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HSP90 INHIBITOR GELDANAMYCIN AS A RADIATION RESPONSE MODIFICATOR IN HUMAN BLOOD CELLS

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□ Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone, involved in the folding, assembly, stabilization and activation of numerous proteins with unrelated amino acid sequences and functions. Geldanamycin (GA), a natural benzoquinone, can inhibit the chaperone activity of Hsp90. It has been shown that GA can produce superoxide anions and increase the intracellular oxidative stress, which, in addition to the direct inhibition of Hsp90, might also contribute to the modifying effects of the inhibitor on the early response in human mononuclear cells exposed to ionizing radiation. The present study shows that GA antagonizes the radiation-induced suppression on MnSOD and catalase, key enzymes of the radical scavenging systems. By significantly up-regulating catalase levels over the entire range of doses from 0.5 to 4 Gy, the inhibitor of Hsp90 exerted adaptive protection and modified the early radiation response of the human blood cells.

Key words: molecular chaperone Hsp90; geldanamycin, human lymphocytes; radiation response; antioxidant enzymes.

INTRODUCTION

Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone, involved in the folding, assembly, stabilization and activation of several hundreds of proteins referred to as Hsp90 "clients" (Zhao *et al.* 2005, Neckers 2007, Wandinger *et al.* 2008, Trepel *et al.* 2010, Samant *et al.* 2012, Sharma *et al.* 2012). Belonging to the clients of Hsp90 are proteins with unrelated amino acid sequences and functions, involved in signal transduction, protein trafficking, receptor maturation and innate and adaptive immunity (Taipale *et al.* 2010) as well as numerous oncoproteins. Hsp90 has been found to be up-regulated in a number of cancer types in comparison with normal tissues and this has been associated with poor prognosis and resistance to therapy (Yano *et al,* 1999, Becker *et al.* 2004). Due to its essential role in the survival of cancer cells, Hsp90 is an important therapeutic target which has led to the development of Hsp90 inhibitors applicable for cancer treatment. As a weak ATP-ase, Hsp90 uses the energy generated in a complex cycle of adenosine triphosphate (ATP) binding and hydrolysis to assist protein folding and

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maturation. Benzoquinones, such as the natural product geldanamycin (GA), and its derivatives 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-demethoxy-17-N,N-dimethylaminoethylaminogeldanamycin (17-DMAG) are inhibitors of Hsp90 and exert their pro-apoptotic effects on malignant cells through inhibition of ATP binding in the ATP/ADP-binding pocket of Hsp90. These inhibitors bind to the ATP-binding center of Hsp90 with higher affinity compared to the natural nucleotides thus blocking its chaperone activity, leaving the substrates of Hsp90 in an unfolded state, prone to proteolytic degradation. Therefore, the inhibition of Hsp90 has been proved selectively lethal to tumor cells (Lin *et al.* 2008), and the inhibitors of Hsp90, including GA and its derivatives have been used either on their own, or in combination with chemotherapy and/or radiotherapy in the treatment of solid tumors such as breast, prostate and lung cancers, malignant melanoma as well as of hematological malignancies like chronic lymphocytic leukemia (CLL), CML, AML and multiple myeloma (Didelot *et al.* 2007, Banerji 2009, Kabakov *et al.* 2010).

In addition to serving as molecular chaperones, Hsp90 and other chaperones have been implicated in autoimmune diseases, antigen presentation, and tumor immunity. Studies have demonstrated that Hsp90 plays a role in generating antigen-specific T-cell responses and activating the innate immune system (Tamura *et al.* 1997, Hauet-Broere *et al.* 2006, Van Eden *et al.* 2007). Hsp90 has been also found to induce the production of pro-inflammatory cytokines in autoimmune and chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus (Ripley *et al.* 2001, Rice *et al.* 2008). Quite recently, in accordance with the effects of the molecular chaperone on the immune system, it has been demonstrated that Hsp90 inhibitors at clinically relevant concentrations can modulate adaptive immune responses both on the level of dendritic cells (DC) activation and T cell proliferation (Trojandt *et al.* 2014).

