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Forum Review

Therapeutic and Clinical Applications of Nitroxide Compounds

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ABSTRACT

Nitroxide compounds have been used for many years as biophysical tools, but only during the past 15–20 years have the many interesting biochemical interactions been discovered and harnessed for therapeutic applications. By modifying oxidative stress and altering the redox status of tissues, nitroxides have the ability to interact with and alter many metabolic processes. This interaction can be exploited for therapeutic and research use, including protection against ionizing radiation, as probes in functional magnetic resonance imaging, cancer prevention and treatment, control of hypertension and weight, and protection from damage resulting from ischemia/reperfusion injury. Although much remains to be done, many applications have been well studied, and some are presently being tested in clinical trials. The therapeutic and research uses of nitroxides are reviewed here, with a focus on the progress from initial development to modern, state-of-the art trials. *Antioxid. Redox Signal.* 9, 1731–1743.

FREE RADICALS AND NITROXIDE ANTIOXIDANTS

NITROXIDES, ALSO KNOWN AS AMINOXYLS, are chemical compounds containing the tertiary amine (R_3N^+-O^-) functional group that are oxidized to form relatively stable nitroxide radicals. Many of these compounds have been synthesized and described, but it has only been during the past 15–20 years that many of the interesting biochemical interactions been discovered and exploited for medical use. The mechanism underlying the biologic activity of these compounds is related to modification of oxidative stress from free radicals and reactive oxygen species (ROS), which are involved in a large number of normal and pathologic processes. Cells and tissues have developed numerous mechanisms to protect against this damage, including enzymatic defense systems such as superoxide dismutase (SOD), catalase, glutathione (GSH), and other protein thiols and intracellular redox couples. These protective mechanisms can be overwhelmed under certain conditions that cause increased oxidative stress, such as exposure to hydrogen peroxide (H_2O_2), radiation exposure, trauma, or infection.

The protective effect of nitroxides was elucidated in a series of experiments beginning in the 1980s (72) and has been shown to be the result of antioxidant activity both *in vitro* and *in vivo*. SOD is a large enzyme that detoxifies superoxide ($O_2^{\bullet -}$) but is too large to cross cell membranes, making it difficult to increase intracellular levels by adding exogenous SOD. As early as 1990, a set of stable, low-molecular-weight nitroxides was identified that possessed SOD-like activity and easily crossed cell membranes. Early experiments revealed that these compounds protected mammalian cells against damage induced by exposure to hypoxanthine/xanthine oxidase, despite having no direct catalase activity against H_2O_2 itself (62). Nitroxides were added to cardiomyocytes in culture that had been treated with H_2O_2 and provided complete protection against damage and death without altering H_2O_2 levels within the cell, suggesting that nitroxides intercept intracellular oxygen radicals and could exert antioxidant effects. Nitroxides were more effective in pro-

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tecting cells exposed to H_2O_2 and other free radical–inducing agents such as streptonigrin than exogenously supplemented catalase or SOD itself (18, 20, 73, 74). Electron paramagnetic resonance (EPR) studies substantiated the catalytic role of nitroxides in the dismutation of $O_2^{\cdot -}$ (43). Additionally, nitroxides were shown to confer catalase-like behavior to heme proteins and to detoxify H_2O_2 (45) and to participate in radical–radical recombination reactions, which can limit the levels of free radicals and protect cells (61).

RADIOPROTECTION AND NITROXIDES

The term radioprotection implies several functions, of which the most literal and fundamental is the protection of individual cells from death after exposure to radiation. From a clinical perspective, however, radioprotection can also include protection against the undesired side effects of radiation including fibrosis, alopecia, mucositis, skin damage, and development of second malignancies. An ideal radioprotective agent would have several characteristics, including minimal toxicity, ease of administration, and selectivity for radioprotection of normal tissues compared with tumor. Several methods of achieving tumor-specific activity have been used in other treatment modalities, but the most consistent and reliable technique is to exploit a biochemical characteristic of tumors to bring about the desired effect. As will be shown, nitroxides are ideal for this application and have the potential for significant benefit.

The use of nitroxides as a radioprotective agent was thought to be possible because much of the damage induced by radiation results from the formation of free radicals within cells. As potent antioxidants, nitroxides should detoxify the radicals and ameliorate the damage. This theory was first tested *in vitro* by using Chinese hamster cells exposed in culture to lethal doses of gamma radiation and treated with the nitroxide tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) in concentrations ranging from 0 to 100 m*M*, as shown in Fig. 1 (60). A significant and dose-dependent protective effect of tempol resulted in protection by a factor of 2.5 in the cells treated with the 100 m*M* concentration as compared with the untreated control cells. Because the cells were grown and irradiated under aerobic conditions, the protective effect of tempol could not be attributed to the induction of intracellular hypoxia, increased intracellular GSH levels, or induction of SOD. Another important finding, shown in Fig. 1, was that the reduced form of tempol, the hydroxylamine tempol-H (TPH), did not confer radiation protection even at a concentration of 100 m*M*, suggesting that nitroxides may more readily react with the radical species produced by radiation than hydroxylamines (93).

The efficacy of several other nitroxides as radioprotectors was tested, and many offered radioprotection in a standard radiation dose–response clonogenic assay (34). Two nitroxides, Tempamine and 3-aminomethyl-proxyl, demonstrated improved protection over tempol, and DNA-binding studies revealed that these two nitroxides bound to DNA with higher affinity. DNA is likely the target of direct and indirect cytotoxic effects of ionizing radiation, potentially explaining the relative increase in protection by compounds that bind more strongly to DNA. Taken together, these studies firmly established nitroxides as true radioprotectors.

The mechanism of this radioprotective effect was further substantiated in a study showing that tempol significantly decreased the mutagenicity of a single 40-Gy fraction of radiation and provided a 3.5-fold protection against cytotoxicity (19). Tempol also increased cell survival and protected against DNA dam-

