Adjunctive Treatment of Murine Neuroblastoma with 6-Hydroxydopamine and Tempol

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INTRODUCTION

Children with metastatic neuroblastoma have only a 5–15% 5-year survival rate, despite maximal surgical, radiation, and chemotherapeutic treatment (1). Clearly, new approaches to this tumor are needed. The studies described herein were designed to test a targeted approach to neuroblastoma, which takes advantage of the dopamine uptake system. Neuroblastoma cells that take up dopamine and its congeners (2, 6) are selectively sensitive to attack by 6-hydroxydopamine (6OHDA) of formation of the disulfide metabolite of WR-2721 and the regimen produced synergistic toxicity in vivo, due to the hastening by 6OHDA of formation of the disulfide metabolite of WR-2721 and the ability of the latter compound to irreversibly inhibit γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis (7, 8).

Our subsequent studies have involved the use of the free radical scavenger Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) as a protective agent during 6OHDA treatment of murine neuroblastoma. Tempol has been shown to be a radioprotective agent for normal tissues and a weak radiosensitizer for tumors. The antioxidant function of Tempol in normal tissues is said to be the result of its activity as a SOD mimic (9). The mechanism of the environment-dependent function of this molecule is not completely known, although it has been proposed that it correlates with the oxygen tension of the tissue to which the drug is distributed (10).

Other studies have suggested that Tempol alters oxygen radical generation and metabolism by additional mechanisms distinct from the dismutation of the superoxide radical. Tempol and other stable nitroxides have been shown to directly react with both carbon-centered and peroxy radicals (11) and to prevent the reduction of hydrogen peroxide to the hydroxyl radical (12). Furthermore, these nitroxides can oxidize reduced transition metals that would otherwise serve to catalyze the formation of the hydroxyl radical via the Fenton reaction (13). Because the oxidative metabolites of 6OHDA include superoxide, hydrogen peroxide, and the hydroxyl radical, and because the generation of the hydroxyl radical from 6OHDA is greatly enhanced in the presence of reduced iron, all of the known mechanisms of the antioxidant activity of Tempol would be expected to contribute to the detoxification of 6OHDA.

The present article describes the adjunctive use of 6OHDA and Tempol in murine neuroblastoma-bearing mice. We further present in vitro data that suggest that the direct reduction of Tempol by 6OHDA, and not the activity of the former compound as an SOD mimic, constitutes the major mechanism by which Tempol protects normal tissues from 6OHDA-induced oxidation.

MATERIALS AND METHODS

Reagents. 6OHDA was obtained from Sigma Chemical Co. (St. Louis, MO). Tempol was obtained from Aldrich Chemical Co. (Milwaukee, WI). 6OHDA was dissolved in normal saline, and Tempol was dissolved in PBS (Life Technologies, Inc., Grand Island, NY), prior to i.p. injection into mice. The concentrations of each were such that the injection volume for each mouse was 0.01 ml/g body weight.
DMPO, ferrous ammonium sulfate, superoxide dismutase, catalase, and horseradish peroxidase were purchased from Sigma.

Animals. Male A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 5–7 weeks of age. Mice were housed five per cage and given free access to food and water throughout the experiment. In all cases, they were accustomed to the animal facility for at least 24 h prior to use.

Cell Culture. Neuro-2A murine neuroblastoma cells were obtained from the American Type Culture Collection (Bethesda, MD). Stock cultures were maintained as adherent cultures in plastic tissue culture flasks and fed with MEM supplemented with 10% FCS (Life Technologies, Inc.). Cultures were incubated at 37°C in an atmosphere containing 5% CO₂.

Establishment of s.c. Tumors in A/J Mice. Neuro-2A cells were harvested with trypsin and suspended in PBS for injection into A/J mice. Cells were counted by hemacytometry, and 10⁶ cells were injected s.c. into the right flank of each mouse. We have previously determined that this regimen results in an 80–100% tumor “take rate” by day 30 (14). Tumor volume was estimated every 1–2 days by direct measurement of the largest (L) and smallest (W) diameter of each tumor and calculating the volume as V = L × W². The mice were euthanized and autopsied for direct visualization of tumors on day 30 after cell injection. Tumors were excised, and their wet weights were determined. Mean weights of the control and various treatment group (see below) tumors were compared statistically using Student’s t-test, with P < 0.05 being accepted as significant.

