Radiation Protection by the Antioxidant Alpha-Tocopherol Succinate

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ABSTRACT

Radiological terrorism and use of nuclear weapons are major concerns for national defense and homeland security. At low doses of radiation, the hazards from these scenarios may not be apparent immediately, but may result in late arising pathologies like cancer and pulmonary fibrosis. At high doses, the hazards vary from incapacitation due to nausea and diarrhea to mortality. Free radical species of oxygen, derived from the interaction of ionizing radiation with critical biological targets and with the aqueous cellular milieu, are implicated in these hazards. Scavengers of free radicals have been shown to be effective protectors from radiation damage. However, many of these protectors are either toxic or cannot be administered orally at doses that are effective. The data presented here indicate that α-tocopherol succinate (TS), a free radical scavenger, can be used as a radioprotector with low toxicity. Tocopherol succinate was dispersed in a vehicle containing polyethylene glycol-400 (PEG) and given orally (PO) to male CD_2F_1 mice. About 22-24 hours later, they were irradiated at different doses of ^{60}Co radiation at a dose rate of 0.6 Gy/min. To maximize the protection, different formulations of the vehicle were used. Mice were monitored for body weight and survival for 30 days. In vitro experiments were done to study the effects of TS on radiation-induced apoptosis of Jurkat cells (lymphoblastoid cell line) using flow cytometry. Although different formulations for solubilizing TS were used, oral formulations based on PEG were found to provide better protection than those based on oil emulsions. The best protection was obtained with a combination of PEG-400 and an emulsifier consisted of benzyl alcohol and ethyl alcohol. The PEG and emulsifier combination provided 60-70% protection at 9 Gy, a radiation dose at which only 0-13% of the animals treated with the vehicle survived. Other available analogues/derivatives of tocopherol were not protective with any of the formulations. In vitro experiments indicated that TS promoted radiation-induced apoptosis in human lymphocyte cell cultures. In vitro apoptosis experiments were not done with other tocopherol analogues/derivatives. The results with bone marrow cells were not conclusive. Protection of irradiated mice by oral TS but not by other analogues probably indicates the need for amphipathic groups on tocopherol to obtain protection by oral administration. A hemisuccinate moiety on TS may satisfy this requirement. However, the increase in apoptosis in in vitro experiments suggests that the role of the hemisuccinate moiety is only in facilitating the transport of TS across the intestine and that the in vivo protection is probably not due to the whole TS molecule but only the tocopherol released from TS in vivo.

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1.0 INTRODUCTION

Military personnel and civilian population are at risk of exposure to radiation from nuclear or radiological attack. Use of improvised nuclear devices (IND), also known as "dirty bombs," is not a question of if; it is a question of when [Kimery 2003]. Therefore, radioprotectors for use prior to exposure has been identified as one of the highest priority areas for research [Pellmar 2005]. Depending on the dose rate and dose of exposure, the effects of radiation range from nausea and vomiting to immune system compromise to death from either radiation-induced tissue damage or infection. Although it is believed that the effects of exposure to moderate doses (1–8 Gy) of γ-radiation result in hematopoietic dysfunction, the net injury is a result of a dynamic process involving cell killing, altered cell-to-cell communication, inflammatory responses, compensatory tissue hypertrophy and repair [Stone 2003, Stone 2002]. Higher radiation doses compound these effects with gastrointestinal (GI) and neurovascular tissue damage [Weiss 1988, Coleman 2004].

Ionizing radiation triggers the formation of free radicals, which interact among themselves and with critical biological targets with the formation of a plethora of newer free radicals. It is generally believed that production of these free radicals is the main mechanism through which radiation induces biological damage at lower radiation doses [Weiss 1988]. Some of these stable free radicals may be responsible for the genomic instability mediated by the microenvironment [Barcellos-Hoff 2001]. The cells of the immune and blood-forming systems are particularly sensitive to changes in oxidant/antioxidant balance because of a high percentage of polyunsaturated fatty acids in their plasma membranes. The oxidant/antioxidant balance is an important determinant for both immune and blood-forming functions, not only for maintaining the integrity and function of the plasma membrane, cellular proteins, and nucleic acids, but also for control of signal transduction and gene expression [Aw 1999].

