CCM-AMI, A POLYETHYLENE GLYCOL MICELLE WITH AMIFOSTINE, AS AN ACUTE RADIATION SYNDROME PROTECTANT IN C57BL/6 MICE

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Abstract—Acute radiation syndrome results from radiation exposure, such as in accidental nuclear disasters. Safe and effective radioprotectants, mitigators, and treatment drugs must be developed as medical countermeasures against radiation exposure. Here, the authors evaluated CCM-Ami, a novel polyethylene glycol micelle encapsulated with amifostine, for its radioprotective properties after total-body irradiation from a ^{60}Co source. Male C57BL/6 mice (6–8 wk old) were intravenously injected with 45 mg kg^{-1} of CCM-Ami 90 min before exposure to 7.2 and 8.5 Gy irradiation at a dose rate of 0.04 Gy min^{-1}. Both survival benefit and hematopoietic protection were observed after prophylactic CCM-Ami administration when compared with the effects measured in excipient control and amifostine groups. Pharmacokinetic results showed that after the intravenous injection, the plasma concentration of WR-1065, the active form of amifostine, was higher in CCM-Ami-treated mice than in amifostine-treated mice. These findings suggest that CCM-Ami-mediated hematopoietic protection plays a key role in enhancing survival of mice exposed to radiation toxicity and thus indicate that CCM-Ami is a radioprotectant that can be used safely and effectively in nuclear disasters.
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Key words: ^{60}Co; exposure, cumulative; gamma radiation; mice

INTRODUCTION

Since the Fukushima Daiichi nuclear power plant disaster of 11 March 2011, developing countermeasures against radiological damage has become a crucial medical goal.

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The authors declare no conflicts of interest.

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Moreover, in addition to the chance of nuclear accidents, the probability of international terror attacks has increased substantially in recent years (Andrew Newman 2009). Consequently, the risk of emergency responders, the military, and civilians being exposed to ionizing radiation doses sufficient to cause acute radiation syndrome (ARS) has increased.

The U.S. Centers for Disease Control and Prevention clearly defines ARS as a severe illness that occurs when a person is exposed to extremely high levels of radiation, typically over a short period. The major causes of ARS-induced death are infections and internal bleeding that result from bone marrow damage. ARS comprises four subsyndromes—hematopoietic, gastrointestinal, neurovascular, and cutaneous subsyndromes—and, according to clinical signs and symptoms, ARS progression is categorized into four phases: prodromal phase, latent phase, manifest illness, and recovery or death (Dainiak et al. 2003; Waselenko et al. 2004). Medical treatment is available to limit or eliminate internal contamination, depending on the type of radioactive material involved; however, only a few agents are recommended, such as potassium iodide, Prussian blue, diethylene triamine pentaacetate, and Neupogen (Shleien et al. 1983; Melo et al. 1994; Pospisil et al. 1999; Kazzi et al. 2012). Therefore, it is urgent that radioprotectants, mitigators, and treatment drugs for use as medical countermeasures to radiation exposure, which are safer and more effective than currently available drugs, be developed (Weiss and Landauer 2009).

Amifostine is the first drug approved to provide protection and diminish side effects in cancer patients undergoing chemotherapy or radiotherapy (Sasse et al. 2006). Amifostine is a prodrug featuring an organic thiophosphate compound that was synthesized by the U.S. Army in studies conducted at the Walter Reed Institute of Research. Amifostine is dephosphorylated to its active form, WR-1065, by alkaline phosphatase, and WR-1065 distributes between normal tissue and tumors at distinct concentrations (Kouvaris et al. 2007). As a scavenger of oxygen free radicals generated by ionizing radiation, WR-1065 exhibits considerable potential in ARS protection. However, because of its pharmacokinetics, the

clinical application of amifostine is restricted to amelioration of chemotherapy and radiotherapy side effects. The average distribution half-life of amifostine is 0.88 min, and amifostine removal from plasma predominates and is complete within 6 min after drug administration (van der Vijgh and Korst 1996; Korst et al. 1997). Amifostine is administrated via several routes, and new formulations of the drug have been developed, such as oral capsules or subcutaneous injection, in order to enhance convenience and to increase the oxidation-resistant duration (Cassatt et al. 2002). Without compelling evidence from animal or human experiments, no innovations involving amifostine use have been approved by regional regulators.

