

# Pharmacological Countermeasures for the Acute Radiation Syndrome

Mang Xiao\* and Mark H. Whitnall

*Radiation Countermeasures Program, Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD 20889, USA*

**Abstract:** The acute radiation syndrome (ARS) is defined as the signs and symptoms that occur within several months after exposure to ionizing radiation (IR). This syndrome develops after total- or partial-body irradiation at a relatively high dose (above about 1 Gy in humans) and dose rate. Normal tissue injuries induced by IR differ depending on the target organ and cell type. Organs and cells with high sensitivity to radiation include the skin, the hematopoietic system, the gut, the spermatogenic cells and the vascular system. Exposure to IR causes damage to DNA, protein, and lipids in mammalian cells, as well as increased mitochondria-dependent generation of reactive oxygen species (ROS), with subsequent cell cycle checkpoint arrest, apoptosis, and stress-related responses. DNA double strand breaks (DSBs) are a primary lethal lesion induced by IR. The cellular response to damage is complex and relies on simultaneous activation of a number of signaling networks. Among these, the activation of DNA non-homologous end-joining (NHEJ) and homologous recombination (HR), and signaling pathways containing ataxia telangiectasia mutated (ATM), play important roles. The transcription factor NF $\kappa$ B has emerged as a pro-survival actor in response to IR in ATM and p53-induced protein with a death domain (PIDD) cascades. Although radiation-induced ARS has been well documented at the clinical level, and mechanistic information is accumulating, successful prophylaxis and treatment for ARS is problematic, even with the use of supportive care and growth factors. There is a pressing need to develop radiation countermeasures that can be used both in the clinic, for small-scale incidents, and outside the clinic, in mass casualty scenarios. In this review we summarize recent information on intracellular and extracellular signaling pathways relevant to radiation countermeasure research.

**Keywords:** Acute radiation syndrome, ionizing radiation injury, radiation countermeasures, apoptosis, DNA repair, free radical scavengers, radioprotection, cytokines, growth factors, p53, ATM.

## INTRODUCTION

Today, more than 50% of cancer patients receive radiotherapy at some time during their disease [1]. In addition, nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials make incidents involving radiation injuries increasingly likely. However, the mechanisms through which radiation injury become manifest are not well understood [2]. Medical approaches to ARS aim to prevent or ameliorate radiation injury with radioprotectants or radiation mitigators, or by stimulating regeneration of tissue with post-radiation treatment [1-5]. After decades of work on radiation countermeasures [3], no pharmaceuticals are currently approved for ARS. In this review, information on specific intracellular and extracellular signaling pathways relevant to radiation countermeasure research will be discussed.

ARS, also referred to as radiation sickness, is an acute illness caused by high doses of penetrating IR to most or all of the body in a relatively short time period. High doses of internalized radionuclides can also cause ARS. The first organs to be affected by systemic irradiation are those with relatively rapid cell proliferation, and these cells and their respective organs are among the most sensitive to radiation injury [2, 4, 5]. The most radiation sensitive organs include the hematopoietic [6], gastrointestinal (GI) [7], skin [8], spermatogenic [9], and vascular systems [10, 11]. The moderate dose range (1-7 Gy in humans) of exposure to IR poses a risk of damage to the hematopoietic system, leading to decreases in blood cell and platelet counts and increased susceptibility to infection and hemorrhage [2, 5]. Radiation also induces loss of intestinal crypts and breakdown of the GI mucosal barrier. The acute generalized GI syndrome appears after high-dose (about 8 Gy or more) whole-body irradiation. IR induces GI hemorrhage, endotoxemia, bacterial infection, anorexia, nausea, vomiting, diarrhea, and loss of electrolytes and fluid [12]. Cutaneous injury from thermal or radiation burns is characterized by loss of epidermis and dermis. Injuries to the skin may cover small areas but extend deeply into the soft tissue, even reaching underlying muscle and bone [13]. IR not only affects the proliferative capacity of skin stem cells, but also modu-

lates the communication between cells, leading to impaired cutaneous integrity [8]. Injury to the central nervous system (CNS) and cerebrovascular system after higher doses of IR may be a consequence of damage to the vascular endothelium [10]. The concomitant and interdependent injuries to various organ systems during ARS has led some authors to prefer the term "multi-organ failure" rather than organ-specific "syndromes" [14, 15]. This review will focus on the immediate injury and response mechanisms in irradiated cells exposed to external penetrating radiation, and will not address the physiological networks involved in multi-organ failure, or late effects such as cancer or fibrosis. Moreover, the discussion will be relevant to basic cellular mechanisms occurring in response to radiation, not pharmacological measures aimed at chelating internalized radionuclides, for example.

Radiation qualities can be divided into low linear energy transfer (LET) types (e.g., photons (gamma and X-rays) and beta particles), and high-LET types (e.g., neutrons, alpha particles, cosmic ray heavy particles) [16]. Neutrons and photons can pass through skin and into tissues easily, while alpha and beta particles cannot. A radiation dispersal device (RDD, or "dirty bomb") would have a low likelihood of causing radiation injury, but there is some possibility of exposure to external penetrating low LET radiation from "groundshine", or exposure to low and/or high LET radiation from internalized radionuclides [17]. Covert placement of a radioactive source would involve gamma rays. A nuclear detonation would produce an initial burst of neutrons and gamma rays, but much exposure would occur subsequently due to gamma rays emitted from fallout [18]. A further danger from fallout is beta-emitting particles deposited on the skin or internalized [18]. Astronauts are exposed to high-LET cosmic rays as well as a mixture of high-LET and some photons in solar flares, at doses which may cause late effects but not ARS [19].

The degree of chromosomal damage is proportional to the absorbed dose of radiation, a principal which is useful for cytogenetic approaches to assessing doses of radiation received [20]. High- and low-LET irradiation produce different types of cellular damage: photons (gamma and X-rays) cause most damage indirectly *via* formation of free radicals, while high-LET radiation causes more direct damage [21]. The DNA damage from high-LET irradiation is more complex and difficult to repair than that from low-LET irra-

\*Address correspondence to this author at the SRD-AFRRI, 8901 Wisconsin Ave, Bethesda, MD 20889-5603, USA; Tel: 301-295-2597; Fax: 301-295-0345; E-mail: xiao@afri.usuhs.mil

diation [21-24]. The distinct types of DNA damage from different qualities of radiation may lead to differences in the DNA-damage response pathways triggered [24, 25]. The relative biological effectiveness (RBE) of neutrons compared to gamma differs according to which cell type or tissue is being analyzed [26]. Therefore, the efficacy of different radiation countermeasures may be different for gamma or neutrons, depending on the cell/tissue being targeted, and whether the mechanism of action involves DNA repair mechanisms.

