Mechanism and therapeutic window of a genistein nanosuspension to protect against hematopoietic-acute radiation syndrome

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ABSTRACT

There are no FDA-approved drugs that can be administered prior to ionizing radiation exposure to prevent hematopoietic–acute radiation syndrome (H-ARS). A suspension of synthetic genistein nanoparticles was previously shown to be an effective radioprotectant against H-ARS when administered prior to exposure to a lethal dose of total body radiation. Here we aimed to determine the time to protection and the duration of protection when the genistein nanosuspension was administered by intramuscular injection, and we also investigated the drug’s mechanism of action. A single intramuscular injection of the genistein nanosuspension was an effective radioprotectant when given prophylactically 48 h to 12 h before irradiation, with maximum effectiveness occurring when administered 24 h before. No survival advantage was observed in animals administered only a single dose of drug after irradiation. The dose reduction factor of the genistein nanosuspension was determined by comparing the survival of treated and untreated animals following different doses of total body irradiation. As genistein is a selective estrogen receptor beta agonist, we also explored whether this was a central component of its radioprotective mechanism of action. Mice that received an intramuscular injection of an estrogen receptor antagonist (ICI 182,780) prior to administration of the genistein nanosuspension had significantly lower survival following total body irradiation compared with animals only receiving the nanosuspension (P < 0.01). These data define the time to and duration of radioprotection following a single intramuscular injection of the genistein nanosuspension and identify its likely mechanism of action.

Keywords: soy isoflavone; hematopoietic–acute radiation syndrome; total body irradiation; estrogen receptor antagonist

INTRODUCTION

Accidental exposure to high-dose radiation can lead to a variety of potentially lethal syndromes. Hematopoietic–acute radiation syndrome (H-ARS) is a primary medical concern for individuals exposed to total-body irradiation. In humans, death occurs within 30–60 days following >2 Gy total-body exposure due to hematopoietic insufficiency [1–3]. The development of countermeasures to prevent radiation toxicity, especially H-ARS, has been a focus of research for over 50 years. ‘Radioprotectants’ are defined as agents that provide a benefit to irradiated subjects when given prior to radiation exposure, whereas ‘radiation mitigators’ are agents that are administered after exposure. In both cases, the agents are given prior to the development of overt evidence of injury [4]. Both protectants and mitigators can be utilized for personnel at risk of accidental radiation exposure (e.g. military personnel responding to a nuclear attack, first responders to radiation incidents, or astronauts exposed to space radiation during periods of high solar activity), but also have applications in clinical oncology to minimize the toxicities
of radiotherapy without interfering with the anti-cancer effects of radiation.

Although three cellular growth factor drugs (filgrastim, pegfilgrastim and sargramostim) have been repurposed as radiation mitigators to accelerate the recovery of radiation-induced bone marrow failure (such as is experienced in H-ARS), there are no FDA-approved radioprotectant drugs to protect an individual from the onset of H-ARS. Two intravenously administered radioprotectors, palifermin and amifostine, have however been approved by the FDA to decrease the incidence and duration of severe oral mucositis in conjunction with clinical radiotherapy for head-and-neck cancer [5,6]. Unfortunately, these drugs have significant adverse effects, and neither has been approved by the FDA for prevention of H-ARS. Palifermin administration can induce dermatitis at the radiation site, anemia, hypokalemia, dysgeusia and vomiting [5]. Amifostine has severe dose-limiting side effects, including nausea, vomiting and pronounced hypotension, with performance-degrading toxicity in animal models [7–10]. These side effects in conjunction with an intravenous route of administration would make these drugs unacceptable for use by healthy military or civilian populations. [11,12]. Therefore, there is an urgent need to develop non-toxic radiation protective agents that can be easily administered prior to potential radiation exposure.

