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An oral Hemokine™, α -methylhydrocinnamate, enhances myeloid and neutrophil recovery following irradiation *in vivo*

Douglas V. Faller^{1,7}, Serguei A. Castaneda¹, Daohong Zhou⁴, Merriline Vedamony⁵, Peter E. Newburger³, Gary L. White², Stanley Kosanke², P. Artur Plett⁶, Christie M. Orschell⁶, Michael S. Boosalis¹, and Susan P. Perrine^{1,7}

¹Cancer Center, Boston University School of Medicine, Boston, MA

²Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

³Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA

⁴Division of Radiation Health, Department of Pharmaceutical Sciences, University of Arkansas, Medical School, Little Rock, Arkansas

⁵Armed Forces Radiobiology Research Institute, Bethesda, MD

⁶Indiana University, Indianapolis, Indiana

⁷Phoenicia BioSciences, Inc., Weston, MA

Abstract

An oral therapeutic which reduces duration of cytopenias and is active following accidental radiation exposures is an unmet need in radiation countermeasures. Alpha methylhydrocinnamate (ST7) prolongs STAT-5 phosphorylation, reduces growth-factor dependency of multi-lineage cell lines, and stimulates erythropoiesis. Here, ST7 and its isomers were studied for their effects on myeloid progenitors and hematopoietic stem cells (HSCs) following radiation, in nonhuman primates, and murine irradiation models. Addition of ST7 or ST7-S increased CFU-GM production by 1.7-fold ($p < 0.001$), reduced neutrophil apoptosis comparable to G-CSF, and enhanced HSC survival post-radiation by 2-fold, ($p = 0.028$). ST7 and ST7-S administered in normal baboons increased ANC and platelet counts by 50–400%. In sub-lethally-irradiated mice, ANC nadir remained $> 200/\text{mm}^3$ and neutropenia recovered in 6 days with ST7 treatment and 18 days in controls ($p < 0.05$). In lethally-irradiated mice, marrow pathology at 15 days was hypocellular (10% cellularity) in controls, but normal (55–75% cellularity) with complete neutrophil maturation with ST7-S treatment. Following lethal irradiation, ST7, given orally for 4 days, reduced mortality, with 30% survival in ST7-animals vs 8% in controls, ($p < 0.05$).

§Corresponding author: Susan P. Perrine MD, Boston University School of Medicine, 72 E. Concord St., L909, Boston, MA 02118 & Phoenicia BioSciences Inc, Weston, MA 02493, sperrine@bu.edu, Tel. 617 335-7002, Fax: 617 638-5616.

Authorship Contributions:

The studies were designed by Drs. Perrine, Faller, Newburger, Zhou, and Vedamony, Experiments were performed by Castaneda, Boosalis, Kosanke, White, Vedamony, and Orschell. Drs. Perrine and Faller wrote the manuscript and all the authors reviewed and edited the manuscript.

Conflict of Interest Disclosures

Drs. Perrine and Faller are inventors on patent applications related to this work, which are owned by Boston University, and have equity ownership in Phoenicia BioSciences, Inc. which provided research grant support for this study (R41 HL-87542).

Collectively, the studies indicate that ST7 and ST7-S enhance myeloid recovery post-radiation and merit further evaluation to accelerate hematologic recovery in conditions of radiation-related and other marrow hypoplasias.

INTRODUCTION

The hematopoietic component of the Acute Radiation Syndrome (ARS) is characterized by marrow suppression with multi-lineage cytopenias, and prolonged neutropenia particularly, increases the significant risk of life-threatening sepsis and associated mortality.¹⁻⁴ Radiation following nuclear reactor accidents has caused broad exposures to large populations. Currently available treatment modalities include multiple single-lineage-specific cytokines, which require refrigeration and injection, particularly granulocyte colony-stimulating factor (G-CSF) formulations, including those with minor modifications or hybrid peptides with IL3, and Amifostine, an IV radioprotectant.¹⁻³ In accidental mass radiation exposures, where access to medical treatment is not optimal, a single agent which promotes multi-lineage hematopoiesis, acts following radiation exposure, and is orally bioavailable would be particularly beneficial. We previously found that select orally-active short-chain fatty acid derivatives (SCFADs) have erythropoietic effects, *in vitro* and *in vivo* in phlebotomized baboons and in mice. A small panel of such SCFADs (designated Hemokines™), including α -methylhydrocinnamate (designated “ST7”) stimulated proliferation of erythroid progenitors from human marrow and cord blood (*in vitro* activity).⁵⁻⁷ Treatment of non-anemic mice and anemic phlebotomized baboons with this family of low molecular weight compounds increased reticulocyte counts, total hemoglobin and hematocrit, and, incidentally, WBC counts.⁵ ST7 administration stimulated erythropoiesis in phlebotomized baboons with both oral or parenteral administration.⁵

