

Radioprotection of hematopoietic progenitors by low dose amifostine prophylaxis

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Abstract

Purpose: Amifostine is a highly efficacious cytoprotectant when administered *in vivo* at high doses. However, at elevated doses, drug toxicity manifests for general, non-clinical radioprotective purposes. Various strategies have been developed to avoid toxic side-effects: The simplest is reducing the dose. In terms of protecting hematopoietic tissues, where does this effective, non-toxic minimum dose lie?

Material and methods: C3H/HEN mice were administered varying doses of amifostine (25–100 mg/kg) 30 min prior to cobalt-60 irradiation and euthanized between 4–14 days for blood and bone marrow collection and analyses.

Results: Under steady-state, amifostine had little effect on bipotential and multi-potential marrow progenitors but marginally suppressed a more primitive, lineage negative progenitor subpopulation. In irradiated animals, prophylactic drug doses greater than 50 mg/kg resulted in significant regeneration of bipotential progenitors, moderate regeneration of multipotential progenitors, but no significant and consistent regeneration of more primitive progenitors. The low amifostine dose (25 mg/kg) failed to elicit consistent and positive, radioprotective actions on any of the progenitor subtypes.

Conclusions: Radioprotective doses for amifostine appear to lie between 25 and 50 mg/kg. Mature, lineage-restricted progenitors appear to be more responsive to the protective effects of low doses of amifostine than the more primitive, multipotential progenitors.

Keywords: Amifostine, bone marrow, hematopoietic progenitors, radiation, radioprotectants, WR2721

Introduction

In this era of terrorism and potential catastrophic health consequences of weapons of mass destruction (WMD) deployment, the need to develop to a full array of countermeasures is clear and unambiguous (Coleman et al. 2003, Singh and

Seed 2003, Ferguson et al. 2004, Pellmar and Rockwell 2005, Benjamin et al. 2009). In terms of radiological/nuclear threats, there are few, if any, radioprotectants that are deemed sufficiently safe and systemically efficacious to be used for these non-clinical exposure contingencies (Walker 1988, Weiss 1997, Seed et al. 2002a, 2002b, Seed 2005).

One particular class of radioprotective agents that has been relatively well developed and studied is the phosphorothioate class, whose archetype species is amifostine (WR2721 or Ethyol[®]) (Capizzi 1999, Capizzi and Oster 2000, Khodarev et al. 2004). This class, and especially this agent, was originally identified by the US Army's 'Anti-radiation' drug screening program in the 1950s and has been pursued as a radioprotectant of choice by various researchers, institutions, and agencies over the last five decades: The net result of this rather sizable effort has been mixed (Piper et al. 1969, Davidson et al. 1980, Brown et al. 1988, Giambrarresi and Walker 1989). On the positive side, amifostine is, unquestionably, a potent, systemically active radioprotectant when administered at relatively high doses. Further, amifostine is currently approved by the Food and Drug Administration (FDA) for human use under very select, specific clinical situations, such as mitigation of severe xerostomia (dry mouth and associated pathologic sequelae of the oral cavity) in patients with head and neck cancers undergoing intense, regional radiotherapy (<http://www.medimmune.com/products/ethyol/index.aspref>). However, on the negative side, amifostine is inherently toxic when administered at high, cytoprotective doses. The drug is not only hypotensive in nature, but also produces both upper and lower gastrointestinal disturbances, all of which can yield adverse behavioral responses and decremented performances (Turrisi et al. 1986, Landauer et al. 1987, 1988a, 1988b, Dorr 1998). As a result, amifostine has not been considered as a viable option for radioprotection of special, high-risk service groups, or for the general public at large (Weiss 1997, Seed et al. 2002b).

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A sizable effort has been made by various researchers/research groups to make amifostine more 'user-friendly', largely by attempting to reduce drug toxicity while preserving the drug's overall radioprotective attributes. These efforts include attempts to (a) chemically reengineer amifostine into a new, better tolerated analog (Davidson et al. 1980, Brown et al. 1988); (b) improve methods and vehicles of drug delivery (Fatome et al. 1987, Srinivasan et al. 2002, Pamujula et al. 2004); (c) foster radioprotective synergy by supplementing lower doses amifostine with less toxic (but generally less protective) protectants (e.g., alpha-tocopherol) (Srinivasan et al. 1992); (d) control amifostine toxicity by direct pharmacologic means (i.e., use of antiemetics) (Seed et al. unpublished observations); and (e) use very low, presumably non-toxic doses of amifostine solely for the purpose of protecting against radiation-induced mutagenesis and/or carcinogenesis, while foregoing the drug's cytoprotective attributes that requires much higher, more toxic doses to be delivered (Grdina et al. 2002).

Still, another variant of the latter 'low dose' strategy has been proposed and entails the assumption that even at low doses, amifostine exerts cytoprotective effects on selected targeted tissues and although the level of protection might be insufficient for absolute survival protection, it might well be sufficient to protect fractionally vital targeted tissues and that this partial protection can be effectively leveraged by post-exposure therapies (Patchen and MacVittie 1994, Seed 2005, Seed et al. 2002b).

Therefore, this paper describes our attempt to detail the radioprotective attributes of relatively low doses of amifostine (WR2721) on the hematopoietic system of acutely irradiated mice. Specifically, we asked the question as to how low a dose of amifostine can be used for prophylaxis and still retain a reasonable level of protection of vital hematopoietic progenitors of interest.

Materials and methods

Animals

Male C3H/HEN mice, 5–6 weeks of age, were obtained from the National Cancer Institute (Frederick, MD, USA). Newly purchased mice were quarantined for about 2 weeks and only healthy mice were released for use. At the time of use, mice were approximately 8–12 weeks old. All mice were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were housed in polycarbonate micro-isolator cages (Allentown Inc., Allentown, NJ, USA) with filter tops (Allentown Inc.) on bedding (Harlan Laboratories, Indianapolis, IN, USA) of sterilized hardwood chips, and given standard rodent feed (Harlan Laboratories) and acidified water (pH 2.5–2.8) freely. Animal rooms were subjected to 12-h full-spectrum light/dark cycles and maintained at 20–22°C with 40–70% relative humidity.

All animal-based protocols described were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources – Commission on Life Sciences, 1996), and with authorization of the Institutional Animal Care and Use Committee.