It was previously demonstrated that genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one), a naturally occurring isoflavone found at low levels in soybeans, can function as a radioprotectant in a murine model of H-ARS [13,14]. Genistein was an effective radioprotectant for H-ARS when administered as either a single subcutaneous or intramuscular (IM) injection 24 h prior to radiation exposure in mice [13,14]. Initial reports utilized genistein in its native form, which has low bioavailability. Recently, a pharmaceutically acceptable nanosuspension of genistein was created by utilizing a wet-nanomilling process, improving its water solubility by reducing the average particle size by two orders of magnitude, and increasing its bioavailability [15–18]. This clinically relevant formulation of nanoparticle genistein could also be administered by a single IM injection 24 h prior to radiation exposure to increase survival from H-ARS in mice [15]. Both genistein preparations increased survival correlated with a protection of hematopoietic stem cells within the bone marrow, allowing robust hematopoietic recovery [14,15]. Moreover, the genistein nanosuspension (GEN) was also demonstrated to inhibit the production of inflammatory factors in irradiated mouse bone marrow and spleen cells, which may contribute to the survival of hematopoietic progenitors [15].

Genistein’s mechanism of action as a radioprotectant is not completely understood, although it has been extensively studied due to its beneficial health effects [19]. Studies of genistein have revealed a variety of biological activities, including anti-oxidant and free radical scavenging effects [20–22], anti-inflammatory effects [15,23,24], and cell cycle effects [14], all of which may be relevant for radiation countermeasure effects [25]. Importantly, genistein also has a chemical (benzopyran) structure similar to estrogen and is classified as a phytoestrogen. Estrogens have been demonstrated to reduce mortality from total-body irradiation and to improve hematopoiesis in mice [26–31]. There are two estrogen-binding ligand-dependent transcription factors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) [32]. The ERs are intracellular receptors which, upon ligand binding, dimerize, translocate to the cell nucleus, and bind to estrogen response elements (EREs) in DNA sequences to facilitate site-specific gene transcription. The two transcriptional ERs (α and β) have antagonistic functions in normal/healthy cell types where they are both expressed. ERβ activation primarily induces cellular growth (ERβ is overactive in 50–80% of breast cancers). ERβ is a negative regulator of ERα and represses cell proliferation, a process believed to occur through the transcription of opposing genes rather than a direct inhibition [33]. ERβ’s anti-cellular growth attributes conceptually categorize it as a tumor suppressor, and indeed it is commonly mutated in advanced human cancers [32]. Genistein is a selective agonist of ERβ, with approximately a 20-fold greater affinity compared with ERα, at a physiological concentration for 50% inhibition (IC50) of only 8.4 nM [34]. It is currently not known whether the radioprotective effects of estrogen occur through ERα or ERβ, or whether a significant portion of the radioprotective effects of genistein require activation of ERβ.

In the present study, we investigated various parameters related to radioprotection by a genistein nanosuspension for H-ARS in mice, including: (i) the optimal time of single-dose administration, (ii) the duration of single-dose radioprotection, (iii) the dose reduction factor (DRF) of the genistein nanosuspension formulation for the prevention of lethality due to H-ARS, and (iv) the molecular mechanism of action of genistein protection. Our data show that the genistein nanosuspension has a wide duration for its protective effects, from 48 h through 12 h prior to radiation exposure. In these studies, the genistein nanosuspension was not effective as a radioprotectant when administered after radiation exposure. We confirmed that the genistein nanosuspension selectively activates ERβ in cells, and we provide evidence that a portion of the H-ARS radioprotective effects by genistein requires ER activation.

MATERIALS AND METHODS

Animals

Adult male (12–14 weeks of age) CD2F1 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA). Mice were acclimated upon arrival, and representative animals were screened for evidence of disease. Mice were housed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Animal rooms were maintained at 21°C ± 2°C with 50% ± 10% humidity on a 12 h light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604; Envigo, Dublin, VA, USA) was available freely, as was acidified (pH = 2.5) water to control opportunistic bacterial infections [35]. All animal handling procedures were performed in compliance with guidelines from the National Research Council [36] and were approved by the Institutional Animal Care and Use Committee (IACUC).

Irradiation, drug administration, and survival studies

Mice received total-body irradiation in a bilateral gamma radiation field in a 60Co facility within Lucite jigs [14]. The midline tissue dose to the mice was 9.00–9.25 Gy at a dose rate of 0.6 Gy/min for all survival studies. The alanine/electron spin resonance (ESR)
dosimetry system [37] was used to measure dose rates (to water) in the cores of acrylic mouse phantoms. After irradiation, mice were returned to their home cages. The day of irradiation was considered ‘Day 0’. For 30-day prophylactic survival studies, separate control groups were administered the nanosuspension vehicle at each time point. For these studies, there were 16–36 mice/group. For determination of the DRF, there were 20–40 mice/radiation dose.