A safe compound that would prevent the ablation of immune system function and other radiation-induced oxidative tissue damage would provide an effective medical countermeasure against nuclear or radiological attack. Despite over four decades' effort in this area, no compound has yet been identified and fielded that has broad-spectrum radioprotective attributes necessary to protect populations from the unwanted radiation exposures associated with catastrophic accidental or intentional (terrorist associated) events. Amifostine (WR-2721) is considered a "gold standard" radioprotectant by many investigators, due to its broad spectrum, efficacious nature. Despite the fact that Amifostine is currently FDA (U.S. Food and Drug Administration)-approved and commercially marketed as a normal tissue protectant in cancer patients undergoing intense chemo- and/or radiotherapy, the drug is quite toxic and debilitating when applied at relatively high doses required for radioprotection. Amifostine is, therefore, suitable for use only in a clinical setting and could not be used in a clinically unsupervised scenario such as to protect military or civilian population that has undergone, or is expected to undergo, radiation exposure.

2.0 ANTIOXIDANTS AS RADIOPROTECTANTS

An ideal radioprotectant should offer significant protection against lethality from acute and long-term effects of radiation exposure; be suitable for oral administration and be rapidly absorbed and distributed throughout the body; cause no significant toxicological effects, particularly those on behaviour; be readily available and affordable; and be chemically stable to permit easy handling and storage.

Although the radioprotectants studied earlier do not meet these requirements, some of the recently investi-

gated agents appear to satisfy many of these criteria. Antioxidants are one such class of agents, which are nontoxic and moderately radioprotective. These antioxidants include tocols (tocopherols and tocotrienols), soyisoflavones, vitamin A, β-carotene, selenium (organic and inorganic), zinc, copper, and the enzyme superoxide dismutase and its mimetics [Kumar 1988, Kumar 2002, Kumar 2003, Weiss 2000, Seifter 1984, Weiss 1990]. Among these compounds, vitamin E has attracted considerable attention. The term "vitamin E" refers to a family of 8 tocols—4 each of α, β, γ, and δ tocopherols and tocotrienols (Figure 1).

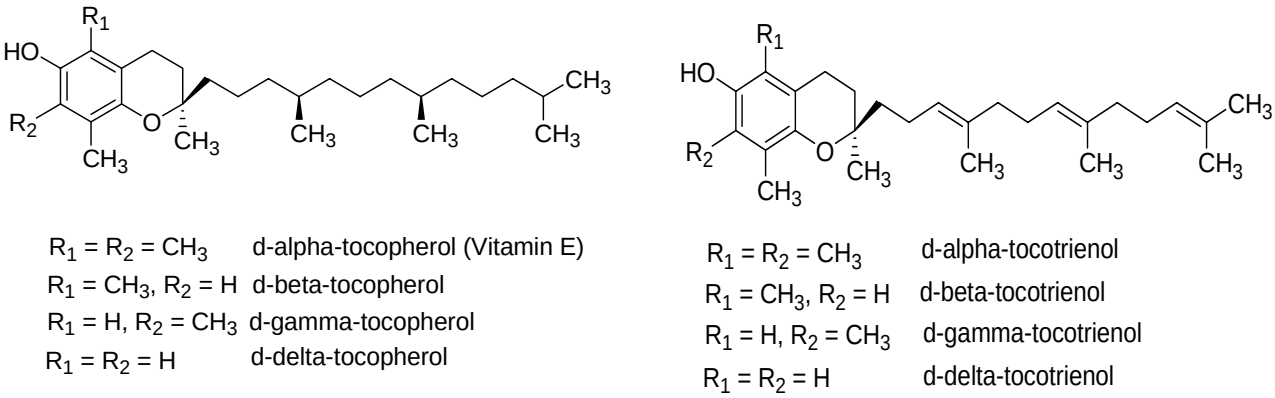

Figure 1: Structure of the eight natural tocols

All reference to vitamin E in this paper will be to α-tocopherol, and these two terms (α-tocopherol and vitamin E) may be used interchangeably. Tocols occur in common human foods at varying levels; α-tocopherol and γ-tocopherol are the most abundant in typical diets. All members of the family are considered generally recognized as safe (GRAS) or self-affirmed GRAS. In a study of the oral toxicity to rats of a mixed preparation containing all 8 tocols, the no-observed adverse effect level (NOAEL) was reported to be 120-130 mg/kg daily [Nakamura 2001]—this would correspond to a human dose of about 700 to 900 mg/day. Vitamin E has a long record of safety in foods as a preservative and nutritional supplement, and nutritional supplements containing mixed tocopherols and tocotrienols are currently being sold in the U.S. and overseas.

