR resonator to avoid artifacts due to variations in the sample volume.

Results

The ability of selected nitroxides (Figure 1) to protect against radiation- or hydrogen peroxide-induced cell death was measured using a clonogenic survival assay in Chinese hamster V79 cells. The radiation dose-response curves are shown in Figure 2a. As can be seen, the nitroxide-treated survival curve lies above the control curve, demonstrating that the tested nitroxides exhibited a radioprotective effect. From the survival curves in Figure 2a, *in vitro* DMFs were calculated for each nitroxide (Table 1). These DMFs are consistent with the previously published observation [31] that 16c, 22c, and 23c were more effective *in vitro* radioprotectors than Tempol or 3-CP. Additionally, the ability of Tempol and 23c to protect against hydrogen peroxide-induced cell death was tested (Figure 2b). Figure 2b shows that 23c protected against hydrogen peroxide cell killing at 250 μM (protection factor (PF) = 1.42 ± 0.26 (standard deviation)) and 1000 μM (PF = 2.00 ± 0.42). Also, the PF of 1000 μM Tempol was 2.73 ± 0.57 , and this value was not statistically different from the PF of 1000 μM 23c.

For *in vivo* radioprotection studies, the mice were administered with the maximum tolerable dose (MTD) of 16c, 22c, 23c, Tempol, or 3-CP. The MTD was determined separately for each nitroxide. The MTD of the nitroxides were: 16c, 350 mg/kg; 22c, 250 mg/kg; 23c, 200 mg/kg; 3CP, 400 mg/kg; and Tempol, 275 mg/kg (Table 1).

Next, the ability of the nitroxides to protect against radiation-induced lethality was measured in female C3H mice. The percent of mice surviving 30 days after radiation treatment is plotted as a function of radiation dose in Figure 3a and Figure 3b. The $\text{LD}_{50/30}$ of the control mice was significantly different from the $\text{LD}_{50/30}$ of the Tempol ($P < 0.05$) and 23c ($P < 0.001$) mice. There was substantial variation between the nitroxides in terms of *in vivo* radioprotection: 16c was the least protective (DMF = 1.08), and 23c was the most protective (DMF = 1.45). Qualitatively, there was little relationship between *in vitro* protection factors and *in vivo* dose modification factors (Table 1). For example, the *in vitro* DMF of 16c was greater than that of Tempol, while the *in vivo* DMF of 16c was less than that of Tempol. Additionally, 16c was the most potent radioprotector *in vitro*, but provided little protection *in vivo*. The nitroxide 23c was the most effective *in vivo* radioprotector among those studied. Notably, when 23c was administered to mice immediately after 11 Gy, the protection was lost, and no mice survived past 30 days (data not shown).

Table 1 shows that the *in vivo* maximum tolerable dose (MTD) and dose modification factor (DMF) varied substantially between the nitroxides studied. In addition, examination of Table 1 shows that there was no correlation between *in vivo* and *in vitro* DMFs. In order to determine factors that contribute to the *in vivo* toxicity and *in vivo* DMF of nitroxides, pharmacokinetic studies of 22c and 23c were conducted. These pharmacokinetic studies

were conducted with a T_1 -weighted MRI scan, which allows indirect measurement of nitroxide pharmacokinetics through their T_1 -shortening effect on local water protons.

Figure 4 shows a set of MR images that were obtained after injection of 22c (80 mg/kg) via a tail vein catheter. Figure 4a shows a T_2 -weighted image of the mouse head with the tongue and submandibular salivary gland outlined in yellow. Figure 4b shows 22c concentration maps overlaid on the T_2 -weighted image in Figure 4a for various time-points before and after injection of 22c. As can be seen in Figure 4b, the concentration of 22c throughout the head rapidly increased immediately after injection of the nitroxide, and as time progressed, the concentration of 22c decayed to 0 mM. The decay of nitroxides observed in these images occurs primarily due to redox reactions between the paramagnetic nitroxide, intracellular antioxidants, and intracellular oxidizing species [18, 33]. These redox reactions result in the overall reduction of the nitroxide into the non-contrast enhancing hydroxylamine [18, 33]. In Figure 4c, the average concentration of 22c within the tongue and salivary gland is shown as a function of time after injection. An exponential decay model was least-squares fit to the data, and the decay rate (i.e. the reduction rate constant, k_r) is shown in the legend of Figure 4c. The calculated reduction rate constants show that the tongue reduces 22c approximately 50% faster than the salivary gland.