Genistein was administered as a wet-milled nanosuspension (average particle size of 200 nm) containing 50 nM phosphate-buffered saline with 5% PVP-K17 and 0.2% Polysorbate 80. This formulation was stable at room temperature and was provided by Humanetics saline with 5% PVP-K17 and 0.2% Polysorbate 80. This formulation was stable at room temperature and was provided by Humanetics Corporation, Edina, MN, USA. All experiments used an IM injection (in the quadriceps muscle) of GEN at a dose of 150 mg/kg in a total volume of 50 μl using a 25-gauge needle attached to a 250 μl syringe (Hamilton, Reno, NV, USA) [15]. The ER antagonist survival studies were based on a previously developed methodology [38]. Briefly, mice received an IM injection (in the quadriceps muscle) of the ER antagonistICI 182,780 (10 mg/kg in 50 μl total volume) (Tocris, Ballwin, MO) or its vehicle (15% ethanol/85% corn oil), once a day for four consecutive days. On the fourth day, mice received a second injection, 2 min after the first injection, with the IM vehicle or the GEN (150 mg/kg). After 24 h, mice received a single total-body irradiation dose of 9.00 Gy 60Co. For survival studies, irradiated animals were monitored two to four times daily for symptoms of morbidity, as described in the Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee (IACUC) policy. When animals displayed designated criteria, humane euthanasia was performed using 100% CO2 inhalation followed by cervical dislocation, in accordance with the American Veterinary Medical Association (AVMA) Guidelines.

Cell-based estrogen-receptor activation assay

Samples were submitted to Indigo Biosciences, Inc. (State College, PA) to assay the ER agonist activation of either unformulated, synthetic genistein (Bonistein® , DSM Nutritional Products, Parsippany, NJ) or the GEN, using a proprietary assay system to quantify agonist activity in cell culture. Chinese Hamster Ovary (CHO) cells (reporter cells) were engineered to stably express the human ERα or human ERβ full-length isoforms, which also contain an engineered receptor-specific genetic response element (GRE) cis-linked to a firefly luciferase gene. ERα- or ERβ-agonist activation was quantified by luminescence. To perform the assay, a suspension of reporter cells were dispensed into wells of a white 96-well assay plate (~1 × 104 cells/well). In parallel, agent-containing test media was diluted to twice the target assay concentration in compound screening medium (CSM). CSM and suspended cells were combined to assay various concentrations of genistein in the non-formulated form or in the nanosuspension. Assay plates were incubated for 24 h in a cell culture incubator at 37°C, 5% CO2 and ~85% humidity. Following the 24 h incubation period, treatment media was discarded and luciferase detection reagent was added. Values of relative luminescence units (RLUs) from each assay well were used to determine receptor activity.

Statistical analysis

A two-tailed Fisher’s exact test was used for analysis of survival data. P < 0.05 was considered statistically significant. The half-lethal dose at 30 days (LD30/30) calculation for reduction factor (DRF) was determined by probit analysis [39].

RESULTS

Genistein nanosuspension administration prior to radiation exposure

GEN was previously shown to protect mice from a lethal dose of radiation when administered by IM injection 24 h before irradiation [15]. The radioprotective time-course for a single IM injection of GEN was investigated. First, the timing of GEN administration before irradiation was examined. GEN was administered 24, 12, 6, 2, 1 or 0.5 h prior to 9.25 Gy total-body irradiation. Separate control groups were administered the nanosuspension vehicle at each time point. The results demonstrated that a single IM injection of GEN administered at 24, 12, 6, 2, 1 or 0.5 h before irradiation resulted in 30-day survival rates of 89%, 64%, 50%, 25%, 6% and 19%.

Figure 1. A single IM administration of the genistein nanosuspension (Gen) 12 h prior to radiation exposure is radioprotective. (A) Thirty-day survival of mice that received a single intramuscular (IM) injection of vehicle (VEH) or Gen (150 mg/kg) either 24, 12, 6, 2, 1 or 0.5 h prior to 9.25 Gy 60Co. *P < 0.01 compared with respective vehicle group. (B) Kaplan–Meir 30-day survival curves. For simplicity, only the −24 h vehicle group is illustrated on the Kaplan–Meir plot.
Figure 2. A single IM administration of the genistein nanosuspension (Gen) provides radioprotection for up to 48 h. (A) Survival of mice 30 days after 9.25 Gy $^{60}$Co irradiation. Mice received a single IM injection of vehicle or Gen (150 mg/kg) either 5, 4, 3, 2 or 1 day prior to irradiation. *$P < 0.01$ compared with respective vehicle group. (B) Kaplan–Meier 30-day survival curves.