Radiation-induced adjustment in cellular tissue homeostasis triggered by various molecular responses related to inter- and intracellular signaling cause both acute and late effects [1]. Acute exposure to IR causes damage to macromolecules, as well as increased mitochondria-dependent generation of reactive oxygen species (ROS), with subsequent cell cycle checkpoint arrest, apoptosis, and stress-related responses in mammalian cells [27]. IR-induced cellular responses involve complex signaling pathways in a cell type-dependent manner. These pathways include the activation of plasma membrane receptors, ceramide synthesis, protein kinases, and regulation of pre- and/or post-transcriptional biological pathways [28]. These pathways act simultaneously after IR. Cell survival or death after IR depends on the amount and severity of lesions, and on the balance between repair and apoptosis regulated by those pathways. The signaling cascades are described in more detail below.

Different mechanistic approaches may be necessary for prophylaxis (given before IR) and therapy (given after IR). The goals of therapy in terms of enhancing short-term survival would be to decrease ongoing injury and cell death and promote regeneration. The goals of prophylaxis may be to set processes in motion that would promote those effects after IR. However, prophylaxis is more often thought of in terms of scavenging free radicals or shifting cells into a state of higher radioresistance. The latter might be accomplished by inducing a state of quiescence, or shifting cells into a part of the cell cycle that is more radioresistant. Higher radioresistance during

some stages [29] might be due to increased activity of genes promoting DNA repair or genomic integrity, or higher levels of anti-oxidant molecules.

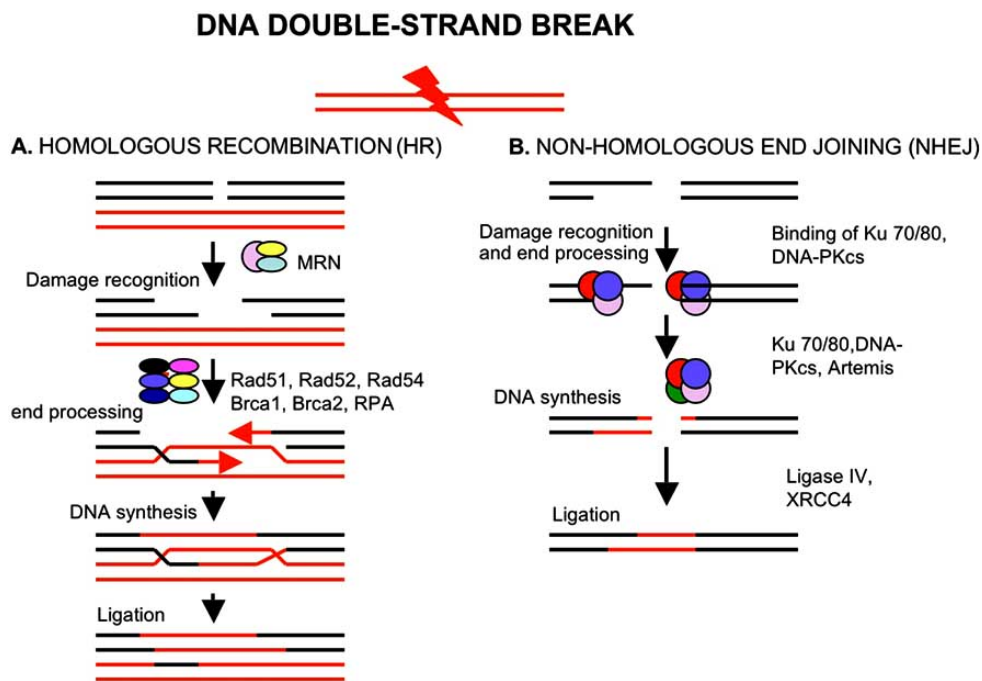
## PATHWAYS FOR IONIZING RADIATION-INDUCED SIGNAL TRANSDUCTION PROCESSES

In this review, we use the phrase “signal transduction” to refer to all signaling cascades within the cell.

### DNA Repair (Fig. 1)

Although recent studies in bacteria after very high doses of IR have pointed to protein damage as being the primary determinant of radiation sensitivity [30], a critical target of IR-induced cell death in eukaryotes has generally been considered to be DNA, including base damage, single strand breaks (SSBs), double strand breaks (DSBs) of varying complexity, and DNA cross links. Among these, DSBs are the major lethal lesion [31, 32]. Jeggo *et al.* [33] recently summarized the radiation-induced DNA damage responses in different cell cycle stages. The role of response mechanisms such as DNA repair and signal transduction regulation is to maintain genomic stability in a multifactorial, cooperative manner, with specific responses dependent upon the cell type and cell state. Two repair pathways, HR and NHEJ, efficiently repair the majority of DSBs [34-36].

The majority (80%-90%) of DSB repair involves NHEJ [37, 38], a process that requires Ku (Ku70 and K80), DNA-protein kinase complex catalytic subunit (DNA-PKcs), Artemis, polymerase, Xrcc4, and DNA ligase IV as core components [34, 39]. Ku is the first protein to recognize and bind at a DSB's free DNA ends [33]. The heterodimeric Ku70/Ku80 protein, once bound to DNA ends (Ku:DNA), improves the affinity of the relevant nuclease (Artemis and DNA-PKcs), polymerase ( $\mu$  and  $\lambda$ ), and ligase (XRCC4: DNA ligase IV) for each DNA end on each strand [40,



**Fig. (1). Simplified overview of DNA double-strand break (DSB) repair pathways.** A. HR repairs DSBs using a homologous stretch on a sister chromatid. DNA damage is first recognized by MRN (Mre11, Rad50, Nbs1) and the DNA ends are subsequently processed as single-strand overhangs. Rad51, Rad 52, Rad 54, RPA, BRCA1 and BRCA2 associate with these overhangs and participate in the repair processing to join molecules by the damaged and undamaged DNA strands. Template-guided DNA synthesis repairs the DSBs in HR.

B. NHEJ repairs broken DNA ends by first bringing DNA ends, Ku 70/80 and DNA-PKcs together and forming a synaptic complex which allow scanning, annealing, and ligation of short homologous patches of DNA.

