The Antioxidant Tempol Reduces Carcinogenesis and Enhances Survival in Mice When Administered after Nonlethal Total Body Radiation

James B. Mitchell1, Miriam R. Anver4, Anastasia L. Sowers1, Philip S. Rosenberg2, Maria Figueroa1, Angela Thetford1, Murali C. Krishna1, Paul S. Albert3, and John A. Cook1

Abstract

There is significant interest in the development of agents that can ameliorate radiation damage after exposure to radiation has occurred. Here we report that chronic supplementation of the antioxidant Tempol in the diet of mice can reduce body weight without toxicity, decrease cancer, and extend survival when administered after nonlethal total body radiation (TBI). These effects were apparent in two different strains of mice (C3H, CBA) exposed to TBI (3 Gy). Notably, delaying administration of the Tempol diet one month after TBI could also enhance survival. Tempol reduced the incidence of hematopoietic neoplasms (lymphomas) in both strains, whereas both the onset and incidence of nonhematopoietic neoplasms were reduced in CBA mice. These results encourage further study of Tempol as a chemopreventive, to reduce the incidence of radiation-induced second malignancies after a course of definitive radiation therapy. Tempol may also find applications to reduce the risk of cancers in populations exposed to nonlethal radiation due to nuclear accidents or terrorist attacks.

Introduction

Intentional or accidental exposure of humans to ionizing radiation (IR) can lead to a variety of acute effects, including lethality or long-term late effects encompassing organ injury (1) and carcinogenesis (2). To protect against these toxicities, there has been an ongoing development of chemical and biologic radiation protectors or mitigators (3), primarily directed to protection against the acute radiation syndrome following total body irradiation (TBI) or for normal tissue protection in cancer patients treated with radiotherapy (4). A terrorist-mediated nuclear attack or accidental damage to nuclear reactors could place large populations of people exposed to nonlethal IR doses at risk for cancer induction. Such events can occur without warning, necessitating the application of interventions post-IR exposure. Likewise, IR-induced second malignancies can occur after a course of definitive radiation therapy (5, 6). Both of these situations suggest a need for safe and effective interventions to reduce these risks to humans after IR exposure.

The development of chemical and biologic radiation protectors or mitigators dates back to the 1940s (7–9). Applications of such agents have been primarily directed to protection against the acute radiation syndrome following TBI or normal tissue protection in radiotherapy patients. Both of these toxicities occur over hours to several weeks post-IR and can be life threatening. Unfortunately IR exposure can also impose late effects including induction of cancer. Most, if not all classical radiation protectors, provide protection only if administered immediately before and during IR treatment (7, 9). Intentional and, in some cases, accidental IR exposure precludes the use of such protectors for IR-induced carcinogenesis. Therapies or approaches administered post-IR for the reduction in IR-induced carcinogenesis are scarce; however, the long latency period of cancer induction post-IR exposure should provide opportunities for intervention (2). IR-induced cancer induction was significantly reduced after radiation treatment by calorie restriction (10–12). These studies used both rats and mice and used either a significant reduction in food intake (up to 40% reduction) or reduction in the caloric content in the food to achieve calorie restriction resulting in significant reductions in body weight. A 25% to 40% reduction in calorie intake is necessary to benefit from calorie restriction in mice; a calorie reduction that would be difficult for most individuals to reach and maintain who are accustomed to modern dietary patterns (13). Preclinical studies conducted in our laboratory have shown that the stable nitroxide-free radical Tempol (14) supplemented in the drinking water or diet of mice results in (i) significant body weight loss without untoward toxicity (15) and (ii) reduction or...
delay in the onset of cancer in ATM, p53, and Fanconi anemia knockout mice (16–18). We hypothesized that chronic Tempol supplementation in the diet immediately after nonlethal total body IR may mimic the effects of calorie restriction by reducing IR-induced carcinogenesis and enhancing survival in mice. To test this hypothesis, the lifespans of 2 different mouse strains were compared after exposure to nonlethal 3 Gy TBI and administration of Tempol or control diets after IR.