Although initial interest in these short-chain fatty acid derivatives was as erythropoietic inducers of fetal hemoglobin for treatment of β -hemoglobinopathies, a few compounds were also found to have proliferative effects on other hematopoietic lineages, in addition to erythroid. Broader actions of ST7 were first observed in multi-lineage hematopoietic 32D cells, which are dependent upon IL-3 or GM-CSF for survival and proliferation, and also respond to EPO and G-CSF.⁸⁻¹² 32D cells undergo apoptotic cell death in the absence of growth factors, and do not proliferate when IL-3 concentrations are reduced by 50-fold below the levels required for growth.⁹ No experimental condition or growth factor had previously been found to abrogate the IL-3 growth factor-dependency of these cells.¹¹⁻¹² However, ST7 restored proliferation of these multi-lineage hematopoietic cells and prevented apoptotic cell death when IL-3 was withdrawn.¹¹ ST7 likely acted in concert with IL-3 and GM-CSF to augment survival signals into proliferative signals. Similarly, the human TF-1 cell line, which is dependent upon GM-CSF or IL-3 or erythropoietin for growth, also proliferated in the presence of ST7 and other “Hemokines”.⁹ This activity was perhaps not unexpected in view of the molecular mechanism through which ST7 and the other Hemokine compounds function. ST7 acts through signaling pathways common to the peptide growth factors IL-3, GM-CSF, and EPO, effectively bypassing their respective cellular receptors. The molecular basis for the mitogenic activity of ST7 was shown to be through prolongation of the phosphorylation of STAT-5 (independently of signaling through

Jak-2), which in turn induces the early growth-related genes *c-myc* and *c-myb*, and ultimately proliferation. ST7 thus co-opts the normal hematopoietic hormone signaling pathway.^{6,13} Further, ST7 and related SCFADs inhibit apoptosis in erythroid progenitor cells which undergo rapid apoptosis or programmed cell death (cultured from patients with β -thalassemia) and stimulate expression of an anti-apoptotic protein, Bcl-x_L, which shifts the over-all ratios of Bcl-family proteins from pro-apoptotic to anti-apoptotic, thus prolonging erythroid cell survival and enhancing cell proliferation.¹⁰ For example, the ratio of the anti-apoptotic Mcl-1_L to the pro-apoptotic Mcl-1_S is shifted in favor of anti-apoptosis by exposure to ST7 and certain other Hemokines, as does erythropoietin.¹⁰

In studies described in this report, one Hemokine™, ST7 and its -S enantiomer, were evaluated for potential proliferative and protective effects in conditions of stable and suppressed myelopoiesis induced by ionizing radiation. ST7 and ST7S were found to inhibit apoptosis of human neutrophils, modestly stimulate human myeloid progenitor growth and hematopoietic stem cell (HSC) survival after irradiation *in vitro*, in multiple distinct assays. In normal baboons without marrow expansion, ST7 and its enantiomers produced modest increases in absolute neutrophil counts and platelets. In sub-lethally irradiated mice, myeloid recovery and recovery from neutropenia was accelerated by ST7 treatment compared to saline controls. In lethally-irradiated mice, oral ST7 treatment begun post-irradiation significantly increased survival compared to controls when administered orally for just 4 days. Taken together with previous studies, these findings strongly suggest that ST7 and its -S enantiomer have reproducible stimulatory effects on proliferation and survival of murine HSC, on differentiated myeloid progenitors, and may increase platelet counts. These diverse actions may offer potential for clinical application for treatment of multi-lineage cytopenias, such as occur following accidental radiation exposures.

METHODS

Test Compounds

The racemate sodium salt of α -methylhydrocinnamate (ST7) was synthesized by Frontage Laboratories (Malvern, PA) and the enantiomers ST7-S and ST7-R (Fig. 1) were synthesized at Colorado State University (Fort Collins, CO). Recombinant human G-CSF (Filgrastim; Neupogen®) was purchased from Amgen (Thousand Oaks, CA). The compound structures are shown in Figure 1.