41]. Subsequently, the Artemis and DNA-PKcs endonuclease cleaves DSB 5' and 3' overhangs, hairpins, gaps, flaps and various loop conformations, and facilitates subsequent repair processes: DNA end modifications on oxidized overhangs, repair of gaps and 5' overhangs by polymerase  $\mu$  and  $\lambda$ , and actions of the XRCC4: DNA ligase IV complex in converting DSB to SSB. Nuclease and polymerase activities fine-tune the remaining DNA strand for ligation. The order of the enzymatic steps and the biochemical properties of the enzymes are crucial in the DSB repair process. Recent reports suggest that Poly (ADP-ribose) polymerase-1 (PARP-1), the abundant nuclear enzyme of eukaryotes, is an additional contributor in SSB and DSB repair [42]. Wang *et al.* [43] demonstrated that PARP-1 binds to DNA ends in direct competition with Ku, and together with DNA ligase III acts as a backup to the pathway of DSB NHEJ. Another key player in DSB repair is ATM, which cooperates with Artemis in 10% of DSB NHEJ repairs and makes a significant contribution to survival after radiation injury [37].

HR represents another important DSB rejoining process [44-46]. In mammalian cells, HR functions primarily in cell cycle S or early G<sub>2</sub> phase using an undamaged homologue from a sister chromatid to repair a DSB. Therefore, HR provides a process which is capable of repair in high fidelity, even if sequence information is lost at the site of the break. Proteins involved in HR are Mre11, Rad50, Nbs1 (MRN) and Rad54, Brca1, Brca2, Rad51B, rad51C, and Rad 51D. The MRN complex is a sensor of DSB involved in

the early steps of HR. Rad51 is the central protein in HR which searches the genome for an intact copy of broken DNA on the sister chromatid.

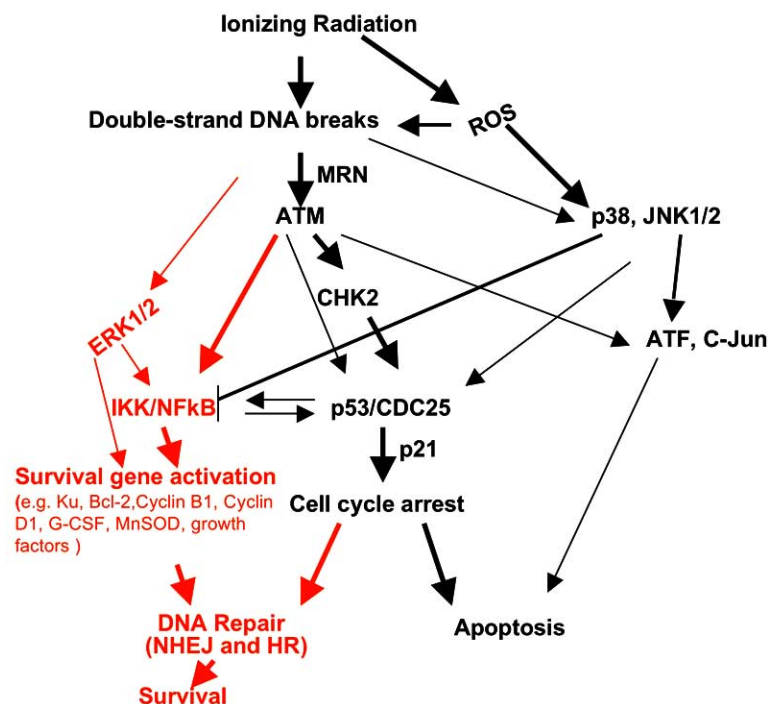
### Damage Response Signal Transduction Pathways (Fig. 2)

Signal transduction mechanisms in responding to radiation damage include cell cycle checkpoint arrest, DNA repair, and apoptosis. ATM, the protein defective in the hereditary disorder ataxia telangiectasia, plays a major role in the signal transduction response to DSBs [47]. DSB-stimulated ATM phosphorylation occurs within minutes and is stable for many hours [48, 49]. ATM regulates cell cycle arrest checkpoints to allow more time for repair, or initiates permanent cell cycle arrest and apoptosis if DNA damage is not repairable.

ATM, as a phosphoinositol 3-kinase like kinase (PIKK), activates cell cycle checkpoint arrest factors that promote arrest at the G<sub>1</sub>/S boundary, inhibition of late origin firing (intra-S), and prevention of entry into mitosis (G<sub>2</sub>/M) [33, 36, 50, 51]. After detection of a DSB by sensor proteins, an early step in ATM and DNA-PK signaling is phosphorylation of the histone variant H2AX at serine 139, generating  $\gamma$ -H2AX [52].

Immunocytochemical assays with antibodies recognizing  $\gamma$ -H2AX have become the gold standard for detection of DSBs, since there is close to a 1:1 relationship between the numbers of DSBs

## Radiation-induced activation of intracellular signal pathways



**Fig. (2). Radiation-induced activation of intracellular signal pathways.** Ionizing radiation (IR) causes DNA DSBs and increased ROS, which induce activation of intracellular signal pathways in mammalian cells. ATM is a central player in the cellular response to IR-induced DNA DSBs. The sensor protein Mre11, Rad50, and Nbs1 (MRN) complex binds directly to damaged DNA and recruits ATM through autophosphorylation and monomerization. Activated ATM induces the downstream checkpoint protein kinase CHK2, as well as p53 activation. CHK2 recruits downstream p53 and CDC25 phosphorylation and subsequent cell cycle arrest checkpoint regulation, which allows more time for repair, or leads to permanent cell cycle arrest and apoptosis if the DNA damage is not repairable. ATM is also required for the activation of NF $\kappa$ B in response to IR-induced DNA lesions, through its effects on the IKK/NF $\kappa$ B pathway. NF $\kappa$ B is a pro-survival factor which induces gene activation and supports DNA repair. Another important signal pathway in response to ROS is the MAP kinase pathway. IR induces ERK1/2 activity, which increases expression of DNA repair factors and cell growth factors. In contrast, other MAPK members, JNK and p38, have been linked to activation of pro-apoptotic factors. IR activates multiple signal pathways simultaneously and induces cross-talk between pro-apoptosis and pro-survival signals.

and  $\gamma$ -H2AX foci formed [53]. Furthermore, the rate of DSB repair correlates with the rate of loss of  $\gamma$ -H2AX foci [37, 53, 54]. Phosphorylated H2AX triggers a signal transduction pathway involving the protein CHK2, subsequent activation of the transcription factors p53 and/or CDC25, and eventual cell cycle arrest through the cyclins and Cdks inhibition. ATM also directly phosphorylates p53, which transcriptionally activates the Cdk inhibitor p21, and arrests cell cycle at G1/S [55, 56]. However, a one-to-one relationship between DSB and  $\gamma$ -H2AX signal is not always obtained with IR-induced DNA damage, so this assay must be used with caution. Bouquet *et al.* [57] suggested that DNA repair and  $\gamma$ -H2AX loss are correlated only when less than 100-150 DSBs are produced per genome. Moreover, Cowell and colleagues [58] demonstrated that, although it is believed that the chromosomal distribution of X-ray induced DSBs is random, the phosphorylation of  $\gamma$ -H2AX occurs in primarily euchromatic regions of the genome following X-ray radiation. Their data indicated a block to H2AX phosphorylation in heterochromatin that is at least partially relieved by ongoing DNA replication.