Prophylaxis

Amifostine, 25–100 mg/kg WR-2721, was dissolved in sterile phosphate buffered saline (PBS) and injected subcutaneously (sc) in small volumes (0.2 ml) approximately ~ 30 min prior to irradiation or sham irradiation (Srinivasan et al. 2002). WR-2721 was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA.

Select control groups received equivalent volumes of the drug vehicle (PBS) alone, representing 0 mg/kg of amifostine.

Irradiation

Mice were bilaterally exposed (whole-body) to ⁶⁰Co gamma rays to total doses of 7 Gy (midline doses) and at dose-rate of 0.6 Gy/min. For select control groups, sham-exposures were carried out. Details of the exposure and dosimetry procedures are reported elsewhere (Carter and Verrillei 1973, Myska et al. 1997, Srinivasan et al. 2002). A single acute, whole-body radiation dose of 7 Gy was used because it is the highest sublethal dose that is possible in this strain of mice without impacting survival over the initial 30+ day post-irradiation period. The LD_{50/30} (dose of radiation expected to cause 50% mortality in the subjects within 30 days) value for C3H/HEN mice is ~ 7.8 Gy, while at 7 Gy the lethality response is less than 5% (steep dose response curve). We used this high, sublethal radiation dose in order to induce maximum hematopoietic suppression without having hematopoietic injury so severe as to cause early hematopoietic tissue-related death. The 'sacrifice-series'-related experimental design and the need to survey early post-irradiation time points (0–30 days post-irradiation) required the use of a suppressive, but non-lethal doses of radiation exposures (Patchen et al. 1988).

For select groups of control mice, sham-exposures were carried out using identical procedures, except the ⁶⁰Co sources remained shielded during the 'exposure' period. These animals served as unirradiated (0 Gy) controls.

Blood and tissue collection

At 4, 10, or 14 days following irradiation or sham-irradiation, subgroups of mice were randomly selected for blood collection and subsequent euthanasia. The animals were deeply anesthetized using inhaled isoflurane (Abbott Laboratories, Chicago, IL, USA). Blood samples (0.5 ml) were quickly collected by cardiac puncture using heparinized 1 ml/27 G insulin syringes and blood was placed in ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St Louis, MO, USA) coated blood collection tubes (Sarstedt, Newton, NC, USA). Immediately following cardiac puncture and collection of blood samples, mice were euthanized via cervical dislocation and both femurs collected by surgical procedures.

Preliminary studies involved additional groups of mice in which blood and marrow samplings (following serial sacrifice) were carried out at 1, 2, 4, 7, 10, 14, 21 and 30 days. These mice were irradiated at high, sub-lethal dose (7 Gy) of whole-body gamma rays, 30 min after drug treatment with amifostine (a single prophylactic dose of 100 mg/kg sc) or the vehicle.

Clinical assays

Complete blood counts (CBC) and blood differentials were determined using an Advia automated hematology instrument (Bayer Corporation, Tarrytown, NY, USA) (Whitnall et al. 2002). Complete clinical chemistry panels (19 analytes) were developed on the blood sera of mice using a J & J Vitros 250 instrument (Ortho-Clinical Diagnostics, Holliston, MA, USA) (Whitnall et al. 2002).

Experimental hematology assays: Bone marrow cellularity and cytomorphology

Marrow samples were collected from surgically excised and flushed femurs of euthanized mice (Ledney et al. 2000). Femurs were collected from individual mice and placed in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Carlsbad, CA, USA) with 5% heat inactivated fetal bovine serum (HI-FBS; Hyclone Labs, Logan, UT, USA) and kept on ice. Bone marrow was flushed from femurs using 0.5 ml IMDM plus 5% HI-FBS per bone and mixed to create single cell suspensions. Nucleated cells were counted using a Coulter Z2 cell and particle counter (Beckman Coulter, Brea, CA, USA), and used to estimate cellularity of femoral marrow. Impression smears of the small fragments of extruded marrow were stained with Wright-Giemsa (Sigma-Aldrich) and examined by light microscopy (Nikon, Melville, NY, USA) for cytological evaluations. From these marrow smears, semi-quantitative estimates of marrow cellularity, fat to cell ratios, myeloid to erythroid ratios, and tri-lineal composition were made (Ledney et al. 2000, Seed et al. 2002a).

Progenitor assays: Multipotential c-Kit⁺Lin⁻ (proto-oncogene tyrosine-protein kinase kit positive, lineage negative) progenitors

c-Kit⁺Lin⁻ progenitors in marrow specimens were quantified by flow cytometry (Orlic et al. 1993) and later modified (Seed et al. 2002a). Primitive hematopoietic progenitors were quantified by flow cytometry using a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) and monoclonal antibodies from BD Pharmingen (San Diego, CA, USA). The procedure is based on the unique phenotype of primitive, marrow repopulating hematopoietic progenitors, in that they have a high surface concentration of c-Kit receptors (a proto-oncogene tyrosine-protein kinase-related cell surface receptor that specifically binds 'Steel Factor', commonly referred to Stem Cell Growth Factor) and very low surface concentrations of all lineage-specific surface receptors. In this regard, samples were analyzed for cells that expressed high levels of CD117 (c-Kit cell surface receptor Cluster of Differentiation 117, CD117 bright) but none of the major hematopoietic lineage specific antigens (Lin⁻). CD117 bright cells were defined as cells with CD117-PE (phycoerythrin) fluorescence at least 10-fold greater than that of the isotype control. The lineage specific antibodies were conjugated to FITC (fluorescein isothiocyanate) and directed towards the following: myelomonocytic cells (CD11b), granulocytes (Ly-6G; lymphocyte antigen 6 complex, locus G), B-lymphocytes (CD45r), erythroid cells (TER119), T-lymphocytes (CD4 and CD8), and natural killer cells (pan NK). Prior to labeling, each sample was incubated with CD16/CD32 to prevent non-specific

binding of antibodies to the bone marrow by their FC (constant fragment) portions. Following labeling, red blood cells were removed from the samples via lysis with BD FACSllyse solution (BD Biosciences).