Materials and Methods

Mice

Female C3H/HenTac-MTV− (Taconic Farms) and CBA/CaJ (Jackson Laboratory) mice were used. A special breeding program for both animal strains was initiated to provide 750 C3H and 600 CBA mice at 4 weeks of age. Mice were housed in a specific pathogen-free (SPF) facility on a 12-hour day/night cycle with standard laboratory chow and water ad libitum. When the mice were 8 to 9 weeks of age, they were divided into various unirradiated control and TBI groups. The groups included 0 Gy control diet (0 Gy C), 3 Gy control diet (3 Gy C), 0 Gy Tempol diet (0 Gy T), 3 Gy Tempol diet (3 Gy T), and 3 Gy Tempol diet, delayed 1 month post-TBI (3 Gy T delayed; C3H mice only). Immediately following TBI, the chow for all groups was switched to bacon-flavored control chow or a bacon-flavored chow containing Tempol and water ad libitum. Powdered Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; supplied by Mitos Pharmaceuticals) was uniformly mixed for all groups was switched to bacon-flavored control chow or a bacon-flavored chow containing Tempol and water ad libitum. Powdered Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; supplied by Mitos Pharmaceuticals) was uniformly mixed for all groups was switched to bacon-flavored control chow or a bacon-flavored mouse chow by a "cold press" technique. Control animals received bacon-flavored chow minus the Tempol. Mice were maintained on these respective chows for their lifespan in climate controlled, circadian rhythm-adjusted rooms (5 mice/cage on Sani-Chip bedding). Average weights of the mice were determined several times a week for the first few weeks post-TBI by weighing the cage of animals and dividing their total weight by the number of animals. Periodic weight assessments were subsequently made approximately monthly post-TBI. Average food consumption was conducted periodically by randomly selecting 5 cages of mice from each group and determining the food consumed over a 2-week period. All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use Of Laboratory Animal Resource, (2008) National Research Council.

Assessment, necropsy, and pathology

Animals were carefully monitored a minimum of 3 times per week for their entire lifespan. The endpoint for the study was tumor formation (not to exceed 2 cm diameter) or until the animal reached a humane endpoint (rapid weight loss, debilitation, diarrhea, red hair coat, hunched posture, labored breathing, lethargy, persistent recumbence, jaundice, significantly abnormal neurologic signs, bleeding from any orifice, proptosis or abnormal appearance of eyes, impaired mobility, or inability to obtain food or water) at which time the animal was euthanized and evaluated for the presence of tumor and cause of death. Mice were euthanized by CO2 inhalation and blood collected from the thoracic aorta for a complete blood count. A comprehensive necropsy examination was conducted on each mouse with descriptions of gross lesions, collection of all major organs, tissues and lesions, and fixation of pathology materials in 10% buffered neutral formalin. Tissues were processed and stained with hematoxylin and eosin. A board-certified veterinary pathologist carried out pathology evaluation. Special stains and immunohistochemistry were carried out on a subset of animals to clarify the major form of hematopoietic neoplasms.

Statistical analysis

Data were analyzed using R (version 2.14), SAS Software version 8.2, and MATLAB. Overall survival probabilities by age were estimated using the Kaplan–Meier method. The log rank test was used to test for significant differences in survival between groups. Cumulative incidence curves by age were calculated for the competing risks of death after hematopoietic neoplasms, death after nonhematopoietic neoplasms, and non-cancer death, using the nonparametric maximum likelihood estimator (19). For mice with both hematopoietic and nonhematopoietic neoplasms, the former was counted as the first event. Relative hazards were estimated using the Cox proportional hazards method. P values less than 0.05 were considered to show a statistical trend.

Gene expression studies

Brain, liver, and muscle tissues (5–8 mice per time point) were collected from Tempol- and control-fed C3H mice after 14 and 60 days and stored at −80 °C. Total RNA was extracted from frozen tissues using TRIzol Reagent (Life Technologies, Inc.) according to the standard protocol. RNA concentration was determined with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Samples were then treated with DNase using the Ambion DNA-free Kit (Life Technologies, Inc.). Amplified antisense RNA was produced from the samples by using the Arcturus RiboAmp PLUS Amplification Kit (Life Technologies, Inc.). The mRNA was labeled and hybridized as previously described (20).

Further details on Materials and Methods can be found in Supplementary Materials and Methods.