Another method to measure DNA damage is the comet assay [32, 59, 60]. With comet analysis software, the comet assay can quantify DNA damage from individual cells. A novel technique combines the comet assay with fluorescence in situ-hybridization (FISH), providing a way to measure DNA damage and repair in specific genomic regions [60].

Recent evidence demonstrates ATM is also required for the activation of NF $\kappa$ B in response to DNA lesions caused by various genotoxic stresses, including IR [61, 62]. Interestingly, anthracyclin-like molecules, as well as TNF and LPS that do not cause DNA breaks, activate NF $\kappa$ B in an ATM-independent way [63-65]. In contrast, the NF $\kappa$ B response to IR in ATM-deficient cells is abrogated, both in cell lines and in primary mouse tissue [65, 66], indicating the critical role of ATM in radiation-induced NF $\kappa$ B activation. Analyzing transcriptional networks, Elkon, Rashi-Elkeles *et al.* [67, 68] observed that NF $\kappa$ B and p53 mediate most of the damage-induced gene activation controlled by ATM. They further determined that the pro-apoptotic pathways mediated by p53 targets, and the pro-survival pathways mediated by NF $\kappa$ B targets, were simultaneously induced by ATM in murine lymphoid tissue in response to IR. Production of phosphorylated ATM (ATM<sup>P</sup>) [55] induced by DSBs stimulates p53 phosphorylation and activation. Phosphorylated P53 further induces PIDD activation [69]. Activated PIDD associates with receptor interacting protein 1 (RIP1) and NF $\kappa$ B-essential modifier (NEMO, also known as IKK $\gamma$ ) [70], and translocates into the nucleus [71]. In the nucleus, ATM<sup>P</sup> binds to and phosphorylates NEMO (NEMO<sup>P</sup>). Subsequently, the complex exits the nucleus and associates with IKK $\alpha$  and IKK $\beta$ . The IKK complex releases NF $\kappa$ B from its inhibitor, I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$ , and unbound NF $\kappa$ B (mostly p65/p50) is then free to move into the nucleus and regulate numerous target genes [72, 73]. The full range of NF $\kappa$ B target genes is yet to be determined. A review article by Ahmed and Li [74] describes the NF $\kappa$ B signaling network, including DNA repair, cell cycle checkpoint regulation, mitochondrial antioxidants, survival and apoptosis, and cytokine and chemokine expression in response to radiation-induced damage.

### Reactive Oxygen Species (ROS) and Growth Factor Receptor Pathways

Ionizing events generated by low-LET radiation in the cytosol amplify and generate large amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) via a mitochondrial mechanism in mammalian cells [75, 76]. Damage due to high-LET radiation such as neutrons is dependent to a greater extent on direct action on molecular targets, rather than on generation of free radicals [77]. Therefore free radical scavengers are useful to prevent damage from photons but not from neutrons or alpha particles [16],

and free radical-triggered pathways may have less relevance for high-LET radiation than for low-LET radiation.

Although ROS and RNS are required for the physiologic function of cells, overproduction of these radicals damages cellular components such as DNA, proteins, and lipids, and activates intracellular signaling pathways. After irradiation by X- or gamma rays, ROS are formed from hydrolysis of water in the cell. ROS in the nucleus cause DNA damage which triggers multiple signaling pathways leading to repair or cell death (see below). However, generation of free radicals in the cytoplasm activates a number of signaling pathways involved in growth, apoptosis, and stress response [78]. These injuries can lead to cell-cycle arrest, transformation, and cell death. The mechanisms of signaling pathway activation downstream of ROS are not well understood, and very little is known about the target genes that are transcriptionally activated by different doses of ROS. Mikkelsen has proposed that RNS are involved in extension of radiation-induced ROS signals in time and space within the cell, since ROS are short-lived and extremely reactive, while RNS are longer-lived and more specific in their reactions [78]. Formation of the peroxyxynitrite anion from reaction of nitric oxide with superoxide exacerbates oxidative stress and has both positive and negative effects on apoptotic pathways [79, 80]. Inhibition of apoptotic molecules can result from S-nitrosylation, but NO can also trigger intrinsic mitochondrial apoptotic pathways [80, 81].

ROS and RNS inhibit protein tyrosine phosphatase (PTPase) activation, resulting in increased tyrosine phosphorylation of multiple proteins, including growth factor receptors (plasma membrane receptors) such as the epidermal growth factor receptor (ERBB) protein family [78, 82]. IR-induced protein receptor activation promotes downstream intracellular signaling including mitogen-activated protein kinase (MAPK) and PI3K pathways [83]. Radiation-induced receptor activation can result both from radiation-induced release of autocrine ligands and from direct ligand-independent activation of receptors [83].

An interesting question with regard to radiation-induced free radicals is how they can produce significant effects when the background levels of these radicals are 100-1000 fold higher than the levels induced by the immediate ionization events [78, 84]. It has been proposed that a reversible permeability transition that releases Ca<sup>2+</sup> is propagated from one mitochondrion to neighboring mitochondria [78]. It is suggested that ROS-induced ROS release accompanies the mitochondrial permeability transition, which propagates and amplifies the redox signal [85].