Progenitor assays: Multipotential granulocyte-erythroid-macrophage-megakaryocyte colony forming units (GEMM-CFU)

GEMM-CFU were assayed using a complete growth media/methylcellulose (Sigma-Aldrich) based colony assay (Cortdy 1995, Seed et al. 2002a). The complete medium was comprised of: methylcellulose (Sigma-Aldrich) in Iscove's MDM (Life Technologies), HI-FBS, bovine serum albumin (Sigma-Aldrich), bovine pancreatic insulin (Sigma-Aldrich), human transferrin (Sigma-Aldrich), 2-mercaptoethanol (Sigma-Aldrich), L-glutamine (Life Technologies), recombinant stem cell factor (rSCF; Pharmingen), recombinant interleukin-3 (rIL-3; R&D Systems, Minneapolis, MN), recombinant human interleukin-6 (rhIL-6; R&D Systems), and recombinant human erythropoietin (rh erythropoietin, R&D Systems). Nucleated bone marrow cells were diluted to 1.5×10^5 cells/ml in IMDM with 2% HI-FBS; 0.3 ml of diluted cells were mixed with 3.0 ml of the complete medium. Cell/media mix (1 ml) was dispensed to each 35 mm plate. GEMM-CFU colonies showing all four lineages were scored 14 days after incubation in a 37°C humidified environment containing 5% CO₂.

Progenitor assays: Bipotential granulocyte-macrophage colony forming units (GM-CFU)

GM-CFU were assayed using a conventional single layer agar assay (Ledney et al. 2000, Seed et al. 2002a). In brief, the procedure involve plating 50,000 bone marrow cells in 2 ml volumes of complete cloning media consisting of CMRL (Connaught Medical Research Laboratories) media with 0.33% agar (Sigma-Aldrich), with or without 5 ng of rmuGM-CSF (recombinant mouse granulocyte macrophage colony-stimulating factor; R&D Systems) using 35 mm culture plates. Culture plates were incubated at 37°C in a humidified environment containing 5% CO₂. After 10 days of incubation, the plates were examined by stereomicroscopy and the numbers of granulocyte/monocyte (GM) colonies (colony > 50 GM cells in growth cluster) plate were counted.

Statistical analysis

Hematology data from drug- and vehicle-treated, irradiated or sham-irradiated animals were compared and evaluated using commercially available statistical software (SigmaStat 5.0; Systat Software Inc., San Jose, CA, USA). For tests of statistical significance, the unpaired Student's *t*-test was applied between matched test and control groups. Significant differences between groups were defined by *p* values less than 0.05.

Results

Preliminary findings

Blood and marrow responses of mice over a 30-day period following prophylactic doses of amifostine (100 mg/kg) and

subsequent whole-body irradiation at maximally high, sublethal doses (7 Gy) are shown in Figures 1 and 2. The well-documented, temporal patterns of initial radiation-induced suppression, reaching of nadirs, and beginning of recovery were noted as major blood and marrow elements of concern. In contrast to the noted response, nadirs and early phases of recovery in circulating blood elements (e.g., neutrophils and platelets) that occurred between ~10–14 days post-exposure, the response nadirs and early signs of recovery of the monitored marrow elements (i.e., marrow cellularity, bipotential and multipotential progenitors) occurred earlier in the 4–7 day range.

Blood responses

Blood responses (CBC and differential counts) are illustrated in Figure 3 for the 'extended study' of still lower doses of amifostine, with numerical values and associated statistics listed in Supplementary Tables I–IV, to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>. We selected the three sampling time-points, namely 4, 10 and 14 days post-exposure, for this follow-up extended study in which multiple low doses (<100 mg/kg) of amifostine were to be tested, specifically for their ability to radioprotect a select number of vitally important hematopoietic progenitor compartments within marrow of test animals. We believe that these three sampling points allowed us to adequately capture and to define the key period associated with amifostine's dose-dependent capacity to 'radioprotect' key marrow progenitors of concern.

Following sham-irradiation, under steady-state conditions, and in the absence of amifostine prophylaxis, white cell counts (WBC) were not significantly altered over the time course evaluated, i.e., 4–14 days (Figure 3; Supplementary Table I, to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). However, with amifostine prophylaxis marginal but statistically significant reductions in circulating WBC were noted generally (Figure 3; Supplementary Table I to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). This pattern of marginal cell depletion, especially at higher prophylactic doses of

amifostine (50–100 mg/kg), was seen most noticeably in the absolute blood lymphocyte counts, less with the polymorphonuclear (PMN) counts, but not with other blood cell types (i.e., red blood cells [RBC], platelets, or monocytes) (Figure 3; Supplementary Tables I and II to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Under the disequilibrium caused by acute, whole-body irradiation, circulating numbers of WBC, WBC subsets, RBC and platelets all markedly declined in the saline (alone)-treated animals and remained significantly suppressed during the 4 to 14 days post-exposure period (Figure 3; Supplementary Tables III and IV to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). Amifostine-treated animals exhibited similar patterns of cell depletion. The extent of depletion tended to be minimized, albeit often marginally, and to the extent of the amifostine dose applied prophylactically. Partial recovery of blood cell values, most notably WBC, platelets, and lymphocytes, occurred 10–14 days post-exposure, especially when higher doses of amifostine were administered (50–100 mg/kg; Figure 3; Supplementary Tables II and IV to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). By contrast, blood levels of erythrocytes and monocytes remained significantly suppressed, and either remained stable or declined still further at days 10 and 14: neutrophil levels started to recover, albeit slightly but significantly, at 10 days post-irradiation and declined marginally again at 14 days. However, similar to the responses of the other blood cell types, the extent of suppression was clearly minimized by amifostine administration of 50 mg/kg or greater (Figure 3; Supplementary Tables III and IV to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Bone marrow responses

Bone marrow responses (total cellularity and select progenitor and maturing subsets) are illustrated in Figures 4 and 5, with numerical values and associated statistics listed in Supplementary Tables V–VIII, to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>.

