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

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ABSTRACT

Nitrooxide compounds are stable free radicals which were previously investigated as hypoxic cell radiosensitizers. The stable nitrooxide 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.

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 radical compounds, 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.

One water-soluble nitroxide, Tempol, 2 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 protect oxic 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 Maximally 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. The controls were injected with PBS only.

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The abbreviations used are: Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; PBS, phosphate-buffered saline; EPR, electron paramagnetic resonance; LD50, 50% lethal dose; LD50/30, dose of radiation at which 50% of mice die at 30 days.
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 $^{125}$I Cs 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 LD$_{50}$ for the Tempol survival curve were also calculated using this logistic model. Confidence intervals for the LD$_{50}$ 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 LD$_{50}$ 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 (Fig. 2). Since the whole blood concentration of Tempol peaked at 5–10 min and since previous in vitro studies indicated that the extent of radioprotection was dependent on nitroxide concentration at the time of irradiation, whole-body radiation was administered 5–10 min after injection.

In Vivo Radioprotection. Tempol was first administered to a group of 20 mice which were subsequently irradiated with a whole-body dose of 8.5 Gy. Treatment of C3H mice with Tempol 5–10 min prior to radiation provided a survival advantage which extended beyond 30 days (Fig. 3). Deaths in the control group occurred between days 14 and 22; this mortality was virtually eliminated in the Tempol-treated group. After demonstrating efficacy for a single radiation dose, the LD$_{50/30}$ was determined by using doses ranging from 7.0 to 14.5 Gy. Data were pooled from several experiments, with each data point repeated at least once and representing a minimum of 40 mice. The LD$_{50/30}$ for Tempol was 9.97 Gy (95% confidence intervals, 9.71–10.27) versus 7.84 Gy (95% confidence intervals, 7.65–8.01) for control mice (Fig. 4). This represents a dose modification factor (radiation dose which caused 50% lethality at 30 days in the Tempol-treated group divided by the radiation dose which caused 50% lethality at 30 days in the control group) of 1.3. The difference in surviving fraction curves between the Tempol- and saline-treated mice was significant ($P < 0.0001$) by logistic regression analysis. No late deaths were
of radiation would be expected to correlate most with radiation
biological measure of bone marrow toxicity (27), the concentra-
radio- Therefore, radiation was administered 5-10 min after drug
be oxidized back to the original state and therefore cannot be
peroxidation (23-26). One or all of these mechanisms may be
suggested that nitroxides also have a role in scavenging carbon-
hydroxyl radicals from hydrogen peroxide (a water radiolysis
effects of Tempol. Tempol has been shown to act as a Superoxide
peroxo radicals and may inhibit lipid
sensitization is unknown; however, it is well documented that ni-
xo compounds which could selectively sensitize hypoxic cells (predom-
nantly within tumors) and protect aerated tissues (predomi-
possible way to circumvent this clinical problem. Any com-
compound with a hypoxic cell radiosensitizer is one
radioprotectors. If data from in vitro work can be extrapolated
to in vivo systems (13), then Tempol, unlike previous classes of

observed in the irradiated Tempol-treated mice, even in those
groups observed longer than 100 days (data not shown). Tempol
was also administered at a dose of 275 mg/kg 24 h prior to
radiation. No radioprotection was observed under these
conditions.

DISCUSSION

The present study identifies the maximally tolerated i.p. dose of
Tempol, provides an estimate of the whole blood pharma-
cology of this agent, and documents in vivo radioprotection in
C3H mice. Radioprotection was observed when Tempol was
administered 5-10 min but not 24 h before radiation.

Mounting evidence suggests that radiation causes cellular
injury predominantly through damage to DNA (20). Of the
total damage to DNA, as much as 80% may result from radi-
induced water-derived free radicals and secondary carbon-
based radicals (21). Previous in vitro work has provided insight
into the potential mechanisms behind the radioprotective
effects of Tempol. Tempol has been shown to act as a superoxide
mutase mimic and to rapidly oxidize Fe(II)-DNA to Fe(III)-
DNA (11). Oxidation of metals could prevent formation of
hydroxyl radicals from hydrogen peroxide (a water radiolysis
product) by the Fenton reaction (22). In addition, it has been
suggested that nitroxides also have a role in scavenging carbon-
centered free radicals or perox radicals and may inhibit lipid
peroxidation (23-26). One or all of these mechanisms may be
at work in the in vivo setting.

Previous in vitro studies suggested that at least 5-10 mM of
Tempol are required to provide radioprotection (13). The max-
imum Tempol EPR signal achieved in the blood corresponds
to a concentration which approaches 3 mM at 5-10 min. It is
possible that Tempol is metabolized to a compound that cannot
be oxidized back to the original state and therefore cannot be
measured by EPR. Nonetheless, the EPR assay does allow for
an estimate of the timing of the peak Tempol concentration.
Therefore, radiation was administered 5-10 min after drug
administration in an attempt to optimize response. Since the
bone marrow is the critical tissue and the LD_{50/30} is a radio-
biological measure of bone marrow toxicity (27), the concen-
tration of Tempol in the bone marrow compartment at the time
of radiation would be expected to correlate most with radiation
Fig. 4. Survival of C3H mice 30 days after varying doses of whole-body
radiation. C3H mice received injections of Tempol (275 mg/kg) i.p. (○) or PBS
(O) and were treated with whole-body radiation ranging in doses from 7.0 to 14.5
Gy. Survival was recorded on the 30th day. The data were analyzed by logistic
regression. Points, survival data from at least 3 experiments, with at least 40
mice/survival dose.
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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