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Improved Total-Body Irradiation Survival by Delivery of Two Radiation Mitigators that Target Distinct Cell Death Pathways

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Abstract

The acute lethality of total-body irradiation (TBI) involves damage to multiple organs, including bone marrow and intestine. Ionizing radiation mitigators that are effective when delivered 24 h or later after TBI include the anti-apoptotic drug, JP4-039 and the anti-necroptotic drug, necrostatin-1. In contrast to effective delivery of JP4-039 at 24 h after TBI, necrostatin-1 is most effective when delivery is delayed until 48 h, a time that correlates with the elevation of necroptosis-inducing inflammatory cytokines and necroptosis-induced serine phosphorylation of receptor-interacting serine/threonine-protein kinase-3 (RIP3) in tissues. The goal of this work was to determine whether administration of JP4-039 influenced the optimal delivery time for necrostatin-1. We measured daily levels of 33 proteins in plasma compared to intestine and bone marrow of C57BL/6NTac female mice over a 7-day time period after 9.25 Gy TBI (LD_{50/30}). Protein responses to TBI in plasma were different from those measured in intestine or bone marrow. In mice that were given JP4-039 at 24 h after TBI, we delayed necrostatin-1 delivery for 72 h after TBI based on measured delay in RIP-3 kinase elevation in marrow and intestine. Sequential delivery of these two radiation mitigator drugs significantly increased survival compared to single drug administration.

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INTRODUCTION

The identification of molecular biological pathways of radiation-induced cellular, tissue, organ and organ system damage led to the discovery of small molecule mitigators of acute radiation syndrome (ARS) (1–5). One pathway initiates with radiation-induced nuclear DNA double-strand breaks, which activate a mitochondrial mechanism of apoptosis (6, 7). This discovery led to the validation of several anti-apoptotic agents as mitigators, including mitochondrial localized GS-nitroxides (1–8), superoxide dismutase (SOD) mimics (9) and inhibitors of mitochondrial nitric oxide synthase (1). One GS-nitroxide, JP4-039, is an effective mitigator of ARS when administered 24 h after total-body irradiation (TBI) (10, 11). Furthermore, JP4-039 confers both systemic and organ-specific radiation protection and mitigation (12–14). The mechanisms of action of JP4-039 involve protection of cardiolipin from oxidative damage, prevention of mitochondrial cytochrome-C release, decrease in mitochondrial membrane permeabilization and inhibition of the caspase pathway (7).

A separate pathway of radiation-induced cell death is necroptosis (2) and is mediated by inflammatory cytokines including TNF- α , which binds to its receptor, and leads to phosphorylation of RIP-3 kinase. In contrast to GS-nitroxides, the necroptosis inhibitor, necrostatin-1, is effective as a radiation mitigator 48 h after TBI, a delay which correlates with the elevation of radiation-induced inflammatory mediators, including IL-1 α , IL-6 and TNF- α (2).

The goal of this work was to determine whether proteins in the plasma of TBI mice served as biomarkers that correlated with apoptosis and necroptosis in the bone marrow and intestine. We administered 9.25 Gy TBI (LD_{50/30}) to C57BL/6NTac mice, and over 7 days, took daily measurements of plasma, marrow and intestinal levels of 33 representative proteins that are associated with the response to ionizing radiation. Administration of JP4-039 at 24 h after TBI delayed RIP3 kinase phosphorylation in intestine and marrow. Using this data, we delayed administration of necrostatin-1 for 72 h after TBI. This sequenced time of delivery of the second mitigator improved survival.

MATERIALS AND METHODS

Animals and Irradiation

C57BL/6NTac adult (30–33 g) female mice received 9.25 Gy TBI (LD_{50/30}) using a Mark I Gamma Cell Irradiator (JL Shepherd & Associates, San Francisco, CA) with filter removed. This action resulted in a dose rate of 343 cGy per minute. To investigate the change in the energy spectrum of the incident radiation field due to the removal of the filter, percentage-depth-dose (PDD) profiles were measured using Gafchromic™ EBT3 radiochromic film [lot no. 06081602 (exp. June, 2018); Ashland® Inc., Covington, KY]. Measurements of the PDD profiles both with and without the filter in place were completed with the films aligned parallel to the beam axis between two 14 × 14 × 0.2 cm³ pieces of poly(methyl methacrylate) (PMMA). The films were centered within the cabinet irradiator for measurements.

The measured PDD profiles are shown in Fig. 1. The profiles were normalized to a depth of 5 cm. The PDD profile measured without the filter decreased more rapidly at shallow depths than did the profile measured with the filter, indicating a greater proportion of low-energy photons detected in the absence of the filter. Beyond a depth of 50 mm, the two PDD profiles were similar, indicating that the high-energy region of the energy spectrum was minimally impacted by the presence of the filter. Therefore, removal of the filter lowered the average energy of the incident radiation, but was not expected to significantly alter the radiation dose received by the animal placed in a Lucite® holding chamber during exposure.

Preparation and Delivery of Radiation Mitigator Drugs

The synthesis of JP4-039, a peptide isostere conjugate of 4-amino-TEMPO (4-AT) nitroxide, was performed as previously described elsewhere (6, 8, 10). The drug was dissolved in F14 emulsion to achieve a concentration of 4 mg/ml. The F14 emulsion has previously been described elsewhere (10). Briefly, F14 is an emulsion developed as an *in vivo* vehicle for JP4-039, and was originally used topically to mitigate radiation-induced damage to the skin (14). This formulation consists of a mixture of 10% sesame oil (Sigma-Aldrich® LLC, St. Louis, MO), 5% soy phosphatidyl choline (Avanti® Polar Lipids Inc., Alabaster, AL) and 85% Dulbecco's phosphate buffered saline (DPBS; Lonza Inc., Walkersville, MD). During the preparation, the active ingredient (JP4-039) is mixed with the other components before making the emulsions. Sonication was performed for 1–2 h using a continuous sonicator at 17–20 W output with ice water cooling and a stream of nitrogen blowing on top (10). JP4-039 was administered intravenously (i.v.) in 100 µl containing 400 µg of drug. In the current experiments, JP4-039 was delivered 24 or 48 h after TBI (1).

Necrostatin-1 was administered as reported elsewhere (2). Briefly, the drug was dissolved in 10% ethanol, 10% Cremophor EL® and 80% deionized water, to obtain a concentration of 0.33 mg/ml. Mice were intravenously injected with 100 µl containing 33 µg of drug at several times after TBI. The drug dose was thus 1.65 mg/kg. In some TBI experiments, necrostatin-1 was delivered after previous administration of JP4-039.

Delivery of Single or Multiple Mitigators to Total-Body Irradiated Mice

The paradigm for TBI and delivery of mitigator(s) is shown in Fig. 2. There were 160 mice in four experiments, designated A–D (n = 40 mice per experiment). Mice in each experiment were sacrificed in groups of five at 8 different time points starting on day 0, which was a preirradiation baseline control, and then daily thereafter for 7 days postirradiation. Three tissues were taken from each animal: plasma, intestine (ileum) and bone marrow (femur and tibia). Thus, a total of 480 samples (120 from each experiment) were removed from 160 mice and tested. Every sample was tested for levels of each of 33 proteins. Since the four experiments (Fig. 2A–D) were performed at different times over four weeks, a separate preirradiation control time point on day 0 was identified for each experiment to serve as a baseline for protein levels for that specific experiment.

The mice in experiment A (n = 40) served as controls and received 9.25 Gy TBI alone. Experiment B animals (n = 40) received JP4-039 at 24 h after TBI. For preparation and drug delivery, JP4-039 (4 mg/ml) combined with 10% sesame oil, 5% soy phosphatidyl choline

and 85% DPBS was placed in ice water with a stream of nitrogen blowing over the top. We then sonicated the preparation 1–2 h, and administered 100 µl containing 400 µg of drug per mouse by i.v. injection. Experiment C animals (n = 40) received necrostatin-1 at 48 h after TBI. The necrostatin-1 was dissolved in 10% ethanol, 10% Cremophor EI and 80% water, and each mouse received 100 µl containing 1.65 mg/kg of drug (33 µg per mouse) by i.v. injection. Experiment D animals (n = 40) received JP4-039 at 24 h and necrostatin-1 at 72 h after TBI (Fig. 2). Mice were sacrificed daily in groups of 5 at one of 8 time points: day 0 (prior to irradiation), 1, 2, 3, 4, 5, 6 or 7 after TBI.