ROS can be eliminated by natural scavengers such as intracellular thiols and antioxidant enzymes. The enzyme superoxide dismutase (SOD, MnSOD or Cu/ZnSOD), converts superoxide anions to H<sub>2</sub>O<sub>2</sub> [86, 87]. H<sub>2</sub>O<sub>2</sub> is subsequently detoxified by catalase or glutathione peroxidase. This redox regulation is essential for protecting cells from apoptosis. Recent studies demonstrated that NF $\kappa$ B is one of the key regulatory molecules in oxidative stress-induced cell activation in which ROS act as second messengers [88]. MnSOD is the primary antioxidant enzyme that regulates the cell response to oxidative stress. Interestingly, the NF $\kappa$ B element has been shown to be essential for induction of MnSOD by TNF and IL-1 $\beta$  [89]. IR-induced growth factor receptor activation can also promote the RAS-RAF-MAPK and RAS-PI3K-AKT pathways [90-92], which regulate cell survival and apoptosis.

The MAPK pathways include both pro-apoptotic and pro-survival regulators. IR-induced extracellular signal-regulated kinase 1/2 (ERK1/2) [93] activity is a typical pro-survival MAPK pathway, which increases expression of DNA repair factors and cell growth factors. In contrast, other MAPK members such as JNK and p38 [94, 95], have been linked to activation of pro-apoptotic factors including BAX and BAK, and the promotion of mitochondrial dysfunction.

PI3K and AKT have been widely studied as cell survival factors. PI3K-AKT signaling can increase expression of multiple anti-apoptotic proteins such as BCL-XL, as well as inactivation of pro-apoptotic proteins, including BAD, BIM, and procaspases [96, 97].

Studies over the past five years have indicated that a novel stress response gene REDD1 (also referred to as RTP801, Ddit4, or dig2) [98-100] is a transcriptional target of p53 and p63 (member of p53 family), and is implicated in regulation of ROS. Treating cells with H<sub>2</sub>O<sub>2</sub> rapidly activated the REDD1 promoter. In contrast, the ROS-reducing enzymes SOD and glutathione peroxidase inhibited promoter activation [101, 102]. In addition, REDD1 is a transcriptional target of hypoxia-inducible factor 1 (HIF-1), and is strongly induced under hypoxic condition in an HIF-1-dependent manner [98, 103]. REDD1 activation subsequently inhibits its downstream protein kinase, mammalian target of rapamycin (mTOR), to regulate cell growth [104].

In summary, IR can cause damage to macromolecules, generate ROS and RNS, and activate multiple interacting signaling pathways. The activation of signaling pathways is dependent on radiation dose, cell type, and cell cycle phase. There is simultaneous activation and interaction of multiple pathways that can either favor or inhibit apoptosis. The end result, survival or apoptotic cell death, depends on whether pro-apoptotic and anti-apoptotic pathways predominate.

## PHARMACOLOGICAL COUNTERMEASURES FOR ARS

Because of the complex molecular damage caused by ionizing radiation, development of pharmacological countermeasures for radiation injury is challenging. The mechanisms through which radiation injury becomes manifest depend on the type (quality), dose, and dose rate of radiation, and vary from tissue to tissue. The signal transduction responses to radiation damage depend on the circumstances of the exposure, such as dose of radiation, dose rate and time of radiation, protraction of exposure, and concomitant exposure to other noxious agents or tissue trauma. After decades of work on radiation countermeasures, no pharmaceuticals are currently approved specifically for ARS. Medical approaches to ARS aim to prevent or ameliorate radiation injury with radioprotectants and radiation mitigators, and to stimulate recovery of damaged tissue such as hematopoietic or GI regeneration with post-radiation treatment. A number of reviews have been published that provide guidance on medical management of ARS [4, 105-107]. Granulocyte-stimulating factor (G-CSF) and Granulocyte-Macrophage CSF (GM-CSF) (Fig. 3) are typically used to treat radiation accident victims off-label [5]. Other standard measures include antibiotics, antifungals, blood transfusions, intravenous fluids, electrolytes, etc., in addition to antiemetic agents, antidiarrheal agents, and comfort measures [5, 105]. Platelet transfusions are necessary to treat radiation-induced thrombocytopenia, as G-CSF and GM-CSF do not stimulate thrombopoiesis [108, 109]. These measures are appropriate for a hospital setting and contribute to recovery of patients from radiation therapy. However, basic research is needed to identify additional targets for small molecule drugs with low toxicity. There is an emphasis on expanding the repertoire of countermeasures to include agents that do not require clinical support and physician supervision, which have been shown to be problematic in realistic analyses of mass casualty scenarios [106, 110]. There is an urgent need to exploit known signaling pathways to identify and develop novel agents. The hematopoietic and GI syndromes are still very important issues in the field of radiation countermeasures, since there are no approved drugs with an indication for treatment of ARS. Even assuming the availability of cytokines, growth factors, and full medical support, there is still an urgent need to identify treatments that will further increase survival during ARS.

According to the report of National Cancer Institute (NCI) workshop held on December 3-4, 2003 [111], the instructions from

the Developmental Therapeutics Program sponsored by NCI specify that pharmacokinetic and pharmacodynamic studies must be used to determine the plasma or tissue drug levels required to have an impact on the target, as well as for endpoints of toxicity. Measurement of target modulation is also necessary for newer molecularly targeted drugs. Selection of appropriate parameters to develop reliable monitors as a measure of biological activity and efficacy are needed. Data from genomics and proteomics studies will be used to design toxicology studies and evaluate the optimal administration for use in preclinical and clinical studies. New drugs often are dropped during development because of animal toxicity (17%), human toxicity (16%), pharmacokinetics (7%) or lack of efficacy (46%) [111]. The studies of efficacy and toxicology must include pharmacokinetics/pharmacodynamics, genomics, and proteomics. It might be possible to separate toxicity from efficacy if they are induced by different mechanisms.