Myeloid progenitor assay

CD34⁺ cells were isolated from de-identified cord blood or adult peripheral blood and cultured in semisolid media with minimal growth factor supplements (IL-3, G-CSF, Stem Cell Technologies, Vancouver B.C.), with or without ST7 or its enantiomers. Cord blood was generously provided by Dr. Ludy Dobrila from the National Cord Blood Program (Howard P. Milstein Cord Blood Center, New York Blood Center, New York, NY). Briefly, CD34⁺ cells were enriched by negative selection of non-CD34⁺ cells using specific antibodies (RosetteSep reagent, Stem Cell Technologies, Vancouver, BC, Canada), using a Ficoll-paque density gradient, and further enriched by positive selection using EasySep (Stem Cell Technologies). The enriched cells were cultured in H4230 medium (Stem Cell

Technologies) containing 1% Methylcellulose in Iscove's MDM, 30% fetal bovine serum, 2 mM L-glutamine, 1% Bovine Serum Albumin and 10^{-4} M β -mercaptoethanol, with GM-CSF (20 ng/ml) and IL-3 (20 ng/ml). Cells were cultured in a humidified atmosphere with 5% CO₂, at 37°C and CFU-GM colonies were enumerated on day 14; treated cultures were compared to untreated controls from the same subject. Statistical tests were performed using the GraphPad Prism program; a level of 0.05 was considered significant.

Cobblestone area forming cell assays

The cobblestone area forming cell (CAFC) assay, an assay for HSCs, was performed as previously described.^{11,12} Feeder cell stromal layers were prepared by seeding 10^3 /well FBMD-1 stromal cells in each well of flat-bottomed 96-well plates (Falcon, Lincoln Park, NJ). One week later, bone-marrow mononuclear cells (BM-MNCs), irradiated to 2 Gy or left non-irradiated for controls, were resuspended in CAFC medium (Iscove's MDM supplemented with 20% horse serum, 10^{-5} M hydrocortisone, 10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin), with or without ST7 (50–200 μ M), or ST7-S or ST7-R (50 μ M), and were overlaid on these stromal layers in 3-fold dilutions. Cultures were fed weekly by changing one-half of the medium, including addition of ST7 or enantiomers in the appropriate cultures. The frequencies of day-35 CAFCs representing mouse HSCs were analyzed to assess survival of HSCs with or without exposure to ST7 in comparison with non-irradiated control cells.

Neutrophil apoptosis assay

Peripheral blood was collected and assays were performed with the approval of the Institutional Review Board of the University of Massachusetts School of Medicine. Normal neutrophils were harvested from healthy volunteers, purified by sedimentation through 6% dextran, placed in culture in HBSS containing 1% human serum albumin serum, and incubated with ST7 at 200 μ M, ST7-S at 50 μ M, or G-CSF at 100 U/mL, at 37°C in a shaking water bath. Samples were analyzed after 24 and 43 hrs of exposure; apoptotic cell fraction was assayed by Annexin V binding (BD BioSciences; San Jose, CA) and a TUNEL assay using FITC-dUTP (Chemicon; Billerica, MA).

Hematopoietic studies in stable baboons

These studies were performed with the approval of the Institutional Animal Care and Use Committee of the University of Oklahoma Health Science Center. Non-irradiated, 2–3 year old juvenile baboons (*Papio hamadryas anubis*) were studied as previously described.⁵ Complete blood counts were performed, using an automated hematology analyzer (Scil Animal Care Company; Gurnee, IL), including platelet counts and absolute neutrophil counts (ANC), prior to and with administration of the test compounds. Platelet counts were determined in two nonanemic baboons (GG24, FM83) treated with ST7, ST7-S, or ST7-R intravenously at 100 mg/kg, once daily, twice per week during the first week, and five times per week in the second week.

Sub-lethal irradiation studies in mice

These studies were performed with the approval of the Institutional Animal Care and Use Committee of Boston University School of Medicine. A radiation dose of 6 Gy was established to be a marrow-toxic, but sublethal, dose of gamma irradiation in female FVB mice.¹⁵ Female FVB mice, six to eight weeks of age, were placed in a pie cage and treated with a single uniform total body dose of 6 Gy of gamma radiation, using a ¹³⁷Cs source (GammaCell 40; Nordion International, Kanata, Ontario, Canada) at an exposure rate of 70 cGy/min $\pm$ 1.8 cGy on day zero. All mice were given acidic water to decrease infections. Although oral bioavailability was previously established for ST7, intraperitoneal administration was utilized due to avoid inadvertent esophageal perforation in irradiated animals who were expected to develop radiation-associated mucositis. ST7 (at 300 mg/kg or 500 mg/kg), or an equal volume of normal saline (vehicle), administered daily by *ip* injection, was begun following radiation. The treatments were given once daily, seven days per week for two weeks, followed then five days per week for another two weeks or until neutropenia resolved. Complete blood counts (CBCs) determinations were performed manually from nicked tail veins. Enumeration of leukocytes and absolute neutrophils was performed using the Unopette micropipettes for dilution and hemocytometry. CBCs were determined three days prior to radiation and three times per week following radiation.