In this study, a new radiation-exposure animal model was created in C57BL/6 mice, which is the most reported and recommended strain for testing radiomitigators or radioprotectants (Williams et al. 2010; Chua et al. 2012; Plett et al. 2012). This radiation model was developed based on the continuous radiation procedure, in which 4-h radiation accumulation is used to mimic real-world scenarios such as disasters caused by dirty bombs or nuclear power plant explosions. Here, the authors used CCM-Ami, a novel polyethylene glycol micelle drug carrier encapsulated with amifostine, in order to alter pharmacokinetic profiles, and the results indicated that it may be used for ARS protection or treatment, although additional experiments need to be executed to support any treatment effect. The results of measuring 30-d survival and peripheral blood-cell counts indicated that CCM-Ami provides hematopoietic protection and thereby increases survival after total-body irradiation in the ARS mouse model. The findings of this study might facilitate the development of the optimal CCM-Ami formulation and dosage for pivotal efficacy studies in animals.

MATERIALS AND METHODS

Animals and radiation

This study was conducted across several testing sites, including National Tsing Hua University, I-Shou University, and Chung-Hwa University of Medical Technology. Male C57BL/6 mice (BioLASCO Taiwan Co., Ltd.) 7 ± 1 wk of age were divided into groups of 5 to 7. All described experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at each site. All irradiation was conducted at the ^{60}Co gamma-radiation facility of National Tsing Hua University (Nuclear Reactor & Radiation Isotope Lab, Hsinchu, Taiwan), and the potency of the radiation source is validated annually with certification by the Atomic Energy Council at Taiwan. Mice were irradiated in well ventilated cages at a dose rate of 0.04 Gy min^{-1} to reach the 6.9, 7.2, 8.3, 8.5 Gy of γ radiation exposure and at a dose rate of 0.9 Gy min^{-1} and 0.99 Gy min^{-1} to reach 8.3 Gy and 6.9 Gy exposure, respectively.

An electronic dosimeter (Thermo Scientific EPD-G; Thermo Fisher Scientific, Berkshire, UK) was used to measure the radiation dosage, which was validated regularly by the Institute of Nuclear Energy Research (INER), Atomic Energy Council, Taiwan. Effects on the irradiated CCM-Ami-treated groups were compared with excipient- and amifostine-treated groups and are presented in the results. After irradiation, mice were returned to their original cages with access to food and water ad libitum.

Drug formulation and administration

CCM-Ami (patent publication number: US8785569), the polyethylene glycol blocked with polyglutamic acids micelle encapsulated with amifostine, was provided by Original BioMedicals Co., Ltd. (Tainan, Taiwan). Light yellow lyophilized powder CCM-Ami was dissolved in sterilization water for injection and sterilized using a 0.22-μm Millipore membrane filter. CCM-Ami is a 20–30-nm micelle particle with a polydispersity index < 0.3 and a zeta-potential < 0 mV. The encapsulation efficiency of CCM-Ami is $> 95\%$, and the purity of each batch is $> 99\%$. CCM-Ami (45 mg kg^{-1} body weight) was administered intravenously 90 min before irradiation; the amifostine groups were administered intravenously with the equivalent dose of CCM-Ami (based upon equivalent analytical potency values) before irradiation in the same time window.

Peripheral complete blood count test

Blood samples were obtained from the orbital sinus and collected into EDTA tubes at predose (day 1 was defined as the day of dosing and irradiation) and days 8, 14, 21, and 32 after radiation exposure, and the blood samples were conducted to complete a blood count by using a hematology analyzer (Diatron Abacus Junior Vet, MA, USA). White blood cell (WBC), red blood cell (RBC), lymphocyte (LYM), and granulocyte (GRA) counts are presented as percentage change of predose count.

