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Nitroxide TEMPO: A genotoxic and oxidative stress inducer in cultured cells[☆]

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Abstract

2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) is a low molecular weight nitroxide and stable free radical. In this study, we investigated the cytotoxicity and genotoxicity of TEMPO in mammalian cells using the mouse lymphoma assay (MLA) and in vitro micronucleus assay. In the absence of metabolic activation (S9), 3 mM TEMPO produced significant cytotoxicity and marginal mutagenicity in the MLA; in the presence of S9, treatment of mouse lymphoma cells with 1–2 mM TEMPO resulted in dose-dependent decreases of the relative total growth and increases in mutant frequency. Treatment of TK6 human lymphoblastoid cells with 0.9–2.3 mM TEMPO increased the frequency of both micronuclei (a marker for clastogenicity) and hypodiploid nuclei (a marker of aneugenicity) in a dose-dependent manner; greater responses were produced in the presence of S9. Within the dose range tested, TEMPO induced reactive oxygen species and decreased glutathione levels in mouse lymphoma cells. In addition, the majority of TEMPO-induced mutants had loss of heterozygosity at the *Tk* locus, with allele loss of 34 Mbp. These results indicate that TEMPO is mutagenic in the MLA and induces micronuclei and hypodiploid nuclei in TK6 cells. Oxidative stress may account for part of the genotoxicity induced by TEMPO in both cell lines.

Keywords

Nitroxide; Mouse lymphoma assay; Micronucleus assay; Loss of heterozygosity; Glutathione; Oxidative stress

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Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

The biological activity of nitroxides has been recognized for more than four decades. Nitroxides have a reducible nitroxide group (N–O) as part of a five- or six-carbon ring (Soule et al., 2007; Wilcox and Pearlman, 2008). Piperidine nitroxides are a diverse group of stable free radicals classically used as spin probes in electron paramagnetic resonance spectroscopy (Matsumoto et al., 2004), as paramagnetic contrast agents in nuclear magnetic resonance imaging (Bennett et al., 1987), and as oxygen-sensitive probes in biological systems (Strzalka et al., 1990). TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl, C₉H₁₈NO, MW 156.25), one of the most commonly used members of the nitroxide family, is a stable nitroxyl radical that has a variety of uses related to its role as radical scavenger and stabilizer. TEMPO is also broadly employed in organic synthesis for the oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones. Recently, TEMPO catalytic systems (e.g., ruthenium/TEMPO and copper/TEMPO) were reported to enable efficient oxidation of a broad range of primary alcohols, which facilitated their widespread use in synthetic chemistry (Dijksman et al., 2001; Hoover and Stahl, 2011).

By undergoing one-electron-transfer reactions, nitroxides are readily reduced to hydroxylamines or oxidized to oxoammonium cations (Fig. 1). The hydroxylamines can function as typical reducing agents, like vitamin C or E, to scavenge oxidants (Krishna et al., 1998). Therefore, TEMPO has been considered an antioxidant because it can mimic superoxide dismutase, inhibit hydroxyl radical generation, act as a hydrogen donor antioxidant, neutralize carbon-centered free radicals, and mimic the enzymatic activity of catalase (Castagna et al., 2009). TEMPO is employed extensively in cell and animal models; it has the advantages of being water-soluble, low-molecular weight, membrane-permeable, and commercially available. Studies have demonstrated the protective properties of TEMPO in various pathological situations, including radiation injury (Cuscela et al., 1996) and ischemia/reperfusion injury (Gelvan et al., 1991).

TEMPO, however, also can be converted into the highly oxidizing oxoammonium cation that consequently can mediate the selective catalytic oxidation of alcohols (Israeli et al., 2005). Therefore, under certain conditions, TEMPO can have pro-oxidant activity (Fig. 1). Thus, nitroxides may induce adverse effects (Israeli et al., 2005), producing toxicity mediated by nitroxide free radicals, as has been reported in studies conducted with both bacteria and mammalian cells. For example, nitroxyl compounds, including TEMPO, are mutagenic in *Salmonella typhimurium* strains TA104 and TA100 (Sies and Mehlhorn, 1986; Gallez et al., 1992). Some nitroxides manifest pro-oxidant effects by increasing the cellular hydrogen peroxide concentration (Voest et al., 1992; Offer et al., 2000). In addition, oxidation of nitroxides by superoxide radicals could produce oxoammonium species that may yield reaction products, i.e., oxoammonium adducts, which can oxidize many organic and inorganic molecules found in biological systems (Dragutan and Mehlhorn, 2007).