The process outlined in Figure 4 was used to quantify the dynamic concentration of 22c and 23c in the kidney, liver, oral muscle, leg muscle, tongue, brain, blood, and the submandibular salivary gland (Figure 5). Figure 5 shows the average concentration of 22c and 23c in these tissues as a function of time after injection. For the data in Figure 5, the reduction rates (k_r) and the peak concentrations of nitroxide are found in Table 2 and Table 3 respectively. Taken together, Figure 5 and Tables 2 and 3 show that, with a few exceptions, the rates of nitroxide reduction and the peak tissue concentration did not differ between the nitroxides 22c and 23c. For both nitroxides, the reduction rates varied from 0.2 min^{-1} in oral muscle and 0.7 min^{-1} in the brain. In terms of peak nitroxide concentrations, values varied between 0 mM in the liver and 4 mM in the brain.

Because clinical contrast agents such as gadolinium chelates are not blood brain permeable, the ability of 23c to pass the blood brain barrier is of interest. Figure 6 shows a representative set of images for 23c in the mouse brain. Figure 6a is an illustration of the mouse brain and spinal cord, with the location of imaging slices indicated. The five T_2 -weighted images in Figure 6b correspond to the five image slices indicated in Figure 6a. In Figure 6b, the spinal cord and brain are outlined in green. The images in Figure 6c correspond to slice 4 of Figure 6a–b, and are concentration images overlaid upon T_2 -weighted images, with each image corresponding to a different time-point before or after the injection of 23c. As can be seen from Figure 6c, the concentration of 23c in the brain increases rapidly after injection of the nitroxide, and decays to 0 mM by approximately 4 minutes after injection. Next, redox maps were generated of the healthy mouse brain (Figure 6d). Redox maps are obtained by spatially smoothing the entire dynamic imaging time course, and fitting an exponential curve to the time-course of each individual voxel. The decay rate of each voxel time-course is then inserted into the corresponding voxel in the redox map. The redox images (Figure 6d) show that there is considerable variation in the reduction rate of 23c across the brain. In general, it was found that the spinal cord and the ventral region of the brain reduced 23c 50% more rapidly than dorsal regions of the brain (Figure 6e). These data suggest that there are inherent spatial variations in the metabolism and clearance of 23c within the mouse brain.

The signal decay represented by Figures 6d–e results from a combination of reduction and physical clearance of the nitroxide 23c. To test the extent to which physical clearance causes 23c signal decay, electron paramagnetic resonance spectroscopy was used to measure the

total nitroxide concentration (oxidized plus reduced) in *ex vivo* brain samples at three time-points after injection (Figure 6f). As can be seen in Figure 6f, the total concentration of 23c in the brain decreases by about 40% between 1 and 4 minutes after injection. During this same period, the concentration of oxidized nitroxide in the brain, as measured by MRI, decreases by about 90%. These observations suggest that the signal decay rate of 23c in the brain is due to both reduction and clearance, but that the strongest contributor to signal decay is reduction.

Finally, the images acquired during this study demonstrated the surprising result that 23c accumulates in high concentrations in the mouse myocardium (Figure 7, red arrows). It was observed that in the myocardium, the accumulation as well as the reduction and/or clearance of 23c occurred very rapidly: myocardial enhancement had mostly disappeared by one minute after injection. High levels of nitroxide in the myocardium were also observed with 22c, but not with Tempol or 3-CP (images not shown).