Results

Animal weights and food consumption

Figure 1A and C show the average weight per mouse for both the CBA and C3H strains over the study. Both strains of mice maintained on the Tempol diet gained significantly less weight compared with controls, yet remained healthy throughout their lifespan as previously reported for C3H mice (15). For CBA mice 0 Gy C and 3 Gy C groups exhibited similar weight profiles to approximately 50 weeks (Fig. 1A). Three Gy CBA mice exhibited lower weights than 0 Gy C mice beyond 50 weeks post-IR (P < 0.02). The Tempol diet resulted in reduced weight gain starting at approximately 10 weeks compared with mice on the control diet (P < 0.001). There was no statistical difference in the weights of 0 Gy T or 3 Gy T mice throughout the study. C3H mice exhibited similar weight profiles (Fig. 1C).
as observed for CBA mice. Weights of 0 Gy C C3H mice increased rapidly to 25 weeks, peaked at 60 weeks (~37 g), and then slowly decreased as the animals aged (Fig. 1C). The 0 Gy T group of C3H mice gained weight similar to control animals up to approximately 5 weeks, followed by a plateau in weights to approximately 50 weeks (~27 g), and then decreased slowly. The 0 Gy C mice had significantly higher weights ($P < 0.001$) than 0 Gy T mice from approximately 10 to 80 weeks with a maximum difference of approximately 38% at 55 weeks. Weights for 3 Gy C C3H mice were similar to the 0 Gy C to approximately 50 weeks. The weights of the irradiated mice decreased more rapidly from 50 to 80 weeks than unirradiated mice ($P < 0.005$). Irradiated mice on the Tempol diet (both those placed on the Tempol diet immediately after IR or 1 month after IR) exhibited near identical weights to 0 Gy T controls throughout the study. The decreased weight gain of both mouse strains receiving the Tempol diet could not be explained by reduced food consumption as shown in Fig. 1B and D. Food consumption monitored from weeks 23 to 80 (CBA) and weeks 20 to 77 (C3H) showed no statistical difference among any of the groups.

**Survival and mortality assessment**

Figures 2A and 3A show Kaplan–Meier survival plots. Whereas Tables 1 and 2 list the median survival times for CBA and C3H mice, respectively. Log rank analysis of the survival curves showed no significant difference in the lifespan of 0 Gy C versus 0 Gy T (CBA, $P = 0.91$; C3H, $P < 0.06$). Overall, chronic Tempol food supplementation was well tolerated by both mouse strains with little untoward toxicity. In the Tempol-fed C3H mice toward the end of their lifespan (>98 weeks), transmural proximal duodenal ulcers and peritonitis were noted. This was not observed in the CBA mice. There was a substantial reduction in the lifespan after 3 Gy TBI compared with unirradiated controls for both mice strains; 34 weeks less for CBA mice ($P < 0.001$) and 38 weeks less for C3H ($P < 0.001$) mice. Tempol diet supplementation initiated immediately after TBI resulted in a significant survival advantage compared with the control diet for both CBA and C3H mice (CBA, $P < 0.003$; C3H, $P < 0.001$), representing an extension in survival of approximately 18 weeks and 34 weeks for CBA and C3H mice, respectively. Interestingly, delaying the Tempol diet one month post-TBI in C3H mice also provided a significant survival advantage (23 weeks) compared with the 3 Gy C ($P < 0.001$). However, there was no statistical difference between the 3 Gy T and 3 Gy T delayed groups (see Fig. 3).