TEMPO is widely used throughout chemistry and biochemistry as process intermediates. Although there is no available information about the extent of human exposure, it is expected that the use of TEMPO will increase in the manufacturing process. Therefore,

additional toxicity data from studies using standard assays with validated protocols would be useful in a weight-of-evidence evaluation of TEMPO for human health risk assessment.

In the present study, we investigated the genotoxic potential of TEMPO using the mouse lymphoma assay (MLA) and the micronucleus (MN) test in TK6 human lymphoblastoid cells, two assays conducted using the Organization for Economic Co-operation and Development (OECD) Test Guidelines (TGs) (OECD, 1997, 2010). The MLA, which uses the thymidine kinase (*Tk*) gene of L5178Y/*Tk*^{+/-}-3.7.2C mouse lymphoma cells as a reporter of gene mutation, is currently the mostly widely used in vitro mammalian cell gene mutation assay, principally because it is able to detect a wide variety of genotoxic mechanisms. TK6 cells, derived from a patient with chronic myelogenous leukemia, are often used for evaluating the ability of a test agent to induce chromosomal damage that is expressed by the formation of MN. Based on its potential for prooxidant activity, oxidative stress induced by TEMPO also was determined in this study. In addition, we conducted loss of heterozygosity (LOH) analysis at four microsatellites spanning mouse chromosome 11 to explore the underlying mechanisms for the induction of *Tk* mutants by TEMPO.

2. Materials and methods

2.1. Materials

2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO, molecular formula C₉H₁₈NO, CAS# 2564-83-2), dimethyl sulfoxide (DMSO), cyclophosphamide, benzo[*a*]pyrene, 4-nitroquinoline-1-oxide (4-NQO), and trifluorothymidine (TFT) were purchased from Sigma (St. Louis, MO). 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was obtained from Life Technologies (Carlsbad, CA). Liver post-mitochondrial supernatant fraction (S9) prepared from Aroclor 1254 induced male Sprague–Dawley rats, Fischer's medium, and the cell culture supplies used for the MLA were obtained as previously described (Guo et al., 2011). X-rays were generated with an RS-2000 Biological Irradiator (Rad Source Technologies, Suwanee, GA). RPMI-1640 medium and supplies for TK6 cell culture and for conducting the MN assay, were obtained as described in Ali et al. (2011). PCR Master Mix, proteinase K, and the GSH-Glo[™] glutathione assay kit were obtained from Promega (Madison, WI). The primers used for detection of LOH in mouse chromosome 11 were synthesized by Integrated DNA Technologies (Coralville, IA).

2.2. Mouse lymphoma assay

2.2.1. S9 mix preparation—The S9 fraction was mixed with a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system. An S9 mix was prepared by mixing 180 mg/ml of glucose-6-phosphate, 25 mg/ml of NADP, 150 mM KCl, and rat liver S9 in the ratio of 1:1:1:2. The final concentration of S9 in the treatment medium was 1% (0.38 mg S9 protein/ml).

2.2.2. Cells and culture conditions—The L5178Y/*Tk*^{+/-}-3.7.2C mouse lymphoma cell line was used for the assay. Cells were grown and pre-existing *Tk*^{-/-} mutants were cleansed periodically according to the methods described in our previous study (Mei et al., 2005). The basic medium was Fischer's medium for leukemic cells of mice with L-glutamine

supplemented with pluronic F68 (0.1%), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). The treatment medium (F5p), growth medium (F10p), and cloning medium (F20p) were the basic medium supplemented with 5%, 10%, and 20% heat-inactivated horse serum, respectively. The cultures were gassed with 5% (v/v) CO₂ in air and were maintained in a shaker incubator at 37 °C.

2.2.3. Cell treatment with TEMPO—TEMPO working solutions (100×) were prepared just prior to use by dissolving TEMPO in DMSO. 6×10^6 cells in a total volume of 10 ml F5p were exposed to different concentrations of TEMPO in the absence and in the presence of 1% S9 (1–3 mM TEMPO without S9 and 1–2 mM TEMPO with S9) for 4 h at 37 °C. Positive controls were 0.3 µg/ml benzo[*a*]pyrene with S9 and 0.1 µg/ml 4-NQO without S9. After treatment, the cells were centrifuged and washed twice with fresh medium, and then were resuspended in F10p at a density of 3×10^5 cells/ml. The culture tubes were gassed with 5% CO₂ in air and placed on a roller drum (15 rpm) in a 37 °C incubator to begin the standard 2-day phenotypic expression.