Discussion

Radiation exposure is often undesirable, because it promotes carcinogenesis and toxicity in otherwise healthy tissue. Exposure can occur unexpectedly during a nuclear attack or power plant meltdown, but can also occur in a controlled medical environment during cancer radiotherapy. In each of these situations, countermeasures that protect radiosensitive organs from radiation damage may improve the outcome of the exposed individuals. In the current study, the nitroxide 23c was identified as an effective *in vivo* radioprotector, with an *in vivo* dose modification factor of 1.45 at the LD_{50/30} dose. To the authors' knowledge, 23c is currently the most potent known nitroxide radioprotector.

A unique feature of nitroxide radioprotectors is that their pharmacokinetics can be measured non-invasively with magnetic resonance imaging (MRI). Using MRI, the pharmacokinetics of 22c and 23c were measured in the blood, liver, submandibular salivary gland, anterior leg muscle, oral muscle, brain, kidney, and tongue. It was found that both the peak concentration and rate of nitroxide metabolism and clearance varied substantially between tissues. However, for a given tissue, the peak concentration and reduction rate constant were usually the same for 22c and 23c. Additionally, due to the high BBB permeability of 23c, experiments were conducted that demonstrate the utility of 23c as a brain redox imaging contrast agent.

A useful question in radioprotection research is: "for a given set of radioprotectors, is there a correlation between the pharmacokinetic behavior of the radioprotector and the biological properties of the radioprotector?" For example, identification of correlations between peak tissue concentration on the one hand and toxicity or radioprotective potency on the other may lead to insights that will help to identify less toxic and more effective radioprotectors. The current study and a previous study [32] together provide complete pharmacokinetic, radioprotection, and toxicity data for the nitroxides Tempol, 3-CP, 22c, and 23c. Based on these data, it is possible to identify or dispel correlations between the pharmacokinetic and biological properties of the nitroxides. It should be noted that although the discussion below is valid for the four nitroxides studied thus far, the correlation may not hold if additional nitroxides are considered. Table 4 ranks the nitroxides Tempol, 3-CP, 22c, and 23c in the order in which they exhibit various biological or pharmacokinetic properties. For example, the row labeled "a" ranks the four nitroxides in order of their maximum tolerable dose during i.p. injection of the nitroxide (MTD). As can be seen from Table 4, the nitroxides fell in the order of 23c < 22c < Tempol < 3-CP in terms of their MTD. Notably, the order of MTD (Table 4, row a) exactly followed the order of normalized brain dose (Table 4, row c; the normalized dose for a given nitroxide is the peak brain concentration divided by the

injected dose). That is, nitroxides with higher normalized brain dose were found to be more toxic than nitroxides with lower normalized brain doses. This observation, taken with the additional observation that overdosed mice die from seizure, implicates blood brain barrier permeability as a factor in nitroxide toxicity. Interestingly, the rate of nitroxide reduction by the brain (Table 4, row e) did not correlate with toxicity.

Another important conclusion from Table 4 is that the radioprotective potency of each nitroxide (row “b”) did not correlate with its whole-body peak tissue concentration (row “d”). The whole-body peak tissue concentration was calculated separately for each nitroxide by taking the average peak tissue concentration (Table 3, ref [32]) of the submandibular salivary gland, tongue, anterior leg muscle, liver, brain, and kidney. The whole-body peak tissue concentration thus reflects the average accumulation of nitroxides in six different tissues. As can be seen from Table 4 row “d”, the normalized whole-body peak nitroxide concentrations are roughly the same for all four nitroxides, and the whole-body concentration metric therefore cannot account for differences in radioprotective potency. Because 23c is the most potent radioprotector, the current study suggests that the variations in radioprotective potency were not determined by systematic differences in nitroxide accumulation in tissue. Thus, at first sight there might seem to be a discrepancy between the nearly identical pharmacological data of 22c and 23c and the significantly different *in vivo* DMF of the two drugs.