Delivery of Radiation Mitigator Combinations

In other experiments (not shown in Fig. 2) we evaluated the survival of mice in groups of 20, that were given a single compound or a combination of the two mitigators at the same or different times after 9.25 Gy TBI. These animals were followed for survival only and no tissues were excised for protein measurements from these experiments.

Collection and Storage of Plasma, Intestine and Bone Marrow for Luminex Assay and Western Blot for Protein Levels

Plasma was collected according to methods reported elsewhere (10). Intestine (ileum) was removed and placed on dry ice to freeze prior to storage at –80°C. Bone marrow (femur and tibia) was removed, centrifuged at 2,000 rpm and 4–6°C for 10 min, with the supernatant discarded, leaving a concentrated pellet and stored at –80°C.

Protein Immunoassay by Luminex

Plasma was thawed and vortexed to remove particulate matter prior to micropipetting for dilution for the Luminex® protein assay. A 4-mg sample of intestine was dissected, weighed and homogenized in 1 ml of 0.1% Tween® 80 in PBS to prevent protein clumping and stored at –80°C. Prior to use in the Luminex protein assay, intestine homogenate was thawed to room temperature and centrifuged at 2,000 rpm and 4°C for 10 min and protein quantitated using the Biomed protein assay (Bio-Rad® Laboratories, Hercules, CA). The bone marrow pellet was reconstituted via serial pipetting and homogenized in 0.5 ml of 0.1% Tween 80 in PBS and stored at –80°C. Prior to use in the Luminex protein assay, the bone marrow homogenate was thawed to room temperature, centrifuged as described above and protein quantitated.

Standardization of Plasma, Intestine and Bone Marrow Protein Levels for Luminex Assays

A list of the 33 proteins assayed and the known primary function of each is shown in Table 1. Selection of these 33 proteins was based on the pathways involved in the known inflammatory and radiation-induced damage responses summarized in Table 2. A Bio-Rad protein assay was run for each tissue type (plasma, intestine, bone marrow) and experimental group [9.25 Gy irradiation only (control); 9.25 Gy with JP4-039; 9.25 Gy with necrostatin-1; and 9.25 Gy with JP4-039 at 24 h and necrostatin-1 at 72 h after TBI, as well as other delivery times for the 2 drugs] using a Biotek® Epoch™ Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT). We utilized the TGF β1 Single Plex Magnetic Bead Kit, as well as the 32-Multiplex Mouse Cytokine/Chemokine Magnetic Bead Panel

(EMD Millipore, Billerica, MA) that tested protein concentrations for eotaxin, G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2, regulated upon activation normal T cell expressed and secreted (RANTES), TNF- α and VEGF.

Each kit (MCYTOMAG-70K and TGFBMAG-64K-01) provided reagents for use in the Luminex Protein Immunoassay, which included one vial of mouse cytokine/chemokine standard, two vials mouse cytokine/chemokine quality controls, one vial serum matrix, 96-well plate, 30 ml assay buffer, 60 ml 10 $\times$ wash buffer, one 3.2-ml bottle of mouse cytokine detection antibodies and one 3.2-ml bottle of streptavidin-phycoerythrin. The 32-Multiplex Kit (MCYTOMAG-70K) provided one 3.5-ml pre-mixed 32-plex beads. The TGF β 1 kit (TGFBMAG-64K-01) provided the above reagents, as well as 5 ml sample diluent, 1.0 ml 1.0 N HCl, 1.0 ml 1 N NaOH and 3.5 ml anti-TGF- β 1 beads.

Preparation of Reagents for Immunoassay Beads, Quality Controls, Wash Buffer and Serum Matrix

Antibody-immobilization beads were sonicated for 30 s and then vortexed for 1 min prior to use. Quality controls 1 and 2 were reconstituted with 250 μ l of assay buffer. The 60 ml 10 $\times$ wash buffer was diluted 1:10 with 540 ml deionized water. Using the Luminex method, 32 proteins were assayed. TGF- β 1 (the 33rd protein) was assayed by a different immunoassay technique.

For the 32-multiplex assay, serum matrix for plasma samples was reconstituted in 2.0 ml of assay buffer, and allowed to sit at room temperature for 10 min.

Preparation of Standards for Protein Luminex Assays

Mouse cytokine standards were reconstituted in 250 μ l deionized water, inverted to mix, vortexed and then transferred to a polypropylene microfuge tube and labeled as “standard 6.” Serial dilutions were performed, by which 50 μ l of standards 6, 5, 4, 3 and 2 were micropipetted to a microfuge tube containing either 200 μ l of assay buffer (for 32-multiplex) or 150 μ l of assay buffer (for TGF- β 1), until standard 1 was obtained.

Determination of Protein Concentration

Intestine and bone marrow homogenates were tested for protein concentration using the Bio-Rad protein detection assay by diluting the detection solution 1:5 in deionized water. Each sample and standard (2 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0 mg/ml) was plated in triplicate on a 96-flat-bottom-well plate with 5 μ l of sample/standard combined. The 1:5-diluted protein detection solution (250 μ l) was added to each well, mixed and run for analysis on the spectrophotometer at a wavelength of 595 nm.

The average optical density value for each sample and standard was calculated, and a standard curve was formulated based on the averages of the standards. This standard curve was used to obtain the actual protein concentrations of each sample, which was used

standardize the Luminex assay. Immunoassay results for plasma were converted from “pg/ml” to “pg/mg protein” for comparison to intestine and bone marrow.

Dilution of Samples

The 32-multiplex plasma samples were diluted at 1:2 by combining 25 µl of plasma with 25 µl of assay buffer. TGF-β1 plasma samples were diluted at 1:5 by combining 12.5 µl of plasma with 50 µl sample diluent. After dilution, 25 µl of each 1:5-diluted sample was plated on a 96-well plate and treated with 2.0 µl of 1.0 N HCl and tested to ensure pH was below 3.0. The plate was sealed and covered with aluminum foil and shaken at room temperature for 15 min. The acid-treated plasma samples were then further diluted at 1:6 by combining 10 µl of acid-treated plasma with 50 µl of assay buffer, leading to a final dilution of 1:30.

Procedures for the 32-Multiplex Luminex Immunoassay

Mice were sacrificed according to institutional IACUC protocol using carbon dioxide inhalation; blood was retrieved in heparinized tubes. Ileum and bone marrow from femur and tibia were obtained from the animals immediately after sacrifice and processed for the Luminex assay as described above. Beginning on the day of (but prior to) TBI (day 0) and daily for the next 7 days after irradiation, specimens were collected for a total of 8 time points for each experiment, as shown in Fig. 2. Intestine was washed free of blood prior to preparation for Luminex assay. Data were presented for each as pg/mg protein.

All plasma, intestine and bone marrow samples were assayed in 96-well plates, which were conditioned first by adding 200 µl of wash buffer to each well. Plates were then sealed and covered on a plate shaker for 10 min at room temperature. Wash buffer was then removed via suction.

Prepared standards and controls (25 µl of each) were added to the appropriate well, followed by 25 µl of assay buffer to the background sample wells. Either serum matrix (for plasma; 25 µl) or 0.1% Tween® 80 in PBS (for intestine and bone marrow; 25 µl) was added to each background, standard and control well. Then, 25 µl of sample was added to each sample well. The bottle of pre-mixed beads was vortexed and then 25 µl was added to each well. The plates were then sealed and covered and incubated overnight at 2–8°C.