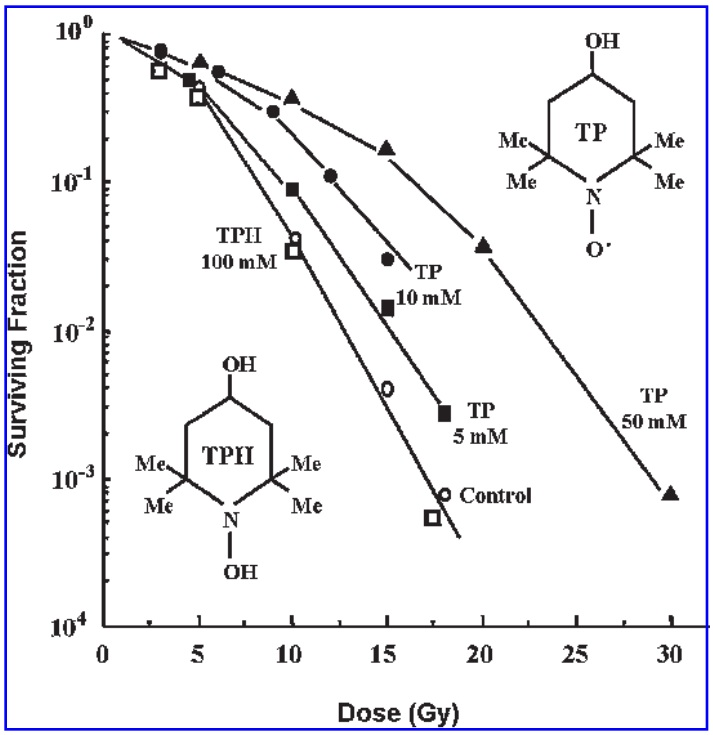

FIG. 1. Effect of tempol and tempol-H on radiation-induced cell death *in vitro*. Irradiated Chinese hamster cells treated with tempol (TP, 5 m*M* —■—, 10 m*M* —●—, 50 m*M* —▲—) had an increased survival compared with the control group (—○—) not treated with tempol. The reduced form of tempol (hydroxylamine, TPH, —□—) did not confer radiation protection even at a concentration of 100 m*M*. (Adapted with permission from ref. 60.)

age induced by the mutagen neocarzinostatin (NCS), which had previously been shown to induce its mutagenic activity *via* a GSH-dependent mechanism (21). NCS is reduced to its active form by sulfhydryls, and it is this reduced form that interacts with tempol, resulting in inactivation of NCS. Tempol was also found to protect against radiation-induced chromosomal damage in peripheral blood lymphocytes (39), and two nitroxides demonstrated significant radioprotection of irradiated bone marrow mononuclear cells and were hypothesized to be suitable for support of accelerated hematopoietic treatment after radiation exposure (42). Induction of apoptosis by ionizing radiation is one of the mechanisms implicated in radiation-induced cytotoxicity, particularly in hematopoietic cells. In another study, tempol prevented cell death in lymphoblastoid cells, which undergo apoptosis in response to radiation exposure, but interestingly had no effect on the induction of radiation-induced apoptotic products such as cleaved caspase-3 or cleaved PARP (76). Tempol did, however, inhibit a radiation-induced increase in p53 observed in untreated control cells. The antioxidant *N*-acetylcysteine, which is not a nitroxide compound, was found to decrease cleaved caspase-3 and cleaved PARP levels, but did not offer any significant protection from cytotoxicity.

In a related experiment, nitroxides were found to protect against lipid peroxidation (75). Radiation-induced ROS formation can result in the peroxidation of lipids and the consequent degradation of liposomes, membranes, and other lipid-based cellular machinery, which contributes to aging and other pathologic conditions. The higher the degree of unsaturation of the acyl chain, the more sensitive it is to damage from ionizing radiation. Nitroxides were able to protect even the most susceptible lipids from oxidation while engendering no damage of their own.

The therapeutic potential of nitroxides as radioprotectors also depends on their safety and efficacy *in vivo*. Several doses of tempol were administered to C3H mice *via* intraperitoneal (i.p.) injection, and the pharmacokinetic properties were evaluated (33). The mice were then exposed to total body irradiation, and the $LD_{50/30}$ (the dose of radiation that caused 50% lethality at 30 days) for the nontreated mice was observed to be 7.84 Gy, whereas the $LD_{50/30}$ for the tempol-treated mice was significantly higher at 9.97 Gy, as shown in Fig. 2. This was the first evidence that tempol would function as an *in vivo* radioprotector. A second study confirmed that tempol protected mice against death from radiation compared with untreated mice (52). It was also found that this effect was additive to the protective effect of stem cell factor, which is given to aid in the reconstitution of the bone marrow after large doses of radiation.

One problem encountered with the administration of tempol was that, even at doses below the maximal tolerated dose, animal subjects demonstrated a substantial and sometimes deleterious decrease in their arterial blood pressure (29). This was thought to be the result of increased bioavailability of NO, leading to systemic vasodilatation (31). The reduced form of tempol (tempol-H) had been tested and found not to confer any radioprotectivity *in vitro* (60). It was hypothesized that tempol-H administration would not cause the same severity of side effects seen after tempol administration, but would be oxidized *in vivo* to the active, radioprotective form. This hypothesis and the pharmacology of tempol-H were tested in a study in which tempol-H was administered to C3H mice before whole-body irradiation (30). The maximal tolerated dose and limiting toxicities were similar to those of tempol. EPR spectroscopy demonstrated that tempol-H is rapidly oxidized to tempol; however, the peak level of the oxidized form is lower than when tempol is administered directly. The *in vivo* radioprotective activity of tempol-H is shown in Fig. 2. Although only a small fraction of control mice survived, >60% of the tempol-H–treated mice survived an 11-Gy radiation dose, and >10% were alive at 30 days after exposure to a 13-Gy dose. Tempol-H provided significant *in vivo* radioprotection on a par with that demonstrated previously for tempol and, as hypothesized, resulted in only a mild decrease in arterial blood pressure, demonstrating that reduced nitroxides could be safely administered and would be oxidized *in vivo* to the active compound able to provide significant radioprotection.

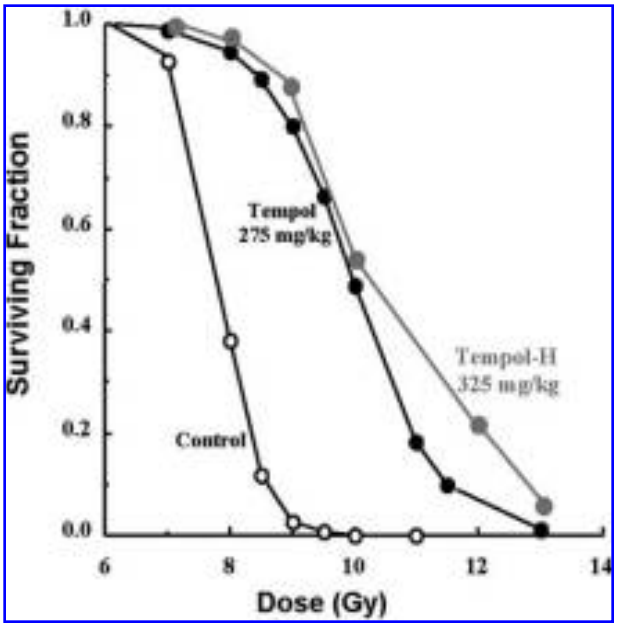

FIG. 2. Radioprotection in mice with tempol and tempol-H. The radiation $LD_{50/30}$ (the dose of radiation that caused 50% lethality at 30 days) for the nontreated mice was 7.84 Gy, whereas the $LD_{50/30}$ for the tempol-treated mice was significantly higher at 9.97 Gy. Tempol-H conferred similar radioprotective benefit *in vivo*. (Adapted with permission from refs. 30 and 33.)

