Abstract

Purpose: The nitroxide free radical, Tempol, was evaluated for potential differential radiation protection of salivary glands and tumor using fractionated radiation. Mechanistic information was explored by monitoring the presence and bioreduction of Tempol in both tissues noninvasively by magnetic resonance imaging (MRI).

Experimental Design: Female C3H mice were immobilized using custom-made Lucite jigs for localized irradiation (five daily fractions) either to the oral cavity or tumor-bearing leg. Tempol (275 mg/kg) was administered (i.p.) 10 min before each radiation fraction. Salivary gland damage was assessed 8 weeks after radiation by measuring pilocarpine-mediated saliva output. Tumor growth was assessed by standard radiation regrowth methods. Dynamic T1-weighted magnetic resonance scans were acquired before and after Tempol injection using a 4.7T animal MRI instrument.

Results: Tempol treatment was found to protect salivary glands significantly against radiation damage (≈60% improvement); whereas no tumor protection was observed. Intracellular reduction of Tempol to the nonradioprotective hydroxylamine as assessed by MRI was 2-fold faster in tumor compared with salivary glands or muscle.

Conclusions: Tempol provided salivary gland radioprotection and did not protect tumor, consistent with the hypothesis that differential radioprotection by Tempol resides in faster reduction to the nonradioprotective hydroxylamine in tumor compared with normal tissues. The unique paramagnetic properties of Tempol afforded noninvasive MRI monitoring of dynamic changes of Tempol levels in tissue to support the finding. These data support further development and consideration of Tempol for human clinical trials as a selective protector against radiation-induced salivary gland damage.

Differential Radiation Protection of Salivary Glands versus Tumor by Tempol with Accompanying Tissue Assessment of Tempol by Magnetic Resonance Imaging

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The consequences of head and neck radiotherapy can greatly affect the quality of life of 350,000 patients every year worldwide. Around 41,000 of these patients are treated in United States (1, 2). Normal tissue present in the radiation field can be severely damaged, salivary glands being particularly sensitive to ionizing radiation. Consequently, ≈80% of patients that undergo radiotherapy for head and neck cancers show marked salivary hypofunction (3–6). The lack of saliva leads to considerable morbidity, including dental caries, mucosal infections, dysphagia, and extensive discomfort. The existing management approaches for reduced salivary flow remain symptomatic, palliative, and generally unsatisfactory (e.g., artificial saliva and intense dental care). Therefore, preventive approaches are currently being tested as potential alternatives. Amifostine [WR-2721; 2-[(3-aminopropyl)amino]ethylphosphorothioic acid], although a Food and Drug Administration–approved radioprotector for xerostomia (7, 8), still remains controversial in its ability of protecting tumor as well as normal tissue (9, 10).

We have shown previously that i.p. and s.c. administration of Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a stable nitroxide, protected single-dose radiation-induced damage to murine salivary glands (11, 12). Our preclinical studies to date have used a single radiation dose. Because radiation therapy is delivered in a multifraction fashion in the clinic, the present study uses fractionated radiation delivery such that multiple applications of Tempol before each radiation fraction can be evaluated. Based on our single application studies, we would hypothesize that multiple applications of Tempol will protect against radiation-induced salivary gland damage.
Protection of normal tissues by Tempol would be markedly compromised if the tumor were protected as well. This study also examines whether Tempol given during the course of multiple radiation fractions protects tumor. Last, given that Tempol is paramagnetic and gives $T_1$ contrast with magnetic resonance (MR) imaging (MRI), differential reduction rates of Tempol were assessed in salivary glands and tumor. Our results indicate that Tempol protects against fractionated radiation-induced salivary gland damage but does not protect tumor. Further, MRI studies show that there is faster reduction of Tempol in tumor compared with salivary glands. These data support the further development and consideration of Tempol for human clinical trials as a selective protector against radiation-induced salivary gland damage as well as a MRI contrast agent, which can reflect tissue redox status.

Materials and Methods

Chemicals. Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Aldrich) was recrystallized from diethylether: orange-yellow needles obtained from the supersaturated solution were filtered and air dried. Recrystallized Tempol was stored at 4°C in sealed bottles and protected from light until use. The gel form of Tempol was prepared by Starks Associates, Inc. by adding Tempol to a 1.5% hydroxyethyl cellulose base gel solution at a final Tempol concentration of 470 mmol/L.