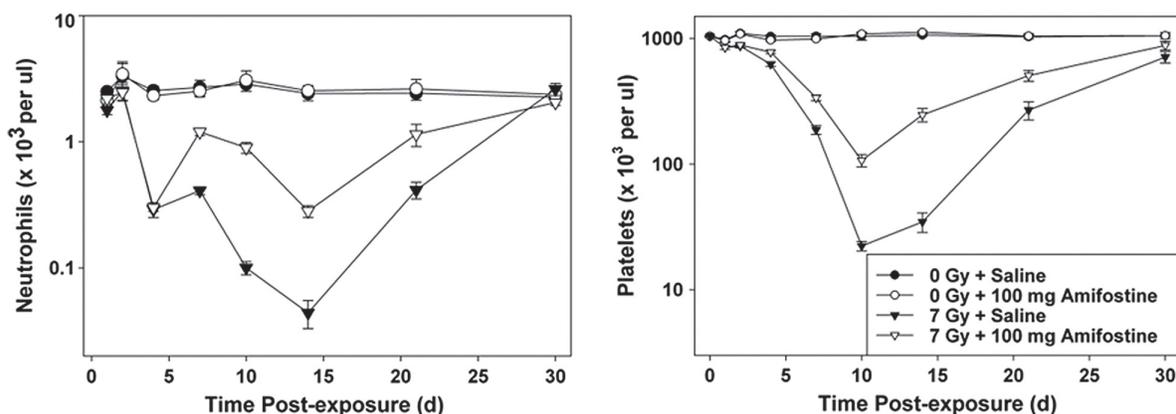


Figure 1. Blood responses of mice prophylaxed with either a single dose of amifostine (100 mg/kg) or the drug-vehicle alone and either acutely irradiated (7 Gy) or sham-irradiated (0 Gy) mice at 1, 2, 4, 7, 10, 14, 21, and 30 days following treatments. Responses of blood neutrophils (left panel) and blood platelets (right panel) are shown. Error bars on data points represent standard error of the means. Radiation and prophylactic treatments are listed in the figure key.

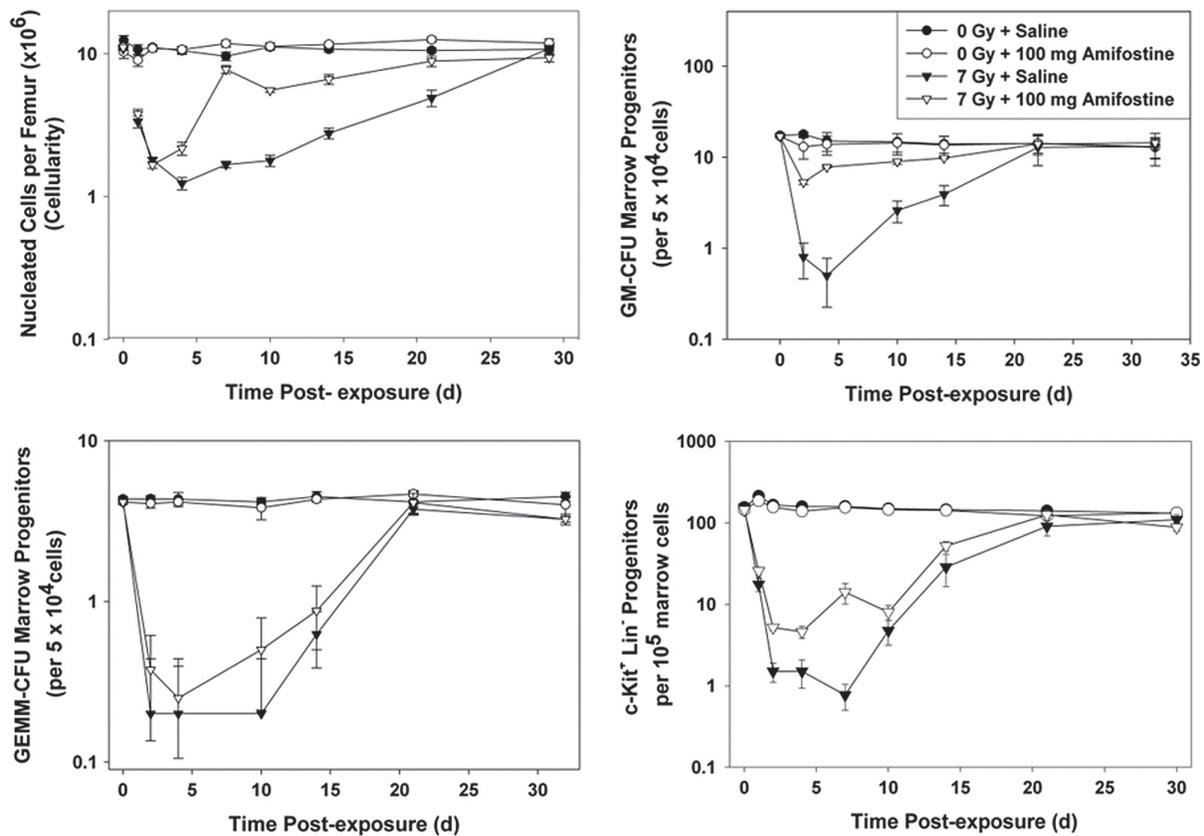


Figure 2. Bone marrow response of mice prophylaxed with either a single dose of amifostine (100 mg/kg sc) or the drug vehicle alone and either acutely irradiated (7 Gy) or sham-irradiated (0 Gy) mice at 1, 2, 4, 7, 10, 14, 21 and 30 days following treatments. Total bone cellularity (upper left panel), bi-potential marrow GM-CFU (upper right panel), multi-potential GEMM-CFU (lower left panel) and primitive, multi-potential progenitors bearing a c-Kit⁺ Lin⁻ phenotype are presented. Error bars on data points represent standard error of the means. Radiation and prophylactic treatments are listed in the figure key.

Total marrow cellularity

Marrow cellularity remained relatively constant within all unirradiated control groups; i.e., sham-irradiated, saline- and amifostine-treated groups (Figure 4; Supplementary Table V to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). By contrast, the 7 Gy TBI resulted in significant marrow cell depletion at 4 days post-exposure and was followed by marginal, but significant recovery at the 10 and 14 days post-exposure time points (Figure 4; Supplementary Table VII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). Amifostine-pretreatments of 100 mg/kg significantly minimized magnitude of depletion and hastened recovery (Figure 4; Supplementary Table VII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

GM-committed progenitors

Under steady-state, bone marrow content of GM-committed progenitors (GM-CFU) was not significantly altered by time, or by either saline- or amifostine-pretreatments (Figure 5; Supplementary Table V to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Following acute irradiation, bone marrow content of GM-progenitors within the saline-treated animals, markedly declined to ~1% of the age-matched, sham-irradiated control values (i.e., to ~1% of the steady-state value by 4 days), with subsequent slight, but gradual recovery thereafter (i.e., to