Pharmacokinetic study

The plasma disposition of WR-1065 was studied in 7 ± 1 wk old male C57BL/6 mice (n = 3–7 per each time point) after intravenous injection of 45 mg kg^{-1} of CCM-Ami and amifostine. The blood samples were collected from the orbital sinus after 10, 30, 60, 90, 120, 240, and 360 min after administration of CCM-Ami and amifostine. Three to seven mice were sampled once at each time point. Blood samples were extracted by 1 mM EDTA and 0.5 M perchloric acid in a ratio of 1:1 (v/v), immediately after sampling. The mixture was then centrifuged at 16,000 g for 3 min at 4°C, filtered by 0.22 μm PVDF membrane filter, and then incubated in 50°C water bath for 90 min.

Plasma concentrations of WR-1065 were measured by a liquid chromatography-mass spectrometry (LC-MS) assay. A 10-μL aliquot was injected into the LC-MS system for quantification. Likewise, all samples were kept on ice

during the entire procedure in order to minimize the hydrolysis of amifostine to WR-1065. Analysis was performed on a Waters H class core system with QDa detector. The analytes were separated by a Waters Atlantis T3 (C18) column (3 μm, 2.1×150 mm) at 35°C under Ion Pair Reagent (MPA) (10 mM Undecafluorohexanoic acid) and acetonitrile (MPB) at a flow rate of 0.4 mL min^{-1}. The mass spectrometer was operated at positive mode with an electrospray voltage of 0.8 KV and capillary temperature of 600°C. The area under the curve (AUC) represents the total drug exposure over time and was plotted by the concentration of drug in blood plasma against time.

Statistical analysis

The statistical significance among survival study groups was analyzed using a Log-rank (Mantel-Cox) test. The results of WBC and RBC counts and the plasma WR-1065 concentrations were expressed as means ± standard error of the mean (SEM). Group comparisons, through variance analysis, were examined using an ANOVA and post hoc Tukey test. Differences in plasma WR-1065 concentrations between CCM-Ami and amifostine groups at each time point were analyzed using a Student's t test. The significant level was set at 5% for each test ($p < 0.05$).

RESULTS

Radiation dose rate response of C57BL/6 mice

To determine the relationship between radiation dose rate and the survival response, male C57BL/6 mice (7 ± 1 wk old) were exposed to radiation doses of 0.99 or 0.04 Gy min^{-1} within approximately 10 min or 4 h in order to accumulate total-body irradiation of 6.9 or 8.3 Gy, respectively, and then analyzed for overall survival rates for 30 d. Mice irradiated with the low radiation dose rate (long radiation exposure time) were more radioresistant than were mice irradiated with the high radiation dose rate (short radiation exposure time). A statistically significant survival advantage was observed following the long exposure at 8.3 Gy when compared with the short radiation exposure time [$p < 0.05$, Log-rank (Mantel-Cox) test] (Fig. 1).

Protective effects of CCM-Ami in C57BL/6 mice after radiation exposure

In this study, a new radiation-exposure model was created in order to mimic real-world scenarios such as nuclear bomb explosions, nuclear power plant accidents, or space radiation, which are characterized by low radiation dose rates and long radiation exposure times. Male C57BL/6 mice were intravenously injected with 45 mg kg^{-1} of CCM-Ami, amifostine, or excipient 90 min before exposure to a total-body irradiation of approximately 7.2 and 8.5 Gy of γ-radiation by using a ^{60}Co source (radiation dose rate, 0.04 Gy min^{-1}). The results showed that CCM-Ami treatment improved survival rates and median survival to a
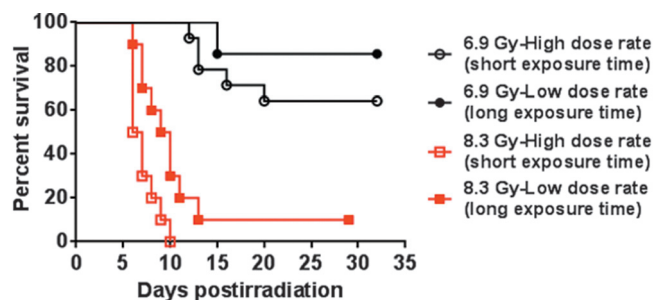
TBI	6.9 Gy		8.3 Gy	
Irradiation exposure	High dose rate	Low dose rate	High dose rate	Low dose rate
Irradiation rate (Gy/min)	0.99	0.04	0.90	0.04
Irradiation time	7 min	3 h	9.2 min	4 h
Survival rate (D29 or32)	64 %	86 %	0 %	10 %
Median survival	Undefined	Undefined	Day 6.5	Day 9.5
Statistics (P value)	ns (p=0.32)		* (p=0.017)	