Treatment with 6OHDA and/or Tempol. Tumor-implant-bearing animals were divided into four treatment groups (see Fig. 2 for n) receiving, respectively, vehicle alone in both injections, Tempol followed by vehicle, Tempol followed by 6OHDA, or vehicle followed by 6OHDA. The second injection (either vehicle or 6OHDA) was given 10 min after the first injection. This schedule was chosen because Tempol concentrations peak between 10 and 15 min after i.p. injection (10). Tempol injections were uniformly given in the right side of the abdomen; 6OHDA injections were uniformly given in the left side of the abdomen. This, coupled with the rapid absorption of Tempol into the bloodstream, made it unlikely that adjunctive effects would be related to direct interaction between Tempol and 6OHDA at the injection site or in the peritoneal cavity.

Grading of Animals for Ptosis and Activity Level. Ptosis was assessed as a measure of interference with the sympathetic nervous system, as we have described previously (15). Briefly, animals were graded as follows by two observers blinded to the treatment history of the animal: 0, normal; 1, oval palpebral fissures; 2, slitlike palpebral fissures; and 3, closed fissures; 1, oval palpebral fissures; 2, slitlike palpebral fissures; and 3, closed palpebral fissures. The activity level was graded in a similar fashion using the following scale: 3, normal activity; 2, lethargic but ambulatory; 1, nonambulatory but alive; and 0, dead. Statistical analysis of the values obtained at each time point was performed using the Mann-Whitney U test for nonparametric data (16). Interobserver concordance was excellent, and for each of the studies described herein, a representative plot of these parameters is shown.

RESULTS

Protective Effect of Tempol in Tumor-bearing A/J Mice Treated with 6OHDA. Preliminary studies were performed to determine the likely optimal dose of Tempol for protection against the toxicity of 6OHDA. An initial experiment in which mice (n = 5/group) were treated with Tempol alone demonstrated that from 1 h on after a single i.p. injection of 250 mg/kg Tempol, mice were indistinguishable in activity level from vehicle-injected mice (activity score, 3 in both groups). Mice treated with 300 mg/kg Tempol exhibited an early significant decrement in their activity score (2 at 10 min and 2.5 at 1 h) but reverted to normal activity (activity score, 2.9) by 5 h after injection. Mice treated with 350 mg/kg Tempol alone developed a decrement in activity (activity score, 1.5) by 10 min after injection that persisted for the 5-h duration of the study. On the basis of this experiment, it was decided to perform subsequent, adjunctive studies at doses of Tempol at or below 250 mg/kg, so that any observed decrement in activity could be ascribed to the addition of 6OHDA to the regimen.

To determine whether doses of Tempol at or below 250 mg/kg were effective in ameliorating the toxicity of 6OHDA, an initial experiment was performed in which mice (n = 5/group) were treated with either 125 or 250 mg/kg alone or in combination with 300 mg/kg 6OHDA. An additional control group was treated with 300 mg/kg 6OHDA alone. Mice treated with 125 mg/kg Tempol prior to treatment with 6OHDA were indistinguishable in activity score from those treated with 6OHDA alone (activity scores, 1.4 and 1.7, respectively, 6 days after 6OHDA injection). In contrast, mice treated with 250 mg/kg Tempol prior to treatment with 6OHDA were indistinguishable in activity score from those treated with Tempol alone (activity scores, 2.6 and 3.0, respectively, at 6 days after 6OHDA injection). All subsequent studies, therefore, were performed with a Tempol dose of 250 mg/kg.

Groups of five mice each were treated with Tempol, followed 10 min later with 6OHDA (350 or 400 mg/kg), both by i.p., injection 24 h following the s.c. injection of murine neuroblastoma cells. These doses of 6OHDA were chosen because they are just beyond the maximum tolerated dose of this drug given alone (7), and because we wished to demonstrate the ability of Tempol to facilitate tolerance of these ordinarily toxic doses. All mice were scored for sympathetic nervous system impairment (ptosis) and activity level, as detailed in “Materials and Methods.” Note that the retained level of function improves with an increasing activity score and with a decreasing ptosis score. Furthermore, the activity score is a cumulative score; i.e., for animals that succumb to treatment, a score of 0 is included in the average for the group from the point of its death through the end of the experiment. On the other hand, the group ptosis score is the average of the ptosis scores for the living animals at each particular time point. The study was performed in duplicate, and a representative plot is shown for each parameter.