Lethal irradiation studies in mice (LD_{50/30}) with subcutaneously administered ST7-S

These studies were performed with the approval of the Institutional Animal Care and Use Committee of Indiana University. Beginning on day four after irradiation and continuing until the end of the study (day 30), all mice were provided laboratory chow wetted with autoclaved acidified water in petri dishes set on the bottom of the cage. Sixteen to 18 female and 16 male C57BL/6 mice (32–34 mice per group), twelve weeks of age, were randomly assigned to one of two treatment groups (normal saline or ST7-S). The randomization schedule for each cage of mice was prepared by a study statistician using a SAS program Version 9.1 (SAS Institute Inc., Cary, NC). The mice received a single, uniform, total body dose of 7.76 Gy of gamma radiation (LD_{50/30}, 50% lethality in 30 days), using a ¹³⁷Cs source (GammaCell 40; Nordion International, Kanata, Ontario, Canada) at an exposure rate of 65–69 cGy/min $\pm$ 2.5 cGy on day zero. All mice were injected *subcutaneously*, after irradiation, with either normal saline or ST7-S at 100 mg/kg. The treatments were given once daily, five times per week for four weeks. Femurs were collected for bone marrow histology evaluation from 3 mice per group (2 females and 1 male) at day 18 after radiation.

Lethal irradiation studies in mice (LD_{70/30}) with oral administration of ST7

These studies were performed with the approval of the Institutional Animal Care and Use Committee of the Armed Forces Radiobiology Research Institute (AFRRI) using the principles outlined in the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Inbred male CD2F1 mice (Harlan Laboratories, VA), 8–9 weeks old were maintained at the AFRRI vivarium. These animals were evaluated for microbiological, serological, and histopathological tests by the veterinary staff and determined to be pathogen-free during the quarantine period. Healthy animals were housed 4 per box in conventional sterile polycarbonate boxes with filter covers (Microisolator, Lab Products Inc.,

Seaford, DE) and autoclaved hardwood chip bedding. Mice had access to Harlan Teklad Rodent diet 8604 (Purina Mills, St. Louis, MO) and acidified water (pH 2.5–3.0) *ad libitum*. The animal rooms were maintained at $21 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity with 10–15 cycles of fresh air hourly and a 12:12 h light:dark cycle. A dose-ranging toxicology study was first performed to identify maximally-tolerated doses of ST7 using two doses, 1200 and 3600 mg/kg doses; no toxicity was identified by clinical observations and histopathology, and 1000 mg/kg was selected as a dose for further testing. One dose of ST7 (1000 mg/kg PO) and PBS at the same volume (0.2 ml) were evaluated. The treatment schedule in one group was days 1–4 pre-irradiation and in another group, days 1–4 post-irradiation. Unanesthetized mice were irradiated bilaterally at AFRRI's Cobalt-60 γ -irradiation facility. During irradiation, the animals were placed in well-ventilated plexiglass chambers made specifically for mouse irradiation. The mid-line dose to the animals was delivered at a dose rate of 0.6 Gy/min. Animals were monitored daily for 30 days.

RESULTS

ST7 enhances production of myeloid progenitors and survival of normal neutrophils

In the absence of added cytokines, colony growth was negligible, with 10% or less of normal colony numbers, although addition of (racemic) sodium ST-7 increased the number of colonies under all culture conditions compared to control. The effects of ST7 on myeloid progenitors (CFU-GM) were studied here using a minimal required cytokine cocktail (GM-CSF and IL-3). Addition of ST7-S increased colony numbers most significantly above untreated control cultures from the same subject, with a mean of 1.7-fold higher CFU-GM, $p < 0.001$) shown in Fig. 2A.