This apparent discrepancy may be partly resolved by noting that the mice in the current study died of bone marrow toxicity. In general, the cause of death of mice exposed to whole body irradiation can be deduced from the time-course of mouse survival and the total whole-body dose. In terms of the survival time-course, death from cerebrovascular syndrome occurs usually within 24–48 hours, death from gastrointestinal syndrome occurs between 3–4 days, and death from hematopoietic syndrome (bone marrow death) peaks between 10–15 days after exposure [3]. In the current study, of the 100 mice treated with 23c, 67 died of radiation exposure, and of those that died, 58 (85%) died between 7–15 days after exposure (data not shown). In terms of whole-body dose, 7 Gy results in 50% of mice dying from bone marrow toxicity before 30 days after exposure (i.e., the LD_{50/30} for mice is 7 Gy) [3]. In the current study, the LD_{50/30} of the untreated control mice was 7.9 ± 0.15 Gy, which is suggestive of bone marrow toxicity. Thus, the predominant cause of death in the current study was apparently bone marrow toxicity, suggesting that in the surviving mice, 23c acts primarily by protecting the bone marrow.

Based on the conclusion that 23c protects mice from radiation-induced bone marrow toxicity, an attempt was made at measuring the pharmacokinetics of 22c and 23c in the bone marrow with MRI. The largest regions of marrow that could be identified were in the iliac crest and femur, but the size of these marrow compartments is at best comparable to the maximum resolution of the T₁ weighted MRI scan, and it was therefore not possible to reproducibly measure the 23c and 22c levels in the bone marrow using MRI. Nonetheless, the survival timeline implicates the bone marrow as the site of 23c radioprotection over the radiation dose range studied (7–12 Gy).

The substantial difference in DMF between 16c, 22c, and 23c implies a structure-activity relationship for *in vivo* nitroxide radioprotection. An *in vivo* structure-activity study is well beyond the scope of this study, but it is worth speculating about what factors may connect the molecular structure of these nitroxides to their *in vivo* DMF. In theory, the *in vivo* DMF of nitroxides may be influenced by their subcellular compartmentalization and/or metabolism. In terms of compartmentalization, DNA damage is a major cause of radiation toxicity [3], and it follows that the degree to which a nitroxide non-covalently associates with DNA may affect its DMF. Association of nitroxides with DNA may occur due to

residual positive charge on the nitroxide, which would promote an electrostatic attraction between the nitroxide and negatively charged DNA. In the case of 16c, 22c, and 23c, the nitrogen atoms on the pyrroline ring substituent exhibit a residual positive charge, which may promote their association with DNA. However, 16c, 22c, and 23c each have a residual charge on the nitrogen, and so the existence of a positive charge alone cannot account for the difference in the *in vivo* DMFs of these three nitroxides. The difference between the DMF of 22c and 23c might be explained by the molecular structure of the positively charged ring substituent. For 22c, the substituent is the relatively planar N-methyl pyrrolidine group, while the substituent for 23c is the non-planar N-methyl piperidine. Because the non-planar substituent of 23c is capable transforming between “boat” and “chair”-like conformations, both with a protruding positively charged nitrogen atom, 23c may form a stronger attractive electrostatic interaction with DNA than 22c. This may result in greater proximity of 23c than 22c with DNA molecules, which may contribute to the greater *in vivo* DMF of 23c. This theory does not, however, explain why 16c and 22c are potent radioprotectors *in vitro*, but not *in vivo*.

The difference between the *in vivo* and *in vitro* DMFs of 16c, 22c, and 23c may be due to enzymatic metabolism of these nitroxides *in vivo*. That is, 23c may be metabolized by yet to be identified enzymatic reactions into a radioprotective molecule, or alternatively, 16c and 22c may be metabolized into non-radioprotective species. Because many enzymes catalyze chemical reactions in a kinetically rapid and substrate-specific manner, the involvement of enzymes in the metabolism of these three nitroxides may have caused the observed differences in their *in vivo* DMFs.