Respectively (Fig. 1). The survival rates for the vehicle-treated groups at the corresponding time points were 11%, 28%, 25%, 6%, 19% and 25%, respectively. Only the groups that received GEN 24 h or 12 h before irradiation had significantly ($P < 0.01$) increased survival rates compared with their respective vehicle control group.

Next, the time of GEN administration was extended so that mice received a single administration (150 mg/kg) either 5, 4, 3, 2 or 1 day before 9.25 Gy irradiation. The 30-day survival rates were 44%, 6%, 6%, 81% and 100%, respectively. The 30-day survival rates for the corresponding vehicle control groups were 38%, 19%, 12%, 6% and 31%, respectively. In this experiment, only the groups administered genistein either 2 days (48 h) or 1 day (24 h) before irradiation exhibited significantly ($P < 0.01$) higher levels of survival when compared with their vehicle control group (Fig. 2). Together, these data indicate that GEN has a wide window for time of administration for effective radioprotection, from 48 h through 12 h before radiation exposure, with the optimal time point being 24 h prior to radiation exposure.

**Post-irradiation exposure administration of the genistein nanosuspension**

We investigated the efficacy of GEN as a radiation mitigator, i.e. a drug capable of mitigating radiation injury if administered after exposure to radiation. Accordingly, GEN (150 mg/kg) was administered as a single IM injection 0.5, 1, 2, 4, 6 or 24 h after 9.25 Gy irradiation. (Fig. 3). In this experiment, vehicle or 150 mg/kg of GEN were also administered 24 h before irradiation to serve as negative and positive control groups, respectively. Mice administered GEN 0.5, 1, 2, 4, 6 and 24 h after irradiation had 30-day survival rates of 45%, 40%, 45%, 50%, 50% and 44%, respectively. These rates of survival for post-irradiation administration of genistein can be compared with the survival rate of 90% ($P < 0.01$) for the positive control group, which received GEN 24 h prior to irradiation. These findings indicate that a single IM injection of GEN at any of these time points after irradiation did not induce a significant increase in survival (Fig. 3). This suggests that GEN is not an effective radiomitigator for H-ARS when given as a single IM injection at the radiation dose used in this animal model.

**Dose reduction factor for IM-administered genistein nanosuspension**

Based on the optimal GEN dose (150 mg/kg) and the optimal time for IM administration (24 h before irradiation), the DRF for GEN given by a single IM administration was determined using probit analysis (Fig. 4). The vehicle group received a single radiation dose of 8.00, 8.25, 8.50, 8.75 or 9.00 Gy. Gen-treated mice received a single radiation dose of 9.50, 9.75, 10.00, 10.25, 10.50 or 10.75 Gy. The DRF was calculated as the ratio of the LD$_{50/30}$ dose for mice injected with the GEN to that for the vehicle-treated animals. Based on the survival data, the LD$_{50/30}$ for vehicle was 8.78 Gy and the LD$_{50/30}$ for GEN was 10.18, yielding a DRF of 1.16.

**Effects of the estrogen receptor antagonist on genistein-induced radiation protection**

We wished to evaluate the contribution of ER activation in vivo to the radioprotective effects of genistein. To determine whether genistein provided radioprotection through an ER-dependent mechanism, mice were treated with the ER antagonist ICI 182,780 (ICI) (also known as fulvestrant), which binds ER$\alpha$ and ER$\beta$ indiscriminately with a high affinity, but does not have detectable estrogenic activity [40]. Mice were treated with GEN or vehicle with or without co-treatment with ICI (Fig. 5). The survival rate for the combination of the ICI and GEN vehicles (V-V) was 25%, indicating that neither the ICI vehicle nor the GEN vehicle had any radioprotective effects. The survival rate for the ICI plus vehicle-GEN group (ICI-V) was also 25%, demonstrating that the ER antagonist, ICI 182,780, by itself was not radioprotective. The 30-day survival for the GEN positive control group (V-GEN) was 90%, significantly higher than that for either of the control groups ($P < 0.01$). The group that received the ER antagonist before the administration of genistein nanosuspension (ICI-GEN) had a 30-day survival of 45%.
This was a significant reduction in survival compared with the group that only received GEN ($P < 0.01$). These data indicate that the radioprotection mediated by GEN was significantly reduced by the ER antagonist, ICI-182,780. This suggests that ER activation is necessary for radioprotection by genistein.