Specific mortality analyses (cancer) for both mouse strains are described in Tables 1 and 2 and Figs. 2B–G, 3B–G. The spectrum of neoplasms was not changed in either mouse strain by Tempol food supplementation (see Supplementary Tables
S1 and S2), consistent with previous reports (15–18). Over their lifespan, 61% of 0 Gy C CBA mice had malignant neoplasms (hematopoietic and nonhematopoietic neoplasms), compared with 39% (P < 0.005) for 0 Gy T mice. As anticipated, TBI increased malignant neoplasms by 22% and 17% for 3 Gy C and 3 Gy T CBA mice, respectively. Hematopoietic neoplasms were primarily lymphomas of B cell origin (data not shown). Table 1 also lists the percentages of neoplasms for both malignant and benign tumors. For unirradiated mice, the Tempol diet lowered the incidence of all forms of neoplasms (nonhematopoietic neoplasms: 37%, hematopoietic neoplasms: 60%, and benign: 14%). Likewise, 3 Gy T mice reduced the incidences of malignant neoplasms compared with 3 Gy C mice (non-hematopoietic neoplasms: 27%, hematopoietic neoplasms: 55%). Temporal analyses of the cumulative incidence of malignant hematopoietic neoplasms and nonhematopoietic neoplasms, and noncancer-related deaths for CBA mice are shown in Figs. 2B–G. Tempol treatment delayed the onset of both hematopoietic neoplasms and nonhematopoietic neoplasms in IR and unirradiated mice. The onset for hematopoietic neoplasms death for 0 Gy C versus T mice changed from 87 to 106 weeks (P = 0.008), and for 3 Gy C versus T mice from 39 to 55 weeks (P = 0.03). Similarly, Tempol treatment delayed the onset of nonhematopoietic neoplasms death for 0 Gy C versus T from 84 weeks to 111 weeks (P < 0.001) and for 3 Gy C versus T from 55 weeks to 77 weeks (P < 0.001). Thus, TBI shortened the lifespan of mice and Tempol treatment delayed the onset of radiation-induced neoplasms for CBA mice. Tempol treatment increased the noncancer deaths for CBA mice as they approached the end of their lifespan. As was seen for CBA mice, Tempol exhibited significant reductions in hematopoietic neoplasms and nonhematopoietic neoplasms for unirradiated C3H mice (Table 2, Fig. 3B–G). In this strain, lymphomas were also the most common hematopoietic neoplasms. Over their lifespan, 51% of 0 Gy C C3H mice had malignant neoplasms, compared with 33% (P < 0.001) for
0 Gy T mice (Table 2). TBI significantly increased malignant neoplasms in C3H mice by 25% ($P < 0.01$) from the 0 Gy C, whereas the increase was 41% for 3 Gy T mice and 35% for 3 Gy T delayed. In contrast to the CBA mice, however, the number of C3H mice with benign neoplasms on Tempol diet increased (49%, 0 Gy C to 81%, 0 Gy T, $P < 0.01$, Table 2). The increase in benign neoplasms was dominated by increases in hepatocellular adenomas (30% 0 Gy C vs. 74% in 0 Gy T mice ($P < 0.001$); see Supplementary Table S4). It is well known that C3H mice are susceptible to hepatocellular tumors (21), which was not as prominent in CBA mice (Supplementary Table S3). However, it is important to note that the number of hepatocellular carcinomas in unirradiated C3H mice was not increased with the Tempol diet (9%–6%), suggesting that Tempol was likely not facilitating the conversion of adenomas to carcinomas.

There were several differences between the 2 mouse strains with respect to IR-induced neoplasms. Supplementary Table S5 shows that the normalized ratio of hematopoietic neoplasms to nonhematopoietic neoplasms after 3 Gy IR was approximately 4.5-fold higher in C3H mice as opposed to CBA mice, in which the ratio remained unchanged compared with 0 Gy C mice. Tempol reduced both hematopoietic neoplasms and nonhematopoietic neoplasms incidence in the 0 Gy C groups of both mouse strains; however, its effects were greatest on hematopoietic neoplasms (compare 0 Gy C with 0 Gy T, Supplementary Table S5). A second major difference in the 3 Gy C groups between CBA and C3H mice was the nonhematopoietic neoplasm incidence, which increased in CBA mice by 14% and decreased in C3H mice by 7%. Nonhematopoietic neoplasms in C3H mice did increase after 3 Gy in the Tempol-fed mice from 30% to 47% (Table 2). No significant differences
with respect to either hematopoietic neoplasms or nonhematopoietic neoplasms were observed in the 3 Gy T delay group compared with the 3 Gy T group (Table 2). Lastly, the rate of hematopoietic neoplasm incidence was accelerated in C3H mice compared with CBA mice (compare hematopoietic neoplasm incidence in Fig. 3C vs. Fig. 2C).