3.0 PREVIOUS RADIOPROTECTION STUDIES WITH A-TOCOPHEROL

Vitamin E fed at 3 times the normal mouse requirement for 1 week before and for 30 days following an 8.5-Gy dose of ^{60}Co radiation led to 60% 30-day survival, whereas 100% of control animals succumbed. At 7.5 Gy, the control survival was 10% and 100% of the vitamin E-treated animals survived [Srinivasan 1983]. We have observed that subcutaneously administered vitamin E provided 79% survival in mice exposed to the supralethal dose of 10.5 Gy; oral administration was ineffective in this study [Kumar 2002]. In mice exposed to 1 Gy of whole-body ^{60}Co radiation 2 hr before or 2 hr after oral administration of vitamin E, both bone marrow polychromatic erythrocyte micronucleus formation and chromosome aberrations were significantly suppressed [Sarma 1993]. Chromosome damage suppression by vitamin E has also been demonstrated in mouse cells [Konopacka 1998] and in human lymphocytes [Konopacka 2001]. The dose reduction factor (DRF) for vitamin E in mice (30-day survival as the end point) has been variously reported to be in the range of 1.06 [Srinivasan 1983] to 1.23 [Seed 2002]. It is interesting to note that at a dose of Amifostine that minimizes side effects, the DRF of Amifostine drops to 1.2—within the range reported for vitamin E. Even at this dose Amifostine continues to have significant side effects, especially when the testing is done with large animal models

(Seed and Kumar, unpublished findings). Vitamin E does not have any toxicity at the doses that give a DRF of about 1.2. In humans even higher doses have been tolerated without any toxicity.

4.0 CURRENT EXPERIMENTS

In order to develop an oral preparation of tocopherol for radioprotection, we have tested α-tocopherol for its radioprotective efficacy after oral administration and, as pointed out in an earlier section, it was not protective [Kumar 2002]. Because tocopherol is a hydrophobic chemical, we tested a hemisuccinate ester of tocopherol, the chemical structure (Figure 2) of which indicated its amphipathic nature.

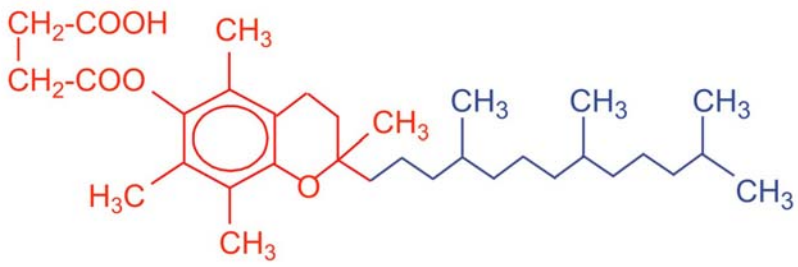

Figure 2: Chemical structure of tocopherol succinate

In these experiments, tocopherol succinate at doses of 400 mg/kg and 800 mg/kg bodyweight was dispersed in a mixture of polyethylene glycol (PEG-400) and a proprietary emulsifying agent provided by Stuart Products, of Texas, USA. TS in this vehicle was given as a single oral gavage (PO) to CD2F1 male mice 22-24 hr before exposure to whole-body gamma radiation at doses of 9, 9.5, and 10 Gy. In an attempt to maximize the efficacy of TS, it was dispersed in various formulations using oil-based and PEG-based formulations and tested for their radioprotective efficacy. In general, for radioprotection studies, mice were monitored every day for survivors and every other day for weight loss/gain. The percent of mice surviving after 30 days was taken as an index of survival-protection.