Because 23c readily passes the blood brain barrier (BBB), 23c was evaluated as brain redox imaging agent. To be a viable redox imaging agent, a nitroxide must accumulate in measurable quantities in the target organ, and its rate of washout must be substantially less than the rate of reduction. If these conditions are met, then it is reasonable to assume that signal decay constants are informative of tissue redox status. With regards to accumulation of 23c in the brain, it was found that the peak concentration of 23c in the brain was 3.6 ± 0.4 mM, which provided more than sufficient contrast on T₁ weighted images. With regards to washout, it was found that over a 3 minute period washout alone (measured invasively) accounted for only 40% of signal loss, while washout and reduction together (measured with MRI) resulted in 90% signal loss over the same period (Figure 6f). These data suggest that the signal decay constant of 23c in the brain is related to the redox status of the tissue.

Using 23c as a brain redox imaging agent, spatial variations in brain redox status were identified. In particular, the ventral regions of the brain exhibited a decay constant k_r that was 50% greater than surrounding brain regions (Figure 6d–e). The regions with a more reducing environment were the spinal cord, thalamus, hypothalamus, and lower midbrain. Spatial variations in brain redox status are not surprising given that metabolic rates and antioxidant capacity varies spatially within the rodent brain. For example, spatial variations in glutathione levels within the rodent brain have been noted [34–35], and glutathione is known to influence the rate of nitroxide reduction *in vivo* [19–20, 36]. Additionally, spatial variations in the rate of glucose metabolism have been observed in the rodent brain [37–39]. Because glucose can feed into the pentose phosphate pathway (PPP), and because the rate of nitroxide reduction correlates with PPP activity *in vitro* [40–41], the rate of glucose metabolism may correlate with the rate constant of 23c reduction in mice.

The ability of 23c to provide redox data in the mouse brain may prove to be useful in future studies, because many neurocognitive disorders and stressors are associated with oxidative stress. Examples include sleep deprivation [35], MDMA (Ecstasy) neurotoxicity [42–45], and phencyclidine (PCP)-induced schizophrenia [46]. Furthermore, in the MDMA exposure

[45] and schizophrenia [46] models, the levels of oxidative stress varied between different brain structures. Because nitroxide imaging allows spatial mapping of tissue redox status with high resolution ($\sim 0.2 \times 0.2 \times 1$ mm) (Figure 9 and refs [32, 47–48]), the novel nitroxide 23c may allow brain-region specific assessment of oxidative stress in preclinical models of neurocognitive damage.

An unexpected observation made in the current study was that high levels of 23c and 22c briefly accumulated in the myocardium after injection (23c is shown in Figure 7). This was unexpected, because in a previous study, myocardial accumulation was not observed for the nitroxides Tempol and 3-CP [32]. Further experiments are necessary to better understand the mode of 23c delivery (e.g. via the ventricle or via the coronary arteries) and the cause of 23c signal decay (e.g. clearance and/or reduction). Depending on the mode of delivery and cause of decay, 23c may allow localization of a thrombus in the coronary arteries and/or the resulting myocardial infarct.

In summary, the current manuscript reports several novel findings. First, it is reported for the first time that 23c is a potent *in vivo* nitroxide radioprotector. Second, the pharmacokinetics of 22c and 23c were characterized in various healthy tissues using magnetic resonance imaging. It was found that 22c and 23c exhibit almost identical pharmacokinetics in the tissues studied, with the important exception that 23c accumulated in the brain at a greater concentration than 22c. Third, it was found that the exponential decay rate of 23c in the mouse brain reflects the redox status of the underlying tissue, and that inherent redox differences exist in the mouse brain. Finally, the observation was made that 23c accumulates in the myocardium at high concentrations relative to other nitroxides, and that this effect may be useful for functional imaging of the mouse heart. These findings highlight the versatile biomedical applications of the nitroxide 23c.

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List of Abbreviations

BBB	blood-brain-barrier
DMF	dose modifying factor
EPR	Electron paramagnetic resonance
LD_{50/30}	the dose resulting in 50% of mice dying by 30 days after radiation exposure
MRI	magnetic resonance imaging
MTD	maximum tolerable dose
PR	protection factor (for hydrogen peroxide exposure only).

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A

Figure 1.
The molecular structure of the nitroxides studied in the current report.

Figure 2.