The next step was to examine whether nitroxides protected normal tissues while leaving tumor cells vulnerable to the cytotoxic effects of radiation. Radiation-induced fibrosarcoma (RIF-1) tumor cells were injected into the legs of C3H mice (32). Once tumors had developed, the mice were injected with either PBS or tempol solution 10 min before irradiation of the tumor at doses ranging from 10 to 60 Gy. Thirty days after irradiation, tumor growth rates were the same in mice treated with tempol as in the control group (Fig. 3A). Furthermore, the radiation dose that resulted in 50% local control at 30 days was 36.7 Gy in tempol-treated mice compared with 41.8 Gy in the control group and was not statistically different. These results showed that tempol administration had no effect on tumor growth after irradiation and, therefore, would not compromise

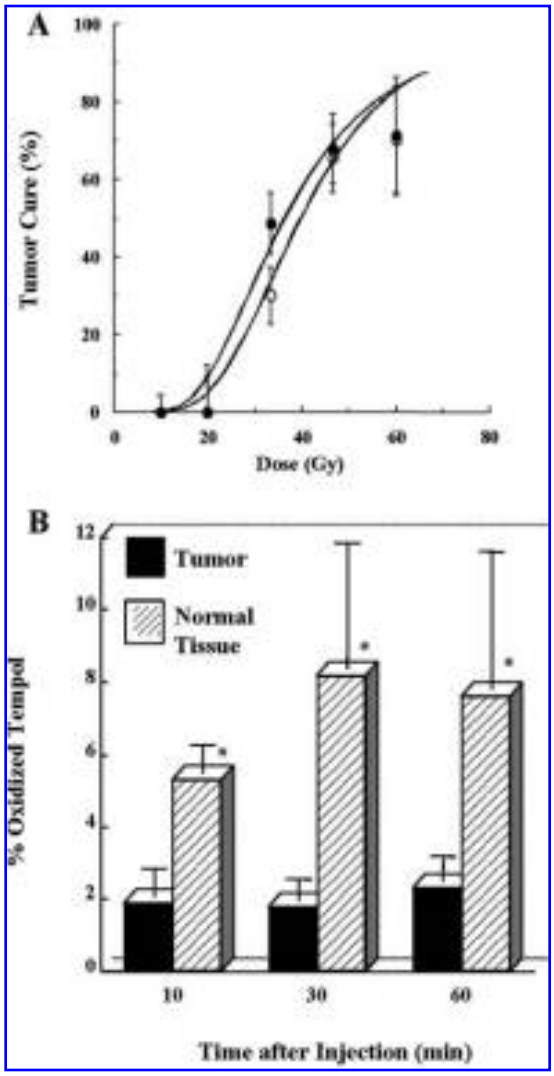

FIG. 3. Tempol protects normal tissue but not tumor. (**A**) Mice were injected with either PBS or tempol solution before irradiation. After 30 days, tumor growth rates and the radiation dose that resulted in 50% local control were the same in mice treated with tempol (—●—) compared with the control group that did not receive tempol (—○—). (**B**) Tempol remains in the oxidized form in normal, well-oxygenated tissues (bone marrow) (▨) but is reduced in hypoxic tissue, such as tumor (■), to the inactive form of the compound. (Adapted with permission from ref. 32.)

radiation treatment of tumors. This differential effect results from the fact that tempol remains in the oxidized form in normal, well-oxygenated tissues but is reduced in hypoxic tissues, such as tumor, to the hydroxylamine form, which is not radioprotective (see Fig. 3B).

Further to evaluate the radioprotective aspects of nitroxides, several compounds were administered *via* i.p injection to C3H mice that were exposed to a single radiation dose and then followed up for 30 days (29). The nitroxide-treated groups showed significantly improved survival, ranging from 35 to 100%, depending on the compound used. Mice that received 3-carbamoyl-proxyl showed essentially unchanged survival up to a dose of 9 Gy, and a small fraction survived 30 days after receiving 11 Gy, demonstrating a significant degree of radioprotection.

A clinically important undesired consequence of radiotherapy is the occurrence of xerostomia, decreased saliva production in the treated area, resulting in dental caries, infection, dysphagia, and significant discomfort. The selective radioprotective effects of nitroxides for normal tissues prompted a trial of tempol against damage to the salivary glands from irradiation (90). A single dose of ionizing radiation was administered to C3H mice with or without an i.p. injection of tempol, and pilocarpine-induced salivary gland output was measured 8 weeks after radiation exposure. A dose-dependent decrease in saliva production was found in the untreated cohort that was prevented by tempol that was thought to result from the scavenging of free radicals including hydroxyl radical (OH) and secondary radical species (91). The pharmacokinetics of the protective effect of tempol was tested by administering the agent *via* several routes and evaluating development of xerostomia (Fig. 4) (12). Tempol was administered to C3H mice by the i.p. route, already established to be effective (90), and compared with administration by intramuscular (i.m.), intravenous (i.v.), or subcutaneous (s.c.) injection and topical administration by using a gel and mouthwash. All administration methods except i.m. were effective in reducing radiation-induced salivary hypofunction by 50–60%, suggesting that tempol may be used clinically to protect salivary glands in patients undergoing radiotherapy. Attempts have been made to prevent xerostomia by using other systemically administered radioprotectors such as amifostine, a thiol compound, which is now being evaluated in clinical trials. Tempol has the advantage of being a well-demonstrated radioprotector that is selective for normal tissue, an issue that continues to be debated with regard to amifostine (7, 8, 86, 95–97).

One of the first clinical applications of nitroxides to be evaluated in humans was the ability to protect against radiation-induced alopecia. In an experimental animal study, tempol was applied topically to guinea pigs 15 min before a 30-Gy single dose of ionizing radiation or during a fractionated radiation schedule (13, 28). Tempol significantly increased the rate and extent of new hair growth compared with untreated skin and was not detectable in the blood or brain tissue. Although both tempol-treated and untreated guinea pigs demonstrated hair loss, the hair density remained significantly greater in the tempol-treated group, and hair recovery was more rapid (Fig. 5). The clinical application of tempol is currently being studied at the University of Pennsylvania. A pilot study was performed enrolling 11 patients treated with topical tempol before irradiation for brain metastases (59). Topically applied tempol was well tolerated and resulted in a significant reduction in hair loss (see Fig. 5).