~9% and ~19% by 10 and 14 days post-exposure, respectively). By contrast, amifostine prophylaxis with doses of 50 mg/kg or greater, limited the extent of early marrow depletion (i.e., to ~3–4% of the sham-irradiated control level at 4 days post-irradiation), and significantly promoted levels of subsequent recovery seen at 10 and 14 days post-exposure (i.e., to ~21–23% and 54–64% by 10 days and 14 days post-exposure, respectively) (Figure 5; Supplementary Table VII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Multipotential GEMM-progenitors

Similar to the responses of the GM-committed progenitors, the levels of multipotential GEMM-progenitors within steady-state marrow were largely unaffected by time or by prophylaxis (i.e., with amifostine or PBS) (Figure 5; Supplementary Table V to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Following acute irradiation and its associated hematopoietic disequilibria, GEMM-progenitors declined in the saline-treated animals to extremely low levels (below assayable levels) at 4 days post-exposure, but progressively recovered 10–14 days following exposure, at which values reached ~10% and ~28.4% of the age-matched, sham-irradiated controls (Figure 5; Supplementary Table VII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). Although recovery of the more

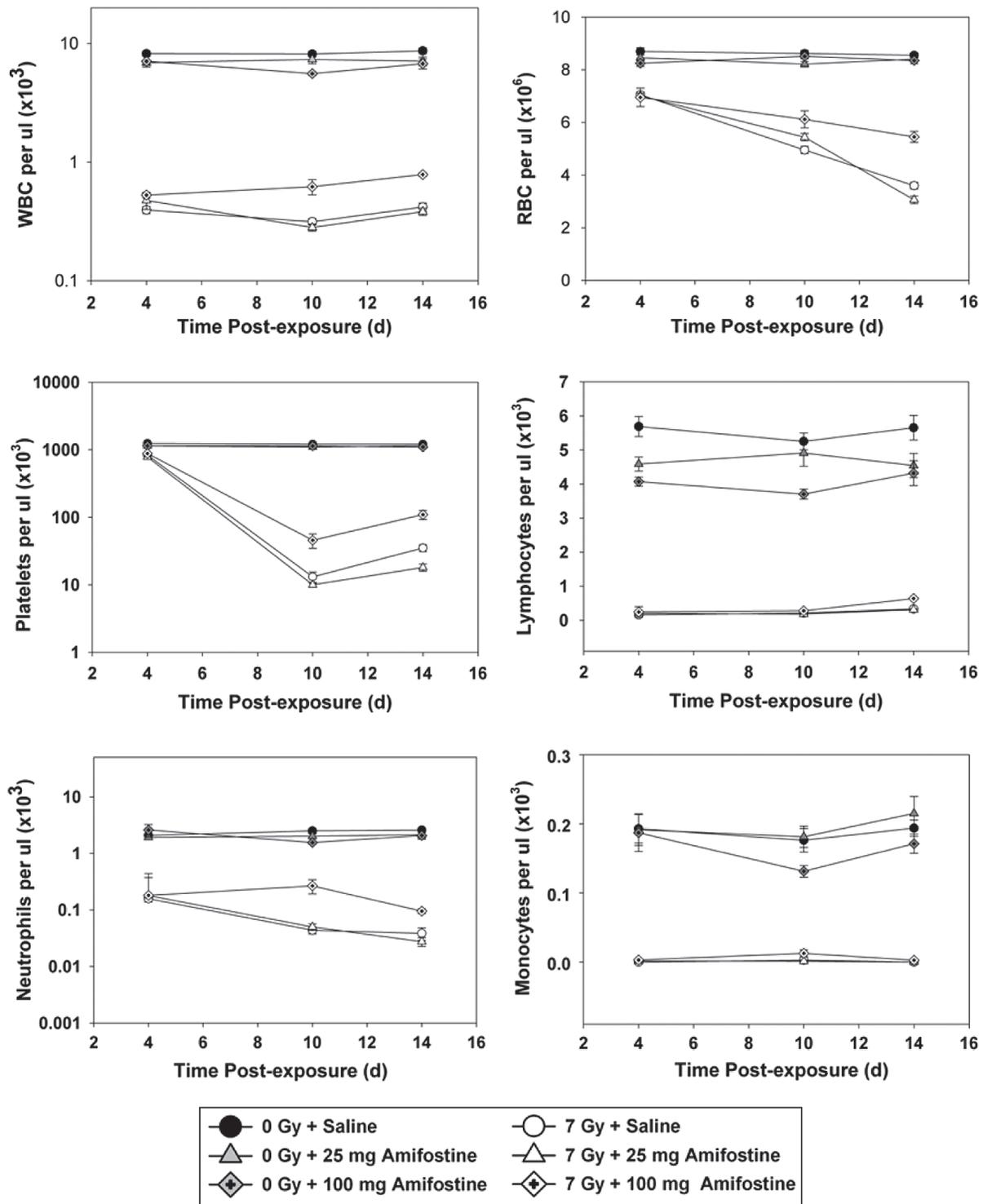


Figure 3. Blood responses of prophylaxed (0, 25 and 100 mg/kg amifostine), acutely irradiated (7 Gy) or sham-irradiated (0 Gy) mice at 4, 10 and 14 days following treatments. Data for the additional low amifostine doses tested, 50 and 75 mg/kg, are provided in the supplemental files. White blood cells (WBC), erythrocytes (RBC), platelets, lymphocytes, neutrophils and monocytes are shown. Error bars on data points represent standard error of the means. Radiation and prophylactic treatments are listed in the figure key.

primitive GEMM progenitor marrow compartment was less robust than the more mature GM-progenitors, the general recovery pattern was comparable, especially at the later sampling times of 10 and 14 days.