Between 16–18 h later, each plate was placed on a magnet, the well contents were removed via suction, and the plates were then washed twice with 200 µl of wash buffer, as described in the first step. Detection antibodies (25 µl) were then added to each well, and the plate was sealed, covered and incubated for 1 h on a plate shaker at room temperature. After incubation, 25 µl of streptavidin-phycoerythrin was added to each well, and the plate was again sealed, covered and incubated for 30 min on a plate shaker at room temperature. After incubation, well contents were removed via suction, and the plates were washed twice with 200 µl of wash buffer, as described above. After the two washes, 150 µl of wash buffer was added to all wells, the plate was placed on a plate shaker for 5 min and then read using the Luminex 100/200™ analyzer (Luminex Inc., Austin, TX).

Data Analysis and Standardization of Intestine and Bone Marrow Results

We reported the protein concentrations by Luminex assay in pg/ml. A protein concentration assay was run on each sample. For each intestine and bone marrow sample, the data from the Luminex assay for that sample were divided by the determined protein concentration to give standardized concentrations of pg/mg protein.

TGF- β 1 Immunoassay

Intestine and bone marrow samples for the TGF- β 1 immunoassay required an acidification step. Tissue samples (25 μ l of each) were plated onto a 96-well plate, treated with 2.0 μ l of 1.0 N HCl and tested to ensure pH was below 3.0. The plate was sealed and covered with aluminum foil, and shaken at room temperature for 15 min. The acid-treated samples were then neutralized with 2 μ l of 1.0 N NaOH prior to adding to the sample well.

For the TGF- β 1 assay of plasma, a serum matrix was reconstituted in 1.0 ml deionized water and 4.0 ml assay buffer, and was allowed to sit for 10 min at room temperature. Then, 0.1 ml of the reconstituted serum matrix was micropipetted and diluted in 0.5 ml assay buffer, for a final dilution of 1:30. The next procedures with the 96-well plate assay were then performed as described above for the 32-multiplex Luminex immunoassay.

Western Blot Analysis of Representatives of the 33 Proteins and Phosphorylation of p-RIP3 Kinase

As an independent method to confirm the validity of the data collected by Luminex assay, which showed changes in protein levels over 7 days after TBI, Western blot analysis was used. We also measured RIP3 kinase phosphorylation as a marker for necroptosis. C57BL/6NTac female mice received 9.25 Gy TBI. The bone marrow and ileum were collected from 5 mice per time point, preirradiation, and daily for 7 days after irradiation (8 time points). The bone marrow was centrifuged at 1,500 rpm for 5 min, and the pellet resuspended in ACK Lysing Buffer (Quality Biological Inc., Gaithersburg, MD) for 10 min on ice to lyse the red blood cells. The cells were centrifuged at 1,500 rpm for 5 min with the pellet resuspended in PBS and transferred to 1.5 ml Eppendorf tubes, and microcentrifuged at 2,500 rpm for 5 min. The pellet was resuspended in 1 ml of cell lysis buffer (1% NonidetTM P-40, 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin), placed on a rotator, rocked gently at 4°C for 30 min and then centrifuged in a microcentrifuge at 13,000 rpm and 4°C for 10 min. The supernatant was removed and placed in a new Eppendorf tube and stored on ice.

The ileum was excised from the mouse, a 100-mg piece was placed in a round-bottom tube, 1 ml of cell lysis buffer added and placed on ice. Each mouse ileum sample was homogenized using a Polytron® PT 3000 (Brinkmann Inc., Riverview, FL), placed on ice for 2 h, transferred to a 1.5-ml Eppendorf tube, centrifuged in a microcentrifuge for 15 min at 13,000 rpm and 4°C, and then the supernatant was transferred to a new Eppendorf tube and stored on ice. Protein concentration was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA). The protein samples were added to 4 $\times$ Loading Buffer (Bio-Rad) with 10% 2-mercaptoethanol, vortexed and heat at 95–100°C for 5 min.

The samples were loaded on 4–12% Bis-Tris Criterion XT Precast Gels (Bio-Rad) at 145 volts for 70 min using XT MES as the running buffer (Bio-Rad). The proteins were transferred to PVDF membranes (Bio-Rad) at 0.45A for 90 min using Tris/Glycine buffer (Bio-Rad) with an ice block. Nonspecific binding was blocked by incubating the membrane in 5% nonfat milk for 30 min. For confirmation of data collected with plasma, intestine and bone marrow protein levels by Luminex assay, we measured bone marrow levels of two proteins over 7 days after 9.25 Gy TBI by Western blots, using two antibodies: rat anti-mouse IL-6 and goat anti-mouse TNF- α from Santa Cruz Biotechnology, Dallas, TX. As the loading control, we used anti-beta-actin mouse monoclonal antibody, (Cell Signaling Technology, Danvers, MA). The Western blots were left in 5% nonfat milk overnight at 4°C and then washed three times with TBS (Bio-Rad) for 5 min. The secondary antibody [either goat anti-rabbit IgG-HRP (cat. no. sc2004), goat anti-mouse IgG-HRP (cat. no. sc2005) or donkey anti-goat IgG-HRP (cat. no. sc2020); all from Santa Cruz Biotechnology] was diluted 1:10,000 in 5% non-fat milk, incubated with the membrane for 1 h at room temperature and washed three times with TBS for 5 min. The proteins were detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, Pittsburgh, PA). Western blot quantification was performed using LabWorks™ Management System (Lablogics Inc., Mission Viejo, CA).

For Western blot measuring RIP-3 kinase phosphorylation in intestine (ileum), the membrane was incubated with the primary antibody (anti-phospho RIP3 antibody, Abcam®, Cambridge, MA).

Graphical and Statistical Analysis

The mean and standard error of the mean for each time point was determined and graphed, using GraphPad Prism software (LaJolla, CA), to compare each experimental group for all 33 proteins.

Student's unpaired *t* test was used to compare the time points of each group that received mitigating drugs to the corresponding time points in the control group, as well as to compare all days in each experimental group (including the control) to day 0 (nonirradiated baseline) of the control group.

RESULTS

Pattern of Changes in Protein Levels in Plasma, Intestine and Bone Marrow over Seven Days after TBI

We first analyzed the data for plasma, intestine and marrow proteins in irradiated mice not given mitigator drugs (Fig. 2, experiment A). We compared overall patterns of increased (red color) or decreased (green color) levels of each protein over 7 days after TBI. We first assessed the protein levels occurring at specific time points in response to TBI alone. At 24 h after TBI, there were significant increases in plasma levels of multiple inflammatory cytokines, including G-CSF, MCP-1 and IP-10 (Fig. 3A). Most of these changes were sustained for 7 days. At the same 24-h time point after TBI, there were significant decreases in plasma levels of IL-5, IL-1 α , LIX and IL-13 (Fig. 3A).

Protein levels in the intestine at 24 h after TBI were different from those in plasma. In intestine (ileum), there was a relatively modest overall change in protein levels after TBI (Fig. 4A), although there was a detectable increase in LIF and decrease in IL-1 β . Protein levels in bone marrow at 24 h after TBI were different from both plasma and intestine. In bone marrow, there was an increase in KC, and decrease in IL-5, MIG and RANTES (Fig. 5A). The data indicate that plasma showed a broader spectrum of proteins upregulated at 24 h after TBI compared to intestine or marrow. The elevated protein levels in plasma may have reflected the production of these proteins by organs other than intestine or bone marrow, which released these proteins into the systemic circulation. Alternatively, endothelial cells, or circulatory immunocytes, neutrophils and macrophages, may have contributed to plasma protein level increases. The data establish that there are clear differences at 24 h after TBI in the plasma levels of the 33 proteins assayed compared to those same proteins in intestine and bone marrow.