In other experiments, because apoptosis has been reported to be one of the mechanisms of radiation damage and that TS is pro-apoptotic, we tested the effect of TS on radiation-induced *in vitro* apoptosis of Jurkat cells (lymphoblastoid) as well as in *ex vivo* bone marrow cells. In apoptotic cells, the membrane phospholipids phosphatidyl serine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca^{+}-dependent phospholipids binding protein that has high affinity for PS, and binds to cells with exposed PS [Raynal 1994]. Annexin V may be conjugated to fluorochromes such as phycoerythrin (PE). Because externalization of PS occurs in the earlier stages of apoptosis, annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes [Vermes 1995]. Staining was carried out using an Annexin V-PE apoptosis-detection kit (BD Pharmingen). In brief, human lymphoblastoid Jurkat (or mouse bone marrow) cells were washed twice with cold PBS and then resuspended in binding buffer at a concentration of 1×10^6 cells/ml. One hundred µl of suspension was transferred to another tube (1×10^5 cells). Annexin V was added and vortexed, and incubated for 15 min at room temperature. Then 400 µl of binding buffer was added to each tube. Analysis was carried out in flow cytometer (Bection Dickinson, San Jose, CA) using CellQuest software

5.0 RESULTS

5.1 Survival Protection

Although several formulations of TS with different vehicles were tested for increased radioprotective efficacy of TS, none of them was as effective as a combination of PEG-400 with the emulsifying agent. Therefore, this formulation was used in all further studies reported here. In all these studies, TS was given as an oral gavage 24 hr before irradiation.

Tested at 2 different doses (400 mg/kg and 800 mg/kg body weight), TS protected mice from radiation-induced lethality based on the radiation exposure doses (Figure 3). At both doses of TS, protection from irradiation at 9 Gy was almost the same (60-70%; $p < 0.01$ compared to vehicle treated mice); only 0-13% of vehicle treated animals survived. When radiation was increased to 9.5 Gy, 63% of the mice treated with TS at a dose of 800 mg/kg bodyweight survived ($p < 0.01$ compared to vehicle treated); only 13% of mice (although significantly different from vehicle treated mice) exposed to 10 Gy survived with this dose of TS. Higher doses of TS were not tested. None of the vehicle treated mice survived 9.5 or 10 Gy radiation.

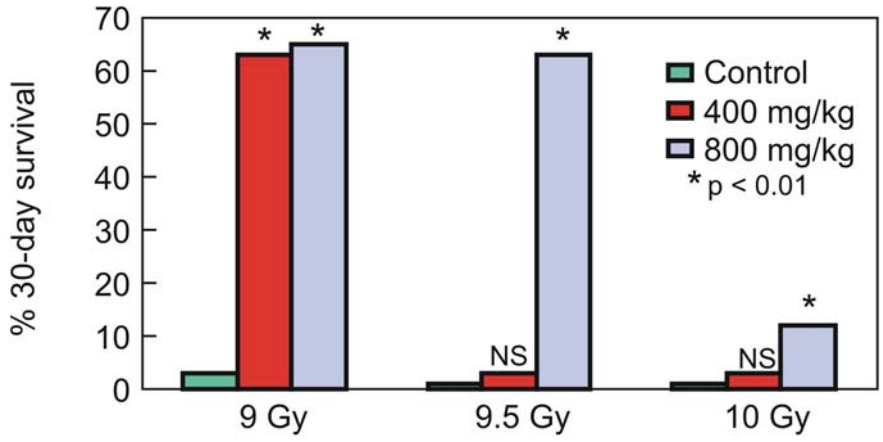

Figure 3: Protection of irradiated mice given TS by oral gavage 24 hr before irradiation at 9-, 9.5-, or 10-Gy radiation

5.2 Effect of TS on Apoptosis of Jurkat Cells *In Vitro*

Jurkat cells were cultured in plates having 6 wells each in fetal bovine serum-supplemented RPMI 1640 media. Various doses of TS were added to culture, and the plates were incubated in a CO_2 incubator for 24 hr at 37°C. At the end of incubation, cells were collected and washed three times with PBS and stained with annexin-V as described above. Figure 4 shows annexin-V positive apoptotic cells in control and various treated groups. Results demonstrate the dose-dependent increase in apoptosis induced by TS in Jurkat cells.