Fig. 1. Survival curves obtained for various radiation dose rates in C57BL/6 mice. Mice were exposed to a high (0.90–0.99 Gy min^{-1}) or low (0.04 Gy min^{-1}) radiation dose rate in order to accumulate total-body irradiation (TBI) of 6.9 and 8.3 Gy within 7–9 min or 3–4 h: (a) Kaplan-Meier survival curves show the survival rate measured over 30 d following exposure to high and low radiation dose rates at 6.9 and 8.3 Gy; (b) the summary of median survival, survival rate at D29 or D32, and statistical results; Ns, not significant, and *$p < 0.05$, compared with the short-exposure group, Log-rank (Mantel-Cox) test.

greater extent than did treatments with the corresponding dose of amifostine and excipient control (Fig. 2a and 2b). The CCM-Ami group showed significantly enhanced survival compared with the amifostine group when the radiation dose was 7.2 Gy (86% with CCM-Ami vs. 0% with amifostine; $p < 0.01$), and compared with the excipient control group when the dose was 8.5 Gy (80% with CCM-Ami vs. 0% with excipient control; $p < 0.05$). These results indicated that a single injection of 45 mg kg^{-1} of CCM-Ami before total-body irradiation improved the survival rate and median survival of the C57BL/6 mice and, compared with amifostine, yielded superior protection.

Peripheral blood counts after radiation exposure in C57BL/6 mice treated with CCM-Ami and amifostine

Male C57BL/6 mice were intravenously injected with 45 mg kg^{-1} of CCM-Ami or amifostine or the excipient before being exposed to 7.2 Gy of γ-radiation, and then peripheral WBC, RBC, LYM, and GRA counts were determined at days 8, 14, 21, and 32 after radiation exposure. At day 8, WBC, RBC, and LYM counts were diminished uniformly in the irradiated excipient control, amifostine, and CCM-Ami groups. However, WBC, RBC, LYM, and GRA counts showed higher trends in the CCM-Ami group than in the excipient control group at days 8, 14, and 32 (Fig. 3a–d). RBC counts at day 14 and LYM counts at day 32 were significantly higher in mice treated with CCM-Ami than in mice treated with the excipient control (RBC, $p < 0.01$; LYM, $p < 0.05$)
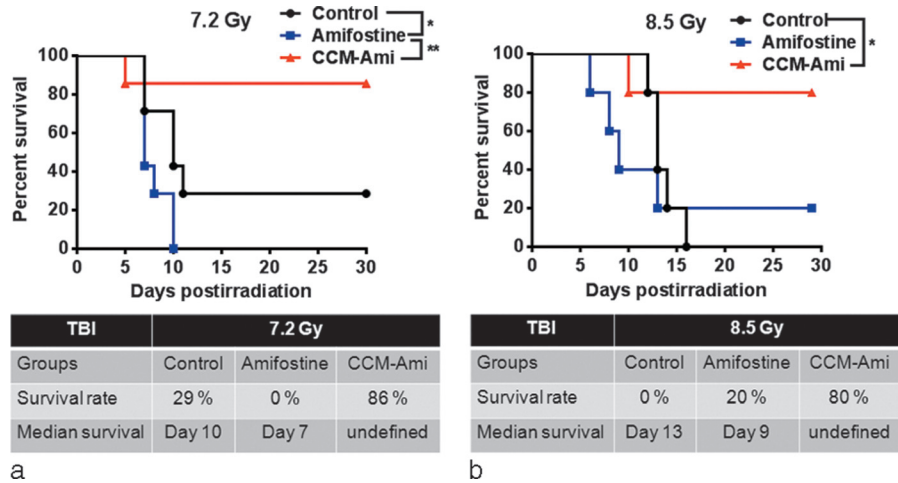
Fig. 2. Protective effects of CCM-Ami (45 mg kg^{-1}) in C57BL/6 mice after 7.2- and 8.5-Gy radiation exposure. Survival curves of the CCM-Ami-treatment group compared with those of the excipient- and amifostine-treatment groups over (A) 30 d following 7.2-Gy radiation exposure and (B) 29 d following 8.5-Gy exposure; *$p < 0.05$ and **$p < 0.01$, compared with excipient control or amifostine group, Log-rank (Mantel-Cox) test.