Salivary gland studies. Female C3H mice, bred in the National Cancer Institute Animal Production Area (Frederick, MD), were used for this study. The mice were 7 to 9 weeks of age at the time of experimentation and weighed between 20 to 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 Animal Resource. (1996) National Research Council. Ionizing radiation of salivary glands was accomplished by placing each animal into a specially built Lucite jig in such a way that the animal could be immobilized without the use of anesthetics. Additionally, the jig was fitted with a Lucite cone that surrounded the head and prevented head movement during the ionizing radiation exposure. Five daily fractions (Monday-Friday) of 6 Gy were delivered to only the animal’s head by a Therapax DXT300 X-ray irradiator (Precision X-ray, Inc.) using 2.0 mm Al filtration (300 kVp) at a dose rate of 1.9 Gy/min. Groups of four animals were used and all in vivo experiments were done twice. Tempol was injected i.p., 275 mg/kg (in 100 μL sterile water), 10 min before each ionizing radiation fraction. Tempol gel was also administered topically to the oral cavity of mice before each ionizing radiation fraction. Briefly, mice were anesthetized and 50 μL Tempol gel (or placebo gel) was applied to a sterile cotton ball, small enough to fit into a mouse’s oral cavity. This impregnated cotton was left in place for 20 min before each radiation fraction. For either, i.p. or gel treatment, one set of mice received Tempol without ionizing radiation.

Immediately after ionizing radiation, animals were removed from the Lucite jig and housed (four animals per cage) in a climate- and light-controlled environment and allowed free access to food and water. To determine salivary flow rate, saliva samples were collected 8 weeks after ionizing radiation (12). Mice were weighed and mild anesthesia was induced with a solution of ketamine (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (20 mg/mL; Phoenix) in sterile water, given i.p. (1 μL/g of body weight). Whole saliva was collected after stimulation of secretion, using pilocarpine (0.5 mg/kg of body weight) administered s.c. Saliva collection began within 2 min of pilocarpine administration. Animals were positioned with a 75-mm hematocrit tube (Drummond) placed in the oral cavity and whole saliva was collected in preweighed 0.75 mL Eppendorf tubes for 10 min. The amount of saliva collected was determined gravimetrically. Immediately afterwards, anesthetized animals were euthanized.

Radiation tumor regrowth studies. Two murine tumor models were used. SCC VII/SF (SCC) was derived from spontaneous squamous cell cancer (obtained from Dr. T. Phillips, University of California San Francisco, San Francisco, CA) and propagated in C3H/Hen mice. For radiation regrowth delay studies, $2 \times 10^5$ viable SCC cells suspended in 100 μL PBS were injected into the subcutaneous space of the right hind leg of 7- to 9-week-old female C3H/Hen mice. Human colon adenocarcinoma cells (HT-29) were purchased from American Type Culture Collection and 1.0 × 10⁴ cells were injected into the subcutaneous space of the right hind leg of 5- to 6-week-old female athymic nude mice. Tumor growth was followed until the diameter of tumor reached 0.6 to 0.8 mm as measured by caliper. At this point, animals were divided into four groups: control, fractionated radiation, Tempol control, and Tempol plus fractionated radiation. Fractionated radiation treatment for the SCC tumor was five daily 3 Gy fractions (Monday-Friday; total radiation dose of 15 Gy) and the HT-29 tumor was treated with five daily 2 Gy fractions (Monday-Friday; total radiation dose of 10 Gy). Identical to the salivary gland radiation studies, Tempol was injected i.p., 275 mg/kg (in 100 μL sterile water), 10 min before each ionizing radiation fraction. Selective irradiation of the right leg was accomplished by placing each unanesthetized animal into a specially built Lucite jig that immobilized the animal and facilitated ease of extension of the right hind leg into the radiation field (same X-ray unit described above). Lead shields designed as a part of the Lucite jigs assured that only the right hind limb of the immobilized animal was irradiated. Immediately after irradiation, the animal was removed from the Lucite jig and housed (five animals per cage) in a climate- and light/dark–controlled environment and allowed free access to food and water. Tumor measurements were made two to three times per week.

