Tempol, a Stable Free Radical, Is a Novel Murine Radiation Protector

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INTRODUCTION

The search for agents that protect against ionizing radiation is important to those at risk by virtue of environmental exposure or health-related treatment and for scientific study of the mechanism of radiation injury and cytotoxicity. Cysteamine, a sulfur-containing compound, was one of the earliest radioprotectors identified (1). Its discovery prompted the Department of Defense to sponsor the synthesis and systematic screening of over 40,000 compounds to find a useful, more effective, in vivo radiation protector (2). The major finding of this monumental undertaking resulted in the discovery of a few radiation protectors such as the aminothiol compound, WR-2721 (3–5). More recently, superoxide dismutase, interleukin 1, and granulocyte-macrophage colony-stimulating factor have been studied and shown to be radiation protectors (6–8). When the above agents were compared, WR-2721 was the most promising because it showed the most substantial and selective protection of normal tissues (3). However, when used to protect patients who were being treated with radiation for cancer, concern over inherent toxicity and nonselective protection of the cancer dampened enthusiasm for the use of WR-2721 (5).

Nitroxides, stable free radicals, were found to be low-molecular-weight, membrane-permeable, metal-independent superoxide dismutase mimics (9–11) which protect mammalian cells (11) and cardiomyocytes (12) from oxidative stress. To extend these observations from the cellular level to the whole animal, the toxicity, pharmacology, and in vivo radioprotective effects of Tempol were studied in C3H mice. The maximum tolerated dose of Tempol administered i.p. was found to be 275 mg/kg, which resulted in maximal Tempol levels in whole blood 5–10 min after injection. Mice were exposed to whole-body radiation in the absence or presence of injected Tempol (275 mg/kg) 5–10 min after administration. Tempol treatment provided significant radioprotection (P < 0.0001); the dose of radiation at which 50% of Tempol-treated mice die at 30 days was 9.97 Gy, versus 7.84 Gy for control mice. Tempol represents a new class of in vivo, non-sulfur-containing radiation protectors. Given the potential for hypoxic radiosensitization and aerobic cell radioprotection, Tempol or other analogues may have potential therapeutic application.

ABSTRACT

Nitroxide compounds are stable free radicals which were previously investigated as hypoxic cell radiosensitizers. The stable nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Tempol) has recently been shown to protect aerated cells in culture against superoxide generated from hypoxanthine/xanthine oxidase, hydrogen peroxide, and radiation-induced cytotoxicity and to modestly sensitize hypoxic cultured cells. To extend these observations from the cellular level to the whole animal, the toxicity, pharmacology, and in vivo radioprotective effects of Tempol were studied in C3H mice. The maximum tolerated dose of Tempol administered i.p. was found to be 275 mg/kg, which resulted in maximal Tempol levels in whole blood 5–10 min after injection. Mice were exposed to whole-body radiation in the absence or presence of injected Tempol (275 mg/kg) 5–10 min after administration. Tempol treatment provided significant radioprotection (P < 0.0001); the dose of radiation at which 50% of Tempol-treated mice die at 30 days was 9.97 Gy, versus 7.84 Gy for control mice. Tempol represents a new class of in vivo, non-sulfur-containing radiation protectors. Given the potential for hypoxic radiosensitization and aerobic cell radioprotection, Tempol or other analogues may have potential therapeutic application.

One water-soluble nitroxide, Tempol, was shown to protect cultured mammalian cells exposed to ionizing radiation (13). In this same study, Tempol was also shown to modestly sensitize hypoxic cells to radiation (13). Interestingly, other nitroxides were also investigated in the past as hypoxic cell radiosensitizers (14–16). Much work has been invested to identify compounds that will selectively sensitize hypoxic cells or selectively protectoxic cells. The combination of hypoxic cell sensitization and oxic cell radioprotection in one compound is thus appealing in the field of therapeutic radiation oncology.

It is in this context that in vivo studies of Tempol were initiated. Multiple mechanisms have been proposed for nitroxide protection against oxidative stress and ionizing radiation (11–12). In the current study, Tempol was administered to C3H mice, and its toxicity, pharmacology, and in vivo radioprotection were assessed. These findings extend the in vitro results and demonstrate that Tempol is an in vivo radioprotector.

MATERIALS AND METHODS

Materials. Tempol was purchased from Aldrich. PBS and Hanks’ balanced salt solution were purchased from Biofluids, Inc. (Rockville, MD).