(Fig. 3b and c), and the GRA count after irradiation was increased in the CCM-Ami group relative to control (Fig. 3d). Moreover, the peripheral complete blood counts were similar in C57BL/6 mice treated with 45 mg kg^{-1} of CCM-Ami before exposure to 8.5- and 7.2-Gy irradiation (data not shown). These results suggested that a single injection of CCM-Ami before lethal total-body irradiation protected the mice from hematopoietic ARS and thus enhanced their survival.

Pharmacokinetic analysis of intravenously injected CCM-Ami and amifostine in C57BL/6 mice

To compare the pharmacokinetics of CCM-Ami and amifostine, male C57BL/6 mice were injected intravenously
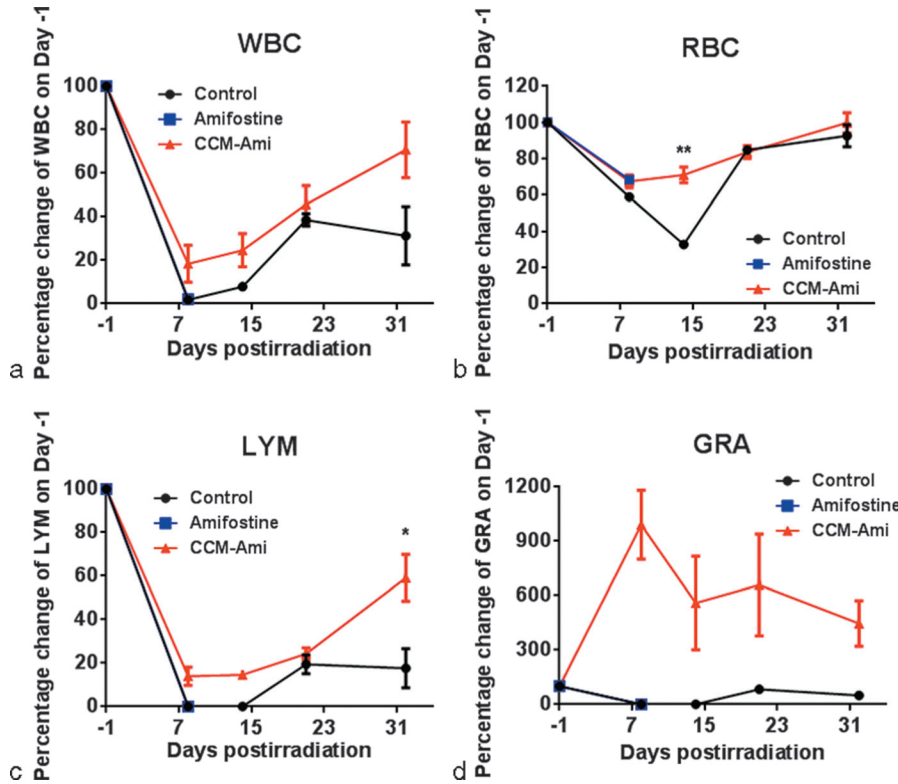

Fig. 3. Peripheral blood counts following CCM-Ami, amifostine, and excipient control treatments in 7.2-Gy-irradiated C57BL/6 mice: (a) WBC, (b) RBC, (c) LYM, and (d) GRA counts at Days 8, 14, 21, and 32 after 7.2-Gy irradiation; *$p < 0.05$ and **$p < 0.01$, compared with the excipient control group, ANOVA test and post hoc Tukey test.

with 45 mg kg^{-1} of CCM-Ami and amifostine and then blood samples were collected from the orbital sinus at 10, 30, 60, 90, 120, 240, and 360 min. All blood samples were extracted and processed before being subjected to UPLC/MS analysis. The results showed that at all time points, the plasma WR-1065 concentration was higher after CCM-Ami injection than after injection of the corresponding dose of amifostine, and the area under the curve (AUC) of WR-1065 in the CCM-Ami groups was also higher than that in the amifostine groups (Table 1). This finding suggests that the antioxidative capacity of CCM-Ami as radioprotector might be higher than that of amifostine.