Cytokine growth factors, including G-CSF, are well-established to promote the survival of myeloid progenitors and differentiated myeloid cells, through receptor-mediated activation of STAT-5. To determine whether ST7 could similarly affect survival of mature myeloid cells, human neutrophils purified from peripheral blood were cultured in the presence of racemic ST7, the S-enantiomer of ST7 (ST7-S), or G-CSF (as a positive control). The number of viable (non-apoptotic) cells under control conditions decreased dramatically by the 43 hour time point (Fig. 2B). This apoptotic cell death was efficiently prevented by the presence of ST7 or ST7-S, which essentially was as active as G-CSF in promoting neutrophil survival.

Effects of ST7 on mouse HSC survival

Analysis of day-35 cobblestone area-forming cells (CAFCs) provides an estimate of hematopoietic function corresponding to primitive hematopoietic stem cells (HSCs) with long-term repopulating ability.^{15–15} Exposure of bone-marrow mononuclear cells (BM-MNCs) to 2 Gy of ionizing radiation typically results in more than 70% reduction in day-35 CAFC frequency ($p < 0.001$ versus vehicle control).^{14–16} To assess the potential radioprotective effects of ST7 on HSCs, the number of day-35 CAFCs were determined in irradiated cultures, with or without exposure to ST-7 (at 50–200 μM), or ST7-R or ST7-S (at 50 μM). Culture in the presence of ST7-S significantly increased the survival fraction of HSCs after irradiation by 156% vs. vehicle treated cells ($p = 0.028$) (Fig. 3). ST7-R did not

have activity in the CAFC assay (data not shown). No significant effect of ST7-S or ST7-R was observed in control HSC cultures which were not irradiated.

ST7 and ST7-S stimulate neutrophil and platelet counts in vivo in stable baboons and irradiated mice

Hematopoiesis in the baboon closely mimics human blood cell production. Treatment with racemic ST7, and ST7-S, administered at 100 mg/kg IV, resulted in modest increases in absolute neutrophil and platelet counts in different baboons without prior marrow manipulation (shown in Fig 4A–D; ANC increased in ST7-S treated baboon, up to 3-fold over baseline, Fig. 4A–B. Absolute neutrophil counts (ANC) increased by doubling to -4-fold above baseline counts. With ST7 or ST7-S treatment, platelet counts, which had been stable, increased by 50% -100% of baseline; the higher change, from 400,000 to 950,000 was noted incidentally in a baboon which was undergoing chronic phlebotomy (Fig 4D).

ST7 treatment promotes myeloid recovery and survival after irradiation

G-CSF accelerates recovery of neutrophils after marrow damage secondary to gamma irradiation. To determine whether ST7 has similar activities, the effects of the ST7 or its enantiomers on marrow recovery from sublethal irradiation were studied. In saline-treated control animals, the ANC nadir declined to <200 neutrophils/fl by day 4 following radiation, and remained below 200 for 7 days (Fig. 5A). ST7 treatment (ip) effectively prevented the decline in ANC <200, and ANC in the treated animals recovered to baseline 12 days before the saline controls recovered; ANC did not return to baseline for 18 days in saline controls, compared to 6 days in the ST7-treated cohorts. Higher doses of gamma irradiation ablate marrow elements.

The effects of ST7 on marrow myeloid cellularity was also studied after mid-lethal (LD_{50/30}) irradiation. In these studies, ST7-S or saline (as control) was administered daily five days/week, subcutaneously, for four weeks, beginning 24 hr after the irradiation. Histological examination of femoral bone marrow at day 15 revealed marked hypocellularity, with no mature myeloid elements, in saline controls (Fig. 5B). In contrast, the marrows from the ST7-S-treated animals were normocellular, and exhibited complete neutrophil maturation at day 15.

ST7 enhances survival after lethal irradiation

ST7 (racemic) or PBS was administered orally (at 1000 mg/kg) for a brief 4 day treatment course beginning 24 hours after lethal irradiation prolonged survival and resulted in a modest but significant increase in survival for oral ST7-treated animals compared to PBS-treated controls (Fig. 6). 40% of the lethally-irradiated animals treated with ST7 animals beginning one day after radiation survived compared to 10% of the controls at 15 days post-radiation; 30% of ST-7 treated mice survived compared to 8% of PBS controls at 20 days post-radiation; the difference in survival of the post-radiation orally-treated ST7 animals compared to PBS controls was significant ($p < 0.02$, paired t-test). In animals treated beginning 4 days pre-radiation, survival at 20 days was 20% vs 8% in saline controls. Subcutaneous administration of ST7 did not result in a survival advantage, whereas oral treatment did.