At 48 h after TBI (day 2), there were elevations in some plasma proteins that persisted out through day 7 (Fig. 3A). Plasma showed consistent elevations in G-CSF and MCP-1, and new increases in IL-6, KC and IP-10 (day 3). Furthermore, there were decreases in several other plasma cytokines, including RANTES, TNF- α , IL-7, IL-9, IL-3 and IL-13. The decreased levels of IL-9 on days 2, 3, 5 and 7 may have indicated apoptosis in other organs, given that IL-9 levels inversely correlate with intensity of apoptosis (18). In the plasma of irradiated mice, there were late increases at day 5 in IL-5 and at day 7 in MIP-1 β , MIP-2 and IL-10 (Fig. 3A).

Protein levels in the intestine at 48 h after irradiation showed elevation in LIF persistent to day 3, and new increases in MCP1 and RANTES. There were decreases in the intestine at day 2 in the levels of IL-10, IL-2, IL-1 α , IL-12 (p40) and IL-9. Over the next 5 days, there was a persistent decrease in the levels of IL-2, IL-4, IL-12, (p40), IL-9 and IL-13 (Fig. 4A). Decreases in intestinal IL-12 (p40) were detected at day 4 and persisted to day 7. There was a late decrease in intestinal IL-13 at day 4 persisting to day 7 (Fig. 4A).

Protein levels in bone marrow at day 2 after TBI (Fig. 5A) were different from levels in both plasma (Fig. 3A) and intestine (Fig. 4A), and were striking with respect to the magnitude and diversity of changes. There were significant increases in KC, eotaxin, IL-12, (p70), LIX, TNF- α and IL-9. Increases in these protein levels in marrow persisted over days 2–7 with KC, eotaxin, IL-12 (p70), TNF- α and IL-9 (Fig. 5A). There were also persistent decreases beginning at day 2 in IL-6, IP-10, MIP-1 β , MIP-2, IL-1 β , IL-2 (p70), IL-17, VEGF, IFN- γ , IL-4, IL-1 α , LIF, RANTES, TGF- β , IL-12 (p40) and IL-3 (Fig. 5A). Decreases in the levels of IL-5, IL-10 and MIG continued to day 4.

The protein levels in bone marrow after TBI revealed an intense and persistent pattern of changes compared to the changes observed in the intestine or plasma. The greater number and intensity of changes in the protein levels in marrow may have reflected the intensity of the marrow response to 9.25 Gy TBI, and are consistent with marrow being a target organ of ARS and specifically the hematopoietic syndrome. Alternatively, lower plasma levels of some proteins may have reflected clearance of initially elevated plasma levels by kidney or liver, or to other stabilizing effects of TBI in other organs.

Western Blot Confirms Luminex Assay for Protein Changes in Tissues of TBI Mice

Western blot analysis was used to validate the Luminex assay as a reliable indicator of differences in protein expression. We performed Western blot assays for bone marrow proteins, which changed after TBI (Fig. 6 and Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14787.1.S1>). The data for levels of two representative proteins in bone marrow (TNF- α and IL-6) over 7 days after TBI correlated very well with data in the color-coded tables with Luminex assays for bone marrow proteins (Fig. 5A). TNF- α levels increased in both the Western blot and Luminex assays, while IL-6 levels decreased from the preirradiation (day 0) levels in bone marrow. These data confirm, and extend by Western blot, the validity of the magnitude and changes in protein levels detected by Luminex assays in bone marrow over the 7 days after TBI.

Effect of GS-Nitroxide, JP4-039, on Protein Levels in Plasma, Intestine and Bone Marrow

We next evaluated the effect of JP4-039 administration at 24 h after TBI on protein levels in plasma, intestine and bone marrow. After delivery of JP4-039 [100 μ l containing 20 mg/kg delivered in F14 formulation according to methods reported elsewhere (10)], there was a further increase in plasma signatures for several proteins (Fig. 3B). Increased plasma G-CSF and MCP, as well as IL-6 and KC, were accompanied by increases in eotaxin and in MIP-1 β starting at day 2 and persisting through day 7 (Fig. 3B). Furthermore, LIF levels increased from days 3–5 (Fig. 3B). Some protein levels, which were initially decreased after TBI, returned to preirradiation levels after delivery of JP4-039, including TNF- α , IL-9, IL-3 and IL-13 (Fig. 3B).

Administration of JP4-039 at 24 h after TBI was associated with less alteration in protein levels in the intestine (Fig. 4B) compared to the changes observed in plasma (Fig. 3B). There was an increase in the intestine of levels of IP-10 on days 2–6, IL-10 on days 3, 5 and 6, while MIG and LIF levels remained relatively unchanged. There was return to preirradiation levels of proteins that had been decreased by irradiation alone (Fig. 4A), including IL-12 (p40), IL-9 and IL-3 (Fig. 4B).

The effects of JP4-039 on bone marrow protein levels at 24 h after TBI revealed a return to preirradiation levels of many proteins that had been elevated by irradiation alone (Fig. 5A), including IL-6, IL-2, IL-4 and LIF (Fig. 5B). In bone marrow, there was a decrease in the level of IL-12 (p70), which went from the preirradiation baseline level to a decrease over days 2–7 (Fig. 5B). There was also a significant change in the TBI-induced rapid elevation of IL-9 over days 2–5 (Fig. 5A), which then showed a delayed elevation in IL-9 level at days 6 and 7 (Fig. 5B). Overall, there was a greater effect of JP4-039 administration after TBI on protein levels in the irradiated bone marrow compared to that observed in plasma or intestine.

Effect of Necrostatin-1 on Protein Levels in Plasma, Intestine and Bone Marrow

Mice that received the anti-necroptosis drug, necrostatin-1, at 48 h after TBI were studied. Mice received i.v. administration of 100 μ l of F14 emulsion containing 1.65 mg/kg necrostatin-1. These animals showed a very different protein response pattern in plasma, intestine and marrow. In contrast to the results with administration of JP4-039 on day 1,

necrostatin-1 administration on day 2 downregulated many radiation-induced plasma proteins (Figs. 3C), and reduced levels of more proteins to below preirradiation levels (Fig. 3C). The radiation-induced increases in G-CSF, MCP-1, IL-6, KC and IL-10 were less significant if necrostatin-1 was given at day 2, compared to that observed after JP4-039 administration at day 1 postirradiation. There were decreases in more plasma proteins in necrostatin-1-treated mice (Fig. 3C) than in JP4-039 treated animals (Fig. 3B). Plasma protein decreases after necrostatin-1 administration included those from day 3 onward in: MIP-1 β , MIP-2, IL-10, IL-15, IL-12 (p70), IL-1 β , IL-2, IFN- γ , IL-1 α , LIF, M-CSF, LIX, RANTES, IL-7, MIP-1 α , TGF- β , IL-9, IL-3 and IL-13 (Fig. 3C). There was a late decrease in plasma GM-CSF at day 5 persisting through day 7 (Fig. 3C).

The intestines of irradiated mice treated with necrostatin-1 at day 2 showed increases in MCP-1, MIP-1 β , IL-12 (p70), IL-17, IFN- γ , GM-CSF, LIF, M-CSF, RANTES, TNF- α , TGF- β and IL-3 (day 4) (Fig. 4C). There was normalization to preirradiation levels of the radiation-induced decreases in IL-3 and IL-4 (Fig. 4C).

The bone marrow of necrostatin-1-treated mice showed a very different pattern of changes in protein levels compared to that seen in JP4-039-treated mice (Fig. 5C). From day 3 on, there were significant elevations in levels of G-CSF, MCP-1, IL-6, KC, IP-10, eotaxin, MIP-1 β and MIG (Figs. 5C). There were elevations in IL-2, GM-CSF, IL-1 α , MIP-1 α and IL-9. The IL-9 levels remained elevated. Elevation in IL-9 has been associated with a decrease in apoptosis (18). There was elevation in the TGF- β levels at days 3–4 (Fig. 5C).

These results establish that necrostatin-1 administered at 48 h after TBI reduced the levels of more plasma proteins than did administration of JP4-039 at 24 h after TBI, but increased the level of other proteins in bone marrow. The effects of necrostatin-1 at 48 h after TBI on bone marrow were significant, with many protein level increases (Fig. 5C), and were the reverse of effects on marrow detected after JP4-039 administration at 24 h, when there were multiple decreases in protein levels (Fig. 5B).