In vivo MRI studies. For these studies, SCC tumor cells (2 × 10⁵) were injected into the subcutaneous space of the right shoulder (cleidobrachial muscle area). Placing the tumor in this region enabled simultaneous MR assessment of tissue levels of Tempol in the tumor, salivary gland region, and other normal tissues (muscle). Tumor-bearing mice (~1 cm in diameter) were anesthetized and secured on a special mouse holder by adhesive skin tape, stomach side down. The tail vein was cannulated for the injection of Tempol. The mouse was then placed in the resonator, which was warmed previously by a warm water cycling pad. The resonator unit including the mouse was placed in the 4.7 T magnet. MR measurements were started after the animal’s body temperature was equilibrated to 37°C. Before the experiments, multislice, multiecho–based $T_1$ mapping was done. The spoiled gradient echo–based $T_1$-enhanced image data sets were repeatedly scanned for 20 min. The contrast agent Tempol was injected (1.5 μmol/g of body weight) via the tail vein cannula 20 min after starting the scans.

MRI scanner and pulse sequences. MRI measurements were done at 4.7 T controlled with ParaVision 3.0.1 (Bruker BioSpin MRI GmbH). $T_2$-weighted sequences were used to collect the scout images to localize the regions of interest in the tumor, salivary glands, and muscle. To do $T_2$ mapping, spin echo images were obtained using a multislice, multiecho sequence with TR of 4,000 ms and a 16-echo train with 15 ms echo times. The scan time for a $T_2$ mapping image set ($N_{EX} = 1$) by the multislice, multiecho sequence was 5 min. A slice containing the salivary gland, tumor, and muscle was chosen from the scout images. The spoiled gradient echo (also referred as gradient echo fast imaging; TR = 75 ms, TE = 3 ms, FA = 45°, and $N_{EX} = 2$) was used for the acquisition of $T_1$-weighted images. The scan time for an image set (which included two slices) by the spoiled gradient echo sequence was 20 s. Other image parameters were as follows: image resolution of phase-encoding dimension was 130 and gradient encoding dimension was 256; field of view was 32 × 32 cm; pixel resolution was 256 × 256; and slice thickness was 2.0 mm.

Image analysis. The MRI data were analyzed using the Image software package (a public domain Java image processing program for medical imaging, written at the National Center for Supercomputing Applications, University of Illinois, Urbana-Champaign). The contrast agent Tempol was identified by a paramagnetic signal with a $T_1$ contrast of 150 ms and the relaxation times $T_2$ of 20 ms. The signal corresponding to Tempol in the tumor was easily distinguishable from that in the normal tissue. The contrast agent Tempol was identified by a paramagnetic signal with a $T_1$ contrast of 150 ms and the relaxation times $T_2$ of 20 ms. The signal corresponding to Tempol in the tumor was easily distinguishable from that in the normal tissue.
inspired by NIH Image that can be extended by plug-ins\(^3\)). \(T_2\) mapping was calculated using a plug-in (MRI analysis calculator, Karl Schmidt, HypX Laboratory, Brigham and Women’s Hospital) available in ImageJ.

**Statistical analyses.** Statistical differences were estimated with \(t\) test function using Microsoft Excel XP. The suitable ‘type’ and ‘tail’ for the test was selected according to the correspondence and variance of the data. Significances were estimated when \(P\) values were <0.05.

### Results

**Protection against radiation-induced salivary gland damage.** Our previous studies used a single 15 Gy dose that resulted in approximately 40% to 50% reduction in salivary gland production 2 months after irradiation (11). To approximate the same biological effect with fractionated irradiation (five fractions), biological effect doses were calculated using an \(\alpha/\beta\) ratio of 3 Gy (13). The calculation resulted in a fractionation protocol of five daily 6 Gy fractions. As can be seen in Fig. 1A and B, 5 × 6 Gy resulted in a reduction of saliva production of 54% and 68% as assessed in two independent experiments. The reduction in saliva production induced by the fractionation protocol was in relative agreement with the 15 Gy single-dose data predicted by the biological effect dose calculation. Five daily treatments of Tempol (i.p.) alone exhibited no toxicity and had no effect on salivary gland function (Fig. 1A and B); however, Tempol (i.p.) provided significant protection \((P < 0.001)\) against radiation-induced salivary gland damage in replicate experiments. Topically applied Tempol gel also provided significant protection \((P < 0.001)\) against radiation damage compared with placebo gel/radiation.

