Potential Use of Nitroxides in Radiation Oncology¹

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Abstract

The identification of radioprotectors is an important goal for those involved in radiation oncology and for those interested in the investigation of the mechanisms of radiation cytotoxicity. Recently, a new class of in vitro and in vivo radioprotectors, the nitroxides, has been discovered. The nitroxides are low-molecular-weight stable free radicals which are freely membrane permeable and which have been shown to act as superoxide dismutase mimics. Further investigation of these compounds has shown that a water-soluble nitroxide, Tempol, protects cultured Chinese hamster V79 cells from the cytotoxicity caused by superoxide, hydrogen peroxide, and .Notification added: [3-aminomethyl-Proxyl; LD₅₀/₃]l. dose of radiation that causes 50% lethality within 30 days after radiation.

Historical Background. Sulfhydryl compounds were among the first radioprotectors to be identified (1). Patt et al. (1) found that pretreatment of rats with i.v. cysteine protected them from the lethality of whole body radiation. Subsequently, Bacq et al. (2) found that cysteamine also protected animals from whole body radiation. The mechanism by which these sulfhydryl compounds protect appears to involve their ability to scavenge radiation-induced free radicals and/or donate reducing equivalents to oxidized molecules.

These discoveries prompted the Department of Defense to sponsor the synthesis and screening of compounds to find a relatively nontoxic and potentially more effective radioprotector (3). The result of this massive undertaking was the discovery of a thiophosphate compound, S-2-(3-amino-propylamino)ethylphosphorothioic acid (WR2721). WR2721 has been studied extensively and appeared to be promising because it showed substantial and selective protection of normal tissues (4–7). Yuhas and Storer (6) showed that WR2721 could protect normal tissues but not tumor. The basis for this observation was the slow penetration of WR2721 into tumor tissue (7). Phase I studies of WR2721 have defined the maximally tolerated dose under different treatment schemes (8–10). No tumor protection has been reported in any of these studies, but substantial toxicity has been described. Phase II clinical studies have yet to confirm the exciting preclinical results, however. The toxicity of this compound and the possible protection of tumor tissues has dampened enthusiasm for its use (11).

Nitroxides have also been investigated as radioprotectors (12, 13). These compounds differ from the sulfhydryl compounds because they are not believed to act by changing the inherent radiosensitivity of individual cells. Rather, they are believed to protect by more quickly restoring hematopoietic function after radiation exposure. Interleukin 1, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor have all been shown to modestly protect mice from the lethality of whole body radiation (12, 13).

Introduction

Radiation is an important modality in the treatment of cancer and in some instances may be the single best "agent" for treatment. However, for many solid tumors, cure with radiation remains elusive. The radiation therapy of cancer depends upon achieving a therapeutic differential between cancer cell cytotoxicity and normal tissue toxicity. Therapeutic differential may be achieved with technical factors such as conformational therapy. Alternatively, chemical radiation sensitizers or protectors may enhance the therapeutic differential. The development of radiation protectors is important not only to enhance the effectiveness of cancer treatment but also for the study of the underlying mechanisms of radiation cytotoxicity. Some radioprotectors are known to protect by a direct effect on the cellular targets of radiation. Alternatively, other radioprotectors protect by altering physiological functions that interfere with the effects of radiation, such as agents that cause vasoconstriction and hypoxia. Lastly, some radioprotectors act by enhancing the recovery or repair of normal tissues. This review will focus on a novel class of non-thiol radioprotectors which may also have application as general antioxidants. Additional work is necessary to screen other nitroxides for in vivo radioprotection and toxicity as well as to fully evaluate the extent to which these compounds protect tumors.

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³The abbreviations used are: OXANO, nitroxide 2-ethyl-2,5,5-trimethyl-3-oxazolidine-1-oxyl; OXANO, oxanoidaminoylhydroxylamine; SOD, superoxide dismutase; Tempol·H, reduced form Tempol (hydroxylamine); 3AM, 3-aminomethyl-Proxyl; LD₅₀/₃, dose of radiation that causes 50% lethality within 30 days after radiation.
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![Basic structure of selected nitroxides. The six-membered and five-membered ring nitroxides are shown. Data from Ref. 28 is used with permission.](image)

In a more recent study, Melhorn and Swanson (27) have shown that nitroxides induce a catalase-like conversion of H$_2$O$_2$ to O$_2$ in the presence of hemoglobin and phenols through the oxoammonium intermediate.