2.2.4. The Tk microwell mutant assay—Mutant selection was performed as described previously (Mei et al., 2005). Briefly, the cells were counted and the densities were adjusted daily using fresh F10p following exposure. After 2 days of expression, mutants were enumerated by adding 3 µg/ml of TFT to cells suspended in F20p at a concentration of 1×10^4 cells/ml. The cells then were seeded into four 96-well flat-bottom microtiter plates using 200 µl per well. For the determination of plating efficiency, the cultures were adjusted to 8 cells/ml in F20p without TFT, and aliquoted at 200 µl per well into two 96-well flat-bottom microtiter plates. All plates were incubated at 37 °C in a humidified incubator with 5% CO₂ in air. After 11 days of incubation, the colonies were counted by visual inspection and the mutant colonies were categorized as small colony (SC) or large colony (LC) mutants. SCs are defined as those occupying <25% the diameter of the well. Mutant frequencies (MFs) were calculated using the Poisson distribution. Cytotoxicity was measured using relative total growth (RTG), which includes measurements of cell growth during the treatment (4 h), expression (2-day), and cloning (11-day) phases of the assay (Chen and Moore, 2004).

2.3. Micronucleus assay

2.3.1. Cells and culture conditions—TK6 human lymphoblastoid were purchased from the American Type Culture Collection (Manassas, VA). TK6 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air.

2.3.2. Cell treatment with TEMPO—MN assays were conducted as described in OECD TG487 for tests not employing cytochalasin B (OECD, 2010). To initiate an assay, 3×10^5 cells in 5 ml of growth medium were transferred to T-25 flasks and incubated overnight. The cells then were pelleted by centrifugation at 200g for 5 min, and resuspended in serum-free RPMI-1640. Dose range finding experiments were first conducted to establish a useful concentration range for conducting the assay and to provide data for reproducibility of the responses. For the main experiment, the cultures were treated for 3 h in duplicate with 1.4–2.3 mM TEMPO for the treatments without S9 and 0.9–1.5 mM TEMPO for the treatments

with S9. Cultures also were treated in duplicate with 2.5 µg/ml cyclophosphamide or exposed to 0.75 Gy of X-rays as positive controls, with and without S9 activation. As with the MLA, the chemical test agents were dissolved in DMSO, and delivered to give a final concentration of 1% DMSO in the treatment medium. S9 mix was prepared as described by Machanoff et al. (1981) and used at a final concentration of 0.4 mg S9 protein/ml in the treatment medium. After treatment, the cells were centrifuged and washed twice with calcium-magnesium-free phosphate-buffered saline (PBS), resuspended in 5 ml of growth medium, and incubated for 32 hr so that the vehicle controls would go through 1.5–2.0 cell divisions. A small volume of cells was removed from the vehicle controls (plus and minus S9) after washing (i.e., at the beginning of the culture period) to establish the initial cell concentration. After the 32 h manifestation period, all cultures were counted in order to insure that the vehicle control cultures had replicated adequately and to estimate the cytotoxicity of the treatments.

2.3.3. Flow cytometry—The MN analysis was performed essentially as described for the High Content Protocol 1 in the instruction manual for the *in vitro* MicroFlow™ Kit (Litron Laboratories, Rochester, NY). The volume of culture containing 5×10^5 cells was determined for the vehicle controls and this volume was withdrawn from each cell culture and placed into 15 ml centrifuge tubes. The cells then were processed and stained as described in the kit instruction manual. The samples were analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). The stopping gate was set at 10,000 intact nuclei and threshold parameters were set as recommended in the instruction manual. The P1 gate was used to capture hypodiploid events and estimate the induction of aneuploidy.

2.3.4. Cytotoxicity analysis—Treatment-related cytotoxicity was estimated by relative population doubling (RPD) as recommended by OECD TG487 (OECD, 2010). Information on cell death (apoptosis and necrosis) was gathered from the flow cytometer as described previously (Bryce et al., 2007).

2.4. Measurement of reactive oxygen species (ROS)

DCF-DA staining was used to determine the time course for intracellular ROS production (Karlsson et al., 2008). For each dose, 2×10^5 mouse lymphoma cells/ml were suspended in phenol-red free medium containing 5 µM DCF-DA and incubated at 37 °C in the dark for 30 min. Unincorporated DCF-DA was removed by washing the cells with PBS. Then the cells were resuspended in phenol-red free medium with 1–3 mM TEMPO, and immediately were transferred into a 96-well plate at a concentration of 1×10^4 - cells/well (4 wells per dose). The plate was incubated at 37 °C, and fluorescence was measured at 1–6 h and 24 h using wavelengths of 485 nm for excitation and 528 nm for emission.