DISCUSSION

In this study, a new radiation model was designed by using C57BL/6 mice in an effort to mimic radiation exposure after a genuine nuclear disaster and to evaluate the radioprotection provided by CCM-Ami. Researchers in this field have previously focused on various radiation dosages, tissue and organ effects, and animal species; however, the radiation dose rate might also play a critical role when attempting to mimic nuclear disasters such as a nuclear power plant meltdown or a dirty-bomb explosion (Williams et al. 2010). Providing medical protection for a high radiation dose rate, such as 0.8–2 Gy min^{-1}, is probably not appropriate in the real-world scenario, but prolonging and limiting the radiation dose rate to within 0.8–3 Gy h^{-1} is realistic and useful for emergency responders. This study demonstrated that survival was higher in mice irradiated with a low radiation dose rate (long exposure time) than in mice irradiated at a high radiation dose rate. This outcome might result from damages caused to the server cells in irradiated mice by the exposure to a high radiation dose rate in a short period and by the inability of the surviving proliferating cells to replenish the full complement of blood cells, which would increase the mortality of these mice.

CCM-Ami treatment provided a survival advantage when compared with amifostine or excipient control treatment in mice irradiated with > LD70/30 radiation dosage at a dose rate of 0.04 Gy min^{-1} over approximately 4 h. The amifostine treatments showed a significant and insignificant difference compared with the control groups at 7.2 and 8.3 Gy, respectively. These outcomes might result from the inject time point of amifostine (90 min before irradiation) and the damages caused randomly by irradiation-induced free radicals in mice. Previous study indicated that the distribution half-life and removal of amifostine is < 10 min (van der Vijgh and Korst 1996; Korst et al. 1997). Therefore, as administered 90 min prior to irradiation, amifostine could not provide protection, as did an equivalent amount (potency) of CCM-Ami in this mice model. CCM-Ami, which is a polyethylene glycol micelle encapsulated with amifostine, exerted a stronger radioprotective effect than amifostine alone did. The results of pharmacokinetic analysis indicated that CCM-Ami, a novel nanosized drug carrier, exhibits unique properties that enhance drug dispersion in a liquid state and prolong drug half-life within the body, which might ultimately improve the therapeutic index. The radioprotection mechanisms of CCM-Ami are based on the actions of the active pharmaceutical ingredient amifostine, which protects mature and primitive hematopoietic cells against radiation toxicity (List et al. 1996; Romano et al. 1999). Therefore, when this drug-delivery system is

Table 1.

(a) Plasma WR-1065 concentration[a,b,c]   unit: μM

Groups	$C_{10\ min}$	$C_{30\ min}$	$C_{60\ min}$	$C_{90\ min}$	$C_{120\ min}$	$C_{240\ min}$	$C_{360\ min}$
CCM-Ami	123.03 ± 46.67	28.31 ± 2.62	16.17 ± 3.43	6.94 ± 1.36	3.82 ± 1.21	2.34 ± 0.70	0.77
amifostine	27.17 ± 4.54	5.80 ± 0.55	1.50 ± 0.10	0.95 ± 0.19	0.58 ± 0.25	0.24 ± 0.14	0.13
CCM-Ami /amifostine	4.53	4.88	10.76	7.34	6.53	9.65	5.95
Statistics	ns	**	*	*	ns	*	ns
(p value)	($p = 0.16$)	($p = 0.001$)	($p = 0.012$)	($p = 0.012$)	($p = 0.06$)	($p = 0.041$)	($p = 0.46$)

[a]Pharmacokinetic analysis of intravenously injected CCM-Ami (45 mg/kg) and amifostine (45 mg/kg) in C57BL/6 mice.
[b]The values are means ± SEM. ns, not significant; *$p < 0.05$; **$p < 0.01$, compared between CCM-Ami and amifostine groups.
[c]The ratio of CCM-Ami and amifostine was represented as CCM-Ami/amifostine.