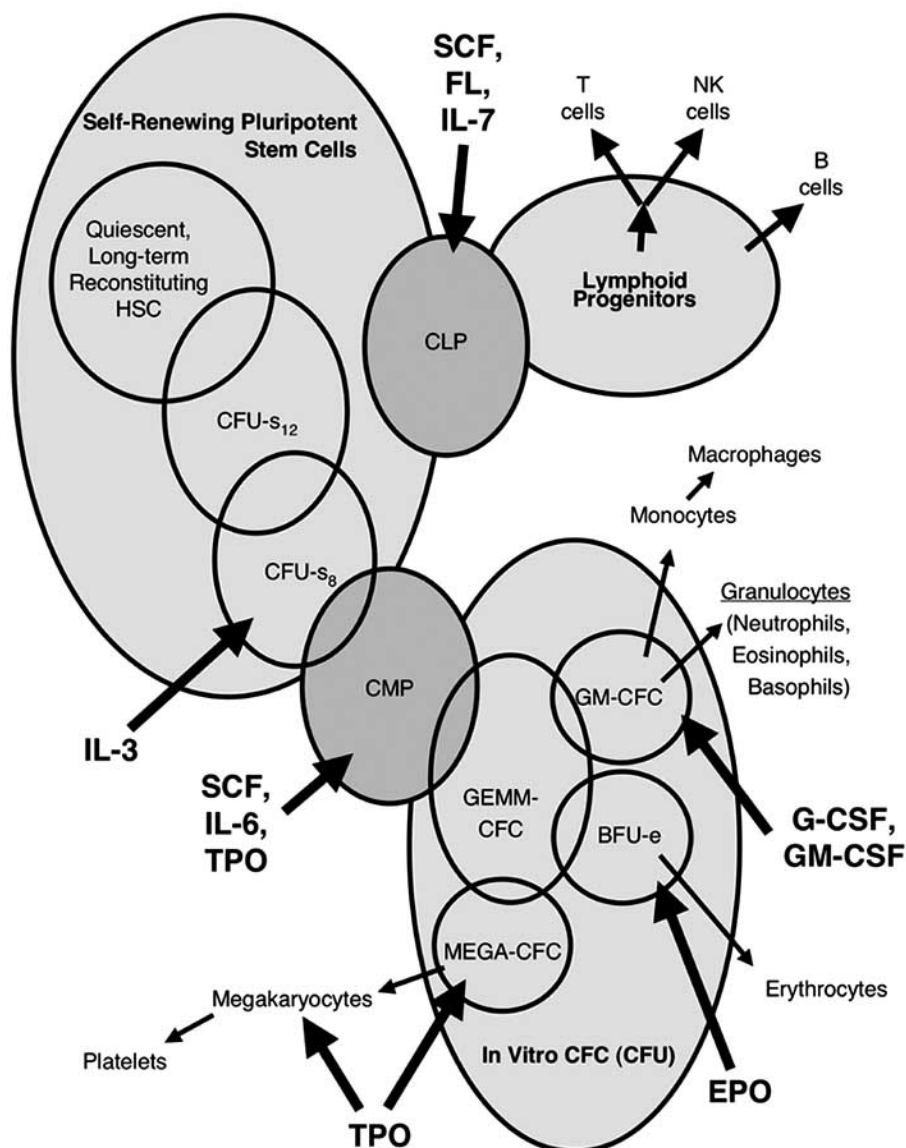
Current treatment guidelines are covered elsewhere [2, 4, 5]. Here we will describe some recent trends in pharmacological radiation countermeasure research. Our orientation is accident victims and military and terrorist attacks, although some drugs designed for these indications might also be useful to protect or treat patients undergoing radiation therapy. The listing is not meant to be comprehensive, but to provide examples of how known molecular changes occurring during radiation injury and recovery can be targeted by pharmacological agents.

## Hormones, Growth Factors, and Cytokines

Early apoptotic cell death within the first days plays a critical role in IR-induced hematopoietic stem cell depletion [107, 112]. The massive apoptosis involves almost all cell compartments through direct and indirect effects [113]. Cell fate depends on initial DNA breaks, cell cycle arrest, and regulation of many pro- and anti-apoptotic signals. Therefore, reduction of IR-induced apoptosis using hematopoietic growth factors and cytokines (Fig. 3) is a promising radiation countermeasure approach. The anti-apoptotic growth factors G-CSF and GM-CSF are typically used to treat radiation accident victims. Recent reports by Herodin *et al.* [15] demonstrated that the effects of anti-apoptotic cytokine combinations restored hematopoietic stem cells after IR. Using SCF + Flt-3 ligand (FL) + thrombopoietin (TPO) + interleukin-3 (IL-3) after IR, they obtained 60% survival in mice (versus 5% in controls) after a lethal dose (9 Gy) of total-body-irradiation (TBI) [15]. Stem cell factor (SCF) can block radiation-induced apoptotic signals through the FAS pathway in hematopoietic cells [114]. Keratinocyte growth factor (KGF) or fibroblast-growth factor 2 (FGF2) given before TBI ameliorated cell death in the gut as well as in epithelial thymic cells, and increased survival in irradiated mice [113, 115]. In combination with hematopoietic stem and progenitor cell (HSPC) transplantation, KGF is capable of restoring immune status after TBI [116]. Additionally, given after irradiation, KGF reduces incidence of oral mucosal ulceration [117]. IL-11 also exerts pleiotropic effects and might be efficient at promoting GI mucosa recovery [118, 119]. FL is another pleiotropic cytokine that exhibits immunorestorative properties involving thymodependent and thymoindependent [120] pathways. In addition, EPO, IL-1, IL-4, IL-11 and angiopoietin-1 have been reported to provide protection against lethality and/or crypt cell depletion [112].

It is assumed in many studies that cytokines and growth factors are acting in large part by inhibiting apoptosis in progenitor cells in hematopoietic tissue. This belief is based on *in vivo* and *in vitro* studies of cytokine actions on hematopoietic lineages [121-124], but the relative roles of inhibiting apoptosis and stimulating proliferation of stem and progenitor cells need to be examined at various times after irradiation and cytokine administration. Surviving beyond the period of ARS may unmask late effects. Moreover, when apoptosis is inhibited, it is possible that some irradiated cells with

### Hematopoiesis



**Fig. (3). Growth factors and cytokines in regulation of hematopoiesis.** This is a simplified schematic showing a few of the relevant factors. Growth factors and cytokines act synergistically at multiple levels of hematopoiesis.

compromised DNA or genomic instability may survive and proliferate, leading to later deleterious consequences, since the natural role of apoptosis is to remove these cells. An example of an unwanted consequence of apoptosis inhibition is mitotic catastrophe in the GI tract following p53 inhibition in irradiated mice [125]. However, growth factors do not simply block apoptosis. For example, cytokine activation of Gadd45 proteins [126] would have other beneficial actions such as promoting DNA repair and genomic stability [127].

#### Parathyroid Hormone (PTH)

Fifty years ago, parathyroid hormone (PTH), in one injection of Lilly's bovine parathyroid extract, was found to greatly increase 30-day survival of X-irradiated rats when given from 18 h before to as long as 3 h after irradiation [128]. This was the first indication that PTH might stimulate hematopoiesis and protect hematopoietic cells

from IR damage. Osteoblasts, which constitute an important component of the hematopoietic niche [129, 130], produce hematopoietic growth factors in response to parathyroid hormone (PTH). In mice, PTH administration (80 µg/kg, ip, 5 times per week for 4 weeks) significantly increased survival when combined with BM transplantation [131]. The peptide operates through a cyclic AMP-mediated burst of Jagged 1 (Notch receptor ligand) expression on osteoblastic cells, and Notch receptor activation on stem cells [131, 132]. PTH-triggered cyclic AMP signals also directly stimulate proliferation of hematopoietic stem cells.