Genistein is a selective agonist of ER$\beta$

Estrogen is recognized as having effects as a radiation countermeasure [26–31], and based on the previous experiment we hypothesized that some portion of the radioprotective effects of genistein may be due to its activity as an ER agonist. The molecular structure of genistein is similar to that of estrogenic steroids, which allows for its agonist activity, particularly its selective binding to ER$\beta$ [41]. Biochemical studies previously demonstrated that genistein exhibits a 20-fold selective binding to ER$\beta$ over ER$\alpha$ [34]. We compared the ability of genistein to activate ER$\beta$ over ER$\alpha$, which would reflect the capacity of genistein to biologically activate the receptors, using a CHO cell reporter system that utilized luciferase. We also compared the activity generated by the GEN formulation with that generated by native genistein solubilized in dimethyl sulfoxide (DMSO). The results demonstrated that cells treated with either DMSO-solubilized native genistein or GEN only activated ER$\alpha$ at high concentrations, with EC$_{50}$ values for ER$\alpha$ at ~2000 nM (Fig. 6). The reference agonist, 17$\beta$-estradiol, had an EC$_{50}$ of 0.012 nM for ER$\alpha$. Activation of ER$\beta$ by either genistein formulation appeared to be biphasic, which we interpreted as effectively two dose response curves, each with an EC$_{50}$ value. EC$_{50,LOW}$ corresponds to the inflexion in the curve at lower concentrations, and EC$_{50, HIGH}$ corresponds to the inflexion point at higher concentrations. The EC$_{50,LOW}$ for both the genistein formulations was 0.9 nM, and the EC$_{50, HIGH}$ was 3000 nM. Activation of ER$\beta$ by 17$\beta$-estradiol had standard kinetics, with an EC$_{50}$ of 0.012 nM. Notably, the relative selectivity of both genistein formulations for activation of ER$\beta$ over ER$\alpha$ was ~2000-fold, confirming that both standard genistein and GEN are selective agonists of ER$\beta$.

**DISCUSSION**

Research for the discovery and development of radioprotectants has been ongoing for decades, but no drugs are yet approved to prevent radiation syndromes such as H-ARS. Our current work found that a nanosuspension of genistein (150 mg/kg) can be administered in a single IM injection between 48 h to 12 h prior to radiation exposure for the prevention of H-ARS. At the dosage used in these studies, no protection was observed when GEN was given in a single IM
Figure 5. An ER-dependent mechanism facilitates genistein’s radioprotective bioactivity. CD2F1 male mice were administered an IM injection of the estrogen receptor antagonist ICI 182,780 (ICI) (10 mg/kg) or with the ICI vehicle (V; 15% ethanol: 85% corn oil) once a day for 4 consecutive days. On the 4th day, mice received a second IM injection, 2 min after the first injection, with the genistein nanosuspension vehicle (V) or genistein nanosuspension (Gen; 150 mg/kg). Mice received a single dose of 60Co total body irradiation 24 h following the second injection. (A) Gen significantly increased 30-day survival (90%), compared with vehicle (V) control levels (25%). The estrogen receptor (ER) antagonist ICI alone had no protective effect, yielding the same survival rate (25%) as vehicle. The radioprotective effect of Gen (90%) was significantly reduced by the estrogen receptor antagonist ICI, resulting in a survival rate of 45% (*P < 0.01). (B) Kaplan–Meier survival curves for the four treatment groups.