**In Vitro Studies.** The discovery that the nitroxides were free radical scavengers led to in vitro investigations that evaluated whether these compounds could protect cultured cells against a variety of oxidative stresses. Using EPR spectroscopy, it was found that nitroxides partition into the intracellular space in an energy-independent fashion (28–30). In addition, initial cytotoxicity studies showed that at 10 mM concentrations, most of the nitroxides did not cause a reduction in plating efficiency of Chinese hamster V79 cells.

Monolayered V79 cells were exposed to the superoxide-generating system hypoxanthine/xanthine oxidase. Several of the nitroxides provided protection when applied immediately before the exposure to superoxide (29). In addition, catalase which detoxifies hydrogen peroxide and desferrioxamine, a ferric ion chelator, also provided protection. SOD, exogenously applied, conferred no protection. This was not unexpected because SOD is unlikely to enter the intracellular space in substantial concentration. Additional studies showed that several of the nitroxides also protected V79 cells against hydrogen peroxide cytotoxicity (29). Since it is believed that hydrogen peroxide cytotoxicity is mediated by reduced transition metals via the production of the hydroxyl radical (31, 32), it was speculated that nitroxides were protecting V79 cells by oxidizing reduced transition metals. This action of nitroxides was confirmed in a cell-free system (29). Further work showed that the nitroxides could protect V79 cells from the cytotoxicity of t-butyl hydroperoxide, which is independent of superoxide and hydrogen peroxide and produces alkyl, alkoxyl, and allylperoxy radicals (33). The reaction of these radicals with nitroxides has been documented in previous studies (20–23). From these in vitro studies, it was concluded that selected nitroxides protected cells from a variety of oxidative stresses and that such protection was probably mediated by multiple mechanisms including oxidation of reduced transition metals, superoxide dismutase-like activity, and scavenging of free radicals.

Despite the protection of cultured cells against some oxidative insults, it remained unclear whether these compounds could protect against radiation-induced cytotoxicity in vitro. Monolayered V79 cells were exposed to varying concentrations of one water-soluble nitroxide, Tempol, and then exposed to increasing doses of radiation (14). Tempol protected V79 cells against radiation in a dose-dependent fashion (Fig. 2A). The reduced form Tempol (Tempol-H), the hydroxylamine, did not provide aerobic radioprotection (Fig. 2B). Interestingly, however, Tempol did not provide protection for hypoxic V79 cells (Fig. 2C). On the contrary, Tempol produced a modest radiosensitization of hypoxic cells, consistent with previous findings (34). This observation may be important since the nitroxides are reduced to hydroxylamines under hypoxic conditions, perhaps explaining the lack of radioprotection observed under these conditions (35).

Additional studies showed that aerobic radioprotection by Tempol was not the result of induction of hypoxia, an increase in intracellular glutathione concentration or an induction of intracellular SOD mRNA (14). The mechanism of differential radiation effects of Tempol under aerobic and hypoxic conditions is, at present, unclear. Fig. 3 shows that radiation under aerobic and hypoxic conditions produces several species in common (cross-over area of the figure), in addition to carbon-centered free radicals (R·). Tempol could directly react with and thus detoxify radiolytically produced -OH, -H, or aminated electrons; however, these products are produced by radiation independent of oxygen, and Tempol only protected aerobic cells. Tempol may react with R· under hypoxic conditions to result in radiosensitization by producing a non-repairable lesion. Since R· is also produced under aerobic conditions, sensitization would be expected to occur also; however, the sensitization might be overshadowed by other Tempol-mediated protective mechanisms. For example, the reaction between Tempol and radiolytically produced alkoxy-radicals (RO-, ROO-, etc.) might serve to inhibit these radiation-induced lesions (see Fig. 3) (14). These possibilities are being further pursued in our laboratory.