A) *In vitro* radiation survival curves of Chinese hamster V79 cells treated with 10 mM Tempol, 3-CP, 16c, 22c, and 23c. B) *In vitro* survival curves for cells treated with hydrogen peroxide, demonstrating the antioxidant activity of Tempol and 23c.

Figure 3.

A) *In Vivo* radiation survival curves for control mice (injected with PBS), and mice treated with Tempol, 3-CP, 16c, and 22c. B) *In Vivo* radiation survival curves for control mice and mice treated with Tempol or 23c.

Figure 4.

Images from an experiment where MRI was used to measure the pharmacokinetics of the nitroxide 22c. A) a T_2 weighted image of the mouse head with the tongue and submandibular salivary gland (abbreviated as “sal” in the figure) outlined in yellow. B) Images of the dynamic concentration of 22c throughout the head region of the mouse. These images show that immediately after injection, 22c accumulates in the mouse head at concentrations exceeding 1.5 mM, and then decays toward 0 mM within minutes. C) The average concentration of 22c in the regions outlined in parts “A” and “B”. The exponential decay constant for the tongue was 0.40 (0.36, 0.43) min^{-1} and 0.26 (0.22, 0.30) min^{-1} for the salivary gland (mean and 95% confidence interval).

Figure 5. Pharmacokinetics of 22c and 23c in the brain, anterior leg muscle, oral muscle, kidney, salivary gland, tongue, and blood.

Figure 6.

Images showing the accumulation and reduction of 23c in the mouse brain. A) A representation a mouse brain and spinal cord with the location of image slices indicated. B) T_2 -weighted images of the mouse brain, corresponding to the image slices shown in part "A". The brain and spinal cord are outlined in green. C) An overlay of the T_2 -weighted image and concentration maps of 23c. These images correspond to slice 4 in parts "A" & "B". The brain and spinal cord are outlined in yellow. D) Redox maps of corresponding to the T_2 -weighted images in parts "A" & "B". Redox maps are calculated by fitting the entire imaging time-course with an exponential decay function on a voxel by voxel basis. The decay constant of 23c within a voxel is then placed in the corresponding voxel of the redox

map. The brain and spinal cord are outlined in green. E) A ROI analysis of dorsal and ventral regions of the mouse brain. The reduction rate constants were $k_r = 0.93$ (0.89, 0.97) min^{-1} ($N = 4$ mice) in the ventral region, and $k_r = 0.63$ (0.60, 0.66) min^{-1} ($N = 4$ mice) in the dorsal region. The inset is a typical T_2 -weighted image of the mouse brain, shown with dorsal (red) and ventral (blue) ROIs. F) Measurements of the oxidized and total (oxidized plus reduced) concentration of 23c in the mouse brain. Total nitroxide concentrations were measured invasively, and the oxidized concentrations were measured non-invasively with MRI.

Figure 7. Images showing the accumulation of ^{23}C in the mouse myocardium. The uptake and subsequent clearance occurred very rapidly, within 40 seconds after injection. The yellow ROI encompasses the entire heart, and the red arrows point to the enhanced myocardium.

Table 1

Dose Modification Factors (DMFs) in cells and mice

Nitroxide	<i>In Vitro</i> DMF V79 clonogenic cell survival	<i>In Vivo</i> LD _{50/30}	<i>In Vivo</i> DMF C3H mouse survival	<i>In Vivo</i> Toxicity Max. Tolerable Dose (MTD) [†]
Tempol	1.38 ± 0.02	9.11 ± 0.02 Gy	1.15	275 mg/kg
3-CP	1.48 ± 0.06	9.5 Gy [‡]	1.10	400 mg/kg
22c	1.52 ± 0.26	— [¥]	— [¥]	250 mg/kg
23c	1.55 ± 0.11	11.47 ± 0.13 Gy	1.45	200 mg/kg
16c	2.1 ± 0.28	8.6 Gy [‡]	1.08	350 mg/kg

Values > 1.0 signify that the compound was radioprotective.

[†]The MTD was used as the nitroxide dose during the *in vivo* radioprotection study

[‡]Only one LD_{50/30} experiment was conducted for 3-CP and 16c, so no error estimate is included.