2.5. Measurement of glutathione (GSH) levels

Cell viability and the intracellular GSH production were measured immediately after the mouse lymphoma cells were treated with different concentrations of TEMPO for 4 h. In order to exclude possible confounders for the GSH assay, the trypan blue dye exclusion assay was conducted by using an automated cell counter (Cellometer Auto T4, Nexcelom Bioscience, Lawrence, MA) to determine the number of viable cells present in the treated

cell suspensions; viability was expressed as a percentage of viable cells among the total number of cells counted. Cellular GSH levels were measured as described in the GSH-Glo™ kit instruction manual. After the 4 h treatment, 1×10^4 cells were dispensed into wells of a 96-well white flat-bottomed plate (8 wells per dose) and 50 μ l of freshly prepared GSH-Glo™ Reagent 2x (containing Luciferin-NT and Glutathione S-Transferase diluted 1:50 in GSH-Glo™ Reaction Buffer) were added into each well. The plate was incubated at room temperature for 30 min. After incubation, 100 μ l/well of Luciferin Detection Reagent were added to each well and luminescence was read with a Synergy 2 multi-mode microplate reader (Biotek Instruments, Winooski, VT).

2.6. LOH evaluation of Tk mutants

LOH analysis was conducted on 48 LC and 48 SC mutants resulting from the treatment with 2 mM TEMPO with S9 and a like number of SC and LC mutants resulting from treatments with 3 mM TEMPO without S9. The mutant cells from each treatment were transferred directly into a 96-well plate from TFT-selection plates and washed once with 200 μ l of PBS by centrifugation. The cell pellets were quickly frozen and stored at -20°C . The procedures for genomic DNA extraction, PCR, and agarose gel electrophoresis have been described previously (Guo et al., 2011). One band was scored as LOH and retention of two bands was scored as non-LOH at the given microsatellite locus.

2.7. Data analysis

For the micronucleus assay, genotoxicity was expressed as the percent micronuclei relative to intact nuclei, and presented as the mean and range of duplicate assays per dose. Data evaluation was conducted as described in OECD TG487 (OECD, 2010). Positive responses were characterized by a concentration-related increase in MN frequency, at least one response in excess of the 95% confidence interval for the historical negative control ($0.59 \pm 0.40\%$ MN with S9, $n = 34$; $0.60 \pm 0.36\%$ MN without S9, $n = 37$). For the MLA, the data evaluation criteria developed by the MLA Expert Workgroup of the International Workgroup for Genotoxicity Tests (IWGT) were used (Moore et al., 2003). Weighted sums of the number of LC and SC mutants were used in the comparison of LOH patterns and mutation spectra between different treatments. LOH patterns were compared using the computer program described previously (Mei et al., 2005; Mei et al., 2006). All the ROS and GSH values are presented as mean $\pm$ standard deviation (SD). Differences between groups were evaluated by the one-way analysis of variance (ANOVA) followed by pairwise comparisons to the vehicle control.

3. Results

3.1. Cytotoxicity and mutagenicity of TEMPO in the MLA, with and without metabolic activation

Dose range-finding tests were conducted before the main experiment in mouse lymphoma cells. According to the results of the dose-range finding tests, 5 doses of TEMPO were selected, and the main experiments were performed with and without S9. Table 1 shows the mean RTG and MF data from the main experiments. In the absence of S9 activation, the RTG produced by 3.0 mM TEMPO was approx. 20%, with the MF (208×10^{-6}) marginally

exceeding the global evaluation factor (GEF) of 126×10^{-6} . A final concentration of 1% S9 was used in assays testing the influence of metabolic activation on the cytotoxicity and mutagenicity of TEMPO. In the presence of S9, TEMPO resulted in dose-responsive decreases in RTG and increases in MF (Table 1), and MFs were positive by GEF for TEMPO doses of 1.25–2 mM. Compared with the treatment conducted without S9, the treatment of 2.0 mM TEMPO with S9 induced significantly higher levels of cytotoxicity and mutagenicity ($p < 0.05$). MFs with S9 were not determined for doses higher than 2 mM due to the induction of severe cytotoxicity and low plating efficiency.

3.2. MN induction by TEMPO in TK6 cells, with and without metabolic activation

Dose range-finding studies before the main assays indicated that 0.9–2.3 mM TEMPO produced a useful range of cytotoxicity for conducting the MN assay in TK6 cells. In the main study (Table 2), TEMPO induced dose-dependent increases in MN frequency (Table 2), with positive increases measured at doses 1.4 mM (without S9) and 1.0 mM (with S9). The 1.8 mM treatment without S9, which produced an RPD 51% (Table 2), induced a 4.5-fold increase in MN frequency over the vehicle control and a net increase of 1.95% micronuclei. 1.3 mM TEMPO with S9 resulted in a mean RPD of 45% and a 5.4-fold increase in MN frequency over the control with a net increase of 2.63% micronuclei. Based on evaluation criteria of OECD TG487 (OECD, 2010), TEMPO produced positive responses in the TK6 cell MN assay, both with and without S9.