To extend these observations, six additional water-soluble nitroxides with different structural features were studied (28). Several additional nitroxides were found to protect V79 cells from radiation-induced cytotoxicity (Fig. 4). Two of these compounds, 3-aminoethyl-3AM and Tempamine (Fig. 1), were found to be the best protectors with protection factors of 2.3 and 2.4 at the 10% survival level. Treatment with 10 mM Tempol provided a protection factor of 1.3. One nitroxide, 3-carboxy-PROXYL, did not provide substantial radioprotection (Fig. 4). Speculation regarding the reason for this differential radioprotection involved differences in structure (Fig. 1). Both 3AM and Tempamine contain amino groups and are more likely to be positively charged at a neutral pH. 3-Carboxy-PROXYL is more likely to be negatively charged under those same conditions. These charge differences could alter the affinity of the nitroxide for the presumed target of radiation, DNA, which is negatively charged. Nonequilibrium dialysis studies with single-stranded DNA showed that Tempamine and 3AM had a greater affinity for DNA than did the other nitroxides. These findings suggested that since the target of
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Radiation is felt to be DNA (36), the differences in radioprotection of the compounds were related to the ability of the individual nitroxides to associate with DNA (28).

Additional in vitro studies have been performed to evaluate the direct protection of DNA by nitroxides. Tempol was evaluated for antimutagenic activity against hydrogen peroxide and superoxide (37). Mutations were assessed using the XPRT forward mutation assay in CHO AS52 cells. Tempol (10 mM) provided nearly complete protection from the mutagenic effects of hydrogen peroxide and superoxide and was not itself mutagenic (37). X-ray- and neocarzinostatin-induced mutagenicity or DNA double-strand breaks were also evaluated in CHO AS52 or V79 cells (Fig 5; Ref. 38). DNA double-strand breaks were measured with field inversion gel electrophoresis. Tempol (50 mM) provided protection against X-ray- and neocarzinostatin-induced mutagenicity and double-strand breaks (38).

Preliminary studies have also shown that Tempol reduces radiation-induced chromosomal aberrations in human peripheral blood lymphocytes.

In Vivo Studies. It was in the context of in vitro radioprotection that in vivo studies of nitroxide activity were initiated. Tempol was administered to C3H mice i.p., and the toxicity, pharmacology, and radioprotective properties were assessed (15). The maximally tolerated dose of Tempol was found to be 275 mg/kg. Above this dose, restlessness and seizure activity were noted, often leading to death. A steep dose-lethality curve was noted. No long term toxicity was found when Tempol was administered at or below 275 mg/kg (15).

Using EPR spectroscopy, whole blood levels of Tempol were measured after i.p. injection. A peak whole blood concentration of 600 μg/ml was found 5–10 min after injection (Fig. 6) (15). This corre-

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Radiation-Induced Species

Aerobic

Hypoxic

Fig. 3. Species produced by radiation under aerobic and hypoxic conditions. The area of overlap of the two circles contains those species produced under both conditions.

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Fig. 2. Full X-ray dose-survival curves for Tempol and Tempol-H. A, Chinese hamster V79 cells were pretreated 10 min prior to X-irradiation with Tempol at the concentrations shown under aerobic conditions. Increasing radioprotection is observed with increasing Tempol concentration. B, V79 cells were pretreated 10 min prior to X-irradiation with Tempol-H (100 mM) under aerobic conditions. No radioprotection is observed. C, V79 cells were pretreated with Tempol 10 min prior to X-irradiation under hypoxia. Modest radiosensitization is observed. Data from Ref. 14 is used with permission.

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Fig. 4. Full X-ray dose-survival curves for selected nitroxides. V79 cells were pretreated 10 min prior to X-irradiation with selected nitroxides at a concentration of 1 mM under aerobic conditions. 3AM and Tempamine provide the greatest radioprotection. □, control; △, 3-carboxy-PROXYL; ○, Tempol; △, 3-carbamoyl-PROXYL; ○, Tempamine; ■, 3-AM. Data adapted from Ref. 28 is used with permission.