DISCUSSION

We previously established that a small panel of select short-chain fatty acid derivatives (Hemokines™) can stimulate erythropoiesis *in vitro* in cultures of primary hematopoietic progenitor cells, and *in vivo* in mice and anemic baboons.^{5,6,10–11} As previous studies demonstrated that ST7 prolongs phosphorylation (and therefore the activity) of STAT-5 in hematopoietic cells, a mechanism similar to that of the cytokine hematopoietic growth factors G-CSF, EPO and TPO, we investigated whether ST7 might exhibit multi-lineage activity, rather than being erythroid-specific. Evidence for this potential multi-potency was initially observed in the 32D and TF-1 cell lines, which are dependent for survival upon IL-3 or GM-CSF/IL-3 and erythropoietin, respectively, but which proliferated in the presence of added "Hemokines" such as ST7.^{6,11} The studies here confirm that exposure to sodium α -methylhydrocinnamate (ST7, the racemic compound) and the S-enantiomer produced stimulatory activity in the myeloid lineage and suggest effects on platelets. ST7 increased the production of human myeloid CFU-GM from cord and peripheral blood, enhanced survival of mature human neutrophils, and enhanced murine hematopoietic stem cell (HSC) survival after irradiation *in vitro*. In irradiated mice, marrow recovery was profoundly accelerated by ST7-S. In normal baboons, ST7 and enantiomer produced increases in absolute neutrophil counts and platelets in the absence of marrow stress. ST7-S was the more active enantiomer (producing 2–2.5 fold increases above baseline vs. 50% increases with ST7-R, (data not shown). In sub-lethally irradiated mice, ST7 treatment reduced the neutropenic nadir and accelerated neutrophil recovery. In lethally-irradiated mice, oral ST7 treatment for just 4 days post-irradiation significantly increased survival compared to PBS control cohorts.

Because signaling through STAT-5 enhances the survival of hematopoietic cells, cytokines such as erythropoietin and G-CSF have anti-apoptotic actions in addition to their proliferative effects. We had previously reported that some SCFAD "Hemokine™" compounds, particularly ST7, increase the expression of pro-survival effector, Bcl-xL (a member of the Bcl-2 family of anti-apoptotic proteins), and Mcl-1L, in pan-hematologic cell lines and in progenitors which undergo rapid apoptosis due to beta thalassemia.⁶⁵ Apoptosis is induced during bone marrow damage by radiation exposure or chemotherapy. The anti-apoptotic Bcl-2-family proteins have been shown to protect hematopoietic cells from radiation damage.¹⁷ We demonstrate here that the physiological rapid apoptotic program inherent in neutrophils is suppressed by exposure to ST7 and both isomers, to a degree comparable to the actions of G-CSF. Further evidence of the anti-apoptotic effects of this compound is provided by the CAFC assays, wherein ST7-S exposure enhanced the survival of HSCs after cytotoxic irradiation. It is likely that the anti-apoptotic activity of ST7, as well as its proliferative effects, contributed to the enhanced bone marrow recovery and enhanced survival in animals subjected to lethal irradiation. In the irradiation studies, a brief 4-day oral administration regimen enhanced survival in a LD90/20 model while a lower dose, 100 mg/kg, given subcutaneously did not affect survival. Whether the difference in route of administration contributed to the difference in survival is not clear, but may be related to pharmacokinetic factors, and importantly, the subcutaneous and ip routes were shown to

enhance neutrophil recovery, and the oral route would be far more useful than parenteral in large accidental radiation exposures.

The tri-lineage activity and oral bioavailability of ST7 may be particularly applicable to certain type of cytopenias characterized by general bone marrow cytotoxicity, such as radiation exposure or cytotoxic chemotherapy. Exposure to ionizing radiation (IR) as the result of accidental exposure has become an increasingly frequent risk, such as following the 2011 earthquake and tsunami which damaged the Fukushima nuclear reactor in Japan. A recent interagency workshop on the radiobiology of nuclear terrorism has concluded that, in such an event, many victims may receive moderate doses of ionizing radiation (IR) in the range of 1 to 10 Gy.^{18–19} Exposure to these doses of IR would cause significant civilian casualties due to IR-induced damage to normal tissues. Without treatment, approximately half of all people exposed to a dose of more than 350 rad (3.5 Gy) die within 60 days.^{20–29} In this context, radiation countermeasures that can be used as mitigating agents or treatments for post-radiation rescue therapy, particularly oral therapies, are needed. Within a few hours or days after exposure to a significant dose of total body irradiation (TBI), a series of characteristic clinical complications termed the acute radiation syndrome (ARS) appear.^{24–30} Significant injury as a function of increasing IR doses indicates that the hematopoietic system is the most radiosensitive tissue of the body, followed by the gastrointestinal system, and finally neural and cardiovascular tissues. The severity and duration of bone marrow (BM) suppression is dose-dependent at TBI greater than 1 Gy. Acute and transient myelosuppression typically results from exposure to TBI less than 3 Gy, which primarily damages hematopoietic progenitor cells (HPCs).^{28–30} With TBI greater than 3 Gy, persistent myelosuppression, or late bone marrow failure occurs as a result of severe injury to hematopoietic stem cells (HSCs).^{32–35} This can be complicated by the gastrointestinal (GI) syndrome if the IR dose is further increased.^{1,2,36} Because BM failure and GI toxicity are the primary life-threatening injuries after exposure to a moderate dose of IR, rescuing these tissues from IR injury is a priority for saving lives after radiation exposure accidents and countermeasures.