TEMPO treatment increased the frequency of hypodiploid nuclei, with the induction being greater in the presence than in the absence of S9 (Table 2). Applying similar criteria for positive responses recommended by TG487 for MN induction, TEMPO was positive for the induction of hypodiploid nuclei in TK6 cells.

3.3. ROS levels in mouse lymphoma cells after TEMPO treatment

In order to evaluate the oxidative stress induced by TEMPO, intracellular ROS production was measured in mouse lymphoma cells at 1–6 h and 24 h after adding 1–3 mM TEMPO to the media (Fig. 2). The intracellular ROS level in 2, 2.5, and 3 mM treatments reached the peak level at 2 h treatment with about 4-fold increase, while 1 and 1.5 mM treatments increased to the maximum ROS level after 4 h and 3 h treatments, respectively. The ROS level in all treatments was sharply increased after adding TEMPO to the media, and gradually reduced after the peak and returned to the background level at 24 h. In addition, it appeared that TEMPO exposure resulted in a dose-related increases in the early stage of the treatments (i.e., before 3 h), and there were no differences in ROS levels between the different doses after 3 h treatment.

3.4. GSH levels in mouse lymphoma cells after TEMPO treatment

Intracellular GSH levels were measured after the mouse lymphoma cells were exposed to TEMPO for 4 h in the absence of S9. Within the dose range tested, TEMPO produced significant dose-dependent decreases in GSH levels compared to the vehicle control (Fig. 3). To exclude any bias caused by reduced cell numbers or cell death that occurred as a result of the TEMPO treatments, cell viability and cell concentrations were determined immediately after the 4 h treatment. The results showed that the cell viability in all the treatment groups

was >90%, and that there was no significant difference in cell concentration between the treatment groups and the vehicle control (data not shown).

3.5. LOH analysis of TEMPO-induced Tk mutants

DNA was extracted from 48 SC mutants and 48 LC mutants induced by 2 mM TEMPO with S9 and a like number of LC and SC mutants induced by 3 mM TEMPO without S9 (both treatments had similar levels of cytotoxicity) and then analyzed for LOH at four microsatellite loci (*Tk* locus and *D11Mit36*, *D11Mit20* and *D11Mit74*). These four polymorphic markers are almost evenly distributed along the full length of mouse chromosome 11. The percentages of LOH at the four loci in the LC and SC mutants are shown in Table 3. The percentages of the different types of mutations in the LC and SC mutant colonies combined are displayed in Fig. 4. Statistical analysis revealed that the mutational spectra induced by the two treatment groups were distinctly different from the vehicle control ($p < 0.001$) (Wang et al., 2007). Also, the mutational spectrum induced by TEMPO with S9 was significantly different from that induced by TEMPO without S9 ($p = 0.01$). The comparisons shown in Fig. 4 indicate that, relative to the negative control, TEMPO treatment, both with S9 and without S9, produced high levels of LOH at the *Tk* locus only ($p < 0.01$); also, TEMPO treatment without S9 induced relatively more mutants whose LOH extended to *D11Mit36*.

4. Discussion

The present study evaluated the in vitro genotoxicity of TEMPO using the MLA in mouse lymphoma cells and the in vitro MN test in TK6 human lymphoblastoid cells. In the absence of metabolic activation, TEMPO produced similar levels of cytotoxicity in both assays, with 3 mM reducing the RTG of mouse lymphoma cells to approx. 20% (Table 1), and 2.3 mM resulting in essentially no growth of TK6 cells (Table 2). These results are consistent with a time course analysis of TEMPO cytotoxicity in different types of tumor cells, which indicated that TEMPO treatment resulted in a > 50% decrease in the number of viable cells and a steady increase in apoptosis during the first 2 h of exposure, followed by a considerable increase in necrosis by 3 h (Suy et al., 1998). A 24 h treatment with TEMPO was cytotoxic to HaCaT human keratinocytes, producing an IC_{50} of 2.66 mM using the amido-black-assay (Kroll et al., 1999). It has been reported that TEMPO is approximately 200 times more lipophilic than its derivative 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (4-hydroxy-TEMPO) (Suy et al., 1998), which may facilitate its accumulation in the cell membrane and potentiate its cellular toxicity. In addition, TEMPO is a unique thermosensitizer that synergistically induces cell death when 5–10 mM TEMPO is presented during heating at 44°C in U937 leukemic cells and results in Bax activation and mitochondrial outer membrane permeabilization (Zhao et al., 2010).