[¥]Based on the limited *in vivo* protection afforded by 22c in the dose range 8.5–9.5 Gy, the 22c experiments were terminated before adequate data was obtained to calculate an *in vivo* DMF or LD_{50/30}.

Table 2

In Vivo reduction rate constants (k_r) of 22c and 23c.

Tissue	22c			23c		
	k_r	STDev	N	k_r	STDev	N
Oral muscle	0.17min ⁻¹	0.06min ⁻¹	5	0.20min ⁻¹	0.06min ⁻¹	3
Salivary Gland	0.30min ⁻¹	0.11min ⁻¹	4	0.38min ⁻¹	0.15min ⁻¹	5
Tongue	0.50min ⁻¹	0.06min ⁻¹	6	0.37min ⁻¹	0.07min ⁻¹	4
Whole-brain	0.62min ⁻¹	0.12min ⁻¹	6	0.70min ⁻¹	0.06min ⁻¹	5

Reduction rates of 22c and 23c in muscle, salivary gland, tongue and whole-brain. P-values were calculated based on a student's T-test, and a bold p-value indicates statistical significance.

Table 3*In Vivo* peak concentrations of 22c and 23c

Tissue	22c			23c		
	[22c] _{max}	SEM	N	[23c] _{max}	SEM	N
Liver	0.3 mM	0.4 mM	4	0.0 mM	0.2 mM	3
Salivary gland	1.4 mM	0.3 mM	4	1.1 mM	0.2 mM	5
Anterior leg muscle	1.18 mM	0.04 mM	4	1.1 mM	0.2 mM	5
Oral muscle	1.1 mM	0.2 mM	5	1.6 mM	0.2 mM	3
Whole-brain	1.6 mM	0.2 mM	6	3.6 mM	0.4 mM	5
Kidney	2.3 mM	0.5 mM	3	2.0 mM	0.3 mM	4
Tongue	2.6 mM	0.3 mM	6	3.3 mM	0.9 mM	4

P-values were calculated based on a student's T-test, and a bold p-value indicates statistical significance ($p < 0.05$).

Table 4

summary of toxicity, radioprotection, *in vivo* reduction rates, and *in vivo* peak tissue concentrations of four nitroxides.

Biological property	Ranking of nitroxides			
a) maximum tolerable dose for i.p. injection (toxicity)	23c 200 mg/kg	<	22c 250 mg/kg	<
b) <i>in vivo</i> radio-protection factor	23c 1.45	>	Tempol 1.15	>
Pharmacokinetic measurement				
c) peak concentration in brain (normalized to dose)* mM/(mg/kg)	23c 0.045 mM/(mg/kg)	>	22c 0.020 mM/(mg/kg)	>
d) nitroxide reduction rate in brain min ⁻¹	Tempol 1.5 min ⁻¹	>	23c 0.70 min ⁻¹	≈
e) peak concentration in tissues [†] (normalized to dose)* mM/(mg/kg)	3-CP 0.024 mM/(mg/kg)	≈	23c 0.023 mM/(mg/kg)	≈
			Tempol 0.022 mM/(mg/kg)	≈
			Tempol 0.011 mM/(mg/kg)	>
			22c 0.62 min ⁻¹	†
			3-CP 0.004 mM/(mg/kg)	

The values for Tempol and 3-CP in this table were taken from the data in reference [32], with permission.

‡ Based on qualitative inspection of figure 4. See note “‡” in table 1.

* normalized doses were calculated by dividing the peak concentration of nitroxide within a tissue (Table 3, in mM) by the injected dose (mg nitroxide/kg body weight). Tempol and 3-CP values were obtained from [32], with permission.

† 3-CP did not cross the blood brain barrier at sufficient concentrations to calculate a reduction rate.

‡ The values in row “d” were calculated by averaging the peak dose for each nitroxide in six separate tissues: the submandibular salivary gland, tongue, anterior leg muscle, brain, liver, and kidney. This row thus represents the overall ability of each nitroxide to extravasate into tissue.