It has also been found that GA and its derivatives can produce superoxide anions and increase the intracellular oxidative stress, which might also contribute to the antitumor effect of the inhibitor (Dikalov *et al.* 2002, Sreedhar *et al.* 2003). The pro-oxidant properties of GA have been explained by its electrophilic nature allowing the inhibitor to react with and deplete cellular glutathione (GSH) (Cysyk *et al.* 2006) and to oxidize thiol groups of critical cellular proteins including reactive cysteine residues of Hsp90. As the chaperone has been suggested to play a role in the maintenance of the redox status of the cytosol (Nardai *et al.* 2000), oxidizing cysteines of Hsp90, GA can inhibit the ability of Hsp90 to regulate cellular oxidative stress and disrupt its signaling networks (Clark *et al.* 2009), thus, contributing to cellular oxidative and unfolded protein responses.

Ionizing radiation is a strong inducer of reactive oxygen and nitrogen species - ROS and RNS, respectively (O'Neill and Wardman, 2009). Depending on their concentration, reactivity and distribution, these

species may mediate either adaptive/protective responses or genomic instability in the progeny of irradiated cells and their neighbors (bystander effects) (Azzam and Little, 2004, Brooks 2005, de Toledo and Azzam, 2006). Since GA can produce superoxide anions and increase the intracellular oxidative stress, the pre-treatment of the cells with GA may induce protective adaption in irradiated cells. To test this hypothesis, the present study analyses the impact of Hsp90 inhibition by GA on the early radiation response in human blood cells and provides evidence for a significant, dose-independent up-regulation of catalase in a broad range of doses from 0.5 to 4 Gy indicating induction of adaptive response by the reagent.

MATERIALS AND METHODS

Isolation and treatment of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from 3-5 ml of venous blood from healthy volunteer donors. Isolation was performed by density gradient, as previously described (Stankova *et al.* 2011, Ivanova *et al.* 2010, Stankova *et al.* 2013) and cells were re-suspended in RPMI 1640 medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA). For each experiment, cells from 3-5 donors were pooled right after the isolation, brought to a concentration of 2×10^6 cells/ml and split into samples which were subjected to different treatments: (A) 2 hours of incubation, followed by in vitro irradiation (^{60}Co gamma source, dose rate 7.5 Gy/min, Gitawa Ltd, Sofia, Bulgaria), and incubation for another 2 hours; (B) 4 hours of incubation in the presence of 0.1 μM GA (Sigma St Louis, MO, USA); (C) 2 hours of incubation in the presence of 0.1 μM GA, followed by irradiation (as described), and incubation for another 2 hours; (D) control samples, incubated for 4 hours without GA and non-irradiated. As the GA stock has been dissolved in DMSO (Sigma St Louis, MO, USA), amounts of DMSO corresponding to the volume of the chemical in the treated samples were added to all control samples.

Approval for the studies was obtained from the institutional review body, and informed consent from volunteer donors was obtained in accordance with the Declaration of Helsinki.

Toxicity of treatments with GA and/or radiation exposure

Toxic effects due to the treatment of the cells with GA and/or irradiation were analyzed using the fluorescent dye calcein AM (Molecular Probes Inc., USA), as it has been previously described (Stankova *et al.* 2013). Changes in the cellular viability were determined in three independent experiments and calculated as the percentage ratio of the fluorescence emissions of the treated and/or irradiated samples versus non-treated, non-irradiated controls. The levels of apoptotic cells were determined using the commercially available Caspase 3 colorimetric assay kit (Sigma

St Louis, MO, USA) according to the manufacturer's instructions for the 96-well plate microassay method (Stankova *et al.* 2013). Apoptosis was analyzed 4 and 24 h after treatment with GA and 22 h post-irradiation.

Levels of exogenous ROS after treatment and/or irradiation of PBMC

Changes in the intracellular levels of the reactive oxygen species (ROS) were analyzed by the fluorescent dye 2',7'-dichlorodihydro fluorescein diacetate (DCDHF-DA, Molecular probes, Life Technologies Corporation) as it has been already described by Stankova *et al.* 2013. Data represents relative increase in DCDHF fluorescence, reflecting changes in the intracellular ROS content in comparison to the non-treated, non-irradiated controls.