S9 activation increased the cytotoxicity and genotoxicity of TEMPO in both assays (Tables 1 and 2), indicating that metabolites of TEMPO are more cytotoxic and genotoxic than TEMPO itself. Nitroxides undergo one-electron redox reactions to yield the respective hydroxylamine and oxoammonium cation (Goldstein et al., 2003) (Fig. 1). Under biological conditions, evidence indicates that nitroxides are mostly reduced to stable hydroxylamines

rather than being oxidized to oxoammonium compounds and forming adducts with other radicals (Dragutan and Mehlhorn, 2007). Preincubation of nitroxyl compounds with ascorbic acid in order to reduce the nitroxides to their corresponding hydroxylamines suppressed mutagenic activity (Gallez et al., 1992). In a preliminary study with the MLA, we treated mouse lymphoma cells for 24 h in the absence of S9. Both the 4 and 24 h treatments with TEMPO produced similar, low levels of mutagenicity but the 24 h exposure produced a marked increase in cytotoxicity (data not shown). These results provide further evidence that the mutagenicity of TEMPO mainly is due to metabolic activation by S9 enzymes.

It has been reported that nitroxides might exhibit both prooxidative as well as antioxidative properties (Voest et al., 1992; Balcerzyk et al., 2004), in some cases even at low concentrations (Glebska et al., 2003). In this study, we found that TEMPO exerts pro-oxidant activity and can also induce cytotoxicity and mutagenicity. Awareness of the genotoxicity of TEMPO is important as it may limit the usefulness of this nitroxide as an antioxidant. With regards to ROS, TEMPO produced dose-dependent pro-oxidant effects during the early stage of treatments (0–3 h) at concentrations ≥ 1 mM, based on a time course measurement of intracellular ROS in mouse lymphoma cells (Fig. 2). The increase in intracellular ROS (Fig. 2) and the parallel decrease in GSH levels (Fig. 3) are consistent with the effects of the nitroxide being mediated by oxidative stress. A 30 min treatment of human leukemia U937 cells with 10 mM TEMPO caused an early transient elevation of $\text{H}_2\text{O}_2/\text{O}_2^-$ and a late induction of only O_2^- , with a slight decrease in GSH and 30–50% reductions in ATP levels (Zhao et al., 2006). Intracellular GSH and ROS are two principal parameters for evaluating oxidative stress. GSH, the most abundant intracellular small-molecule thiol, is an important antioxidant and GSH is involved in the detoxification of a variety of electrophilic compounds and peroxides (Townsend et al., 2003). The depletion of cellular antioxidant defenses allows the generation of large quantities of ROS (Armstrong et al., 2002). The pro-oxidant effects of TEMPO in human erythrocytes is accompanied by a decrease in the concentration of intracellular reduced GSH (Balcerzyk et al., 2004); reduced GSH also occurs in human tumor cells where cytochrome P450 and other cellular systems involved in electron transport are believed to mediate the induction of oxidative activity (Voest et al., 1992). It has been suggested that the apparent paradoxical combination of cytoprotective and cytotoxic effects of nitroxides may be because they can act as a free radical and attack cellular components, either directly or by generating ROS when present in excess of intracellular free radicals (Gariboldi et al., 2000).

The L5178Y/*Tk*^{+/-}-3.7.2C mouse lymphoma cells used in the MLA are heterozygous at the *Tk1* locus on chromosome 11. Inactivation of the functional *Tk*⁺ allele (also referred as *Tk1b*) results in TFT resistance, which is the basis for the identification of *Tk*-deficient (*Tk*^{-/-} or *Tk*^{0/-}) mutants in the assay. The MLA detects a broad spectrum of genetic changes from point mutations to various chromosomal mutations. The MLA detects allele loss (Honma et al., 2001; Wang et al., 2009), which usually occurs as a result of LOH. LOH is an important mechanism for functional loss of critical genes (e.g., tumor suppressor genes), and is a common mutational event in the etiology of human cancer. In this study, we screened TEMPO-induced *Tk* mutants for LOH at the *Tk* locus (Table 3). TEMPO with S9 induced a dramatic increase in MF, and the mutants were characterized by an increased percentage of

LOH at the *Tk* locus from 64% in control mutants to 91.5%. TEMPO was less mutagenic in the MLA without S9, and the mutants had a lower percentage of LOH at the *Tk* locus (85.4%), which might be due to a greater contribution of “spontaneous” mutants since TEMPO induced considerably lower MFs in the absence of S9 medium. The LOH patterns of three other polymorphic markers along chromosome 11 also were analyzed in the TEMPO-induced mutants to explore the specificity of LOH induction. As results in Fig. 4 indicate, not only was the frequency and extent of LOH for the mutants induced by TEMPO different from the vehicle control mutants, but also the spectrum of LOH induced by TEMPO with S9 was somewhat different from that induced by TEMPO without S9. These results suggest that different mechanisms might exist for mutation induction by TEMPO with and without exogenous metabolic activation. It is possible that TEMPO-induced oxidative stress (Fig. 2) may contribute to DNA damage, primarily resulting in DNA breakage and deletion. This damage, which could be repaired by recombination and other mechanisms in cells, may result in the LOH that is the primary cause of mutations in the MLA (Mei et al., 2008; Wang et al., 2009) and the clastogenicity measured as MN induction in TK6 cells.