Amifostine prophylaxis with doses of 50 mg/kg or greater again limited (as per the amifostine-associated protective responses of the GM-CFU progenitors) the extent of early marrow depletion of GEMM-progenitors, and promoted the

level of subsequent recovery seen at 10 and 14 days post-exposure (Figure 5; Supplementary Table VII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Multipotential c-Kit⁺Lin⁻ progenitors

Under steady-state conditions, escalating prophylactic doses of amifostine had no significant effect on the number of

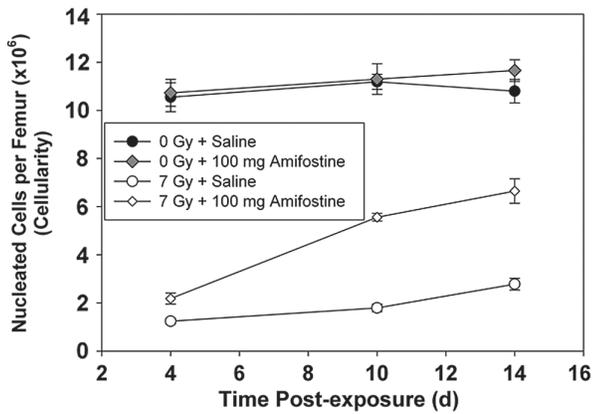


Figure 4. Bone marrow response of prophylaxed (100 mg/kg amifostine), acutely irradiated (7 Gy) or sham-irradiated (0 Gy) mice at 4, 10 and 14 days following treatments. Numbers of nucleated cells per femur are presented in the figure. Error bars on data points represent standard error of the means.

marrow c-Kit⁺Lin⁻ cells at 4 days following sham-irradiation. However 10 and 14 days following sham-irradiation, there appeared to be slight, but non-significant trend of declining progenitor numbers with increasing amifostine dose (Figure 5; Supplementary Table VI to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Under the disequilibria caused by acute, near-lethal irradiation, bone marrow samples from saline-treated control animals had concentration of c-Kit⁺Lin⁻ that precipitously declined to ~0.4% of the non-irradiated control level at 4 days, and subsequently recovered slightly, but significantly to ~1% and ~8% of the control levels at 10 and 14 days, respectively (Figure 5; Supplementary Table VIII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Amifostine prophylaxis, regardless of dosing level, generally failed to elicit consistent and significant initial sparing, or enhanced recovery of the c-Kit⁺Lin⁻ marrow subpopulation (Figure 5; Supplementary Table VIII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>). Nevertheless, at the intermediate sampling time-point of 10 days post-irradiation, three of the four groups of amifostine-treated animals had marrow c-Kit⁺Lin⁻ progenitor levels that were elevated significantly when compared to the saline-treated group. Further, in preliminary studies when a single, relatively high dose of amifostine (100 mg/kg) was tested, a marginal reduction in the response nadir was noted when compared to vehicle-treated responses (Figure 2). Similar to the saline-treated animals, the c-Kit⁺Lin⁻ subpopulation showed significant,

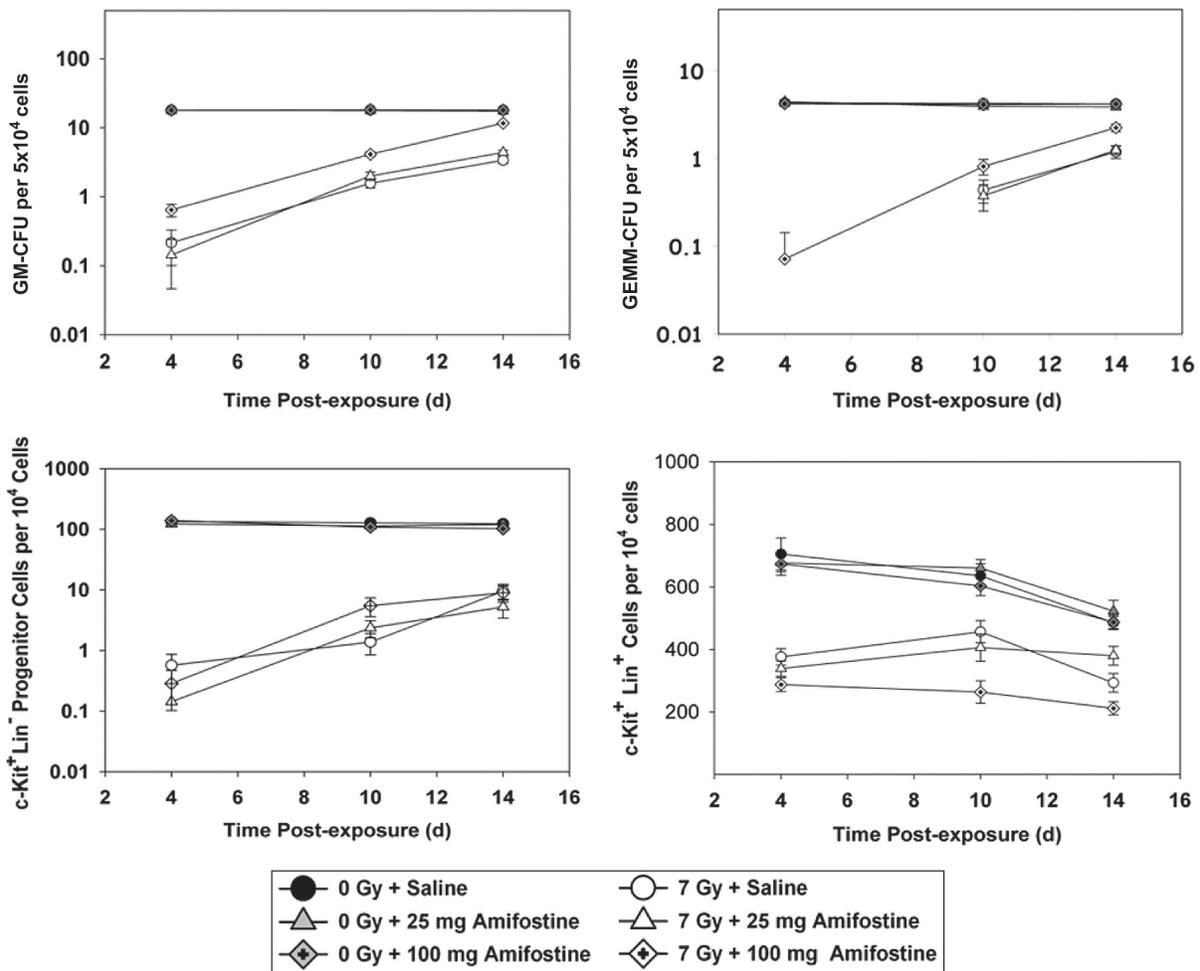


Figure 5. Bone marrow response of prophylaxed (0–100 mg/kg amifostine), acutely irradiated (7 Gy) or sham-irradiated (0 Gy) mice at 4, 10 and 14 days following treatments. Data for the additional low amifostine doses tested, 50 and 75 mg/kg, are provided in the supplemental files. Numbers of bipotential GM-CFU and multipotential GEMM-CFU per 10⁴ bone marrow cells and c-Kit⁺Lin⁻ and c-Kit⁺Lin⁺ progenitors are shown. Error bars on data points represent standard error of the means. Radiation and prophylactic treatments are listed in the figure key.