Overwhelming experimental and clinical evidence speaks to the radioprotective attributes of nitroxides, which stems from their robust affinity for free radicals both *in vitro* and *in vivo*. In light of this, nitroxide radioprotective agents are being studied and developed for the protection of normal tissue in clinical radiation oncology as well as for use in radiologic emergencies such as nuclear power plant disasters or terrorist attacks.

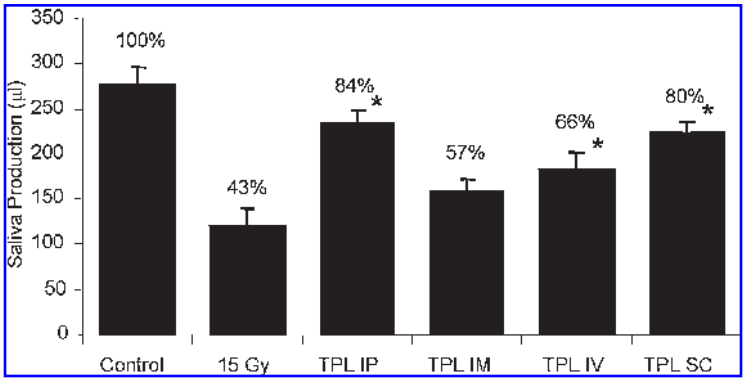

FIG. 4. Effect of tempol administration on salivary production. Saliva production in the radiation-only cohort (15 Gy) was 43% that of the control group. Tempol (TPL, 275 mg/kg) administered *via* intraperitoneal (i.p.) (84%), intravenous (i.v.) (66%), and subcutaneous (s.c.) (80%) routes was found significantly to ameliorate this reduction, but not by intramuscular (i.m.). Columns represent mean saliva production over a 10-min period; values at top represent percentage saliva production compared with control; *significance. (Adapted with permission from ref. 12.)

NITROXIDES AND FUNCTIONAL IMAGING

Biologic functions are exceedingly difficult to monitor in living organisms; however, knowledge of tissue or cellular oxygenation or redox status can be critical to understanding an observed phenomenon. Recent efforts to obtain functional, physiologic, and molecular information by using imaging techniques have enabled noninvasive analysis of blood flow, tissue oxygenation, levels of endogenous metabolites, and metabolism of exogenous tracers, allowing visual mapping of regional differences among studied attributes.

The development of such "functional imaging" techniques has opened the door to a vastly improved understanding of certain diseases. Pairing magnetic resonance imaging (MRI) with positron emission tomography (PET) has allowed clinicians to correlate anatomic abnormalities with functional attributes to better identify metastatic lesions and areas of occult infection in patients. This has led to improvements in patient-specific treatment, as therapy can be directed to treat the appropriate infection or stage of cancer. An extension of this field is the use of EPR imaging, which detects unpaired electrons in such species as transition metal complexes and free radicals (35, 47, 53). Endogenously, unpaired electrons are extremely rare, so little if any EPR signal is detected in biologic tissues. This limits the effectiveness of EPR for normal tissue imaging. However, "contrast" agents containing unpaired electrons, or agents converted to such a compound *in vivo*, can be introduced into a living system and can be detected by using frequencies similar to those used in MRI (50).

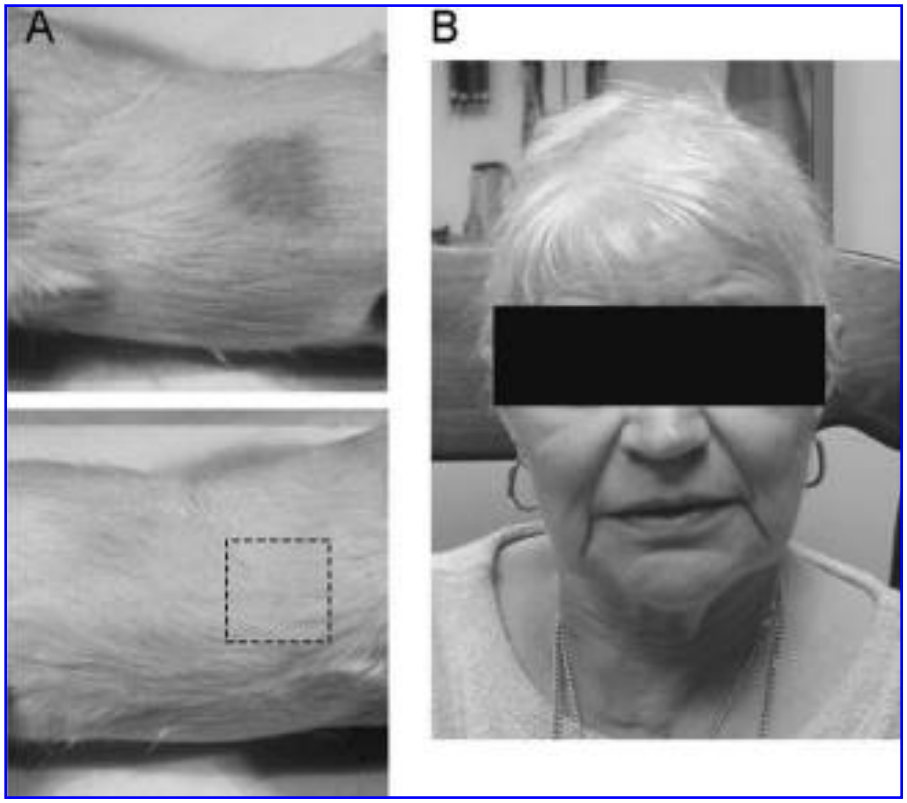

FIG. 5. Prevention of radiation-induced alopecia. (**A**) The flanks of guinea pigs were irradiated to 7 Gy daily for 10 days without (*top*) or with (*bottom*) topical tempol application each day before irradiation. Hair density remained significantly greater in the tempol-treated group (*dashed box*), and hair recovery was more rapid. (**B**) Topical application of tempol resulted in significant reduction of hair loss in this patient who underwent cranial irradiation. (Adapted with permission from ref. 13 and 59.)

Nitroxides are found *in vivo* in an equilibrium between the nitroxide radical form detected by EPR, and the reduced form, the hydroxylamine, which is not detected by EPR because of its diamagnetic nature (71, 87). This equilibrium is dependent on the surrounding environment, specifically the oxygen and redox status of the tissue milieu (44). Cellular redox processes convert the compound between the two states; thus, the ratio of the two states is determined by the redox activity within the cell (Fig. 6). Because only the oxidized form of the nitroxide can be detected by using EPR, signal intensity can be used as a surrogate marker for the relative amounts of the oxidized compound and, therefore, the relative redox activity. In hypoxic cells, the hydroxylamine form is more prevalent, whereas the compound is oxidized to the radical form in well-oxygenated tissues (88). This property of nitroxides makes them ideal compounds for studying intracellular redox metabolism.