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spends to a whole blood concentration of approximately 3.0 mm, which is at the lower limits of drug concentration that provides in vitro radioprotection. With pharmacological data showing a peak concentration 5–10 min after injection, C3H mice were given Tempol at 275 mg/kg i.p. and then exposed to increasing doses of whole body radiation 5–10 min after drug administration. Significant in vivo radioprotection was observed (15). The LD_{50/30} dose was 9.97 Gy in the Tempol-treated mice and 7.84 Gy in the saline-treated mice. The dose modification factor (the LD_{50/30} dose in the Tempol-treated group divided by the LD_{50/30} dose in the control group) was found to be 1.3 (Fig. 6; Ref. 15). When Tempol was administered 24 h prior to radiation, no radioprotection was observed. Thus, a nonlethal dose of Tempol was shown to produce significant in vivo radioprotection. A preliminary evaluation of other water-soluble nitroxides in vivo suggests that 3AM may be a superior radioprotector. Additional full dose-response studies will be needed to confirm these findings.

The survival of C3H mice 30 days after radiation is a radiobiological measure of bone marrow toxicity (39). While it can be assumed that the nitroxides that have been studied provide protection against radiation toxicity to normal bone marrow tissue, it is unclear if tumors would also be protected. Tumor protection, of course, would provide no therapeutic advantage to the administration of nitroxides. Two lines of evidence suggest that Tempol might not radioprotect tumors. The finding that Tempol is a modest radiosensitizer of hypoxic cells in vitro suggests that this compound might protect normal aerated cells and at the same time sensitize hypoxic tumor cells. Secondly, there is evidence that tumor cells have a greater capacity for bioreduction compared to their normal tissue counterparts (40). As a result, greater bioreduction of nitroxides might occur in tumor cells. Since in vitro tissue culture studies have shown that Tempol-H is not an aerobic radioprotector, it might be expected that greater nitroxide reduction in tumor cells would enhance the therapeutic gain from nitroxide administration (14). Preliminary pharmacological studies with Tempol using an EPR assay have shown that this nitroxide is reduced to a substantially greater extent in tumor tissue than normal tissue.

Preliminary studies are underway in our laboratory to assess the effect of Tempol administration upon tumor control using the radiation-induced fibrosarcoma (RIF-1) transplantable tumor cell line (41). This tumor cell line has been used extensively to evaluate the modulatory effect of compounds on tumor control with radiation (41, 42). The preliminary results suggest that no protection of tumor has occurred. Full tumor control experiments are underway to confirm this observation.

Other applications of the nitroxides have been studied that have potential clinical applications. The topical administration of Tempol to guinea pig hair and skin prior to radiation has been shown to protect against radiation-induced alopecia (16). When single doses of up to 30 Gy of radiation were administered, Tempol provided a marked increase in the rate and extent of new hair recovery when compared to untreated skin. Studies are ongoing with fractionated radiation regi-

Fig. 5. Cytotoxicity (top) and mutation induction (bottom) by X-rays in the absence or presence of 50 mM Tempol for CHO AS52 cells. □, □, untreated controls; ○, ○, Tempol-treated. Data from Ref. 37 is used with permission.
mens. Separate studies have shown that the administration of the nitroxide Tempo in an isolated rat heart system protects against postischemic reperfusion injury (43).

**Summary**

Tempol and other nitroxide compounds are *in vitro* and *in vivo* radioprotectors. Tempol is not only an aerobic radioprotector but also a modest hypoxic cell sensitizer *in vitro*. The nitroxides have the potential for *in vivo* use because they readily penetrate the intracellular space and can be administered to animals in nonlethal doses, which provide blood concentrations that are in the range of observed *in vitro* radioprotective activity. Initial studies with transplantable tumors suggest that no tumor radioprotection is observed when Tempol is administered prior to radiation. The concept of using radioprotectors in clinical radiotherapy is appealing, provided agents can be identified that provide differential protection to normal tissues as opposed to tumor. Preliminary preclinical studies with the nitroxides are, indeed, encouraging. Additional *in vitro* and *in vivo* studies will be necessary to identify the most superior nitroxide radioprotectors and to confirm the lack of tumor protection.

**References**

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