**Radiation-induced tumor regrowth delay studies.** Tumor regrowth delay studies using five daily radiation fractions were conducted for two different tumor types as shown in Fig. 2A and B. The Tempol dose (i.p.) and timing of administration were identical to those used in Fig. 1. Tempol treatment alone had no effect on tumor growth for both tumor types. Further, Tempol treatment exhibited no effect on radiation-induced tumor regrowth for either tumor type.

**In vivo MRI studies.** Because Tempol is paramagnetic, its presence and rate of reduction in tissue can be followed using MRI (14, 15). To determine if Tempol levels following injection varied in tumor, salivary glands, or muscle tissues, the tumor was implanted in the shoulder region such that simultaneous

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\(^3\) http://rsb.info.nih.gov/ij/
MR assessment of tissue levels of Tempol for each tissue could be determined (see Fig. 3A). Transverse MR images were acquired to ensure that the target tissues were within the 2-mm slice selected for imaging (Fig. 3B). Dynamic T₁-weighted MR scans were then acquired before and after Tempol injection as shown in Fig. 3C. The green areas show enhancement in MR intensity by Tempol, which quickly appeared at 30 s, peaked at ~1 min, and then gradually disappeared 8 min after injection. To establish the rate of disappearance (reduction) of Tempol in various tissues, selected regions of interest were outlined as shown in Fig. 3D and MR intensity changes by Tempol in normal leg muscle, salivary gland region, and tumor were plotted as a function of time after injection as shown in Fig. 4A. The decay in Tempol-mediated MR intensity was similar for normal leg muscle and the salivary gland region; however, the decay rate was significantly faster in the tumor region as shown in Fig. 4A and summarized in Fig. 4B.

**Discussion**

Our previous studies showed radiation protection of mouse salivary gland damage by Tempol using a single radiation dose (11, 12). More recently, we showed that Tempol can also be used as a MRI contrast agent and its metabolic conversion to the nonradioprotective form can be examined noninvasively in regions where it accumulates (14, 15). These studies show that nitroxides in general and Tempol in particular are more rapidly reduced to the nonradioprotective form in tumors compared with adjacent normal tissue. To examine the protective effects of Tempol against damage to salivary gland and monitor the levels of Tempol after administration to understand the role tumor redox status plays in minimizing the protective effects in tumor selectively, we extended the previous studies to a fractionated radiation regimen with imaging studies conducted in parallel. Tempol administered 10 min before each of five daily fractions of radiation was well tolerated and provided significant protection against radiation-induced salivary gland damage. Likewise, Tempol gel, applied topically 20 min before each radiation fraction, afforded significant protection against salivary gland damage as well. Topical application of Tempol solution has shown efficacy in reducing the extent of radiation-induced alopecia in patients receiving whole brain radiation (16) and a clinical trial evaluating Tempol gel is currently ongoing. Topical application of Tempol to specific anatomic sites would therefore afford the advantage of applying high concentrations of the protector directly to the region of interest, thus reducing the possibility of high systemic concentrations. Preclinical studies (guinea pigs) and clinical studies in humans have shown extremely low systemic levels of Tempol after topical application (16, 17).

Another important issue addressed in this study was whether Tempol treatment would also protect against radiation-induced...
tumor regrowth, a major concern for systemically delivered chemical radioprotectors. It has been shown previously that Tempol administered 10 min before a single-dose radiation dose did not protect RIF tumors based on tumor control doses (TCD_{50}) values (18). In the current study, Tempol administered 10 min before each of five daily radiation fractions did not protect against radiation-induced tumor regrowth delay for two different tumors. The concentration of Tempol and timing of administration was the same as used in the salivary gland studies described previously (11, 12). Thus, within the constraints of the experimental models used, Tempol provided selective normal tissue (salivary glands) radioprotection. Whereas Tempol administration alone did not affect tumor growth (compared with controls; Fig. 2), antitumor effects of Tempol have been reported in brain tumor xenografts (19). These studies used both continuous infusion and i.p. injections of Tempol resulting in possibly higher tumor doses of Tempol than were used in the present studies.