The bioreduction of nitroxides in RIF-1 tumors implanted in mice was compared with that in normal tissue (46). Nitroxides were injected i.v., and the animal was subsequently imaged by using EPR to obtain functional images of *in vivo* cellular metabolism. With this technology, a two-dimensional image of the tumor revealed significant heterogeneity in both nitroxide distribution and rate of reduction. The nitroxides were reduced more quickly in tumor tissue than in normal muscle tissue, and spin-label oximetry confirmed a threefold lower oxygen level within the tumor. Thiols, such as the endogenous reductant GSH, are important in maintaining the intracellular redox balance, so the role of GSH levels on the nitroxide reduction rate has also been examined (37). In mice, the EPR signal of 3-CP, a nitroxide radical, decayed significantly more slowly in normal tissue as compared with implanted RIF-1 tumor tissue. After treatment of tumor with L-buthionine-*S,R*-sulfoximine (BSO), an inhibitor of GSH, 3-CP was also reduced more slowly, implicating GSH in the reduction of nitroxides within cells. In another study, EPR and nitroxides were used to generate redox maps of tumor-bearing mice whose thiol status was modulated by using diethyl maleate (DEM), and the heterogeneity of the redox status within tumor tissue was confirmed (94). Furthermore, the rate of reduction of nitroxides was significantly decreased in tumors of mice treated with DEM. These studies suggest the feasibility of imaging nitroxide distribution *in vivo* to monitor local redox metabolism.

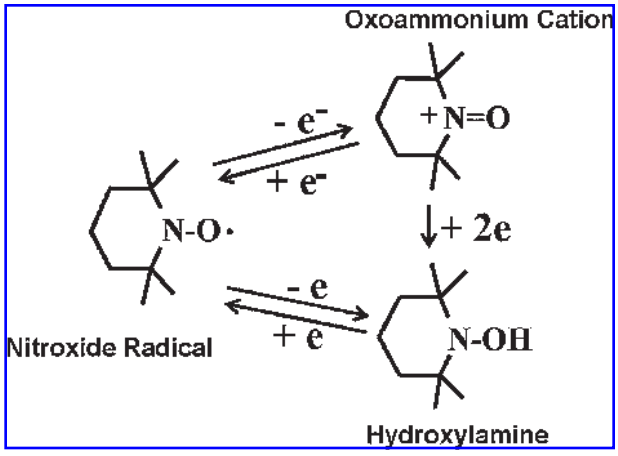

FIG. 6. Conversion of nitroxide radical to hydroxylamine or oxoammonium cation *in vivo*. Nitroxide compounds are found *in vivo* in an equilibrium between the nitroxide radical form, which is detected by EPR, and the reduced form, known as the hydroxylamine, which is not detected. This equilibrium is dependent on the oxygen status and redox status of the tissue milieu. Cellular redox processes convert the compound between the two states; thus, the ratio of the two states is determined by the redox activity within the cell.

For the past several years, nitroxides have been exclusively used in EPR redox imaging. Whereas EPR imaging lacks the ability to coregister anatomy with the redox images (46, 48, 94), MRI provides images with useful spatial and temporal resolutions and, with the use of suitable contrast agents, can provide important functional information pertaining to blood flow, and tissue perfusion (23). Conventional contrast agents used for T_1-contrast enhancement in MRI contain paramagnetic entities such as Gd^{3+} and Mn^{2+} complexes. Nitroxide radicals have a single unpaired electron and can provide T_1-contrast similar to gadolinium complexes. The feasibility of using nitroxides as T_1-contrast agents was examined (4–6) before their use as *in vivo* EPRI probes (2); however, they were not optimal MRI contrast agents (40) because *in vivo*, paramagnetic nitroxide radicals are reduced to the undetectable diamagnetic hydroxylamine (10, 41, 80, 89). Although nitroxides compare unfavorably with Gd^{3+}-containing agents in terms of relaxivity, they are cell permeable and have a larger volume of distribution, meaning that they can provide similar T_1-contrast enhancement per unit volume (81). The major advantages of using nitroxides in MRI as opposed to EPRI include the availability of MRI scanners for both human and small-animal studies, multislice imaging capability, enhanced spatial and temporal resolution, and coregistration of images of tissue redox status with anatomic information inherently available from MRI.

Studies showed that nitroxides provide sufficient contrast enhancement in phantom objects so that metabolic reduction of nitroxides to hydroxylamines can be monitored by T_1-weighted MRI scans as a function of time (55). With this validation that MRI could reliably monitor nitroxide metabolism, the differences in nitroxide (3-CP) metabolism in tumor and normal tissues in mice *in vivo* were investigated. As can be seen in Fig. 7, the MRI signal intensity increased in both normal leg and tumor-bearing leg after 3-CP administration and reached a maximum. However, the subsequent decrease in signal intensity in the tumor region was faster than that seen in normal tissue. The signal-reduction rate in the regions of interest (ROIs) chosen in the normal leg was observed to be ~60% that of the reduction rate in the ROI in tumor (see Fig. 7C). The rate of change of intensity in each pixel was computed, and a parametric image showed that overall tumor reduction was elevated as compared with normal tissue (Fig. 7D). The significant enhancement in image intensity induced by 3-CP administration and the superior temporal and spatial resolution of MRI suggest that it is advantageous to monitor the pharmacokinetic distribution of nitroxides by using T_1-weighted MRI instead of EPRI. To study the sites of reduction of nitroxides, a set of three nitroxides (3-CP, tempol, and carboxy-proxyl) were examined in MRI experiments analogous to those described in Fig. 7. Whereas 3-CP and tempol are cell permeable, carboxy-proxyl is restricted to extracellular regions. Unlike EPR imaging, which is relatively slow, with MRI it was possible to monitor the distri-