Mice. Female C3H mice were supplied through the Frederick Cancer Research Center-Animal Production (Frederick, MD). The animals were received at 6 weeks of age and housed 5/cage in climate-controlled, circadian rhythm-adjusted rooms and allowed food and acidified water ad libitum. The animals were, on average, 60–80 days old at the time of radiation and weighed between 20 and 30 g. Experiments were conducted according to the principles outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

Determination of Maximal Tolerated Dose and Toxicity. Tempol, prepared in PBS (pH 7.4), was administered (100–500 mg/kg) by i.p. injection to female C3H mice. Mice were returned to their cages and observed continuously in the first hour after injection and daily for 30 days for toxicity and survival. For further toxicity studies, Tempol (275 mg/kg) was injected i.p. Control mice received injections of PBS alone. Whole blood samples were obtained from 15 mice and pooled 2 and 7 days after injection. The whole blood samples were centrifuged, and serum was removed for determination of electrolytes, creatinine, blood urea nitrogen, and liver function tests. These tests were performed by the Mid-Atlantic Regional Laboratory (Rockville, MD) and compared to known murine control values. Tissue specimens were obtained from the Tempol- and PBS-treated mice for pathological review. Liver, kidney, brain, lung, and heart specimens were removed and placed in formalin 2 and 7 days after injection. These were sectioned, stained with hematoxylin and eosin, and mounted on slides for review (American Histo Labs, Gaithersburg, MD). Blinded evaluation of the sections was performed by one author (M. S.).

Determination of Whole Blood Tempol Levels. Tempol was dissolved in PBS (pH 7.4) and injected i.p. (250 mg/kg) into C3H mice. At specified times after drug administration, 0.2–0.5 ml of whole blood was obtained and mixed with 0.2 ml of preservative-free heparin. Since nitroxides can be reduced in tissues to an EPR-silent compound (17),
each aliquot of whole blood was first mixed with the oxidizing agent potassium ferricyanide (final concentration, 1 mM) to determine the sum of the oxidized and reduced forms. Blood samples were stored on ice for a short time before being assayed. After mixing, 0.1 ml of blood was drawn by a syringe into a gas-permeable Teflon capillary tube of 0.8 mm inner diameter and 0.025 mm wall thickness (Zeus Industrial Products, Inc., Raritan, NJ). Each capillary tube was folded twice and inserted into a quartz tube which was open at both ends (2.5 mm inner diameter) and then placed vertically into the EPR cavity. EPR spectra were recorded on a Varian E4 (or E9) X-band spectrophotometer with field set at 3357 gauss modulation, frequency 100 kHz, modulation amplitude 1 gauss, and nonsaturating microwave power. A standard curve to allow conversion of the arbitrary units of EPR signal intensity to absolute Tempol concentration was constructed by determining the EPR signal of varying concentrations of an aqueous Tempol solution. Preservative-free heparin was found not to affect the EPR signal of Tempol solutions. For each experiment, an individual time point represents data collected from five mice. Samples from each mouse were measured independently and not pooled. The experiments were repeated in triplicate and were found in all cases to be similar despite the rapid clearance of Tempol from the blood.

Irradiation. A total of 20–80 C3H mice were used per radiation dose. Mice were weighed in groups of five. After preparing Tempol (PBS, pH 7.4), the mice received i.p. injections (275 mg/kg; approximate volume, 0.4–0.7 ml). One-half ml of PBS was injected i.p. into control mice. Groups of 10 mice were then transferred to round (30.5 cm diameter and 10.5 cm height) plexiglass containers with holes for ventilation. Two separate containers were placed in the sample tray of the irradiator, and the mice were irradiated 5–10 min after injection. A 125I gamma cell 40 (Nordion International, Inc., Kanata, Ontario, Canada) was used as the ionizing radiation source. The irradiator was calibrated with thermoluminescent dosimetry chips planted in phantom mice, and the radiation dose was determined according to previously described methodology (18). The dose rate used was 1 Gy/min (100 rad/min). Total time of irradiation varied as a function of the dose delivered. Immediately after irradiation, the mice were separated into groups of five and returned to climate-controlled cages for observation. Mice were assessed daily for survival.