The data presented thus far was analyzed for survival and cumulative neoplasm incidences over the entire lifespan of the mice. To obtain a more refined analysis of the different modes of death for the study, relative hazard (RH) ratios were calculated for both animal strains for 2 intervals; 0 to 104 weeks and beyond 104 weeks (Supplementary Tables S6 and S7). For the first 2 years of life, Tempol provided a significant overall survival benefit for both 0 Gy and 3 Gy groups in CBA mice (RH<sub>CA</sub> = 0.33 and 0.47, respectively, Supplementary Table S6). Beyond 104 weeks there was no overall survival advantage for Tempol treatment for 0 Gy or 3 Gy (RH = 1.14 and 1.05, respectively). For 0 Gy, Tempol treatment reduced both hematopoietic neoplasm and nonhematopoietic neoplasm death over the entire time span (Supplementary Table S6). There was a significant increase in noncancer deaths beyond 104 weeks (RH = 1.92, Supplementary Table S6). The noncancer deaths did not become apparent until after week 139 (Fig. 2F; maximum lifespan = 160 weeks). Similarly, for the 3 Gy T CBA group, there was a highly significant reduction in both hematopoietic neoplasm and nonhematopoietic neoplasm (RH = 0.29 and 0.36, respectively) and an increase in noncancer deaths beginning at week 120 (Fig. 2B; RH = 3.31, Supplementary Table S6). Likewise, Tempol treatment afforded an overall survival benefit in C3H mice for both 0 Gy, 3 Gy, and 3 Gy delayed up to 104 weeks (RH = 0.45, 0.34, and 0.46, respectively; Supplementary Table S7). Unlike the CBA mice, Tempol treatment beyond 104 weeks resulted in a decrease in overall survival for 0 Gy mice (RH = 2.26). The decrease was evident at approximately week 110 (Fig. 3A; maximum lifespan = 136 weeks), contributed predominantly by noncancer deaths (RH = 4.5). Unlike the 0 Gy group, 3 Gy T treatment groups beyond 104 weeks had RH values less than 1.0, indicating that Tempol had beneficial effects in all categories (Supplementary Table S7).

### Early gene expression patterns in tempol-supplemented C3H mice

Gene expression profile studies of selected tissues were conducted to obtain early gene expression changes by Tempol in mice before changes in weight (14 days) and later when animal weights were reduced by Tempol (60 days). Figure 4 shows hierarchical cluster maps monitoring 2-fold gene changes in muscle, liver, and brain (at both time points) comparing Tempol to control mice. The majority of gene changes (both up- and downregulated) resulting from the Tempol diet occurred in the liver (248 genes), followed by the muscle (150 genes) and brain (9 genes). Numbers to the right of each map indicates clustered gene profiles and gene ontology categories are listed in Supplementary Table S8. The prominent genes in cluster I were IGF-1 and Ghr, which were downregulated after 60 days of Tempol diet (I). The Tempol diet upregulated genes related to mitochondrial

### Table 1. Median survival and percentage distribution of neoplasms in CBA Mice

| Number of mice | Number (%) | Benign neoplasm (%) | Malignant neoplasm (%) | CBA group
|----------------|------------|---------------------|------------------------|------------
| 0 Gy Control   | 128.7      | 104.4               | 9.3                    | 1.046      |
| 3 Gy Control   | 94.3       | 95.2               | 0.7                    | 1.145      |
| 0 Gy Tempol    | 134.1      | 135.6               | 0.5                    | 1.056      |
| 3 Gy Tempol    | 117.1      | 115.1               | 0.4                    | 1.031      |

**NOTE:** Statistical comparisons: T:C 0 Gy T to 0 Gy C; C:IR 0 Gy C to 3 Gy C; T:IR-T 0 Gy T to 3 Gy T

**a**CI, 95% confidence interval.

**b**Malignant neoplasms include both hematopoietic neoplasms and nonhematopoietic neoplasms.

**c**Percentages in these 2 categories do not add up to the total percentage of malignant neoplasms because some mice had both hematopoietic and nonhematopoietic neoplasms.
function and fatty acid and/or lipid synthesis in cluster II. Drug metabolizing genes (cluster III) were elevated at both 14 and 60 days of Tempol treatment; the only cluster upregulated at both time points. For muscle, the majority of genes were downregulated with exception to cluster VI, in which a number of mitochondrial genes were upregulated with the Tempol diet.