(b) Plasma WR-1065 AUC[d,e]   unit: μM*min

Groups	$AUC_{10–30}$	$AUC_{30–60}$	$AUC_{60–90}$	$AUC_{90–120}$	$AUC_{120–240}$	$AUC_{240–360}$	$AUC_{10–360}$
CCM-Ami	1513.37	667.24	346.71	161.38	369.78	187.01	3245.49
amifostine	329.65	109.47	36.72	22.95	49.66	22.38	570.83
CCM-Ami /amifostine	4.59	6.09	9.44	7.03	7.45	8.36	5.69

[d]The AUC (area under the curve) represents the total drug exposure over time.
[e]The ratio of CCM-Ami and amifostine was represented as CCM-Ami/amifostine.

used, a slow and continuous release of amifostine from CCM-Ami micelles (Table 1) could substantially reduce toxicity accumulation in the body and prolong the protection of the hematopoietic system against radiation damage, which would enhance survival. In addition to providing radiation protection for emergency responders, the military, and civilians during nuclear accidents and terror attacks, offering radioprotection for astronauts on lengthy space missions is a critical concern. Therefore, it is urgent that drugs that provide radiation protection for prolonged periods of low radiation dosages be developed.

Postirradiation hematopoietic mortality is primarily attributed to the infection and hemorrhaging that result from the loss of bone marrow, which generates the circulating blood cells (Waselenko et al. 2004). After a sufficiently damaging radiation exposure, hematopoietic tissues are depleted of stem cells and proliferative progenitor cells; however, a subpopulation of the quiescent hematopoietic stem cells, which are selectively more radioresistant compared with the other cells, survives, presumably because of these cells being predominantly in the noncycling (G0) state. After radiation exposure, these surviving quiescent, hematopoietic stem cells differentiate and proliferate to replenish the mature functioning compartment (van Bekkum 1991; Inoue et al. 1995; Macia et al. 2011). When the proliferating cells fail to replenish the postmitotic compartment of mature cells that continue to be lost at the regular physiological rate, the major clinical outcome observed is a reduction in blood-cell counts or tissue injury. The measured peripheral complete blood-cell counts indicated that CCM-Ami protects against a radiation-induced reduction of WBC, RBC, LYM, and GRA counts in C57BL/6 mice (Fig. 3), which was also observed in previous studies (van Bekkum 1991; Inoue et al. 1995; Macia et al. 2011). After irradiation, the GRA counts in CCM-Ami-treated mice increased substantially between Days 8 and 32, which might be due to the release into the blood of mature cells (which are not affected by radiation) stored in the bone marrow. This increase of GRA counts might hold prognostic significance and thus could be clinically useful (Dainiak et al. 2003). These results suggest that CCM-Ami could provide protection by reducing radiation-induced blood-cell apoptosis or accelerating cell proliferation, which are possibilities that warrant further investigation. Therefore, the CCM-Ami-induced increase in WBC, RBC, LYM, and GRA counts might counteract hemorrhage, radiation-induced immune suppression, and consequent infections, which are major factors in ARS (Lopez and Martin 2011), and this effect would lead to enhanced survival.

Several radiation countermeasure agents have been developed to prevent, mitigate, and treat the harmful consequences of radiation exposure (Citrin et al. 2010; Dumont et al. 2010). Such agents are developed according to the "Animal Rule" established by the U.S. Food and Drug Administration (FDA), because conducting efficacy studies in humans for this approval is neither ethical nor feasible (Dolgin 2013). The government has authorized the FDA to approve drugs based on adequate and well controlled animal studies, and the FDA has already approved four drugs based on five indications; however, Phase-I clinical safety testing for these drugs remains to be conducted. Currently, promising radiation countermeasure agents are in development, such as the new molecular entity CBLB 502 and the new chemical entity Ex-Rad, and AEOL-10150, which are being evaluated for clinical trials (Traynor et al. 2006; Connolly and O'Neill 2012; Ghosh et al. 2012). CCM-Ami is a micelle encapsulated with amifostine, which is the first agent approved by the FDA as a radioprotector and chemoprotector (Spencer and Goa 1995). Therefore, CCM-Ami could be developed as a radiation-countermeasure agent according to "505(b)(2)," which has been established by the FDA for application approval based on the literature or on an Agency finding of safety and/or effectiveness for an approved drug product. CCM-Ami could then potentially be used as an effective radioprotectant.

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