Western Blot analysis

Protein levels of the antioxidant enzymes MnSOD (manganese superoxide dismutase) and catalase, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and Nrf2 (nuclear factor (erythroid-derived 2)-like 2) were determined by Western blots in whole-cell extracts as described (Ivanova *et al.* 2010, Stankova *et al.* 2013). Blots were visualized with 3, 3'-diaminobenzidine (DAB) peroxidase substrate (Sigma St Louis, MO, USA). Optical densities of the protein bands were analyzed using the Labworks 4.0 software package (UVP Bioimaging Systems, Cambridge, UK). Changes in protein levels are expressed relative to the level of the non-treated, non-irradiated controls and represent the average values of at least five independent experiments performed with different pools of patients' PBMC.

Statistics

All datapoints represent the mean and standard deviation from at least three independent experiments, performed with different pools of patients' PBMC. The experimental data was analyzed by the ANOVA (ANalysis Of VAriance) method. The contribution of each group was characterized by Scheffe test for multiple comparisons. Possible cooperative effects between the factors (GA and radiation) were analyzed by factorial ANOVA, followed by multiple endpoint Post-hoc comparison with the Scheffe test and $p < 0.05$ was considered statistically significant (de Sa 2007).

RESULTS AND DISCUSSION

Effects of GA on the viability of PBMC

Treatment of PBMC with 0.1 μM GA and/or radiation exposure to doses ranging up to 4 Gy did not affect significantly the viability of PBMC

as determined by the calcein AM assay (Figure!1, $F_{5/22}$=2.42, p=0.07). The observation was confirmed by the Caspase-3 activity-based apoptotic test (Figure!2), applied 4 h and 24 h after the *in vitro* exposure of the cells to GA and/or 3 Gy of gamma rays (γ-rays). As seen in Figure 2, apoptosis

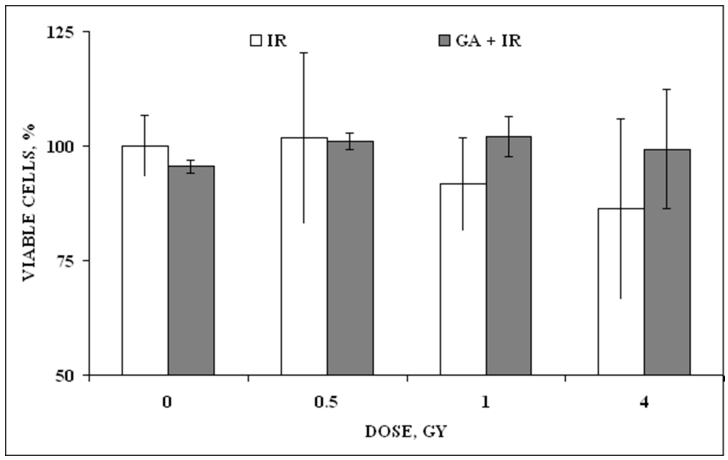

FIGURE 1. Dose-dependence of cellular viability, measured 2 h post-irradiation in lymphocytes pre-treated for 2 h or not treated with GA and exposed to doses ranging from 0.5 to 4 Gy of gamma rays. Exposure to 4 Gy induced statistically significant reduction in the viability of non-treated lymphocytes ($F_{5/51}$=2.54, p=0.0395). Pre-treatment with GA abolished the IR-induced reduction in the viability which remained close to those of the non-treated, non-irradiated controls. Error bars represent the standard deviation (+/-SD) of the mean for n=3 independent experiments performed with different pools of patients' PBMC.