### Discussion

The hypothesis was tested that Tempol food supplementation post-TBI would mimic the effects of calorie restriction in mice by providing a reduction in IR-induced cancers and a

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**Table 2. Median survival and percentage distribution of neoplasms in C3H Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Median survival in weeks (CI)</th>
<th>Malignant neoplasms (%)</th>
<th>Non-hematopoietic neoplasms (%)</th>
<th>Hematopoietic neoplasms (%)</th>
<th>Benign neoplasms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy Control</td>
<td>93</td>
<td>100.9 (97.1–103.6)</td>
<td>9.7 (10.9)</td>
<td>39 (39)</td>
<td>1.1 (1.2)</td>
<td>7 (7.1)</td>
</tr>
<tr>
<td>0 Gy Tempol</td>
<td>170</td>
<td>118.5 (117.8–119.3)</td>
<td>12.5 (12.8)</td>
<td>62 (62)</td>
<td>6.7 (7.2)</td>
<td>7 (7.1)</td>
</tr>
<tr>
<td>3 Gy Control</td>
<td>189</td>
<td>116.7 (115.9–117.6)</td>
<td>13.7 (14.1)</td>
<td>64 (64)</td>
<td>7.6 (8.1)</td>
<td>8 (8.1)</td>
</tr>
<tr>
<td>3 Gy Tempol</td>
<td>202</td>
<td>133.2 (132.5–133.9)</td>
<td>15.9 (16.3)</td>
<td>69 (69)</td>
<td>10.7 (11.2)</td>
<td>9 (9.1)</td>
</tr>
<tr>
<td>3 Gy Tempol delayed</td>
<td>202</td>
<td>133.2 (132.5–133.9)</td>
<td>15.9 (16.3)</td>
<td>69 (69)</td>
<td>10.7 (11.2)</td>
<td>9 (9.1)</td>
</tr>
</tbody>
</table>

**NOTE:** Statistical comparisons: T:C ¼ 0 Gy T to 0 Gy C; C:IR ¼ 0 Gy C to 3 Gy C; T:IR-T ¼ 0 Gy T to 3 Gy T; T:IR T-D ¼ 0 Gy T to 3 Gy T-D.

CI, 95% confidence interval.

Malignant neoplasms include both hematopoietic neoplasms and non-hematopoietic neoplasms. Percentages in these 2 categories do not add up to the total percentage of malignant neoplasms because some mice had hematopoietic and non-hematopoietic neoplasms.

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**Figure 4.** Gene expression patterns in muscle, liver, and brain tissues taken from C3H mice on control or Tempol supplemented diets for 2 weeks or 2 months. Expression patterns were analyzed by hierarchical clustering monitoring 2-fold gene changes in the tissues. The cluster maps were normalized to the 2-week control diet mice (gene expression: green, upregulated; red, downregulated).
survival advantage. Two mouse strains were evaluated having different lifespans, hematopoietic neoplasm incidence post-IR, and the rate and time of hematopoietic neoplasm onset. The data presented in this study clearly show that Tempol reduced the incidence of cancer post-TBI in C3H and CBA mice, thereby providing a significant survival advantage. Tempol treatment (for both unirradiated and irradiated groups) exerted a major effect on hematopoietic neoplasms as shown in Supplementary Table S5. For CBA mice, IR exposure resulted in a near equal proportion of hematopoietic neoplasm to nonhematopoietic neoplasms compared with 0 Gy C, whereas IR-induced hematopoietic neoplasms was approximately 5-fold higher in C3H as compared with CBA mice. Note, hematopoietic neoplasm incidence was elevated approximately 11-fold over nonhematopoietic neoplasms for individuals receiving more than 2 Gy in the Japanese atomic bomb survivors (22). Therefore, the C3H strain used in this study may be a more relevant model for predicting tumor incidence after IR exposure. In addition, Tempol treatment reduced hematopoietic neoplasm incidence in both strains for unirradiated and irradiated mice; however, the overall decrease was greater in C3H mice. These differences could explain the greater survival afforded by Tempol in C3H mice compared with CBA mice. Tempol administered 1-month post-IR exposure was as effective as administration immediately after IR. This latter finding is important in that it suggests that there may be a modifiable time threshold for IR-induced life shortening. Should this finding translate to humans, it would suggest that treatment for nonlethal IR exposure with respect to carcinogenesis might be delayed for some time allowing for IR dose assessment under less chaotic conditions that often accompanies an accident or terrorist event.