In summary, treatments with TEMPO induced dose-related cytotoxicity and genotoxicity in mouse lymphoma cells and TK6 cells, and the genotoxicity of TEMPO was increased in the presence of S9. TEMPO elevated ROS levels and depleted GSH at genotoxic doses, suggesting that oxidative stress may play a role in TEMPO-induced cytotoxicity and genotoxicity. The major type of *Tk* mutation induced by TEMPO, both with and without metabolic activation, involved LOH affecting less than 34 Mbp of chromosome 11. In addition, TEMPO increased MN frequencies (chromosome breakage), along with the frequency of hypodiploid nuclei (aneugenicity). Taken together, these results suggest that TEMPO is genotoxic in mammalian cells, at least partially through the generation of oxidative stress, resulting in large genetic alterations, including chromosome breakage.

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Fig. 1.
TEMPO redox reactions.

Fig. 2.

Analysis of reactive oxygen species (ROS) levels in TEMPO-treated mouse lymphoma cells. The ROS values were measured at 1–6 h and 24 h after treatment. The ROS levels were presented by the fold increase of the vehicle control and expressed as mean $\pm$ standard deviation from three independent experiments with four parallel samples per dose in each experiment. * $p < 0.01$ (when compared to untreated control group).

Fig. 3. Relative glutathione (GSH) levels in TEMPO-treated mouse lymphoma cells. The effect of TEMPO on intracellular GSH levels was presented by the percentage of the vehicle control and expressed as mean $\pm$ standard deviation from 3 independent experiments with 8 parallel samples per dose in each experiment. GSH levels were measured immediately after a 4 h exposure to TEMPO. * $p < 0.01$ (when compared to untreated control group).

Fig. 4.

Comparison of the percentage of TEMPO-induced mutants (large and small colony mutants combined) with LOH at four heterozygous loci in mouse chromosome 11. The mutants were from mouse lymphoma cell assays conducted with 2 mM TEMPO with S9, 3 mM TEMPO without S9, and the solvent control. The data are the weighted sum of mutation percentages from large and small colony mutants (the proportion of small colony mutants for TEMPO with S9 and TEMPO without S9 was 56% and 50%, respectively). The “→” indicates the LOH extends from the *Tk* locus to either *D11Mit36*, *D11Mit20*, or *D11Mit74*. The data for the solvent control are from our previous study (Wang et al., 2007).

Cytotoxic and mutagenic effects in mouse lymphoma cells treated with TEMPO in the absence or presence of metabolic activation (S9).^a

Table 1

Without S9				With S9			
Dose (mM)	RTG (%)	MF ($\times 10^{-6}$)	SC (%)	Dose (mM)	RTG (%)	MF ($\times 10^{-6}$)	SC (%)
0	100 $\pm$ 6	66 $\pm$ 11	49 $\pm$ 5	0	100 $\pm$ 4	82 $\pm$ 18	49 $\pm$ 8
1.0	94 $\pm$ 6	75 $\pm$ 34	53 $\pm$ 7	1.0	58 $\pm$ 10	156 $\pm$ 2	58 $\pm$ 4
1.5	68 $\pm$ 10	126 $\pm$ 58	57 $\pm$ 8	1.25	40 $\pm$ 1	235 $\pm$ 72 ^b	59 $\pm$ 4
2.0	54 $\pm$ 12	139 $\pm$ 34	50 $\pm$ 3	1.5	46 $\pm$ 10	254 $\pm$ 22 ^b	59 $\pm$ 4
2.5	42 $\pm$ 10	199 $\pm$ 56 ^b	51 $\pm$ 7	1.75	28 $\pm$ 12	388 $\pm$ 82 ^b	68 $\pm$ 1
3.0	20 $\pm$ 8	208 $\pm$ 56 ^b	57 $\pm$ 6	2.0	21 $\pm$ 4	502 $\pm$ 104 ^b	67 $\pm$ 11
NQO ^c	69 $\pm$ 11	427 $\pm$ 173 ^b	45 $\pm$ 2	Bp ^c	47 $\pm$ 18	1205 $\pm$ 84 ^b	51 $\pm$ 8

RTG: relative total growth, presenting cytotoxic effects in the mouse lymphoma cells.