amifostine-independent recovery 10 to 14 days following irradiation (Figure 5; Supplementary Table VIII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Lineage marker bearing cells with the c-Kit⁺ epitope

c-Kit⁺ cells bearing an array of lineage markers constituted ~5% of nucleated cells within aspirated and dispersed femoral bone marrow of the young adult, C3H/HEN male mice. Under steady-state conditions, marrow levels of these lineage-restricted, c-Kit⁺ marker bearing cells, were relatively stable initially (4–10 days), but marginally declined by day 14 in the majority of the unirradiated control groups, independent of amifostine prophylaxis (Figure 5; Supplementary Table VIII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Following acute irradiation, during conditions of hematopoietic disequilibria, this marrow subpopulation, c-Kit⁺Lin⁺, was significantly depleted (by ~28–46%) in the saline-treated animals, but the extent of depletion was not as great as was seen in the c-Kit⁺Lin⁻ subpopulations (see Figure 5). The higher prophylactic doses of amifostine appeared to have exacerbated the extent to which this subpopulation was depleted; e.g., at 4, 10, and 14 days post-irradiation, the groups treated with 100 mg/kg of amifostine versus those vehicle-treated groups were 24%, 42%, and 28% lower, respectively (Figure 5; Supplementary Table VIII to be found online at <http://informahealthcare.com/doi/abs/10.3109/09553002.2014.899450>).

Overall hematologic impact of amifostine prophylaxis: statistical analyses

A total of 11 hematologic endpoints (six blood-related endpoints, plus five bone marrow-related endpoints) were evaluated statistically at three time-points (4, 10, 14 days) following drug/saline treatments (5 treatments; 1 saline control, plus 4 escalating levels of amifostine prophylaxis) and acute/sham irradiation. Results of these statistical analyses showed that the impact of amifostine prophylaxis under steady-state hematopoiesis (i.e., sham irradiation) was marginal and largely independent of the drug dosing regimen: Furthermore, when noted, drug-induced responses were generally suppressive in nature. At the drug dosing levels of 25, 50, 75, and 100 mg/kg, statistically significant, suppressive responses recorded were 23%, 37%, 20%, and 27% of the endpoints surveyed respectively.

By contrast, under hematologic disequilibrium resulting from acute irradiation, the drug-induced responses were generally enhancing (protective) by nature and dependent of the drug dosing regimen. Statistically significant enhanced hematologic endpoints of 14%, 56%, 41%, and 61% were noted following drug dosing regimens of 25, 50, 75, and 100 mg/kg, respectively.

Discussion

In general, the results of this study clearly confirm the protective nature of amifostine prophylaxis on the blood forming system of acutely irradiated experimental mice. Previously,

numerous researchers reported finding strong radioprotective action of amifostine when sufficiently high dosing regimens were applied (e.g., >100 mg/kg) to various experimental animal models of acute injury, using a variety of measured end points (Davidson et al. 1980, Fatome et al. 1987, Landauer et al. 1987, Giambrarresi and Walker 1989, Patchen et al. 1990, Weiss et al. 1992, Patchen and MacVittie 1994, Mazur 1996, Seed et al. 2002a, Srinivasan et al. 2002, Stone et al. 2004, Pamujula et al. 2005). In all of these studies there was positive correlation between amifostine's hematopoietic tissue sparing effect and survival. In virtually all of these studies, however, questions concerning 'minimally effective dose' of amifostine, or the nature and distribution of principal cell targets within the hematopoietic system were inadequately addressed. In this regard, this study has yielded new information on amifostine's potency and cell-targeting action.

First, in terms of the minimally effective dose, i.e., for the threshold dose, our results here suggest a value somewhere between 25–50 mg/kg for most of endpoints/targets evaluated: e.g., with circulating blood values (WBC, RBC, platelets), as well as with select bone marrow progenitor types (GM-CFU, GEMM-CFU), the separation between protective and non-protective responses is quite clear and distinct (i.e., no significant protection noted at 25 mg/kg dosing, whereas at doses of 50 mg/kg or greater, the protection was generally significant and pronounced). It should be noted that this tissue-specific threshold value is considerably lower than the purported threshold of slightly under 100 mg/kg for survival protection in acutely irradiated mice (Srinivasan et al., unpublished observations). Also, the threshold value of 25–50 mg/kg is considerably lower than the value noted for amifostine's 'performance decremating' activity (Landauer et al. 1987, 1992). This value of 100 mg/kg which roughly equates to ~2 pmole amifostine per μ l in the blood stream of the mouse at the time of irradiation, when the drug was delivered ~30 min prior to exposure. Assuming that amifostine's pharmacokinetics are such that there is proportionality between the drug dose delivered and blood levels achieved, one can estimate the blood levels achieved for amifostine dosing at the hematopoietic tissue-specific threshold of ~25–50 mg/kg to be ~0.4 to ~0.6 pmoles per μ l of blood. Of course, further testing will be required to confirm these values. Regardless, our findings here suggest that degrees of both tissue and survival protection can be achieved by prophylaxing with amifostine at doses well below those that result in performance decrement.

The second surprising aspect of our results was the apparent selective nature of amifostine's targeting and radioprotecting different types of marrow progenitors. It is commonly thought that amifostine most efficiently radioprotects the most primitive of the hematopoietic progenitor compartments, and is somewhat less effective in radioprotecting the more mature, lineage-restricted progenitor compartments. Our results appear to argue against this concept by showing a drug efficiency gradient based on the relative primitiveness of the hematopoietic cell targets. In this regard, the GM lineage-restricted marrow progenitors (GM-CFU) exhibited a significant and unambiguous degree of sparing following

amifostine prophylaxis when doses ≥ 50 mg/kg were given. Comparing amifostine's sparing/recovery patterns of the bipotential GM-CFU with those of the multipotential GEMM-CFU from irradiated animals, one sees that they are generally similar, but the drug dose-dependent differences were not as distinct, nor as great. Amifostine's dose-dependent sparing effect on still more primitive, less lineage-restricted progenitors (c-Kit⁺Lin⁻ progenitors) was even less clear, and less significant: no clear pattern of a drug dose-dependent sparing effect (or a recovery enhancing effect) emerged from the analyses of this progenitor subtype. It is interesting to note however, that there was an apparent suppressive effect of higher doses of amifostine (≥ 50 mg/kg) on the irradiated marrow's c-Kit⁺ cells following acquisition of lineage associated markers (i.e., Lin⁺). Despite these observations, it is unlikely that 'lineage restriction' *per se* is the sole reason for the noted response differences between various progenitor subtypes; other factors surely must be involved. In this regard, it is reasonable to suspect that 'other factors' might involve amifostine's radioprotective impact(s) on the hematopoietic microenvironment, or perhaps a modulatory role on progenitor cell cycling (Murley et al. 1997, North et al. 2000, Khodarev et al. 2004, Greenberger 2008).