#### Melatonin (N-Acetyl-5-Methoxytryptamine)

Melatonin is another non-toxic radiation countermeasure [133-135]. The endogenous compound synthesized by the pineal gland in human brain was initially recognized as a molecule related to neuroendocrine physiology, especially reproductive physiology. Mela-

tonin is involved in control of circadian rhythms and sleep processes in diurnal species, with circulating levels that reach nadirs during the day and increase to maximal levels at night [136, 137]. Melatonin is a potent antioxidant with direct free radical scavenging action as well as the ability to increase the activity of SOD and glutathione peroxidase and decrease the activity of nitric oxide synthase, a pro-oxidative enzyme. Melatonin interacts with the radiation induced hydroxyl radical ( $\bullet\text{OH}$ ) and forms a very low toxicity indolyl radical (melatonyl). Melatonin restricts lipid peroxidation by preventing the free radical-mediated initiating events as well as interrupting the Chan reaction. Melatonin can also directly scavenge peroxy radical, peroxyxynitrite anion, and singlet oxygen, protecting the cell membrane, proteins in the cytosol, and DNA in the nucleus. Vijayalaxmi *et al.* [136, 138] suggest that melatonin may postpone the saturation of DNA repair enzymes.

### Non-Cytokine Drugs

#### 5-Androstenediol (5-androstene-3 $\beta$ -17 $\beta$ -diol, 5-AED)

5-AED is a naturally occurring steroid developed as a non-toxic radiation countermeasure. 5-AED enhances survival in mice and monkeys exposed to whole-body  $\gamma$ -IR [139-141], and induces hematopoiesis and hematopoietic growth factor expression [139, 142-144]. It is one of only two radiation countermeasures shown to enhance survival in irradiated non-human primates (the other being Cleveland BioLabs' CBLB502), and one of only three countermeasures the FDA has cleared for Phase I human trials under Investigational New Drug (IND) applications (the other two are CBLB502 and BIO300). 5-AED stimulates multilineage hematopoietic recovery in irradiated animals. In sublethally irradiated monkeys (4 Gy  $^{60}\text{Co}$   $\gamma$ ), early administration (3 to 4 h after TBI) of 5-AED (15 mg/kg) once daily for 5 days or once or twice weekly for 3 weeks, prevented severe neutropenia and thrombocytopenia. 5-AED administration also causes increases in circulating monocytes, NK cells, and red blood cells in irradiated animals [139, 141, 143]. Moreover, 5-AED displays beneficial effects after burn injury, trauma, and sepsis [145]. We recently reported that 5-AED exerts survival-enhancing effects on irradiated human hematopoietic progenitor cells *via* induction, stabilization, and activation of NF $\kappa$ B, which results in increased secretion of hematopoietic growth factor G-CSF [146]. 5-AED stabilizes IR-induced NF $\kappa$ B1 (p50) degradation, as well as inducing NF $\kappa$ B gene expression and NF $\kappa$ B activation (DNA binding) in human hematopoietic CD34+ cells. 5-AED also stimulated interleukin-6 (IL-6) secretion. Furthermore, we demonstrated that the effects of 5-AED on survival and G-CSF secretion were abrogated by siRNA inhibition of NF $\kappa$ B gene expression and the NF $\kappa$ B inhibitor MG132.

#### Amifostine

Many promising countermeasure candidates are targeted at inhibiting apoptosis, stimulating protective enzymes, or enhancing regeneration in specific tissues by inducing signaling pathways. However, workers in the field remain interested in the possibility of protecting against initial radiation damage using free radical scavengers. The advantage of this approach would be to avoid having to deal with the specific physiology of various cell and tissue types, since the basic mechanism of physical injury would be blocked. For example, it would not be necessary to know whether the predominant mode of cell death in a particular tissue were apoptosis or necrosis, since the pathways that triggered those processes would be prevented from being initiated by a radical scavenger. Amifostine (WR-2721 [147]) is a clinically approved aminothioli radioprotectant capable of scavenging radiation-induced free radicals before they can interact with macromolecules. WR-1065 [148] is the active thiol form. Although side effects such as hypotension, nausea and vomiting have prevented the use of aminothiols outside the clinic, their remarkable radioprotective efficacy causes them to remain

interesting, and possible approaches to limit toxicity are being explored. Amifostine participates in reductive/oxidative reactions that in turn can affect redox-sensitive cellular processes involving the activation of transcription factors, expression of genes, and activities of proteins [149-151]. Hence the value of amifostine may not be limited to simply preventing the initial damage by scavenging radiation-induced radicals. In particular, amifostine has been observed to be effective in activating the redox-sensitive NF $\kappa$ B and enhancing the expression of the antioxidant gene *MnSOD* [149, 150]. Aminothioli effects appear to be related to a high affinity for DNA, and to the structural similarity of thiol and disulfide metabolites to cellular polyamines [152, 153]. Murley and colleagues demonstrated that 4 mM but not 40  $\mu$ M WR1065 increased human microvascular endothelial cell (HMEC) survival after X-irradiation. However, a delayed radiation protective function was observed 24 h after IR regardless of the dose of WR1065. The mechanisms might involve activation of the redox-sensitive NF $\kappa$ B and elevated expression of the antioxidant gene *MnSOD* [148, 154]. Administration of Helenalin, an inhibitor of NF $\kappa$ B, not only inhibited activation of NF $\kappa$ B by WR1065, but also inhibited the subsequent elevation of *MnSOD* and the delayed radioprotective effect of WR1065. Furthermore, WR1065 protected HMEC against IR-induced  $\gamma$ -H2AX formation and apoptotic death. The frequency of  $\gamma$ -H2AX-positive cells was correlated with radiation dose [155].