As is shown in Fig. 1A, Tempol administration resulted in a decrease and delay in attainment of the maximum ptosis score in mice treated with 6OHDA (Fig. 1A, 0 versus 0, respectively). The mean ptosis score at 24 h for animals given 400 mg/kg 6OHDA with Tempol differs from that with 6OHDA alone, with a P < 0.05, whereas that for animals given 350 mg/kg 6OHDA with Tempol differs from that with 6OHDA alone, with a P < 0.04 (Mann-Whitney U test). Adjunctively treated animals (Fig. 1A, 0 and 0, respectively) exhibited significant recovery of function, as evidenced by the decrease in their ptosis scores after attainment of 60 days.
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Fig. 1. Effects of treatment with 6OHDA and Tempol, alone or in combination, on ptosis score (A), activity score (B), and mortality rate (C) in neuroblastoma cell implant-bearing A/J mice. Tumor cells were injected s.c., and Tempol and 6OHDA were injected i.p. 24 h later. D, Tempol; O, 400 mg/kg 6OHDA; C, 350 mg/kg 6OHDA; Δ, 400 mg/kg 6OHDA + Tempol; □, 350 mg/kg 6OHDA + Tempol. In concert with the known pharmacokinetics of Tempol and 6OHDA and their effects (7, 10), Tempol was administered first, followed 10 min later by 6OHDA administration. Mice were scored for ptosis and activity as described in “Materials and Methods.” At 24 h after injection, when there remained five mice in the adjunctively treated group and three mice in the 6OHDA-treated group, the difference in mean ptosis score between animals receiving 6OHDA (400 mg/kg) with Tempol and those receiving 6OHDA alone was significant at the $P < 0.05$ level (Mann-Whitney U test). At this same time point, when there remained five mice in each group, the difference in mean ptosis score between animals receiving 6OHDA (350 mg/kg) with Tempol and those receiving 6OHDA alone was significant at the $P < 0.04$ level (Mann-Whitney U test). Note that from 3 days through the close of the study, data shown in A and B for 350 mg/kg 6OHDA alone are the raw data for the one remaining mouse. The differences in activity levels between mice receiving 350 or 400 mg/kg 6OHDA alone and their respective Tempol-treated counterparts are significant at the $P < 0.01$ level (Mann-Whitney U test).

maximum ptosis (i.e., at time points more than 1 day after injection). Even more significant was the decrease in mortality rate and improvement in activity level of adjunctively treated mice relative to their 6OHDA-treated counterparts ($P < 0.05$; 24-h activity levels, 350 and 400 mg/kg 6OHDA; Fig. 1, B and C). The increased activity level reflects both a decrease in the mortality rate (Fig. 1C) and the complete recovery of the surviving animals over the 48-h period following drug treatment (Fig. 1B). Note that from 3 days through the close of the study, data shown in Fig. 1, A and B, for 350 mg/kg 6OHDA alone are the raw data for the one mouse that remained alive; the apparent improvement in ptosis score in this group results from this single outlier.

Additional studies were performed using lower doses (100 and 200 mg/kg) of 6OHDA with and without Tempol (250 mg/kg), in an attempt to isolate the antisympathetic effects of 6OHDA (and protection by Tempol therefrom) from its systemic effects. The animals in these studies manifested no obvious systemic effects of 6OHDA (i.e., activity scores of 3 throughout the experiments). Furthermore, these studies demonstrated a trend toward decreased ptosis in the presence of Tempol (mean ptosis scores at 36 h, 2.0 with 200 mg/kg 6OHDA alone and 1.2 with 200 mg/kg 6OHDA + 250 mg/kg Tempol). This decrease did not reach statistical significance, because the ptosis scores at these low doses of 6OHDA alone are extremely low (maximum ptosis scores at 100 and 200 mg/kg 6OHDA, 1.6 and 2.0, respectively). However, the low toxicity of the doses of 6OHDA used in this experiment raises the possibility of lowering the dose of 6OHDA, rather than using it adjunctively with Tempol, as a means of effecting nontoxic tumor suppression.

Antitumor Effectiveness of Adjunctive Treatment. Fig. 2A illustrates the effects of treatment with 6OHDA or Tempol alone and adjunctive treatment on tumor weight and incidence in A/J mice injected s.c. with murine neuroblastoma cells 30 days prior to sacrifice. In all cases, tumor incidence was determined by gross inspection and palpation on dissection; tumor weight was determined as the wet weight of the resected tumor on day 30. All of the tumors were invested with a fibrous capsule and, therefore, were readily separated from surrounding normal tissue.