Lack of Improved Survival by Delivery of Two Radiation Mitigators at Times Known to be Effective for Each Alone

We performed studies on 9.25 Gy TBI mice that were given the two drugs at different times, starting with times indicated in the literature for each drug alone (1, 2). These experiments were designed based on the NIAID/CMCR directive of delivery of radiation mitigators no earlier than 24 h after TBI, and were consistent with the expected time that radiation countermeasures would first become available (Fig. 7A). In previously reported experiments (2), we determined that administration of necrostatin-1 at 48 h was optimal for counteracting the necroptosis-associated elevation in plasma levels of TNF- α , IL-1 α , IL-17 and IL-6 (48 h). Necrostatin-1 given at 48 h enhanced survival to a magnitude comparable to the level observed in mice receiving JP4-039 at 24 h after TBI (2). We tested combined delivery of both JP4-039 and necrostatin-1 at 24 h or 48 h postirradiation (Fig. 7B). In addition, we tested delivery at the respective optimal time for each (i.e., JP4-039 at 24 h and necrostatin-1 at 48 h after TBI). Groups of 9.25 Gy TBI mice received: 1. both drugs at 24 h (best for JP4-039); 2. both drugs at 48 h (best for necrostatin-1); or 3. JP4-039 at 24 h and

necrostatin-1 at 48 h (best for each alone). These groups showed no improvement in survival compared to delivery of one drug at its preferred time (Fig. 7).

JP4-039 Administration Induces a Delay in RIP-3 Kinase Phosphorylation in Irradiated Mice

We tested whether administration of JP4-039 delayed the onset of necroptosis measured by either RIP-3 kinase phosphorylation in intestine and bone marrow or by the plasma level of IL-6 (Fig. 3B) and bone marrow level of IL-1 α (Fig. 5B). Using a separate assay for RIP-3 kinase phosphorylation as a biomarker for the initiation of necroptosis in the intestine and bone marrow, we evaluated this parameter in intestine and marrow from a separate group of mice receiving 9.25 Gy TBI alone, and a subset that were given JP4-039 at 24 h after TBI. The data showed that there was a delay in the onset of necroptosis when JP4-039 was given at 24 h after TBI. As shown in Fig. 8 and Supplementary Fig. S2 (<http://dx.doi.org/10.1667/RR14787.1.S1>), RIP-3 kinase phosphorylation was delayed in onset until 72 h, by administration of JP4-039 at 24 h. These data were in contrast to a previously reported study in which necroptosis started at 48 h after TBI (2). The current data indicate that there was a delay in RIP-3 kinase phosphorylation beyond 48 h postirradiation when JP4-039 was given at 24 h. Furthermore, the data showing a JP4-039-associated delay in RIP-3 kinase phosphorylation after TBI were consistent with the effect of JP4-039 administration on delaying radiation-induced elevations in plasma levels of IL-6 (Fig. 3B) and bone marrow levels of IL-1 α (Fig. 5B).

Effects of JP4-039 with Delayed Delivery of Necrostatin-1 on Protein Levels in Plasma, Intestine and Bone Marrow

We analyzed protein levels in plasma, intestine and marrow of mice that received JP4-039 at 24 h after TBI and a delayed administration of necrostatin-1 at 72 h. Analysis of protein levels in plasma, intestine and bone marrow was performed daily for 7 days. The data revealed a pattern of protein levels consistent with a return toward preirradiation levels. In mice receiving both drugs, plasma levels of several proteins were elevated compared to irradiated controls (Fig. 3D). These plasma levels included a continual (but lower magnitude) elevation in IL-6 and KC, and an increased elevation in G-CSF, MCP-1 and IP-10 (Fig. 3D). There was a continued GS-nitroxide-associated elevation in eotaxin and MIP-1 β . In mice receiving both drugs, there were additional elevations in IL-10, IL-15, IL-12 (p70), IL-2, VEGF, GM-CSF, LIF and IL-7 (Fig. 3D). Plasma protein levels for RANTES, IL-7 and IL-3, which were previously decreased in irradiated controls, were now either normalized or elevated (Fig. 3D).

The intestines of mice receiving both drugs after TBI responded with reductions in protein levels. As shown in Fig. 4D, the levels of proteins detected after administration of either JP4-039 alone or necrostatin-1 alone were reduced in those mice receiving both drugs. Reduction in GM-CSF and decrease in LIF (which was previously elevated after one drug alone) was observed at days 6–7. There was a decrease in induction of MCP-1, MIP-1 β , IL-12 (p70), IL-17, IFN- γ , and GM-CSF, RANTES and TNF- α compared to the levels observed with necrostatin-1 alone. The decrease in levels of IL-17 and IL-1 β seen in JP4-039-treated mice, and decrease in IL-12 (p40) and IL-9 levels seen in necrostatin-1-treated mice, were not observed in mice receiving both drugs.

The results of protein level measurements in the bone marrow of mice receiving both drugs were next compared to those of those mice receiving one drug alone (Fig. 5D). The significant elevation in protein levels that was observed in mice treated with necrostatin-1 alone was reversed by administering both drugs, and the pattern after administration of both drugs (Fig. 5D) was similar to that observed in animals receiving JP4-039 alone (Fig. 5B). An important observation was the effect of the two drugs on the level of IL-9. IL-9 was elevated in bone marrow in irradiated control mice (Fig. 5A) and remained elevated in elevation in those mice receiving JP4-039 (Fig. 5B). In mice receiving necrostatin-1, there was persistent elevation in IL-9 (Fig. 5C). Administration of both drugs reduced levels of IL-9, suggestive of continued apoptosis signals in the bone marrow, since levels of IL-9 inversely correlate with apoptosis (18) (Fig. 5D). Previously published studies have shown a decrease in cellular apoptosis in mice treated with JP4-039 (1). The above results establish that administration of GS-nitroxide at 24 h and necrostatin-1 at 72 h produced a distinct effect on protein levels in a pattern more consistent with JP4-039 alone than with necrostatin-1 alone. Furthermore, administration of the two drugs normalized many of the elevated protein signatures detected at day 3 after irradiation alone.

We analyzed all the data for patterns common to all three tissues (plasma, intestine, and bone marrow) and with all four experimental groups. We analyzed data over days 1–4 after TBI and again for all 7 days after TBI. There were no proteins that followed the same patterns in all tissues and with all experimental groups. This underscores the lack of concordance of levels of specific individual plasma proteins with levels at each time with the same protein in bone marrow or intestine. There was also a lack of concordance of the direction of protein changes with each tissue after TBI alone (Figs. 3A, 4A and 5A). There was a greater concordance of overall protein decreases rather than increases, although not with the same proteins, in plasma, intestine and bone marrow (Figs. 3A, 4A and 5A). While concordance of all three tissues was not observed, the results showed concordance between two tissues (plasma and bone marrow) with some level of KC and eotaxin elevation in both over the 7 days after TBI (Figs. 3–5).

We also analyzed the data focusing on each mitigator delivered separately (Fig. 2, experiments B and C) compared to both mitigators (Fig. 2 experiment D). There were no common patterns of increases or decreases in proteins among the three tissues. In necrostatin-1-treated mice, intestine and bone marrow showed common increases in 9 proteins, which were not observed if JP4-039 had been delivered previously (Figs. 4 and 5). A generally greater concordance between protein levels in intestine and bone marrow suggested that the protein level changes in these tissues reflected common biological responses of these two organs to radiation and drug treatment.

Changes in levels of plasma proteins after irradiation and drug delivery may have reflected the contribution of proteins released by other organs that were not measured, including lung, spleen and muscle. Plasma levels of some proteins (e.g., IFN- γ , TGF- β , IL-15) were suppressed after treatment with necrostatin-1 alone (Fig. 3) in contrast to increases in these same proteins in the intestine (Fig. 4) and bone marrow (Fig. 5) in the same animals. There was a more common decrease in protein levels in intestine and bone marrow after irradiation alone or when JP4-039 was given along with necrostatin-1 (Figs. 4 and 5).