Statistical Analysis. The surviving fractions at 30 days as a function of radiation dose were analyzed using logistic regression (19). Estimates of the LD50 for the Tempol survival curve were also calculated using this logistic model. Confidence intervals for the LD50 data were computed using the profile likelihood method.

RESULTS
Toxicity and Maximally Tolerated Dose. The maximally tolerated intraperitoneal dose of Tempol in C3H mice was found to be 275 mg/kg. At this dose, several minutes after injection, the mice were observed to be restless and hyperkinetic. However, mice treated at this and lower doses survived with no apparent adverse effects. Above 275 mg/kg, Tempol was lethal to varying degrees (Fig. 1). When mice survived 60 min after injection, no mortality was seen. A group of mice treated with the maximally tolerated dose was followed for 3 months, and no additional toxicity was observed. Above 275 mg/kg, restlessness and seizure activity were noted prior to death. The LD50 was 341 mg/kg (95% confidence intervals, 327–355 mg/kg).

Evaluation of serum samples, taken after administration of the maximally tolerated dose, revealed no difference in creatinine, electrolytes, or liver function tests between the control and Tempol-treated mice. In addition, no histological changes were observed in the tissue sections of liver, brain, kidney, heart, or lung of Tempol-treated mice.

Whole Blood Pharmacology. Tempol concentration was measured in whole blood samples. The peak whole blood concentration (~600 μg/ml) of Tempol occurred 5–10 min after injection. Points, survival data from 10–20 mice. The LD50 was 341 mg/kg (95% confidence intervals, 327–355 mg/kg).

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Fig. 1. Lethality induced by Tempol. C3H mice received i.p. injections of Tempol (dose range, 100–500 mg/kg) and were observed for survival. The data were analyzed by logistic regression. Points, survival data from 10–20 mice.

Fig. 2. The time dependence of Tempol concentration in whole blood. C3H mice received i.p. injections of Tempol (250 mg/kg), and at various times after injection Tempol concentration was determined with EPR spectroscopy. Points, average of 3 separate experiments, with a total of at least 15 mice/time point.
of radiation would be expected to correlate most with radiation protection. In the current study, it was assumed that the whole blood pharmacokinetics would closely approximate Tempol bone marrow pharmacodynamics. It also is possible that the concentrations of Tempol in whole blood were not achieved in the marrow space. Such a difference might explain why in vivo radioprotection is not as great as that achieved in vitro, where protection factors as high as 2.2 are observed with a Tempol concentration of 100 mM. It is possible that bioreduction of the nitroxide by the cellular components of blood and cells within the marrow compartment lessen the efficacy of Tempol. If in fact this is the case, another nitroxide which is bioreduced more slowly might provide a greater in vivo therapeutic effect. Preliminary screening of other nitroxides in vitro suggests that this may well be the case.

The nitroxides are also of interest with respect to hypoxic cell radiosensitization (13-16). The mechanism for this sensitization is unknown; however, it is well documented that nitroxides react with carbon-centered free radicals (24). Thus, under hypoxia it is plausible that nitroxides may compete for radiation-induced carbon-centered free radicals, and therefore rather than ameliorate the damage, they fix the damage. A large hypoxic cell fraction in human tumors is thought to contribute to a lack of response to clinical radiation treatment. Pharmacological therapy with a hypoxic cell radiosensitizer is one possible way to circumvent this clinical problem. Any compound which could selectively sensitize hypoxic cells (predominantly within tumors) and protect aerated tissues (predominantly normal tissues) might increase the therapeutic gain from radiation and have clinical utility. Tempol protects aerobic cells and sensitizes hypoxic cells in tissue culture (13). This study documents the in vivo radioprotection of whole mice but does not attempt to address the issue of in vivo hypoxic cell sensitization. We cannot ignore the possibility that Tempol might protect at least a portion of aerated tumor in vivo against the cytotoxic effects of radiation. We have begun studies to explore this possibility.

The current study shows that a nonlethal dose of Tempol in C3H mice resulted in significant radioprotection. This is the first report of in vivo radioprotection with this new class of radioprotectors. If data from in vitro work can be extrapolated to in vivo systems (13), then Tempol, unlike previous classes of
radioprotectors, may provide the potential for selective radioprotection of aerated tissues and sensitization of hypoxic tissues. Further studies to evaluate long-term toxicity, different modes of delivery, normal and tumor tissue pharmacology, and tumor sensitization or radioprotection relative to normal tissues are under way.

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REFERENCES


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