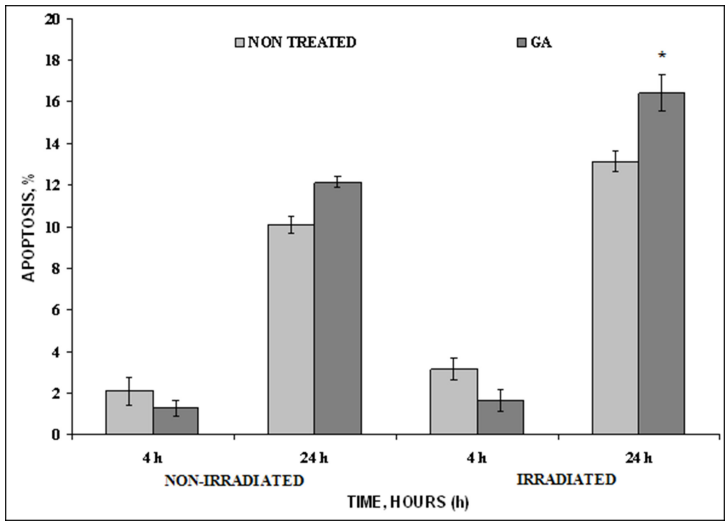

FIGURE 2. Effects of the Hsp90 inhibitor GA on human PBMC. PBMC were treated for 4 h and 24 h with GA and/or exposed to 3 Gy of gamma rays. Factorial ANOVA, followed by multiple endpoint Post-hoc comparison with the Scheffe test revealed statistically significant differences (*p<0.01) between lymphocytes treated with GA and irradiated versus non-treated but irradiated lymphocytes, measured 24 h post-irradiation.

measured 24 h post-exposure, was lowest (10-11%) in the control lymphocytes, not exposed to GA or radiation. *In vitro* irradiation of the cells with 3 Gy slightly increased the apoptotic levels and the magnitude of the effect was dependent on whether the cells were pre-treated with GA or not (Figure 2).

GA increases the cellular oxidative stress and up-regulates key antioxidant enzymes as part of the early radiation response in PBMC

It has been demonstrated that GA can increase the intracellular superoxide formation by enzymatic and non-enzymatic redox pathways (Dikalov *et al.* 2002). Accordingly, we found that 0.1 μM GA elevated the intracellular ROS in PBMC up to 145-150% ($F_{5/12}$=62.068, p<0.0001). Exposure of the cells to different doses of gamma ionizing radiation (IR) ranging from 0.5 to 4 Gy, in addition to their treatment with GA, led to dramatic increase of ROS which reached supra-physiological levels, exceeding several-fold the levels of the endogenous ROS. The effect was linearly dose-dependent (r^2 = 0.99) and statistically significant (Figure!3, $F_{1/16}$ = 9101.914, p<0.0001) as revealed by the linear regression analysis of the data. Cells pretreated with GA, generated higher levels of ROS in the whole dose range from 0.5 to 4 Gy (Figure 3) which, however, were shown to attenuate faster as compared to the decay kinetics of ROS measured in the cells which were not pre-treated with the reagent

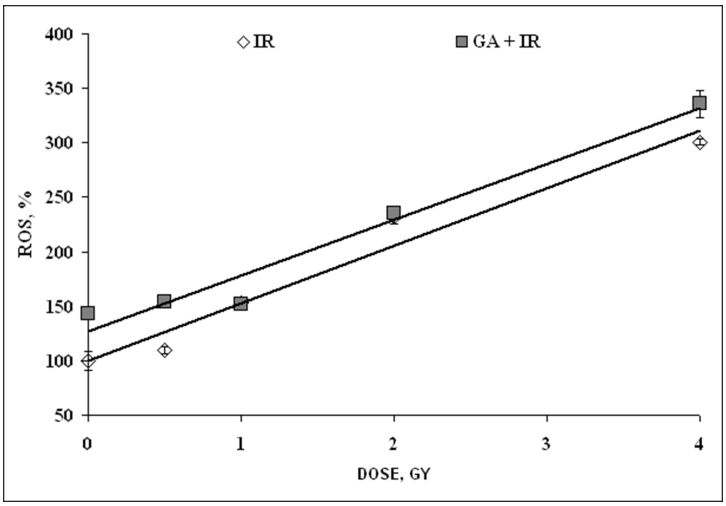

FIGURE 3. Effects of Hsp90 inhibition on intracellular ROS. Dose-dependence of ROS levels in PBMC either pre-treated for 2 h or not treated with GA and exposed to different doses of gamma radiation, analyzed 2 h post-irradiation. Data represents a percentage ratio of the emission of DCDHF-DA at 535 nm (excitation at 485 nm) measured in samples pre-treated with GA and/or irradiated versus the emission of DCDHF-DA in non-treated and non-irradiated controls. Error bars indicate the standard deviation (+/-SD) of the mean for n=3 independent experiments performed with different pools of patients' PBMC.