Analysis of the time of onset of necroptosis using several biomarkers in plasma and intestine suggested that improved survival after TBI might be achieved by delaying delivery of necrostatin-1 to 72 h postirradiation if JP4-039 had been given at 24 h.

Delayed Delivery of Necrostatin-1 after JP4-039 Improves Survival after TBI

Administration of JP4-039 and necrostatin-1 at 24 and 72 h after TBI, respectively, significantly improved survival compared to administration of one drug alone (Fig. 9). These results establish that the administration of two effective radiation mitigators in a modified sequence, based on the onset of each death pathway, can substantially improve survival. Furthermore, the data indicate the importance of correlating plasma with internal organ protein levels when evaluating biomarkers for each of multiple death pathways, particularly when determining optimal times for the sequential delivery of additional radiation mitigators.

DISCUSSION

Recognition of multiple cellular death pathways that occur after TBI (1–4) has led to the discovery of several categories of radiation mitigators (1–7). While each mitigator has been targeted to a specific mechanism of cell death, the potential for one drug to either counteract or supplement the effect of another has been a subject of interest (3). For example, radiation-induced apoptosis involves mitochondrial leakage of cytochrome-C into the cytoplasm to activate caspase-induced cell death by apoptosis (7). Mitochondrial targeted GS-nitroxides block apoptosis (7), but these mitigators have recently been shown to also inhibit ferroptosis (15). In contrast, the necroptosis death pathway involves mitochondrial-independent FAS ligand or TNF- α binding to their respective cell surface receptors and then phosphorylation of RIP-1 and RIP-3 kinases (2). The timing of necroptosis-initiating events after TBI, when TNF- α levels are high, is consistent with the reported effectiveness of necrostatin-1 at 48 h after TBI (2). Whether delivery of a GS-nitroxide mitigator of apoptosis modifies the response of irradiated animals to a mitigator of necroptosis has not previously been reported.

In the current study, we measured TBI-induced changes in 33 proteins in plasma, intestine and bone marrow, and investigated how administration of either JP4-039 or necrostatin-1 modified these levels. While our results were limited to three tissues and 33 proteins, they did reveal differences in radiation response of the target organs, intestine and bone marrow, relative to plasma. The group of 33 proteins measured represents multiple categories of pro-inflammatory and anti-inflammatory cytokines and products of stress response genes and are samples of the major categories of proteins involved in radiation response. These 33 proteins may not be the best of the hundreds of proteins, peptides and other small molecules that also may respond to TBI. Other plasma proteins, including IL-33 [shown to be relevant for ferroptosis as a biomarker (16)] may be added to or substituted for others in the current list of 33 proteins that were studied. The current results also do not take into account other categories of biomarkers of radiation response, which may be more predictive of the successful effect of timing of delivery of two mitigators, such as microRNAs, plasma lipids, oxidized lipids and small molecule peptides including hormones. Other plasma signatures

may be even more predictive of the time to optimize delivery of each of multiple radiation mitigators.

We found that TBI induced an increase in plasma levels of G-CSF, MCP-1 and IL-6. However, these changes did not correlate with similar increases in the intestine or bone marrow. We did not measure protein levels in the lung, liver, spleen or other organs of TBI mice. Elevated levels of some plasma proteins may reflect secretion of these into the plasma from organs other than the bone marrow and intestine. JP4-039 administration after TBI resulted in decreased levels of many marrow proteins. In contrast, administration of necrostatin-1 at 48 h after TBI modulated plasma protein levels, but in the opposite direction compared to intestine and marrow (Figs. 3C, 4C and 5C). There were necrostatin-1-induced elevations in bone marrow G-CSF, MCP-1, IL-6, KC, IP-10, eotaxin, MIP-1 β , MIP-1 α , MIG, GM-CSF, IL-1 α and IL-9 (Fig. 5C). In plasma and intestine of these same necrostatin-1-treated irradiated mice, increases in IP-10, eotaxin, MIP-2, IL-2, IL-4, IL-1 α , LIX, IL-7, MIP-1 α and IL-9 were not observed. The intestinal elevations in TNF- α , IL-17 and IL-13 were observed in a previously reported study of radiation-induced necroptosis (2), but were measured by Western blot and not with the current Luminex chip assay. Thus, the assay methods used to measure proteins must also be taken into account when interpreting the results of different experiments.

The overall pattern of radiation-induced changes in plasma proteins correlated better with those in bone marrow than intestine. However, there were radiation-associated decreases in marrow proteins, including G-CSF, MCP-1, IL-6, MIP-1 β , IP-10, IL-15 and IL-17, which were not seen in plasma or intestine. The data are consistent with the primary bone marrow target of LD_{50/30} (9.25 Gy), which induces hematopoietic syndrome in C57BL/6 mice.

The current data also showed that a radiation mitigator administered after TBI may either elevate or decrease the levels of the same cytokines in bone marrow, and that the direction of change does not predict effectiveness.

When two mitigator drugs were administered at the same time after TBI, either at 24 h (optimal time for JP4-039 alone) or at 48 h (optimal time for necrostatin-1 alone), we detected no additive or synergistic improvement in survival (Fig. 7). We used the time of RIP-3 kinase phosphorylation after TBI in intestine and bone marrow, as well as the time of IL-6 increase in plasma, to direct the time of delivery of necrostatin-1. The time for delivery of necrostatin-1 in TBI mice changed after JP4-039 had been administered. The delay in TBI-induced plasma IL-6 elevation by prior JP4-039 administration correlated with the time of increase in IL-1 α in marrow. Furthermore, the JP4-039-mediated delay in radiation-induced IL-6 in plasma and IL-1 in bone marrow correlated with the delay in the appearance of serine-phosphorylated RIP3 kinase in both bone marrow and intestine. Therefore, we delayed by 24 h after TBI the delivery of necrostatin-1 to 72 h in JP4-039-treated mice, and this sequenced combination improved survival. The delayed administration of necrostatin-1 resulted in return to preirradiation levels, and in some cases, decreases in bone marrow protein levels that had been observed to be elevated by necrostatin-1 alone at 48 h (Fig. 5D). Thus, combinations of effective radiation mitigators may be associated with plasma, intestine and bone marrow protein levels that reflect the same pattern observed when one of

two mitigators was delivered, or an intermediate effect of the two when only one was given. Based on our findings of the 33 proteins studied, a second mitigator may neutralize, downregulate or reverse responses that were induced by a first mitigator.

We conclude that the death pathways of apoptosis and necroptosis, which occur after TBI, may be correlated with levels of the 33 proteins studied to partially explain the increased survival when the two drugs were administered. In bone marrow, JP4-039 inhibited apoptosis and suppressed inflammation, probably via inhibition of the expression of TGF- β , which led to a reduction of TNF- α production and a late onset of necroptosis. In contrast, in intestine, the early onset of necroptosis due to IFN- β/γ was mitigated by JP4-039, which suppressed apoptosis and simultaneously upregulated anti-inflammatory cytokines (e.g., IL-10) and downregulated pro-inflammatory cytokines (e.g., IL-1 β and IL-17). JP4-039 induced the release of chemokines, which may have attracted immune cells to the intestine, including IP-10 and MIG, at 72 h postirradiation. To ameliorate the effects of an excessive inflammatory response, which may have exacerbated damage to the intestinal epithelium, a delayed onset of necroptosis and the release of anti-inflammatory chemokines appeared to delay the optimal treatment window for administration of necrostatin-1. Thus, administration of JP4-039 at 24 h and necrostatin-1 at 72 h increased survival. Further investigation is required to determine whether plasma protein levels can be used to predict appropriate timing of delivery of other categories of radiation mitigators that target yet other cell and organ death pathways, including ferroptosis and lysosomal necrosis.