Are these factors intrinsic or extrinsic in nature? It is well recognized that various cellular compartments within lymphohematopoietic tissues have different radiosensitivities. Such differences are probably best defined experimentally in mice. Meijne et al. (1991) and Ploemacher et al. (1992) reported some time ago that as one moved from the rare, primitive, repopulating progenitors to progenitors in various stages of lineage commitment, the change in radiosensitivity was pronounced: A 'U'-shaped sensitivity curve was described in which peak radiosensitivity recorded for the CFU-S day 7 progenitors (CFU-S₇), with reduced sensitivities with both more mature and less mature progenitor subtypes. Such differences in radiosensitivity were shown to be positively related to survival enhancement following split-doses of radiation exposure, suggesting that sublethal damage (SLD) repair capacity is substantially greater in the more primitive, multipotential progenitors, than in the more mature, lineage-restricted progenitors. Associated with these differences in SLD repair capacity, are corresponding differences in cell cycling status, extent of self-renewal capacity, physical proximity to endosteal bone surface, and the nature of the microenvironmental niche (e.g., degree of oxygenation). In this regard, it is easily envisioned how amifostine, with its pleotropic biochemical effects might differentially interact with these various progenitor targets within marrow in exerting varying degrees of protection. This suggestion is certainly consistent with the current consensus concerning overall mechanisms of amifostine's radioprotective actions; namely, that the drug is not solely limited to free-radical scavenging, auto-oxidation-induced intracellular hypoxia and chemical repair by hydrogen atom donation, but also by its modulatory effects on the transcriptional regulation of genes involved in apoptosis, cell cycle, and DNA repair (Khodarev et al. 2004). It needs to be pointed out, however, that under low doses of ionizing radiation, mammalian cells will upregulate a cascade of antioxidants providing a net radioprotective

effect (Calabrese 2001, Mitchel 2006, Day et al. 2007, Blankenbecler 2010, Brechignac and Paquet 2013).

With regard to the more drug-responsive, lineage-restricted progenitors (e.g., GM-CFU), these cells generally have relatively highly cycling fractions, an abundance of cell-growth signaling surface receptors, but little by way of SLD repair and self-renewal capacities. Further, these progenitor subtypes tend to reside in well oxygenated stromal niches, and have the capacity to efficiently dephosphorylate the parent prodrug, amifostine, in producing oxygen-consuming free thiol (i.e., the active drug, WR-1065) in a dose-dependent fashion. As such, amifostine's dose-dependent oxygen-consumption would serve to foster focal areas of hypoxia and in a radioresistant microenvironment for residing progenitors (Purdie et al. 1983). By contrast, the more primitive progenitors (e.g., MRAs), normally reside in hypoxic microenvironmental niches, and would therefore be less susceptible to the radioprotective action of amifostine. However, there are previous reports that suggest that it is the intrinsic nature of a given progenitor cell type, and not its oxidic microenvironment, that largely dictates its radiosensitivity, or lack thereof (Meijne et al. 1996). Clearly, this issue remains to be fully elucidated. Other possible mechanisms by which amifostine exerts its noted differential radioprotective effect on different subclasses of progenitors include: (a) altering cellular redox pathways, in turn cellular functions (differentiation) of essential cells (adipocytes) comprising the hematopoietic microenvironment (Ramdas et al. 2003, Lechpammer et al. 2005) and (b) differential efficiencies of hydroxyl radical scavenging in different subclasses of progenitors due to oxidic environment and cellular content of polyamines (Held and Awad 1991). Again, these various possibilities need further study.

Relative to our trying to make practical use of the observations here, the current problem of not having safe, effective, systemically active radioprotectors for eminent radiological/nuclear events clearly lies in part with the inherent toxicity associated with amifostine and related aminothiols compounds. If these agents were better tolerated by individuals under high dosing regimens needed for systemic radioprotection, this 'problem' would have been long solved. Unfortunately, this is not the case; at drug doses sufficiently high to radioprotect normal tissues against acute injury and to provide a reasonable degree of survival protection, these agents need to be delivered at relatively high doses that are clearly toxic and that produce significant adverse side-effects. These adverse responses include: significant hypotension, linked with emesis that result in degraded physiological and cognitive capacities and/or performance (Turrisi et al. 1986, Giambrarresi and Walker 1989). In dealing with patients in a controlled hospital setting, limited drug toxicity is generally quite acceptable, assuming that the health benefit (in terms of preventing normal tissue injury) clearly outweighs any or all toxic side-effects that drug may exert. However, in the case of non-clinical situations, in which protection of high risk personnel or the general public at large becomes necessary, drug toxicity takes on a whole new meaning and is generally not at all acceptable, especially if the toxicity is sufficiently strong as to manifest degraded performance. However, if one

acknowledges the very positive, radioprotective attributes of the phosphorothioate class of radioprotectors, than any/all attempts to minimize the dose-dependent toxicity of amifostine takes on additional importance and meaning.

So how does one attempt to minimize amifostine's toxicity, while still retaining its radioprotective effects? Several strategies have been tested, including the one reported here, namely the use of very low, non-toxic doses of amifostine in order to achieve partial, tissue-specific radioprotection. Although, we fully recognize that when this is done, some of amifostine's cytoprotective effectiveness will be lost, especially in terms of absolute survival protection; but, we also recognize that a number of other drug's radioprotective attributes will be still retained; including anti-mutagenic and carcinogenic effects and again, a limited, but specific lymphohematopoietic tissue protection (Grdina et al. 2002). In light of our findings here, this 'low dose' approach to radioprotection might have merit, especially when one considers the possible adjuvant effect low doses of amifostine might have in improving the overall efficacy of post-exposure cytokine therapy (Patchen and MacVittie 1994).

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Supplementary Material available online

Supplementary Tables I-VIII