(Figure!4) and approaching equal levels at later time points post-irradiation (Figure 4). This might reflect some GA-induced stimulation of the cellular antioxidant defense due to up-regulation of certain antioxidant enzymes. Indeed, as seen in Figure!5, the reagent up-regulated MnSOD and catalase levels up to 130 and 115%, respectively. The stimulating effect of GA on the antioxidant enzymes was also well pronounced in the irradiated cells; the reagent effectively counteracted the radiation-induced suppression on MnSOD and catalase over the entire dose range (from 0.5 to 4 Gy). Maintaining the MnSOD levels higher or equal to those in the control (Figure 5a), and dramatically up-regulating catalase (Figure 5b; $F_{4/29}$=4.798, p=0.0043), the reagent efficiently antagonized the radiation-induced suppression on the two enzymes and contributed to the antioxidant capacity of the cells in the early stages of their radiation response. Notably, the stimulating effect of GA on catalase was non-linear and dose-independent, thus suggesting induction of protective adaptation upon pre-treatment of the cells with the reagent.

Since the stress-response NF-κB and Nrf2 pathways contribute to cellular redox homeostasis by inducing the expression of antioxidant genes, including those of MnSOD and catalase (Kensler *et al.* 2007, Ahmed and Li, 2008, Holley *et al.* 2010), and on the other hand Hsp90 is directly involved in the regulation of these two pathways (Hertlein *et al.* 2010, Niture and Jaiswal, 2010), the current study aimed to also analyze the effects of GA and/or irradiation on the protein levels of NF-κB and Nrf2.

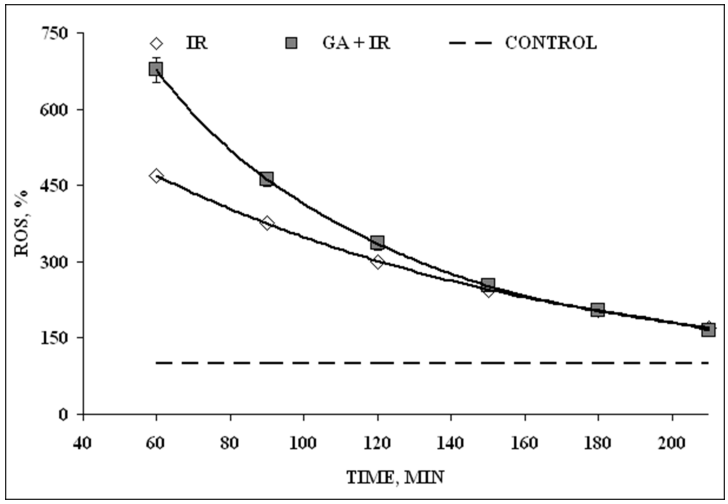

FIGURE 4. Effects of Hsp90 inhibition on intracellular ROS. Kinetics of ROS decay in PBMC, pre-treated or not treated with GA, and exposed to 4 Gy of gamma rays. Data represent a percentage ratio of the emission of DCDHF-DA at 535 nm (excitation at 485 nm) measured in samples pre-treated with GA and/or irradiated versus the emission of DCDHF-DA in non-treated and non-irradiated controls. Error bars indicate the standard deviation (+/-SD) of the mean for n=3 independent experiments performed with different pools of patients' PBMC.