MF: mutant frequency, presenting mutagenic effects per million cells.

SC: the percentage of small colony mutants.

^aThe data are presented as the mean $\pm$ SD from 3 to 5 independent experiments.

^bPositive response in the mouse lymphoma assay, exceeding the global evaluation factor of 126×10^{-6} .

^cPositive controls, 0.1 μ g/ml 4-NQO without S9 and 0.3 μ g/ml benzo[*a*]pyrene with S9.

Micronucleus frequency and induction of aneuploidy in TK6 cells treated with TEMPO in the absence or presence of metabolic activation (S9)^a.

Table 2

	Without S9				With S9			
	Dose (mM)	MN (%)	P1 ($\times 10^{-4}$)	RPD (%)	Dose (mM)	MN (%)	P1 ($\times 10^{-4}$)	RPD (%)
0		0.55 $\pm$ 0.00	9 $\pm$ 0	100	0	0.59 $\pm$ 0.14	28 $\pm$ 3	100
1.4		1.32 $\pm$ 0.31 ^b	33 $\pm$ 19	74 $\pm$ 3	0.9	0.89 $\pm$ 0.06	71 $\pm$ 25	83 $\pm$ 4
1.5		1.47 $\pm$ 0.43 ^b	42 $\pm$ 5	63 $\pm$ 2	1.0	1.41 $\pm$ 0.11 ^b	176 $\pm$ 8	75 $\pm$ 4
1.7		1.70 $\pm$ 0.05 ^b	32 $\pm$ 10	65 $\pm$ 4	1.2	2.16 $\pm$ 0.64 ^b	261 $\pm$ 69	61 $\pm$ 1
1.8 ^c		2.51 $\pm$ 0.32 ^b	61 $\pm$ 6	51 $\pm$ 1	1.3 ^c	3.23 $\pm$ 0.26 ^b	382 $\pm$ 5	45 $\pm$ 17
1.9		4.45 $\pm$ 0.08 ^b	79 $\pm$ 4	40 $\pm$ 5	1.4	4.80 $\pm$ 0.91 ^b	359 $\pm$ 12	35 $\pm$ 2
2.1		3.57 $\pm$ 0.30 ^b	87 $\pm$ 5	37 $\pm$ 8	1.5	5.32 $\pm$ 1.16 ^b	412 $\pm$ 69	21 $\pm$ 2
2.2		4.00 $\pm$ 0.59 ^b	122 $\pm$ 24	26 $\pm$ 3				
2.3		6.31 $\pm$ 0.33 ^b	165 $\pm$ 59	1 $\pm$ 1				
X-ray ^d		6.71 $\pm$ 0.05 ^b	285 $\pm$ 22	63 $\pm$ 1	CPA ^d	5.71 $\pm$ 0.18 ^b	947 $\pm$ 383	57 $\pm$ 2

MN: micronuclei frequency, presented as the percentage of micronuclei relative to intact nuclei.

P1: induction of aneuploidy, presented as hypodiploid nuclei per 10,000 intact nuclei.

RPD, relative population doubling, presenting cytotoxicity.

^aThe data are presented as the mean $\pm$ range of duplicate assays.

^bPositive response in the micronucleus assay according to OECD TG487.

^cResponse at maximum cytotoxic dose.

^dPositive controls, 0.75 Gy X-rays without S9 and 2.5 μ g/ml of cyclophosphamide with S9.

Table 3

Analysis of *Tk* mutants from mouse lymphoma cells exposed to TEMPO for loss of heterozygosity (LOH) at four loci along chromosome 11.

Locus	Position (Mbp) ^a	TEMPO without S9		TEMPO with S9		Control ^b	
		No. of LC (%)	No. of SC (%)	No. of LC (%)	No. of SC (%)	No. of LC (%)	No. of SC (%)
Non-LOH		14 (29)	0 (0)	8 (17)	1 (2)	21 (58)	1 (3)
<i>Tk</i>	117.7	34 (71)	48 (100)	40 (83)	47 (98)	15 (42)	32 (97)
<i>D11Mit36</i>	83.7	21 (44)	14 (29)	17 (35)	9 (19)	11 (31)	16 (48)
<i>D11Mit20</i>	44.6	6 (13)	6 (13)	12 (25)	4 (8)	5 (14)	13 (39)
<i>D11Mit74</i>	5.2	5 (10)	6 (13)	7 (15)	4 (8)	5 (14)	9 (27)
Mutants screened		48	48	48	48	36	33

LC: large colony mutants.

SC: small colony mutants.

^aLocus positions on chromosome 11 according to the updated database (<http://www.informatics.jax.org>).

^bData for the untreated control from our previous study (Wang et al., 2007).