Mice exposed to 3 Gy TBI exhibited a reduced lifespan compared with unirradiated control mice, as evidenced by shorter median survival times (Tables 1 and 2). Tempol food supplementation provided significant median survival advantages post-TBI in both mouse strains. These findings are in agreement with previous studies using cancer-prone mice in which Tempol food supplementation afforded significant extensions in median survival (16–18).

There was no observable effect of Tempol on noncancer deaths following IR in C3H mice. Overall, RH analysis of the C3H data clearly show an advantage for Tempol treatment in mice up to 104 weeks for both control- and IR-treated groups. Beyond 104 weeks, RH analysis shows an advantage for irradiated mice on Tempol; however, in unirradiated mice Tempol exerted a modest adverse effect relative to control with regard to overall survival, nonhematopoietic neoplasms, and noncancer deaths (Supplementary Table S7). The reason for this latter finding is unclear; however, it may reside in compromised or reduced drug metabolism in elderly mice (23). Mice greater than 104 weeks can be considered geriatric with expected perturbations in homeostasis. Alternatively, weights in the Tempol group may have dropped below a minimal acceptable level, which in elderly mice could have contributed to late adverse effects observed as discussed below.

With respect to animal weights, Tempol administration resulted in similarities and differences to calorie restriction. First, Tempol did not significantly extend the lifespan of unirradiated CBA or C3H mice. Calorie restriction has been shown to substantially extend lifespan of a number of mouse strains (24). However, not all mouse strains exhibit this phenomenon (25). It seemed from the Kaplan–Meier plots (Fig. 2A and 3A) that Tempol was indeed extending the lifespan of both strains before the median survival; however, as the animals neared the end of their lifespan, there was an abrupt drop in the survival curve. Whereas the median lifespans for both strains on Tempol were not statistically different from controls, the RHs clearly indicate that Tempol exhibited adverse effects on survival near the end of the lifespan. The reason for this observation is unclear; however, it could relate to the reduced weights in elderly mice. Maintenance of a normal body weight is an important survival parameter in aging. Reduced weight in the elderly is a poor prognostic indicator (26, 27). Furthermore, an increase in mortality was shown when 17- to 24-month-old mice were switched from an ad libitum diet to a calorie restriction diet (25), suggesting that substantial weight loss or dropping below a minimum weight in old age is detrimental. Conversely, when mice were maintained on a calorie restriction diet for 24 months and then switched to an ad libitum diet, the lifespan was extended comparable with the continuous calorie restriction diet (25). Thus, terminating Tempol administration and preventing reduced weight in the last 25% of the lifespan might have extended the lifespan.

Both calorie restriction and Tempol diet supplementation have been shown to result in decreased protein levels of leptin (15, 24) and circulating IGF-1 levels (24, 28, 29). Both of these metabolic hormones have been implicated in the pathogenesis and/or progression of cancer (30, 31). It is interesting to note that gene expression profiles (Fig. 4, cluster I, Supplementary Table S8) showed IGF-1 expression levels downregulated after 60 days on the Tempol diet, consistent with the decreased systemic levels reported (29). Leptin, a cytokine primarily produced by adipocytes, although important in energy balance, weight homeostasis, and food intake, can influence hematopoietic progenitor cell growth and drive myelocytic cell growth (32, 33). Thus, reduced levels of leptin may inhibit or impede carcinogenesis. However, evidence in the literature is not clear as to whether altered leptin levels can inhibit carcinogenesis in experimental models (24). IGF-1 regulates tissue growth and metabolism, and elevated levels have been shown to be associated with increased risk of several types of human cancer (34, 35). Interestingly, restoration of IGF-1 levels in calorie restriction mice removes the antiproliferative effects on leukemia cell growth in calorie restriction mice (36, 37), suggesting that reduced circulating IGF-1 levels are involved in the reduction of carcinogenesis in calorie restriction mice. Collectively, the reduced levels of leptin and IGF-1 in mice on the Tempol diet could explain in part the reduced incidence of neoplasms and delay of onset of neoplasms in this study.