The adjunctive regimen influences both incidence and final weight of the tumors; the effect on tumor weight is significant at the $P < 0.01$ (compared with Tempol alone) level. Although tumor incidence was reduced from 80–100% to 40%, it is not clear whether this is the result of a true reduction in tumor take rate or a reduction in the size of tumors below the limit of gross detectibility. All animals treated with 6OHDA alone were dead by day 30 (see Fig. 1C); there was no
statistically significant difference between the tumor weights or incidence in the control and the Tempol-treated groups.

In a single experiment, the kinetics of tumor growth was examined by direct measurement of tumors every 1-2 days for 22 days. Determination of the initial (days 10-16 after tumor cell injection) slopes of the tumor growth curves revealed that the linear slopes for mice treated with saline alone and Tempol alone were indistinguishable (0.29 and 0.25, respectively). In contrast, in mice treated with Tempol and 6OHDA, tumors remained below the level of detection by palpation throughout the initial 16 days after tumor cell injection. All tumors, including the one first detected on day 18 in an adjunctively treated mouse, continued to grow throughout the 22-day period of direct measurement. This is not surprising, because the treatment regimen involved a single set of injections given well before the establishment of grossly palpable tumors (24 h after tumor cell injection).

As suggested by the toxicity and protection studies described above, another strategy for decreasing the toxicity of 6OHDA in chemotherapy of neuroblastoma might be to simply lower the dose of that agent alone, obviating the need for an adjunctive regimen. However, as is shown in Fig. 2B, doses of 100 and 200 mg/kg 6OHDA were ineffective in reducing tumor size or incidence in this model. Almost all of the mice in this study had tumors by day 30, regardless of treatment with 6OHDA with or without Tempol. In general, the tumors obtained in this experiment were smaller than those in the experiment shown in Fig. 2A; this is related to intrinsic variability in the model itself (11) and necessitates comparison with simultaneously injected animals (e.g., animals receiving Tempol alone) in each experiment. Although the mean tumor weight was lower for mice receiving 200 mg/kg 6OHDA than for those receiving 100 mg/kg 6OHDA, this difference did not reach statistical significance (Student’s t test); furthermore, in no group receiving 6OHDA at these low doses did the mean tumor weight differ significantly from that of animals receiving Tempol alone.

Interaction of Tempol with 60HDA in Vitro. In an effort to explore the mechanism by which Tempol protected mice from the oxygen free radical-mediated effects of 6OHDA, the effects of coinoculation with 6OHDA on the ESR spectrum of Tempol were studied. ESR permits direct measurement of Tempol and reactive oxygen species. Our prior attempts to make such determinations by spectroscopic and colorimetric methods were confounded by the overlapping visible-range spectra of radical-trapping reagents and 6OHDA.

The concentrations of 6OHDA and Tempol used in these studies were chosen on the basis of tissue culture and in vivo data, which demonstrate the ready attainability of these concentrations in dopaminergic nerve terminals and blood, respectively (10, 18). Because of the reports of the dismutation of superoxide by Tempol (9), we also explored by ESR the effects of SOD, catalase, and peroxidase on the interaction of Tempol and 6OHDA.

In the absence of 6OHDA, Tempol gives rise to a characteristic ESR spectrum, which consists of three biphasic signals (Fig. 3C). This three-component spectrum did not change in intensity over a 15-min period of aerobic (Fig. 3A, •) or anaerobic (Fig. 3B, •) incubation. Fig. 3 also shows the effects of 6OHDA (0, 0.5, 2, and 5 mM; •, •, ■, and △, respectively) on the Tempol ESR signal. Coincubation with 6OHDA caused a concentration-dependent decrease in the amplitude of this signal over time; similar results were obtained in aerobic (Fig. 3A) and anaerobic (Fig. 3B) environments.