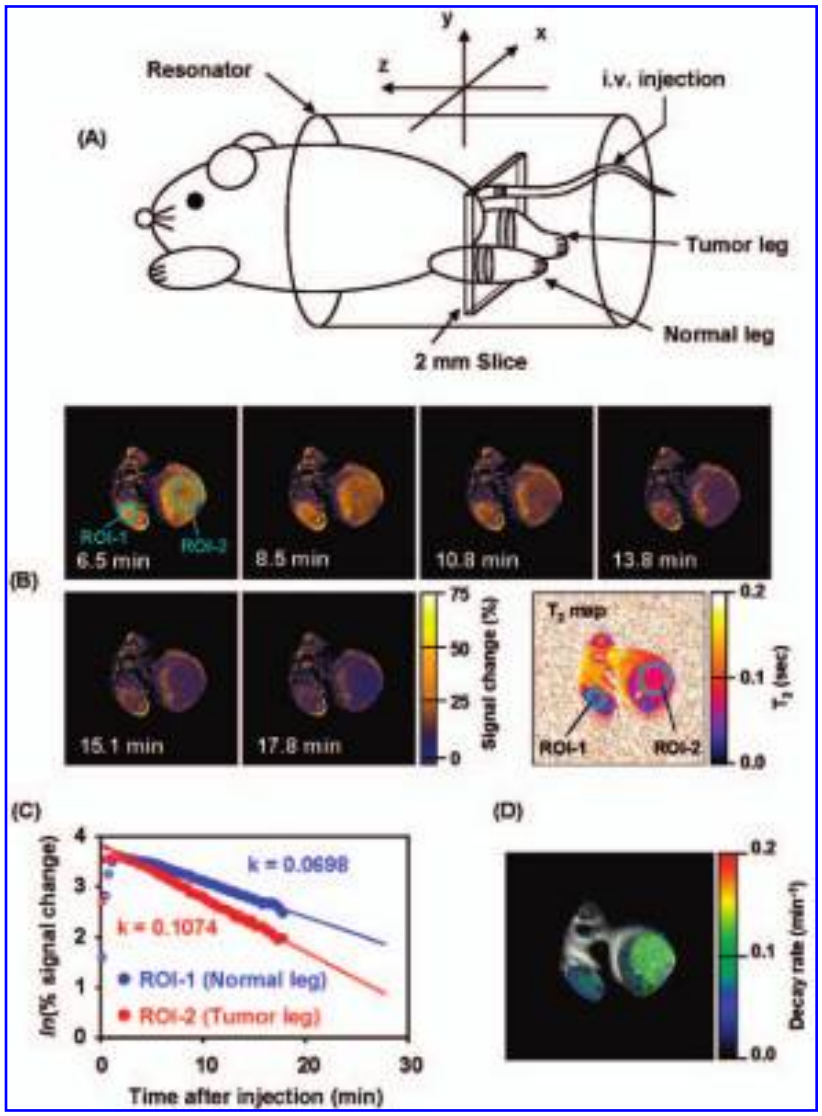

FIG. 7. Nitroxide imaging studies. (**A**) Experimental arrangement of the mouse in the MRI resonator and the slice selected to monitor the nitroxide levels used to examine the differences in nitroxide metabolism in tumor and normal tissue. (**B**) Sequence of T_1-weighted MR images as a function of time after intravenous administration of 3-CP. Signal intensity in normal (ROI-1) and tumor leg (ROI-2) increases after administration and reaches a maximum at 8.5 min. Signal decreases faster in tumor region than in normal tissue. Intensity change plotted as a function of time in the normal leg was observed to be ~60% compared with that in tumor. (**C**) The rate of intensity change in each pixel was computed, and (**D**) a parametric image redisplayed shows that tumor reduction globally is elevated compared with the normal tissue. (Adapted with permission from ref. 55.)

bution and reduction of all three nitroxides. In this study, the reduction rates from ROIs chosen in normal leg and tumor leg were calculated and are shown in Table 1 (36). The two cell-permeable nitroxides, 3-CP and tempol, displayed faster reduction in tumor compared with normal tissue, whereas the cell-impermeable nitroxide, carboxy-proxyl, exhibited no such differences and had the longest half-life of the three agents tested. These results support the notion that nitroxide metabolism reflects the intracellular redox status.

Numerous potential applications of this technology are known. Functional MRI can be used to study noninvasively many phenomena ranging from the effect of ionizing radiation on various tissues to the evaluation of potentially radioprotective or radiosensitizing compounds. Furthermore, MRI could be used in the clinical setting to monitor redox changes in tumor and normal tissue in patients undergoing radiotherapy, and possibly even to monitor the radiation dose being administered to an area of interest. The tremendous gains over the past several years demonstrate that rapid progress is possible, but the use of nitroxides as contrast agents must be developed further before these clinical applications can be realized.

CHEMOPREVENTION AND ANTICANCER ACTIVITY

Cancer treatment and prevention has entered an era of designer drugs targeted at specific biochemical pathways; however, the clinical results thus far are disappointing. Recent studies of nitroxides have yielded some interesting results in this arena as well. By altering the intracellular redox milieu, nitroxides can counter a broad spectrum of oxidative stresses that may overwhelm the cellular defense mechanisms, precipitating sublethal damage that may promote aging, certain neurologic diseases, and the development of cancer.

In the first of a small series of studies, C3H mice were given tempol in their drinking water, and survival and tumor incidence were compared over the course of 2 years (63). A small

Table 1. Decay Rate of Nitroxyl Contrast Agents in Various Tissues

	Decay rate (min $^{-}$1)		
Tissue	*Tempol*	*Carbamoyl-PROXYL*	*Carboxy-PROXYL*
Normal leg	0.319 ± 0.025	0.056 ± 0.013	0.029 ± 0.014
Tumor leg	1.095 ± 0.203†	0.107 ± 0.020*	0.020 ± 0.014

Values are mean ± SD; number of experiments was 3; * and † indicates significances between the normal leg and the tumor leg by *$p < 0.05$, †$p < 0.01$.

but nonsignificant increase in life span and a reduction in tumor incidence from 40% to 10% were noted in tempol-treated mice compared with controls. Tempol was then studied in *Atm*-deficient mice, a murine model for ataxia–telangiectasia, which predisposes to the development of thymic lymphoma (79). Mice that were fed with tempol-containing food had an increased life span from 30.1 weeks to 62.4 weeks because of a delay in the onset of the thymic lymphomas. Tempol also decreased the intracellular generation of ROS, as measured by 2′7′-dichlorodihydrofluorescein (DCF) fluorescence and resulted in decreased proliferation of mitogen-stimulated splenocytes from both *Atm*-deficient and wild-type mice. This study confirmed that ROS play a role in the development of cancer and that nitroxides can potentially ameliorate this tumorigenic drive. The mechanism of this activity was further elucidated in a study using mice deficient in the *p53* tumor-suppressor gene (24). Once again, tempol increased the latency of tumorigenesis in these mice, although this effect was less pronounced than in the *Atm*-deficient mice. Unlike *Atm*-deficient mice, the thymus of *p53*-deficient mice revealed normal levels of oxidative stress that were not significantly altered by tempol. Previous studies have shown that p53 can be phosphorylated in response to oxidative stress (11, 25, 38). Tempol was found to induce the phosphorylation of p53 and resulted in an increase in downstream gene expression including p21.