G-CSF in various formulations (with GM-CSF as an alternative), although not yet approved for this use, is the therapeutic standard after accidental exposure to less than the human LD₅₀ (estimated to be about 4.5 Gy of gamma irradiation), in order to shorten the duration of neutropenia. When administered until neutrophils recovered to > 1,000/uL for 3 consecutive days and with general supportive care measures continued over 60 days, filgrastim has significantly enhanced survival by 38% following mid-lethal dose irradiation in nonhuman primates.^{4,40–46} ST7 treatment, given for just 4 days, in our irradiated murine study increased survival by 2- to 3-fold above controls at 15 and 20 days; neutrophil recovery was accelerated and the nadir was higher in sublethally irradiated animals treated for 2 weeks, and these results together cautiously suggest that longer treatment with ST7 or ST7-S than 4 days may confer added benefit. The rationale is to enhance cell proliferation from residual hematopoiesis. G-CSF is effective in irradiated primates even though it does not stimulate multi-lineage hematopoietic recovery. Moreover, an inevitable civic crisis following a radiation accident, such as in the tsunami disaster, or a terrorism incident, would likely result in delayed administration (with resulting reduced efficacy) of agents which require parenteral administration, as medical personnel are not likely to be widely

available.^{47–48} It has been shown that synergistic effects result from combining cytokines,^{49–53} including stem cell factor (SCF) and erythropoietin (EPO) with G-CSF.⁵⁴ This data suggests that a radiation-mitigating agent, which has multi-lineage effects such as ST7 or ST7-S, may provide additive efficacy with growth factors, compared to sole use of a single-lineage-targeted agent.

The hematopoietic and GI syndromes remain important issues in the field of radiation countermeasures, as there is only one approved therapeutic, Amifostine, approved for treatment of acute radiation, which is administered intravenously, although other candidates which have activity as anti-oxidants or may enhance DNA repair are quite interesting.^{3,55–58} We have presented both *in vitro* evidence that ST7 or its –S enantiomer protect hematopoietic stem cells from radiation and enhance their recovery, and *in vivo* evidence that this compound enhances marrow recovery and mitigates ARS, enhancing myeloid recovery and survival. Because the doses of radiation used in the LD_{90/30} studies was sufficient to induce the GI toxicity component of ARS, the enhancement in survival produced by ST7 may indicate a mitigating effect on the gastrointestinal stem or progenitor populations, and this possibility is under investigation. It is also noteworthy that ST7 enhanced recovery and survival when begun 24 hours after irradiation, a time-frame consistent with target utility following an accidental or unexpected radiation exposure, rather than being solely radioprotective, which requires administration before exposure.

An ideal therapeutic for mitigation of ARS would be an orally active therapeutic which is stable at room temperature, to allow for lengthy storage without refrigeration, and without toxicity over a wide concentration range, to enable safe distribution to persons of different sizes with minimal oversight required. ST7 meets these general criteria, having 90% oral bioavailability in baboons, testing negative for mutagenicity in an Ames test, and induced no significant toxicity at doses up to 3600 mg/kg in rats and dogs; in fact, neutrophilia and monocytosis were cited as dosing signs in dogs. Doses of ST7 and ST7-S activity (50–100 mg/kg in baboons) which produce hematologic activity have corresponded to human equivalent doses (HED) of 15–25 mg/kg/dose for similar short chain fatty acids and maintain plasma levels for several hours which are consistent with drug concentrations sufficient for hematopoietic proliferative activity *in vitro*. Preclinical pilot studies suggest that a standard formulation would produce the desired pharmacodynamic actions at readily tolerable doses in human subjects, based on clinical experience with other short chain fatty acids and subsequent scaling to humans.