To assess the effects of enzymes involved in the metabolism of particular reactive oxygen species on the 6OHDA-dependent decay of the Tempol ESR signal, the intensity of that signal 6 min after the addition of 6OHDA to the mixture was compared with a simultaneously incubated control solution in the presence and absence of each of these enzymes. These studies were performed separately under aerobic and anaerobic conditions. Under aerobic conditions, the 6OHDA-induced decrease in the amplitude of the Tempol ESR signal was not affected by SOD. However, it was significantly inhibited by catalase and horseradish peroxidase (Figs. 3A and 4A). In contrast, none of these oxygen radical-related enzymes altered the 6OHDA-induced signal decrement seen under anaerobic conditions (Figs. 3B and 4B). The addition of peroxidase or catalase following 30 min of incubation of 6OHDA and Tempol (by which time the Tempol signal had decayed to zero) under either aerobic or anaerobic conditions did not reconstitute the Tempol ESR signal. The signal also could not be regained by the addition of the combination of peroxidase and hydrogen peroxide to the incubation system. Incubation of Tempol with
Fig. 3. Effects of 6OHDA and horseradish peroxidase on the amplitude of the Tempol ESR signal under aerobic (A) and anaerobic (B) conditions. The incubation medium for these studies included Tempol (0.5 mM) and 6OHDA (0.05, 0.5, 2, and 5 mM). In addition, under each condition, a sample was included (2) which contained Tempol (0.5 mM), 6OHDA (5 mM), and catalase (1 mg/ml). C. ESR spectra of Tempol nitroxyl radical generated repeatedly over 10 min of incubation with 6OHDA. ESR settings: center field, 320.0 mT; scan range, 10.0 mT; modulation amplitude, 0.05 mT; receiver gain, 10; time constant, 0.01 s; sweep time, 4.0 min; microwave power, 10 mW.

Fig. 4. Effects of SOD, catalase, and peroxidase on Tempol ESR signal intensity in the presence of 6OHDA under aerobic (A) or anaerobic (B) conditions. ESR spectrum magnitudes are given for 6 min after mixing of the reagents. Incubation medium: Tempol, 0.5 mM; 6OHDA, 5 mM; SOD, 0.1 mg/ml; catalase, 0.1 mg/ml; horseradish peroxidase, 0.1 mg/ml. ESR settings were as detailed in the legend to Fig. 3.

Iron-dependent Formation of DMPO-OH Adducts. To determine the potential role of Tempol as a scavenger of hydroxyl radicals formed secondarily from 6OHDA oxidation products in the iron-containing in vivo environment, the effects of Tempol on the formation of a trappable hydroxyl radical from 6OHDA in the presence of iron were studied. The addition of Fe(II), as [Fe(NH4)2(SO4)]2, and the spin trap DMPO to 6OHDA preincubated for 30 min in phosphate buffer (pH 7.4) immediately resulted in the characteristic four-component ESR spectrum of the DMPO/OH spin adduct (Fig. 5, inset). When 6OHDA was preincubated for 30 min with Tempol, the intensity of the DMPO/OH spin adduct signal decreased by a factor of 2.5 (Fig. 5).

DISCUSSION

Currently available therapy for disseminated neuroblastoma affords only a 5–20% 5-year survival rate. This figure has not changed in the past 2 decades (1). We have attempted to design targeted chemotherapy for this disease by exploiting the dopamine uptake system on neuroblastoma cells (7, 18, 19). The dopamine analogue 6OHDA generates cytolytic oxygen radicals in cells, such as many neuroblastoma cells, that can actively take it up. It is, however, predictably toxic both systemically and, especially, to the sympathetic nervous system (2, 7). To abrogate this toxicity, we have used the antioxidant Tempol adjunctively with 6OHDA. Tempol has previously been found to be selectively protective for normal cells relative to tumor cells, although the mechanism of this selectivity is not entirely clear (10).

The present study demonstrates that, unlike single-dose treatment with either Tempol or 6OHDA alone, adjunctive single-dose treatment of mice implanted s.c. with neuroblastoma cells results in a reduction in both tumor incidence and weight, with relative preservation of sympathetic nervous system function and general activity level. Although merely lowering the dose of 6OHDA also results in decreased toxicity, these doses do not reduce tumor incidence or size. This finding not only makes this simpler strategy therapeutically useless; it also makes it likely that the protection afforded by Tempol is not the result of decreased absorption of 6OHDA in the presence of the antioxidant, because 6OHDA in the presence of Tempol is effective as a tumoricidal agent.

Studies of 6OHDA-induced cell and tissue injury have indicated the primary role of reactive oxygen species generated in the process of auto-oxidation of 6OHDA (4, 20). In the absence of iron, the rate of auto-oxidation of 6OHDA is extremely low (K < 10–15/M/s). Almost
certainly, the auto-oxidation of 6OHDA in vivo involves successive reduction of a series of metal-bound oxygen species, the actual transfer of electrons occurring within a ternary reductant-metal-oxygen transition state (21). Indeed, in our system, the generation of the hydroxyl radical from 6OHDA is iron dependent.