Figure 6. Genistein is a selective agonist of ERβ. CHO cells were engineered to express either ERα or ERβ, with receptor-specific promoter elements linked to a firefly luciferase gene. Cells were treated with either genistein, the genistein nanosuspension (Gen) or 17β-estradiol (positive control for both receptors). (A) Activation of ERα quantified by relative light units (RLUs), relative to untreated controls. Both formulations of genistein activate ERα at high concentrations, EC50 = 2000 nM, while 17β-estradiol has an EC50 = 0.3 nM. (B) Both genistein formulations activate ERβ in a biphasic manner. Two EC50 values are reported, corresponding to the low and high concentrations. EC50-LOW = 0.9 nM and EC50-HIGH = 3000 nM. 17β-estradiol activates ERβ with EC50 = 0.012 nM.
injection 30 min to 24 h after irradiation, indicating that in this model genistein is not effective as a post-exposure mitigator for H-ARS. When genistein was administered as a single IM injection at 150 mg/kg 24 h before irradiation, we determined the DRF of GEN to be 1.16. DRF is an important parameter for comparing various radioprotective agents because it is an unbiased predictor of efficacy at various doses of radiation exposure. While other compounds have been reported to have higher DRFs for radioprotection, these agents typically had significant toxic side effects [42].

Several laboratories have also investigated oral administration of genistein. Non-nanosuspension genistein was effective orally when given to mice in multiple daily gavages before irradiation [43–45]. However, a single oral dose (400 mg/kg) of non-nanosuspension genistein given 24 h before irradiation was not protective against H-ARS [44, 45]. Taken together, our findings indicate that genistein is an effective radioprotector when administered by injection or when given orally prior to radiation exposure. Further studies are required to determine the optimal dosing regimen for GEN when given orally prior to radiation exposure.

While we have characterized and validated the prophylactic radioprotective efficacy of GEN, there has been consistent interest in exploring the potential utility of administering a genistein therapy after radiation exposure. Recent work has described how the GEN formulation (BIO 300) was effective in mitigating the delayed effects of acute radiation exposure (DEARE) to the lung (DEARE-lung) in mice when administered daily via oral gavage for up to 6 weeks, starting 24 h after whole-thorax lung irradiation [16, 46]. This work was based on earlier findings by our group, which reported that genistein in a non-nanosuspension formulation protected against total-body irradiation–induced lung damage and weight loss when administered subcutaneously 24 h before irradiation [47]. Because GEN was shown to mitigate DEARE-lung, we explored whether the drug could be administered after radiation exposure to mitigate H-ARS. In the present study, no evidence of such mitigation against H-ARS was observed. We would note several differences between the findings in the lung and our findings with H-ARS in the mouse model. First, the distinct etiology between H-ARS and DEARE-lung. H-ARS occurs rapidly, with symptoms beginning 1–3 days after radiation, and lethality beginning ~10 days post-irradiation exposure. DEARE-lung is characterized by a slow, progressive disease that manifests as potentially lethal pneumonitis ~100 days post-exposure [47, 48]. Second, in the study presented here, a single-dose of GEN was administered, in contrast with published lung studies, where GEN was administered daily for 6 weeks beginning 24 h post-irradiation. Taken together, these findings indicate that GEN is effective as a radiation mitigator for DEARE-lung and as a radioprotector for H-ARS. Additional dose optimization and length of administration studies are warranted to further investigate the potential of GEN as a mitigator of H-ARS.

Our findings indicate that the estrogenic properties of genistein significantly contribute to the mechanism of its radioprotection. A component of estrogenic signaling has been known to be radioprotective since the 1940s [27, 31], and genistein’s classification as a phytoestrogen suggested that some portion of the radioprotective effects of genistein could be due to its estrogenic activity. Genistein was shown to selectively bind ERβ, with an ~20-fold binding preference for ERβ over ERα [34]. A structural comparison of ERα and ERβ bound to genistein indicates that key amino acids of the ligand-binding domain, Leu344 (ERα) vs Met336 (ERβ), differentially interact with genistein, likely contributing to genistein’s selectivity for ERβ [41]. Most notably, the activity of genistein-bound ERα is different from the activity of genistein-bound ERβ, based on the ability of the two genistein-bound receptors to recruit a co-activator, TIF2. Genistein-bound ERα recruited TIF-2 at 0.005% compared with estradiol-bound ERα. In contrast, genistein-bound ERβ recruited TIF-2 at ~60% compared with estradiol-bound ERβ [49]. This work suggested that the functional activities of the two ERs bound to genistein can vary by as much as 10,000-fold [41], even if there is only an ~20-fold difference in genistein binding to each receptor. Based on previous findings in addition to our current results with the CHO ER activity assay, we hypothesize that genistein’s radioprotective effects occur via activation of ERβ. ERβ is found throughout the body of both males and females. Current studies suggest that ERβ may have biological activity other than simply as a negative regulator of classic ERα signaling (for which it was originally attributed) [50]. A potential molecular mechanism for the cellular radioprotective effects of genistein may be provided by studies that demonstrated that genistein-bound ERβ increased the expression of DNA repair genes, including RAD51, FANCA and BRCA1 [51]. Our previous studies of genistein treatment for H-ARS and radiation-induced lung injury demonstrated increased DNA repair in vivo by genistein [47]. DNA repair is central to the efficiency of a radiation countermeasure, and further studies are required to determine whether DNA repair genes are regulated by genistein in vivo via ERβ.