In future studies, sequential administration of multiple categories of radiation mitigators will require further optimization of timing, and should be modified by individual responses to TBI. Furthermore, the possible effects of gender and age, the presence of other therapeutic drugs in the system (polypharmacy) and safety in pregnancy, will all need to be taken into consideration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Rwigema J-CM, Beck B, Wang W, Doemling A, Epperly MW, Shields D, et al. Two strategies for the development of mitochondrial-targeted small molecule radiation damage mitigators. *Int J Radiat Oncol Biol Phys.* 2011; 80:860–8. [PubMed: 21493014]
2. Huang Z, Epperly M, Watkins SC, Greenberger JS, Kagan VE, Bayir H. Necrostatin-1 rescues mice from lethal irradiation. *Biochim Biophys Acta.* 2016; 1862:850–6. [PubMed: 26802452]
3. Williams JP, McBride WH. After the bomb drops: A new look at radiation-induced multiple organ dysfunction syndrome (MODS). *Int J Radiat Biol.* 2011; 87:851–68. [PubMed: 21417595]

4. Kagan VE, Mao G, Qu F, Angeli JPF, Doll S, St Croix C, et al. Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol.* 2016; 13:81–90. [PubMed: 27842066]
5. Kalash R, Epperly MW, Goff J, Dixon T, Sprachman MM, Zhang X, et al. Amelioration of irradiation pulmonary fibrosis by a water-soluble bi-functional sulfoxide radiation mitigator (MMS350). *Radiat Res.* 2013; 180:474–90. [PubMed: 24125487]
6. Jiang J, Belikova NA, Xiao J, Zhao Q, Greenberger JS, Wipf P, et al. A mitochondria-targeted nitroxide/hemi-gramicidin S conjugate protects mouse embryonic cells against gamma-irradiation. *Int J Radiat Oncol Biol Phys.* 2008; 70:816–25. [PubMed: 18262096]
7. Kagan VE, Bayir HA, Belikova NA, Kapralov O, Tyurina YY, Tyurin VA, et al. Cytochrome c/ cardiolipin relations in mitochondria: a kiss of death. *Free Radic Biol Med.* 2009; 46:1439–53. [PubMed: 19285551]
8. Fink M, Macias CA, Xiao J, Tyurina YY, Delude RL, Greenberger JS, et al. Hemigramicidin-TEMPO conjugates: Novel mitochondria-targeted antioxidants. *Crit Care Med.* 2007; 35:5461–70.
9. Belikova NA, Jiang J, Stoyanovsky DA, Greenberger JS, Kagan VE. Mitochondria-targeted (2-hydroxyamino-vinyl)-triphenyl-phosphonium releases NO and protects mouse embryonic cells against irradiation-induced apoptosis. *FEBS Lett.* 2009; 583:1945–50. [PubMed: 19427865]
10. Goff JP, Epperly MW, Shields D, Wipf P, Dixon T, Greenberger JS. Radiobiologic effects of GS-nitroxide (JP4-039) in the hematopoietic syndrome. *In Vivo.* 2011; 25:315–24. [PubMed: 21576404]
11. Greenberger JS, Kagan V, Bayir H, Lazo J, Wipf P, Li S, et al. Mitochondrial targeted small molecule radiation protectors and radiation mitigators. *Front Radiat Ther Oncol.* 2012; 1(article 59):1–12.
12. Berhane H, Shinde A, Kalash R, Xu K, Epperly MW, Goff J, et al. Amelioration of irradiation induced oral cavity mucositis and distant bone marrow suppression in *Fancd2*^{−/−} (FVB/N) mice by intraoral JP4-039/F15. *Radiat Res.* 2014; 182:35–49. [PubMed: 24932534]
13. Shinde A, Berhane H, Rhieu BH, Kalash R, Xu K, Goff J, et al. Intraoral mitochondrial-targeted GS-nitroxide, JP4-039, radio-protects normal tissue in tumor-bearing radiosensitive *Fancd2*^{−/−} (C57BL/6) mice. *Radiat Res.* 2016; 185:134–50. [PubMed: 26789701]
14. Brand RM, Epperly MW, Stottlemeyer JM, Skoda EM, Gao X, Li S, et al. A topical mitochondria-targeted redox-cycling nitroxide mitigates oxidative stress-induced skin damage. *J Invest Derm.* 2016; 10:1–9.
15. Krainz T, Gaschler MM, Lim C, Sacher JR, Stockwell BR, Wipf P. A mitochondrial-targeted nitroxide is a potent inhibitor of ferroptosis. *ACS Cent Sci.* 2016; 2:653–9. [PubMed: 27725964]
16. Martin-Sanchez D, Ruiz-Andres O, Poveda J, Carrasco S, Cannata-Ortiz P, Sanchez-Nino MD, et al. Ferroptosis, but not necroptosis, is important in nephrotoxic folic acid-induced AKI. *J Am Soc Nephrol.* 2016; 28:1–10. [PubMed: 27683895]
17. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. *Front Biosci.* 1997:12–26.
18. Demoulin J, Uyttenhove C, Roost EVAN, Donckers D, Snitck JVAN, Lestre BDE. A single tyrosine of the interleukin-9 (IL-9) receptor is required for STAT activation, antiapoptotic activity, and growth regulation by IL-9. *Mol Cell Biol.* 1996; 16:4710–6. [PubMed: 8756628]
19. Heinzel FP, Hujer AM, Ahmed N, Rerko RM. In vivo production and function of IL-12 p40 homodimers. *J Immunol.* 1997; 158:4381–8. [PubMed: 9127002]
20. Mackay BCR, Leukosite F. Chemokine receptors and T cell chemotaxis. *J Exp Med.* 1996; 184:799–802. [PubMed: 9064339]
21. Lin M, Carlson E, Dianconu E, Pearlman E. CXCL1/KC and CXCL5/LIX are selectively produced by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis. *J Leukoc Biol.* 2007; 81:786–92. [PubMed: 17110418]
22. Escary J, Perreau J, Dumenil D, Ezine S, Brulet P. Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymic stimulation. *Nature.* 1993; 363:361–4. [PubMed: 8497320]
23. Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signaling pathways, biological responses, and therapeutic inhibition. *Trends Pharmacol Sci.* 2001; 22:201–7. [PubMed: 11282421]

FIG. 1.
Comparison of PDD profiles with and without filter.

FIG. 2.

Flow diagram shows the day mice were irradiated (day 0) and the day mice were i.v. injected with JP4-039 only (day 1), necrostatin-1 only (day 2) or the combination of JP4-039 (day 1) and necrostatin-1 (day 3). Panel A: Radiation only. Panel B: Radiation with JP4-039. Panel C: Radiation with necrostatin-1. Panel D: Radiation with JP4-039 and necrostatin-1. All sacrificed mice were from different experiments and had plasma, bone marrow and ileum collected for Luminex assay for inflammatory proteins. Number of mice sacrificed for tissue collection at each time point for each experiment is shown.

FIG. 3.

Radiation induction of protein level changes in plasma. Statistically significant increases and decreases ($P < 0.05$) are indicated in red and green, respectively. Panel A: Induction of protein signature changes by radiation alone. Panel B: Effect of JP4-039 when given at 24 h after TBI. Panel C: Effect of necrostatin-1 when given at 48 h after TBI. Panel D: Effect of combination of JP4-039 at 24 h and necrostatin-1 72 h after TBI. Key below the figure shows statistical significance of each shade of red or green.

FIG. 4.

Radiation induction of protein level changes in intestine. Statistically significant increases and decreases ($P < 0.05$) are indicated in red and green, respectively. Panel A: Induction of protein signature changes by radiation alone. Panel B: Effect of JP4-039 when given at 24 h after TBI. Panel C: Effect of necrostatin-1 when given at 48 h after TBI. Panel D: Effect of combination of JP4-039 at 24 h and necrostatin-1 72 h after TBI. Key below the figure shows statistical significance of each shade of red or green.