In addition to cancer prevention, nitroxides have been studied as a treatment for preexisting tumors. Although tempol was previously shown to have no significant cytotoxicity in a Chinese hamster ovary (CHO) model (1, 19), tempol was found to have an antiproliferative effect on MCF-7 breast cancer cells *in vitro* (25). Cells were found to accumulate in G_1 and then pause in G_2/M phase. Ultimately, DNA fragmentation studies revealed an increase in apoptosis in tempol-treated cancer cells. A later study of *p53*-negative leukemia cells, HL60, revealed that tempol induced a time- and dose-dependent increase in p21 and resulted in G_1 arrest (27, 64). This research culminated in a trial of tempol against glioma cells in an *in vitro* and *in vivo* murine xenograft model (26). Tumors from mice treated with tempol showed increased evidence of apoptosis, and tempol-treated tumors also showed decreased neovascularization on histologic staining. These results suggest that the redox status of certain tumors is significantly altered compared with that of normal tissue and that nitroxides can exploit this difference for use in the treatment of such tumors.

NITROXIDES AND HYPERTENSION

Early animal studies revealed a substantial decrease in mean arterial blood pressure after i.p. injection of nitroxides that initially made their use somewhat problematic (29). Although the mechanism was not known at the time, it was recognized as being similar, and potentially related, to nitric oxide (NO) administration. NO is well known to cause vasodilatation *in vivo*, which results in a decrease in arterial blood pressure. The assertion that nitroxides cause hypotension *via* a NO-related mechanism was supported by studies performed on cultured endothelial cells (101). By using 3-morpholinosydnonimine to induce NO release, the effect of several nitroxides was measured with NO chemiluminescence. Both SOD and tempol increased the signal intensity, indicating that nitroxides increase the amount of bioavailable NO.

In a study using spontaneously hypertensive rats (SHRs), tempol was found to restore normal blood pressure (77). This effect was found to be dependent on the endogenous production and availability of NO. When the NO synthesis blocker N^{w}-nitro-L-arginine methyl ester (L-NAME) was administered, the antihypertensive effect of tempol was abolished. Tempol exerted this antihypertensive effect during both continuous infusion and when administered *via* daily i.p. injection by inactivating $O_2^{\cdot -}$, thus removing the inhibition of NO. In the SHR model, tempol was also shown to correct the blood pressure and the renal vasoconstriction associated with oxidative stress as measured by urinary excretion of 8-iso prostaglandin F2-α (78).

A more comprehensive study of the effect of nitroxides on the cardiovascular system by using Swan-Ganz catheterization in miniature pigs revealed similar results with regard to arterial blood pressure (31). As predicted, systemic vascular resistance decreased, resulting in a decrease in blood pressure. Several doses of the nitroxides were administered, and the recovery time to normal blood pressure and heart rate increased with increasing doses. By using EPR spectroscopy, no direct interaction of nitroxides with NO was observed when the two were co-incubated in a test tube. This confirmed that nitroxides interfere with the NO inactivation by scavenging oxygen free radicals, which results in prolonged NO activity, vasodilatation, and a decrease in arterial blood pressure.

A recent study clarified previous findings that the six-membered ring piperidine nitroxides result in the observed hypotensive response (Fig. 8), but the five-membered ring pyrrolidine nitroxides do not (67). This may be due in part to the

relative differences in lipophilicity, because both are effective SOD mimics *in vitro*. These findings suggest that nitroxides could be formulated for use in the clinical setting of both acute hypertensive crisis and for long-term treatment of endothelial dysfunction, a key player in renal vascular diseases and coronary artery disease.

OTHER APPLICATIONS OF NITROXIDES

Ischemia–reperfusion injury and inflammation

A logical extension of the potential clinical uses of nitroxides is the treatment of ischemia–reperfusion injury. Ischemic tissue undergoes anaerobic metabolism, resulting in the conversion of xanthine dehydrogenase to xanthine oxidase. Reperfusion of the tissue then results in ROS formation, which can cause further tissue injury, which is seen in both acute and chronic medical conditions such as coronary artery disease and diabetes.

The effect of tempol on myocardial infarct size was examined in rat and rabbit models (57). Tempol, administered as an i.v. bolus, reduced the size of infarcts caused by regional ischemia by 30–40%. This beneficial effect was also observed in cerebral-reperfusion injury models. In one study, cerebral ischemia was induced by occluding the common carotid arteries of gerbils bilaterally (16). Ischemic damage was detected post-

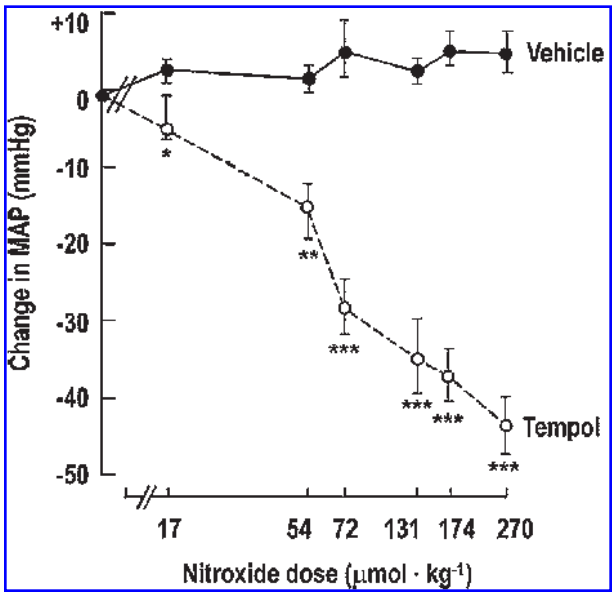

FIG. 8. Tempol reduces arterial blood pressure in a dose-dependent manner. Means ± SEM values for changes in mean arterial pressure (MAP) for SHRs given tempol (- -○- - : $n = 6$) or vehicle (- -●- -; $n = 6$). Compared with vehicle: $*p < 0.05$; $**p < 0.01$; $***p < 0.005$. Tempol was infused intravenously over a 10-sec period. The MAP was recorded over the first 5 min and at 10 and 15 min. Tempol was administered in doses of 17, 54, 72, 174, or 270 μmol/kg under similar conditions. (Adapted with permission from ref. 67.)