The in vitro data presented here confirm that genistein (either solubilized in DMSO or in our nanosuspension) had an ~2000-fold selectivity for the activation of ERβ over ERα. Interestingly, in the case of ERβ, we observed that genistein activated this receptor in a biphasic manner. We cannot unequivocally determine the cause of this biphasic activity; however, biphasic activations have been reported in the literature for estrogen receptors. For example 17β-estradiol has been demonstrated to have concentration-dependent effects on the estrogen signaling in cells (mitogenic at low concentrations and anti–cell growth at high concentrations) [52]. This report and others have noted that non-genomic signaling of estrogen receptors may play an important role in this regulatory biology, and that a significant amount of ER-dependent transcription is regulated by protein–protein interactions. Genistein has been described as a partial-estrogen receptor agonist because of the structural differences in the ERs when bound by genistein, compared with their conformation when bound by 17β-estradiol [33]. Therefore, we can only speculate that the biphasic activation of ERβ that we observed was due to concentration-dependent protein–protein interactions that govern the activity of ERβ as a DNA-binding transcription factor.

In addition to radiation protection for the hematopoietic acute radiation injury described above, genistein alone or in combination with other compounds has been demonstrated to protect a variety of organs, including liver [53], kidney [54], intestine [55], lung [16, 47, 56] and testes [57] from ionizing radiation injury. Genistein as well as other radioprotective agents have been the subject of multiple reviews [42, 58–61]. The dependence upon ER activation by
genistein for radioprotection in other organ systems is currently unknown, and requires additional research.

Genistein is a very well-studied molecule, with over 11 000 articles listed on PubMed using the singular search term ‘genistein.’ However, the pharmacological use of genistein has been limited by its low solubility and bioavailability. Additionally, care should be taken when considering genistein’s potential pharmaceutical applications, as a number of reports of its biological activity utilized high molar concentrations in cell culture that are not relevant in vivo. Moreover, in many instances genistein was dissolved in organic solvents such as DMSO to improve biological activity. These concentrations of DMSO are not pharmacologically applicable, as the clinical delivery of drugs is not typically mediated by dissolution solely in organic solvents. Nonetheless, because of its therapeutic potential, there is significant interest in and focus on improving the bioavailability of genistein using pharmaceutically acceptable formulations. The patented and proprietary GEN described in this paper was developed by physically grinding synthetically prepared genistein into nanometer-sized genistein particles, via a wet nanomilling process. These nanoparticles are carried in a specific pharmaceutically formulated suspension, termed a nanosuspension [17, 18]. Other nano-technologies have been described to improve the biological activity of genistein, such as the use of lipid-based nanovesicles and nanoemulsions that serve as carriers of genistein. These lipid nanoparticles can be prepared using several different methodologies, which vary according to the orientation of the lipid layer(s) to the insoluble drug, as well as the presence of various surfactants, all of which change the molecular characteristics of the lipid nanoparticles [62]. Lipid nanoparticle technology has been applied to genistein in multiple studies for a variety of uses [63–65].

In summary, an ideal radioprotective agent should have several key characteristics: low toxicity, a practical mode of administration such as IM or oral, stability at ambient temperature, an extended shelf life, and a wide window for time of administration for efficacy. The results of the present study support the advanced development of a GEN as an effective medical radiation countermeasure.

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CONFLICT OF INTEREST

M.R.L. is retired from the Federal Government and now serves as a scientific advisor for Humanetics Corporation. M.R.L is a holder of patents for the use of isoflavones for radioprotection. M.D.K. is the Vice President of Research and Development and A.J.H. is an employee for Humanetics Corporation. R.M.D. declares no conflict of interest.

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