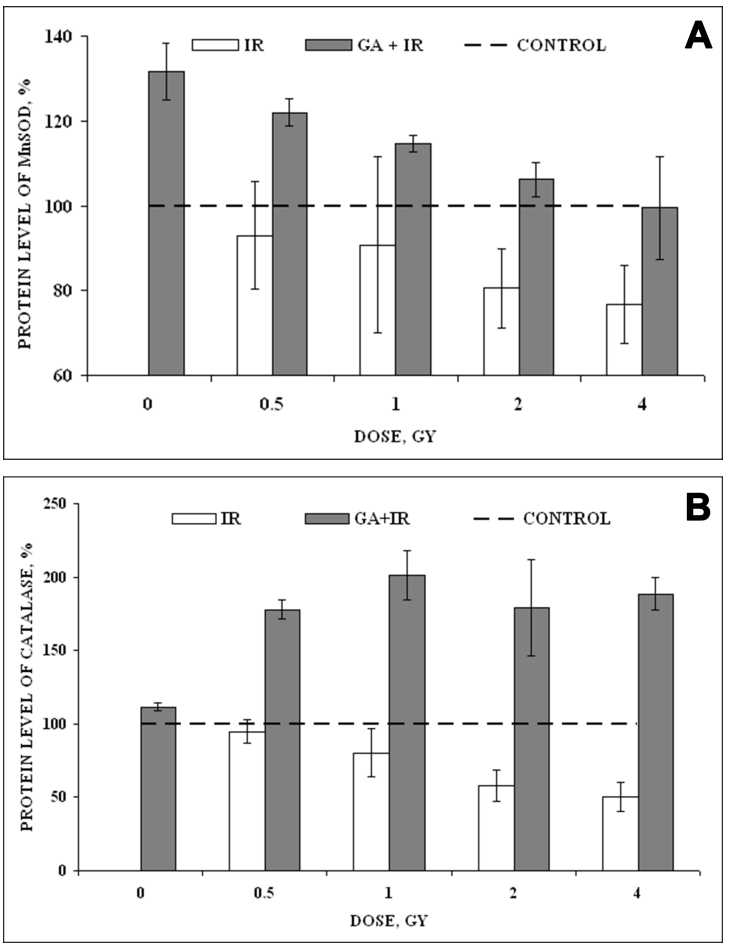

FIGURE 5. Effects of Hsp90 inhibition on radiation-induced changes in MnSOD (5a) and catalase (5b) in PBMC pre-treated for 2 h with geldanamycin. Changes in protein levels are presented relative to the protein levels in non-treated, non-irradiated PBMC which were referred to as 100% (dotted lines). Error bars represent the standard deviation (+/-SD)of the mean for n=5 independent experiments performed with different pools of patients' PBMC.

It was found that NF-κB and Nrf2 levels remained similar to those in the control cells which were not exposed to GA and/or radiation (data not shown), in accordance with other recent studies which have demonstrated that NF-κB and Nrf2 are highly expressed in CLL PBMC but not in the normal PBMC (Hertlein *et al.* 2010, Wu *et al.* 2010). The Hsp90 inhibitor has been identified as a potent inducer of the endoplasmic reticulum (ER) stress and unfolded protein responses leading to disruption of mitochondrial homeostasis (Lawson *et al.* 1998). Thus, pathways, connected with the unfolded protein responses may be predominantly involved in the up-regulation of the antioxidant enzymes MnSOD and catalase observed here. The superoxide, accumulated in the cells pre-treated with

GA, may act as a mediator of adaptive ROS signal which activates pathways linking the cellular antioxidant response to unfolded protein response (Sarbassov and Sabatini 2005) as shown by the concerted up-regulation of key enzymes of the radical scavenging systems such as MnSOD and catalase. This can modify significantly the early dose response and exert protective effects on the blood cells exposed to lethal stress, such as an acute exposure to high doses of ionizing radiation. Similar protective effect has been demonstrated in normal human keratinocytes, where the overexpression of catalase has been shown to decrease significantly the UV-induced apoptosis after reducing the caspase-9 activation and p53 levels (Rezvani *et al.* 2006).

CONCLUSION

We have shown that GA, inhibitor of Hsp90, can induce changes in ROS signaling and protein homeostasis, and promote protective radio adaptation in normal human lymphocytes. GA up-regulates the protein levels of MnSOD and catalase, two key antioxidant enzymes. The elevation in MnSOD levels induced by GA completely compensated the radiation-induced down-regulation of the enzyme levels. The reagent increased dramatically the protein levels of catalase in the irradiated cells exposed to doses up to 1 Gy and the elevation remained constant at higher doses ranging from 1 to 4 Gy, as part of the early dose-response of lymphocytes. This effect of GA on catalase seems of particular interest as it is nonlinear and may suggest a role of GA as a priming agent inducing protective adaptation of the blood cells to radiation exposure and lowering radiation injury.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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