#### Genistein

Genistein, a soy-derived isoflavone, is a phytoestrogen and protein tyrosine kinase inhibitor that affects signal transduction pathways involving AKT, FAK, ErbB-2, Bcl-2, and cytokine secretion [156]. Administration of genistein to mice before irradiation stimulates hematopoiesis and enhances survival with no adverse effects [157-159]. Recently, Song *et al.* [160, 161] compared gene expression profiles in livers of irradiated mice treated with or without soybean isoflavone (SI). They found the JNK/c-Jun-inhibiting genes, *MBIP* and *HERPUDI1*, were significantly up-regulated in irradiated cells after SI treatment. Another gene affected by SI is heat shock 70kD protein 5 (*Hspa5*), a member of the highly conserved heat shock protein 70 family. *Hspa5* is repressed after IR but maintained at normal levels after SI treatment. These results suggest that SI may be an efficient tool to ameliorate radiation damage of the liver through multiple pathways.

#### CBLB502

Recently, Burdelya *et al.* reported a new compound, CBLB502, an agonist of Toll-Like receptor (TLR) 5, as a radiation countermeasure in mouse and primate models [162]. A single injection of CBLB502 (0.2 mg/kg) to NIH-Swiss mice 30 min before 13 Gy TBI protected 87% of mice from radiation-induced death. Administration of CBLB502 24 h before, or up to 1 h post-irradiation, resulted in greater than 90% survival after 9 Gy. CBLB502 is a polypeptide derived from *Salmonella* flagellin that binds to TLR-5 and activates NF $\kappa$ B signaling through phosphorylation and proteasomal degradation of NF $\kappa$ B inhibitor I $\kappa$ B [163]. CBLB502 includes the complete N- and C-terminals of flagellin, and retains its full NF $\kappa$ B-inducing activity. Flagellin derivatives that failed to activate NF $\kappa$ B *in vitro* did not provide radioprotection *in vivo* [162].

### CONCLUSIONS

Understanding of the mechanisms of tissue responses to radiation injury has markedly improved in recent years. Radiation-induced injuries have been well described at the clinical level, and currently available treatments are useful in a limited manner. However, after decades of published work on radiation countermeasures, the only approved drug for countering the effects of external penetrating ionizing radiation is amifostine, limited to use in the clinic to

protect salivary glands during head and neck irradiation. G-CSF is commonly used off-label in the clinic after radiological incidents, and is held in the Strategic National Stockpile for possible use that would require FDA Investigative New Drug approval or Emergency Use Authorization for treatment of radiation-induced bone marrow suppression. There is a pressing need to develop radiation countermeasures that can be used inside and outside the clinic in mass casualty scenarios. Several candidates are emerging that have significant efficacy in terms of enhancing survival after irradiation, with low toxicity. The most promising candidates should target specific molecular pathways related to radiation injury, repair, and recovery.

## ABBREVIATIONS

5-AED	= 5-androstene-3 $\beta$ -17 $\beta$ -diol	I $\kappa$ B	= Inhibitor of $\kappa$ B
AKT	= Family of serine/threonine-specific protein kinases	IKK	= I $\kappa$ B kinase
AMP	= Adenosine monophosphate	IL	= Interleukin
ARS	= Acute radiation syndrome	IND	= Investigational New Drug
ATM	= Ataxia telangiectasia mutated	IR	= Ionizing radiation
BAD	= Bcl-2-associated death promoter, pro-apoptotic protein of the Bcl-2 gene family	JNK	= c-Jun N-terminal kinases
BAX	= Pro-apoptotic protein of the Bcl-2 gene family	KGF	= Keratinocyte growth factor
BAK	= Bcl-2 homologous antagonist/killer, pro-apoptotic protein of the Bcl-2 gene family	LET	= Linear energy transfer
BCL-2	= B-cell lymphoma 2 proto-oncogene, prototype for a family of genes regulating apoptosis. BCL-2 proper is anti-apoptotic.	LPS	= Lipopolysaccharide (endotoxin)
BIM	= PRO-apoptotic protein that inhibits BCL-2	MAPK	= Mitogen-activated protein kinase
c-JUN	= Component (with c-FOS) of the AP-1 transcription factor	MBIP	= MAP3K12 binding inhibitory protein 1
CD	= Cluster of differentiation cell surface molecule	mTOR	= Mammalian target of rapamycin
CNS	= Central nervous system	NCI	= National Cancer Institute
DNA-PKcs	= DNA-protein kinase complex catalytic subunit	NEMO	= NF $\kappa$ B essential modulator
DSB	= DNA double strand break	NF $\kappa$ B	= Nuclear factor kappa B
EPO	= Erythropoietin	NHEJ	= DNA non-homologous end-joining
ERBB-2	= Member of epidermal growth factor receptor (ErbB) family	NK cells	= Natural killer cells (large granular lymphocytes)
ERK1/2	= Extracellular signal-regulated kinase 1/2	•OH	= Hydroxyl radical
FAK	= Focal adhesion kinase	PARP-1	= Poly (ADP-ribose) polymerase-1
FDA	= United States Food and Drug Administration	PI3K	= Phosphoinositide 3-kinases
FGF2	= Fibroblast-growth factor 2	PIDD	= p53-induced protein with a death domain
FL	= Flt-3 ligand	PIKK	= Phosphoinositol 3-kinase like kinase
Gadd45	= Growth arrest and DNA damage gene family	PTH	= Parathyroid hormone
G-CSF	= Granulocyte colony-stimulating factor	PTPase	= Protein tyrosine phosphatase
GM-CSF	= Granulocyte-macrophage CSF	RAF	= Raf-1, a serine/threonine-specific kinase
GI	= Gastrointestinal	RAS	= Membrane-associated G protein, proto-oncogene
H2AX	= One of several genes encoding for histone H2A	RBE	= Relative biological effectiveness
H <sub>2</sub> O <sub>2</sub>	= Hydrogen peroxide	RDD	= Radiation dispersal device (“dirty bomb”)
HERPUD1	= Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	REDD1	= Regulated in development and DNA damage responses 1; HIF-1-responsive protein RTP801
HIF-1	= Hypoxia-inducible factor 1	RIP1	= Receptor interacting protein 1
HMEC	= Human microvascular endothelial cells	RNS	= Reactive nitrogen species
HR	= Homologous recombination	ROS	= Reactive oxygen species
HSPC	= Hematopoietic stem and progenitor cell	SI	= Soy isoflavone
		siRNA	= Small interfering RNA
		SOD	= Superoxide dismutase
		SCF	= Stem cell factor
		SSB	= DNA single strand break
		TBI	= Total-body irradiation
		TLR	= Toll-like receptor
		TNF	= Tumor necrosis factor-alpha, cachexin, cachectin
		TPO	= Thrombopoietin

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