Previous studies have demonstrated that one mechanism of the antioxidant action of Tempol is its function as an SOD mimic (9). However, other investigators have pointed out that the rate of reaction between 6OHDA and superoxide in tissues is already diffusion limited (22), making it perhaps less likely that dismutation of superoxide radicals constitutes the biologically relevant mechanism of Tempol-mediated protection.

We have demonstrated that 6OHDA can directly and efficiently reduce Tempol. The decay of the Tempol ESR signal is identical under aerobic and anaerobic conditions; in neither circumstance is the interaction of Tempol and 6OHDA affected by SOD. This makes it most likely that the direct reaction of Tempol with 6OHDA accounts for the observed decay in the Tempol ESR signal.

One-electron reduction of Tempol results in the oxidation of 6OHDA and the consequent formation of the 6OHDA-semiquinone radical and the hydroxylamine Tempol-OH. In the absence of Tempol-OH, this would in turn result in the accumulation of the superoxide anion and hydroxyl radical. However, Mitchell et al. (23) have demonstrated that Tempol-OH can be oxidized by superoxide to regenerate Tempol and form hydrogen peroxide. The rate constants for these nitroxy1-catalyzed superoxide dismutation reactions are estimated to be in the range of $10^5-10^6$/s. Our studies support the notion of this Tempol/Tempol-OH redox cycling and indicate that, in the presence of these reagents, the oxidation of 6OHDA does not result in the accumulation of superoxide and hydroxyl radicals. Additional studies performed by us using lucigenin-amplified chemiluminescence to detect superoxide indicate that no superoxide accumulates in the course of incubation of 6OHDA (5 mM) with Tempol (0.5 mM) in phosphate buffer (100 mM pH 7.4) containing desferal (100 mM) to chelate adventitious iron in the buffer.

Catalase and peroxidase did not affect the Tempol reduction by 6OHDA under anaerobic conditions but did inhibit its decay under aerobic conditions. This effect could be due to the peroxidase- or catalase-dependent reoxidation of Tempol hydroxylamine to the corresponding nitroxy1 radical, with a resultant restoration of the ESR signal intensity. In this regard, catalase is known to posses a peroxidase-like activity (22). We tested this hypothesis by adding $H_2O_2$ and peroxidase to the incubation medium after complete disappearance of the Tempol ESR signal. This did not restore the Tempol signal under either aerobic or anaerobic conditions, making it unlikely that peroxidase oxidized Tempol hydroxylamine to the nitroxy1 radical. Furthermore, neither catalase nor peroxidase and $H_2O_2$ alone changed the intensity of the Tempol ESR signal over a 15-min incubation, indicating that these enzymes did not convert Tempol to a substance that could not subsequently be reoxidized to Tempol. Rather, catalase and peroxidase most probably oxidize 6OHDA to 6OHDA-quinone and thereby prevent Tempol reduction by 6OHDA.

The dependence of the effects of catalase and peroxidase in our in vitro system on the availability of oxygen raises the possibility that the selectivity of Tempol as a scavenger in normal tissues relative to tumors is a function of the relative hypoxia of solid tumors. This hypothesis has been proposed by previous investigators as well (10).

Although the availability of transition metal complexes in vivo may be a limiting factor in 6OHDA autooxidation and generation of reactive oxygen species, direct interaction of Tempol with 6OHDA may provide protection from these free radicals by decreasing the effective concentration of 6OHDA and thereby abrogating the formation of superoxide and $H_2O_2$. In support of this, our results demonstrate that the formation of the DMPO/OH spin adduct in the presence of Fe(II) was significantly diminished when 6OHDA was preincubated with Tempol.

These studies demonstrate the potential role of mechanisms other than dismutation of superoxide in Tempol-mediated protection from the toxicity of 6OHDA. Experiments are currently underway to determine the in vivo relevance of this mechanistic work. Specifically, we are examining by ESR, in the presence and absence of 6OHDA cotreatment, the concentrations of Tempol in normal tissues and tumors from neuroblastoma-bearing mice.

Our studies further raise the therapeutic possibility of adjunctive treatment of neural crest tumors such as neuroblastoma. The persistent, although greatly attenuated, growth of tumors after a single adjunctive treatment with 6OHDA and Tempol makes it likely that therapeutic application of this approach will necessitate repeated dosing with these drugs. Future studies will focus on the preclinical optimization of a multiple dosing regimen for these drugs.

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