These studies, taken together, with a benign safety profile in screening toxicology studies, suggest potential clinical applications of ST7-S to enhance neutrophil recovery, such as provision to personnel at risk of exposure to radiation (e.g., first-responders, personnel working at nuclear reactors), treatment of large populations after accidental radiation exposure, following therapeutic radiation where large regions of marrow are exposed or following stem cell transplant and chemotherapy. Further evaluation in models of irradiation exposure, perhaps in combination with other candidate therapeutics which act through different mechanisms such as free radical scavenging (Amifostine) or activation of the AKT pathway when given prior to radiation appear indicated.^{56–58}

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Figure 1. Chemical structures of ST7 and isomers

Racemic, -S, and -R enantiomers of sodium α-methylhydrocinnamate; C₁₀H₁₁NaO₂, as designated.

Figure 2A. Effects of ST7 or enantiomers on CFU-GM proliferation

CFU-GM cultured from normal peripheral blood +/- ST7 (0.05 μ M), ST7-S (0.05 μ M), or ST7-R (0.05 μ M), are shown, relative to untreated control colonies cultured from the same subject. The difference in colony number relative to control is significant for the 3 test agents relative to untreated control from the same subjects, ($p=0.01$, (Wilcoxon test for paired differences)). The vertical bars indicate S.D.

Figure 2B. Apoptosis of normal human neutrophils *in vitro*

Purified human neutrophils were cultured under standard (control) conditions, or in the presence of (racemic) ST7 (at 200 μM) or ST7-S (at 50 μM), or G-CSF (positive control). Viable and apoptotic cells were enumerated at 24 and 43 hours. Exposure to ST7 or ST7-S reduced apoptosis in normal human neutrophils as effectively as did G-CSF at 43 hours, whereas untreated neutrophils were largely completely apoptotic.

Figure 3. Effects of ST7-S on survival of murine hematopoietic stem cells (HSCs) after exposure to irradiation *in vitro*

Mouse bone marrow cells were non-irradiated as a control or exposed to 2 Gy of γ -irradiation *in vitro* after the cells were preincubated with Vehicle or ST7-S (50 μ M) for 1 hr. Survival of HSCs was measured by day-35 cobblestone area forming cell (CAFC) assay and expressed as % of non-irradiated control. The data is presented as mean $\pm$ SE of 4 independent experiments.

Figure 4. Effects of ST7 and ST7-S on neutrophil and platelet counts in baboons

4A. Absolute neutrophil counts following administration of ST7 in a baboon treated twice over 2 weeks, as designated by the arrows.

4B. Absolute neutrophil counts following administration of ST7 in a baboon treated over 1 week, as designated by the arrows.

4C. Platelet counts following administration of ST7 orally in 2 baboons (100 mg/kg) as indicated by the inverted arrows.

4D. Platelet counts during administration of ST7-S in a phlebotomized baboon (100 mg/kg IV) as indicated by the arrows. The horizontal bar above the graph designates the period of phlebotomy.

Fig. 5. Effects of ST7 and ST7S on neutrophil recovery and myelopoiesis following irradiation
Fig. 5A, Top panel. Absolute neutrophil counts in irradiated FVB mice. Timing of irradiation (6 Gy) is designated by the symbol. Connected lines show absolute neutrophil counts in animals treated with either ST7 at 300 mg/kg/dose or 500 mg/kg/dose, or Normal Saline, as indicated. The hatched line indicates an ANC threshold of 200/ μL . Absolute neutrophil count did not decline $<200/\mu\text{L}$ in ST7-treated mice, and recovered to baseline within 8 days of nadir, significantly earlier than in saline-treated mice.
Figure 5B. Lower Panel. Bone marrow pathology in irradiated mice following treatment with ST7-S or PBS at day 18 following irradiation. **A)** Marrow in a representative non-irradiated, untreated animal; **B)** Marrow from an irradiated, saline-treated animal, with marked hypocellularity and no myeloid differentiation. **C)** Marrow from a representative irradiated animal treated with ST7-S, at day 18. Complete neutrophil maturation (noted by the arrows) and normal (55–75%) cellularity are observed.

Figure 6.
Percent of mice surviving following LD90/20 irradiation are shown for PBS-treated animals (light circles) and ST-7-treated animals, (dark circles).