Perhaps, the most unique aspect of the study was the ability to monitor tissue levels of Tempol noninvasively using MRI. Tempol, a nitroxide, exhibits several unique properties (20). In addition to being a radiation protector (21, 22), Tempol possesses potent antioxidant properties affording protection against superoxide and hydrogen peroxide cytotoxicity (20, 23). With regard to radioprotection, only the oxidized form of Tempol provides protection; whereas, the reduced form (Tempol-H) does not (21, 24). Interestingly, because nitroxides are paramagnetic, their presence in tissue can be monitored noninvasively by MRI (14). Further, recent studies have shown that the disappearance of nitroxide MR intensity in tissue is a result of intracellular reduction of the nitroxide to the hydroxylamine, thus enabling the evaluation of tissue “redox status” (15). This property distinguishes nitroxides as functional MR contrast agents revealing information about the intracellular redox capacity of cells/tissues. Data presented in Figs. 3 and 4 clearly show that Tempol is reduced ~2-fold faster in tumor compared with muscle and salivary gland tissue. Reduction of Tempol (radioprotective) to the nonradioprotective hydroxylamine (Tempol-H) thus occurs more rapidly in tumor as opposed to normal tissues. Based on a fixed timing of 10 min for Tempol administration before each radiation treatment used in the salivary gland and tumor studies, it is plausible that the selective radioprotection observed in salivary glands compared with tumor resulted because of higher levels of Tempol in the salivary gland as opposed to tumor at the time of radiation. Although the biochemical parameters that mediate nitroxide reduction in cells/tissues requires further clarification, tumor hypoxia has long been known to facilitate nitroxide reduction (25). The SCC VII tumor used in the present study is quite hypoxic (26).

Collectively, the data presented in this study provide the feasibility of evaluating Tempol as a radioprotector in clinical trials for patients with head/neck cancer being treated with radiation. Coupling MRI with such a trial would permit a novel dimension that could provide extremely important information with respect to the timing of Tempol administration and radiation treatment. For example, before radiation treatment, a pilot Tempol/MRI study could be conducted to determine Tempol reduction rates of tumor and normal tissues encompassed in the proposed treatment field. Based on these reduction rates, the optimal timing of Tempol administration with respect to radiation treatment to provide selective radioprotection to normal tissues could be determined. The unique aspect of such an approach is made possible because the therapeutic agent in this case (Tempol) can be visualized by MRI. There are few therapeutic agents used in cancer management (excluding radiolabeled agents) that can be followed by noninvasive imaging. Before such an approach could be considered for clinical trials, more research will be required such as whether Tempol reduction rates in tissues change during fractionated radiation treatment.

A relevant consideration would be why consider using Tempol as radioprotector given that amifostine is Food and Drug Administration approved for clinical use and has shown efficacy in protecting against salivary gland damage and damage to other normal tissues (27). As mentioned earlier, uncertainty remains as to whether amifostine might also protect tumor (10). Both amifostine and Tempol are chemical radioprotectors; amifostine is a reducing agent, whereas, Tempol is an oxidizing agent. Given the reducing nature of tumors (28, 29), particularly tumors with extensive hypoxia (30), Tempol would be expected to be rapidly reduced in tumors to the nonradioprotective hydroxylamine. Indeed, imaging studies presented in the present study support this view. In contrast, selective normal tissue radioprotection by amifostine is thought to reside in its differential delivery to normal tissues compared with tumor hypoxia.

![Fig. 4. A. representative Tempol decay rates after i.v. injection in a mouse for the selected regions of interest shown in Fig. 3D. B. summary of decay rates from the three regions of interest in normal muscle, salivary gland, and tumor (n = 4 for salivary gland and n = 6 for tumor per normal leg).](http://www.aacrjournals.org)
with tumor over a finite time delivery period (31, 32) and/or by differential levels of alkaline phosphatases in the respective tissues (33). The reductive state of amifostine or its radioprotective metabolite, WR-1065, would not be expected to be altered by the tumor microenvironment and thus would be available for radioprotection. Should Tempol have an advantage over amifostine as a selective normal tissue radioprotector will depend on the differences in the reductive state between the two protectors and the reductive capacity differences between normal versus tumor tissue. There is a need for considerably more research to address these issues. Alternatively, use of topically applied Tempol gel to protect site specific tissue may be a more feasible option, as shown in the results presented herein.

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References

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