FIG. 5.

Radiation induction of protein level changes in bone marrow. Statistically significant increases and decreases ($P < 0.05$) are indicated in red and green, respectively. Panel A: Induction of protein signature changes by radiation alone. Panel B: Effect of JP4-039 when given at 24 h after TBI. Panel C: Effect of necrostatin-1 when given at 48 h after TBI. Panel D: Effect of combination of JP4-039 at 24 h and necrostatin-1 72 h after TBI. Key below the figure shows statistical significance of each shade of red or green.

FIG. 6. Confirmation of Luminex assay data by Western blot analysis of bone marrow levels of TNF- α and IL-6. Levels were quantitated by densitometry (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14787.1>, S1) and correlated with the Luminex assay data in Fig. 4A. Day 0 represents the day of irradiation (preirradiation). Days 1–7 follow the day of irradiation.

FIG. 7.

Effect on survival after TBI by simultaneous or sequential delivery of two radiation mitigators, JP4-039 and necrostatin-1, at times known to be effective for individual delivery of each drug alone. Panel A: JP4-039 at 24 or 48 h after 9.25 Gy TBI compared to 9.25 Gy TBI alone ($P = 0.0034$ and $P = 0.0324$, respectively). Panel B: Necrostatin-1 at 48 h after 9.25 Gy TBI compared to 9.25 Gy TBI alone ($P = 0.0009$); JP4-039 at 24 h plus necrostatin-1 at 48 h after 9.25 Gy TBI compared to 9.25 Gy TBI alone ($P = 0.0108$). The combination of JP4-039 at 24 h and necrostatin-1 at 48 h (best time for each) provided no additional advantage to necrostatin-1 alone at 48 h.

FIG. 8.

Administration of JP4-039 at 24 h after 9.25 Gy TBI delays phosphorylation of RIP3 in bone marrow and intestine of C57BL/6NTac mice. At 24 h after TBI, a subgroup was administered JP4-039 (20 mg/kg). Mice were sacrificed at 0, 1, 2, 3 or 4 days postirradiation, protein isolated from bone marrow and intestine (ilium), and Western blot analysis performed for detection of phosphorylated RIP3 protein (p-RIP3), as described in Materials and Methods. In bone marrow (panel A) at day 1, there was increase in p-RIP3 and further increase at day 2. In mice administered JP4-039 on day 1, the increase was delayed to day 4. In intestine (panel B), p-RIP3 expression was increased on days 2, 3, and 4 after 9.25 Gy TBI. Mice treated with JP4-039 showed a delayed expression to days 3 and 4. (Supplementary Fig. 2; <http://dx.doi.org/10.1667/RR14787.1.S1>).

FIG. 9.

Improved survival after TBI by sequential, but delayed delivery of necrostatin-1 after JP4-039. Groups of C57BL/6NTac female mice ($n = 15$) received each drug at indicated times after 9.25 Gy TBI. Survival was plotted as described in Materials and Methods and (10). Survival in animals receiving JP4-039 at 24 h and delayed administration of necrostatin-1 at 72 h was superior to that of animals given each drug alone. Survival in the group that received both drugs at 24 h was no different from those given JP4-039 at 24 h. The group that received both drugs at 48 h was no different from those given necrostatin-1 alone at 48 h. The group that received JP4-039 at 24 h and necrostatin-1 at 48 h was no different from those given JP4-039 alone at 24 h. $P = 0.0173$: JP4-039 at 24 h after TBI compared to TBI alone. $P = 0.0479$: necrostatin-1 at 48 h after TBI compared to TBI alone. $P = 0.0002$: JP4-039 at 24 h and necrostatin-1 at 72 h after TBI compared to TBI alone. $P = 0.0398$: JP4-039 at 24 h and necrostatin-1 at 72 h after TBI compared to JP4-039 at 24 h after TBI. $P = 0.0198$: JP4-039 at 24 h and necrostatin-1 at 72 h after TBI compared to necrostatin-1 at 48 h after TBI.

TABLE 1**Known Major Functions of Proteins Being Studied**

Protein	Function	Reference
TGF- β	Inflammation, pro-fibrotic, controls proliferation and cellular differentiation	(17)
TNF- α	Inflammatory cytokine-stimulates necroptosis	(17)
IL-1 α	Pro-inflammatory	(17)
IL-1 β	Pro-inflammatory	(17)
IL-2	Natural response to microbial infection; regulates activities of white blood cells	(17)
IL-3	Stimulates hematopoietic stem cells to become myeloid progenitor cells	(17)
IL-4	Stimulation of activated B-cell and T-cell proliferation, and stimulates B cells into plasma cells	(17)
IL-5	Stimulates B cell growth and increases immunoglobulin secretion	(17)
IL-6	Pro-inflammatory	(17)
IL-7	Stimulates hematopoietic stem cell differentiation into lymphoid progenitor cells	(17)
IL-9	Stimulates cell proliferation and prevents apoptosis	(17, 18)
IL-10	Anti-inflammatory	(17)
IL-12 (p40)	Stimulates growth and function of T cells, and stimulates IFN- γ and TNF- α	(17, 19)
IL-12 (p70)	Stimulates growth and function of T cells, and stimulates IFN- γ and TNF- α	(17, 19)
IL-13	Mediator of allergic inflammation, and induces MMPs	(17)
IL-15	Induces proliferation of natural killer cells	(17)
IL-17	Recruits monocytes and neutrophils to sites of inflammation	(17)
IP-10	Chemoattractant for monocytes/macrophages, T cells, NK cells and dendritic cells	(17, 20)
KC	Attracts neutrophils	(21)
LIF	Stem cell differentiation	(22)
LIX	Cell migration and activation of neutrophils	(21)
MCP-1	Recruits monocytes, T cells and dendritic cells to sites of inflammation	(17, 20)
M-CSF	Induces hematopoietic stem cells to differentiate into macrophages.	(17)
MIG	T-cell chemoattractant	(17, 20)
MIP-1 α	Recruitment and activation of granulocytes	(17, 20)
MIP-1 β	Chemoattractant for NK cells, monocytes and other immune cells	(17, 20)
MIP-2	Recruits neutrophils and lymphocytes in the intestine	(17, 20)
RANTES	Recruits leukocytes into inflammatory sites	(17, 20)
VEGF	Stimulates vasculogenesis and angiogenesis	(23)
Eotaxin	Recruits eosinophils	(17, 20)
GM-CSF	Stimulates hematopoietic stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes	(17)
G-CSF	Stimulates hematopoietic stem cells to produce granulocytes	(17)
IFN- γ	Activator of macrophages and inducer of class II major histocompatibility complex (MHC) molecule expression	(17)

TABLE 2

Grouping by Radiation Response Characteristic of 33 Signature Proteins

Inflammatory response	Humoral and cell-mediated immunity
TGF- β	IL-2
IL-1 α	IL-4
IL-13	IL-5
TNF- α	IFN- γ
IL-1 β	MIG
IL-6	MCP-1
IL-12 (p40)	IP-10
IL-12 (p70)	IL-12 (p40)
IL-9	IL-12 (p70)
IL-10	
IL-17	
IP-10	
KC	Cell death
MCP-1	TNF- α
MIG	IL-1 β
MIP-1 α	IL-6
MIP-1 β	IL-12 (p40)
MIP-2	IL-12 (p70)
RANTES	IL-9
Eotaxin	MCP-1
IFN- γ	RANTES
Chemoattractant/activator for phagocytic cells, T cells and other inflammatory mediators	
IL-17	
IP-10	
KC	
MCP-1	
MIG	
MIP-1 α	
MIP-1 β	
MIP-2	
RANTES	
Eotaxin	
IFN- γ	
IL-12 (p40)	
IL-12 (p70)	
Stem cell differentiation/proliferation	

Inflammatory response	Humoral and cell-mediated immunity
IL-3	
IL-7	
LIF	
M-CSF	
GM-CSF	
G-CSF	

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