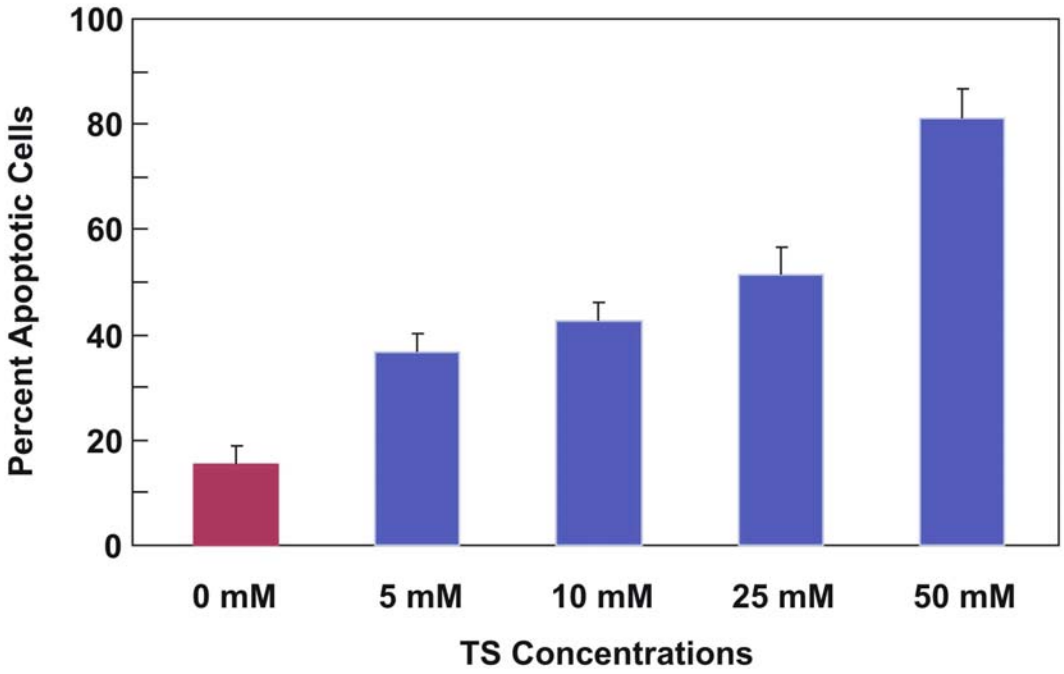

Figure 4: Apoptosis of unirradiated Jurkat cells on exposure to various doses of tocopherol succinate

5.3 Effect of TS on Apoptosis of *Ex Vivo* Mouse Bone Marrow Cells

The effect of TS-induced apoptosis was investigated on mouse bone marrow cells. In the first set, mouse bone marrow cells from unirradiated animals were treated with TS (10 μM) *in vitro*. After 24-hr incubation, cells were stained with annexin-V and analyzed. As shown in Figure 5, TS-treated and untreated cells do not differ significantly for apoptosis.

In the experiment's second set, two groups of mice were administered vehicle and TS (400 mg/kg) and irradiated with 3.5-Gy gamma irradiation 24 hr after drug administration. Mice were bled 24 hr after irradiation and bone marrow cells were stained with annexin-V and analyzed with a flow cytometer. There was no significant difference in apoptosis between the bone marrow cells of TS-treated and untreated control.

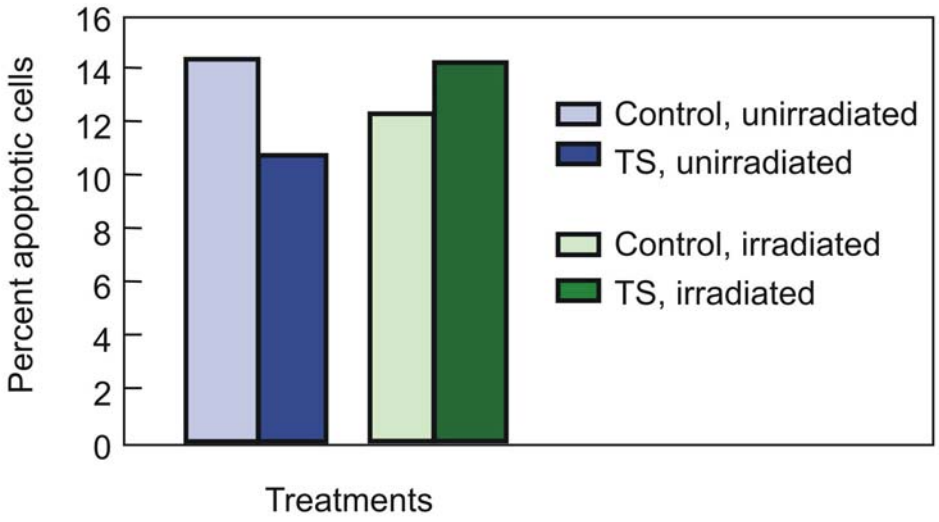

Figure 5: Effect of TS (10 μM) on apoptosis of BM cells from irradiated and unirradiated mice