The Tempol diet significantly exerted dramatic effects on gene expression profiles in the liver shortly after administration (Fig. 4). Because Tempol is not an endogenous molecule, it was of little surprise that drug metabolism/detoxification genes (Fig. 4, Supplementary Table S8) relating to acute phase I and II genes were upregulated in the liver. Gene expression
profiles for times longer than 60 days have not been done. It would be interesting to determine whether elevated steady-state levels of detoxification gene expression occur over the lifespan of the mice, particularly toward the end of the lifespan when the Tempol diet exerted adverse effects. As mentioned above, detoxification enzyme activities in mice have been shown to diminish with aging (38), hence the detoxification of Tempol could have been compromised in the older mice. Tempol has been shown to be as effective as calorie restriction in mice with regard to inhibition of transcriptional markers related to aging (39). Conversely, there was a significant difference between calorie restriction and Tempol with regard to fatty acid/lipid synthetic genes. Calorie restriction has been shown to downregulate SREBP-1c and fatty acid synthetase, whereas 60 days on Tempol significantly upregulated these genes. The reason for this discrepancy is not known, but clearly Tempol, like calorie restriction, reduces adipose tissue.

Tempol has been shown to exert potent antioxidant activity in a variety of in vitro and in vivo models (14, 40). Because Tempol delayed the onset and incidence of cancer, one interpretation of the findings might be that the promotion/progression steps of IR-induced carcinogenesis may involve free radicals or reactive oxygen species (ROS) processes that can be modified by Tempol. Furthermore, this process occurs over time because Tempol provided a survival advantage and decreased the hematopoietic neoplasm incidence in C3H mice when administered 1 month post-IR. In support of this notion are studies in which irradiated bone marrow cells from CBA mice exhibited significant ROS generation 7 days post-IR (41). ROS can inflict DNA damage resulting in chromosomal instability and aberrations leading to carcinogenesis (42). Delayed induction of chromosome damage in BALB/c epithelial cells, reflecting genomic instability over as many as 28 population doublings times post-IR (43) have been reported, suggesting that the progression to IR-induced cancer requires considerable time from the initiating event. In addition, IR can induce chronic inflammation in tissues (44, 45), resulting in cytokine signaling lasting for months post-IR (46). ROS generation often accompanies chronic inflammation and cytokine signaling (47). The role of antioxidants as chemoprevention agents is controversial in that several clinical trials have shown no benefit of antioxidants in reducing or preventing carcinogenesis (48); however, preclinical studies, including this study, have shown that selected antioxidants, protease inhibitors, and dietary supplements can suppress certain IR-induced cancers (49).

With the exception of calorie restriction, many rodent carcinogenesis studies evaluating potential chemopreventive agents or strategies do not include exposure to the agent for the entire lifespan. Chemical carcinogens often used in many of these studies result in tumor formation well before the end of the animal’s lifespan. Thus, potential toxicities of the agents are usually not evaluated for the entire lifespan. In this study, Tempol was evaluated for the lifespan and exhibited no toxicities until near the end of the lifespan. All RH ratios for both mouse strains up to 104 weeks indicated positive effects on overall survival, neoplasms, and noncancer deaths by Tempol (0 Gy and 3 Gy). Maintaining a calorie restriction diet for extended periods would be difficult for humans making a simple food additive or time-release daily capsule a more viable option to reduce IR-induced carcinogenesis. Such approaches should be considered for cancer patients successfully treated with IR, but potentially susceptible for secondary cancers or for individuals accidentally or intentionally exposed to nonlethal IR. Thus, agents such as Tempol might be a reasonable initial therapy option. Further research will have to done to better define the maximum delay after IR for initiating Tempol treatment and the optimal treatment duration to positively impact survival and reduce cancer incidence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Authors’ Contributions
Conception and design: J.B. Mitchell, P.S. Albert, J.A. Cook
Development of methodology: M.R. Anver, P.S. Albert, J.A. Cook
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.B. Mitchell, M.R. Anver, A.L. Sowers
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.B. Mitchell, M.R. Anver, P.S. Rosenberg, P.S. Albert, J.A. Cook
Writing, review, and/or revision of the manuscript: J.B. Mitchell, M.R. Anver, P.S. Rosenberg, M.C. Krishna, P.S. Albert, J.A. Cook
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Study supervision: J.B. Mitchell

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