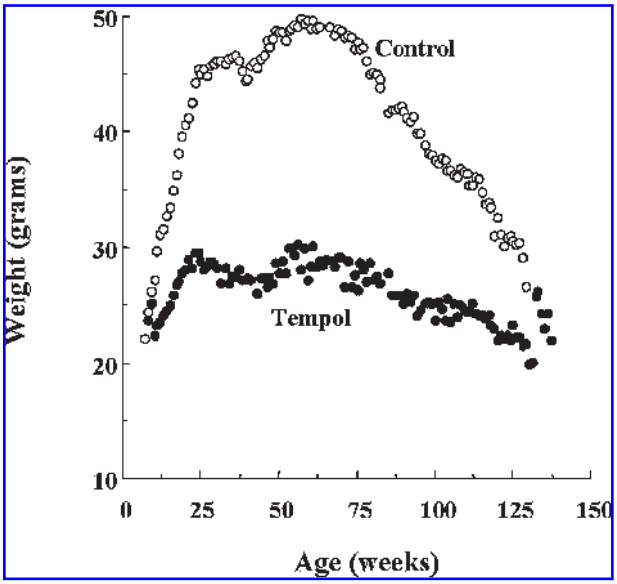

FIG. 9. Tempol controls weight gain. Tempol/sucrose-treated mice (●, 58 m*M* in drinking water) gained weight more slowly and maintained a normal healthy weight, whereas the sucrose-fed controls (○) gained substantial weight very quickly and maintained higher weight throughout their life span. (Adapted with permission from ref. 63.)

mortem by using various immunohistochemical (IHC) stains. Tempol, administered i.p. before and after reperfusion, resulted in a significant decrease in IHC staining and a decrease in lipid peroxidation, as demonstrated by a decrease in cerebral malondialdehyde levels. In another study, i.v. tempol administration during the first 20 min of reperfusion resulted in a significant, dose-dependent decrease in infarct size after occlusion of the middle cerebral artery in rats (68). Similar studies have been performed by clamping the renal pedicle in rats, in which tempol was shown to have a protective effect on kidney function (9).

All forms of shock are the result of poor tissue perfusion and the formation of toxic metabolites including ROS. Toxic shock was induced in rats by using the bacterial antigen lipopolysaccharide (49). Tempol, administered by i.v. bolus, did not prevent the circulatory collapse, but did reduce the associated kidney and liver dysfunction. In another study, severe hemorrhagic shock was induced in rats and then reversed by transfusion (65). Again, tempol protected against multiorgan failure. ROS also play a role in chronic inflammatory diseases. Tempol has been shown to be protective in several models of inflammation including pancreatitis (83), pleurisy (14), arthritis (17), colitis (15), and uveoretinitis (98).

Weight control

An unexpected consequence of long-term nitroxide administration in several animal studies has been weight loss. In one study, C3H mice were given tempol in their drinking water at a concentration of 58 m*M* (63). During the follow-up period from 10 to 50 weeks, tempol/sucrose-treated animals gained

weight more slowly and then maintained a normal healthy weight, whereas the sucrose-fed controls gained substantial weight very quickly, as shown in Fig. 9. Caloric restriction increases life span in mice (56, 66, 92) and decreases the effects of aging. Over a 150-week follow-up period, tempol-treated mice had a significantly increased survival, 123 *versus* 92.6 weeks, and remained more active and healthy as they aged. Coat color and sheen were also superior in the tempol-treated group as they aged. No difference was found in thyroid, liver, or renal function tests, glucose, insulin, or total protein or albumin levels between the tempol-treated and control groups. The level of the hormone leptin, which is produced by adipose tissue and relates to amount of adipose storage, in tempol-treated animals was half that of the control animals. These studies suggest a role for nitroxides in the treatment of obesity, diabetes, and for use in improving performance status in patients with cancer or other chronic diseases.

Neurodegenerative diseases and damage to the lens of the eye

The antioxidant activity of nitroxides has prompted investigators to test their effects in a wide variety of diseases. In Parkinson's disease, dopamine oxidation and ROS formation are thought to contribute to the process of progressively worsening motor function (3, 54, 58, 85, 99). A recent study revealed that tempol protected dopamine-secreting cells *in vitro* from apoptosis induced by 6-hydroxydopamine (6-OHDA) and also protected mice from developing parkinsonian symptoms induced by administration of 6-OHDA (51). A study of several peptides associated with Alzheimer's disease revealed that they oxidize the nitroxide tempone (22). The significance of this finding is difficult to evaluate, however, as they do not spontaneously produce free radicals, and the contribution of ROS to the development of Alzheimer's disease is not well characterized.

The effect of ROS formation and oxygen status in the eye also are being investigated, and studies suggest that cataract formation results from oxidative stress (82), as elevated levels of H_2O_2 in the aqueous humor of cataract patients has been demonstrated (84). An early study used tempol to determine the mechanism by which ROS lead to cataract formation (70). Tempol prevented the H_2O_2-induced inhibition of cell growth, membrane blebbing, and decrease in DNA-repair protein activation, and limited the amount of DNA damage. It was later shown that tempol protected GSH-depleted lens epithelial cells in culture from H_2O_2 toxicity (69). As GSH depletion is known to exist in most forms of cataract, it is argued that tempol may prevent or slow the progression of this condition. A more recent study showed that topical application of tempol-H decreased the formation of cataracts in an *in vivo* model in both rats and rhesus monkeys (100).

CONCLUSIONS

Over the past two decades, research involving nitroxides has flourished. As more is revealed about these fascinating compounds, more potential therapeutic applications are uncovered. It is now known that nitroxides can be used as radioprotectors that are selective for normal tissue. This is known, in part, from imaging studies that exploit the innate polarity of these compounds for use as MRI contrast agents. The field of functional imaging will be enhanced by the ability to image redox status in human patients so that lesions can be targeted and response to therapy evaluated in real time. Furthermore, nitroxides may have the ability to delay cancer formation and progression, and infarcts from myocardial ischemia and cerebrovascular occlusion may be reduced by nitroxide administration. Aging and weight control are also affected by nitroxides, as are disease states involving tissue damage from chronic inflammation.

This wide variety of applications all ties to the ability of these compounds to scavenge oxygen free radicals and alter the redox state. Nitroxides provide an unprecedented opportunity to study the mechanisms underlying many disease states by interfering with this single important cellular metabolic phenomenon.

Several ongoing clinical trials are using nitroxides to prevent the deleterious effects of radiation treatment, and trials for other applications will undoubtedly be initiated in the near future, providing the opportunity to advance the knowledge and treatment of many diseases.

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ABBREVIATIONS

•OH, hydroxyl radical; 6-OHDA, 6-hydroxydopamine; BSO, L-buthionine-*S,R*-sulfoximine; DCF, dichlorodihydrofluorescein; DEM, diethyl maleate; EPR, electron paramagnetic resonance; EPRI, electron paramagnetic resonance imaging; GSH, glutathione; Gy, Gray; H_2O_2, hydrogen peroxide; i.m., intramuscular; i.v., intravenous; IHC, immunohistochemistry; LD, lethal dose; L-NAME, N^w-nitro-L-arginine methyl ester; MRI, magnetic resonance imaging; NCS, neocarzinostatin; NO, nitric oxide; $O_2^{\cdot -}$, superoxide; PET, positron emission tomography; RIF-1, radiation-induced fibrosarcoma-1; ROI, region of interest; ROS, reactive oxygen species; s.c., subcutaneous; SOD, superoxide dismutase; TPH, tempol-H.

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