6.0 DISCUSSION

Results presented here indicate that a single oral administration of tocopherol succinate provides moderate protection from radiation lethality. Oral α-tocopherol (vitamin E), the parent compound from which tocopherol succinate was derived, did not protect from radiation lethality. Because it is known that long-term feeding of α-tocopherol in diet protects mice moderately, it is possible that repeated oral administration of α-tocopherol may probably respond like a single oral administration of tocopherol succinate. The need for long-term feeding indicates that tocopherol is poorly absorbed from the intestine. On the other hand, protection by a single administration of TS indicates that it probably is absorbed better than tocopherol. Thus, the difference in the radioprotection profile of tocopherol and TS by oral dosing may only be a reflection of the respective absorbability of the two drugs. This probably is true because tocopherol is a hydrophobic compound whereas TS is amphipathic by virtue of a free carboxylic acid moiety of the succinic acid molecule esterified to OH group on the chroman ring of tocopherol (Figure 2). Once absorbed, tocopherol succinate is converted via endogenous blood esterases into α-tocopherol and the two will exist in equilibrium. *In vivo*, the active protective ingredient may still be only α-tocopherol hydrolyzed by the esterases from TS. Moreover, it is known that the free OH-group present in α-tocopherol, which is blocked by the ester, accounts for the significant radical scavenging or antioxidant properties of the radioprotectant [Burton 1982].

Free radicals formed from radiation can also induce apoptosis and inhibition of apoptosis by free radical scavengers like α-tocopherol may also account partly for the radiation protection. Vitamin E reduced apoptosis in *in vitro* irradiated human lymphoblastic MOLT-3 cells [Ortmann 2004]. Similarly, Trolox, a water-soluble derivative of vitamin E, completely blocked apoptosis in a human lymphocytic leukemia line MOLT-4 cells irradiated *in vitro* [McClain 1995] if given 4 hr after irradiation but confers no protection if given prior to or during exposure. Neuzil reported that tocopherol is anti-apoptotic in normal cells but induces apoptosis in malignant cells [Neuzil 2001]. These studies and the results presented in this paper show that tocopherol succi-

nate is a potent inducer of apoptosis in *in vitro* cells. However, TS had no effect on bone marrow cells from normal mice or those treated with TS and irradiated. It appears that TS is pro-apoptotic only to transformed cells. What enables TS to distinguish between transformed and normal cells is unknown. In fact, this unique property of TS to distinguish between normal and transformed cells may have far-reaching benefits as far as long-term exposure to chronic low-level radiation is concerned. One of the consequences of low-level chronic exposure to radiation is carcinogenesis. Normal cells altered by exposure to radiation are transformed to become foci for tumor formation. If TS is capable of recognizing such cells for apoptotic destruction, it is quite possible that TS can serve not only as a protector against radiation lethality but also as a safeguard against possible tumor induction by radiation as well.

7.0 FUTURE PLANS

Experiments are in progress to determine the dose reduction factor with TS given SC and PO. It also is important to determine whether there are differences in the pharmacokinetic profile between TS given by these two routes, which may explain the difference in the degree of radiation protection. Studies also are in progress to determine the role of TS in preventing radiation-induced peripheral blood cell cytopenia as well to determine molecular end points of radioprotection by TS.

8.0 CONCLUSIONS

Tocopherol succinate provides moderate protection from radiation-induced lethality when administered 24 hr before exposure. It induces apoptosis in transformed *in vitro* lymphocyte cultures but not in bone marrow cells from normal or irradiated mice treated with TS. Its ability to induce apoptosis in transformed cells but not in normal cells may be beneficial in selectively destroying cells that could be transformed *in vivo* due to exposure to chronic low-level radiation. The combined ability of TS to provide survival-protection from radiation lethality and selective apoptosis of transformed cells makes it a unique non-toxic radioprotectant that can be used against the short- and long-term effects of radiation exposure.

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