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Review

New approaches to radiation protection

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ABSTRACT

Radioprotectors are agents that protect against radiation injury when administered prior to radiation exposure. Mitigators are agents that can protect against radiation injury when given after exposure but before any symptoms appear. Because of concern on a mass casualty situation due to accidental exposure to radiation (*e.g*., Fukushima and Chernobyl nuclear reactor disaster) or intentional exposure (*e.g*., nuclear device or "dirty bomb"), development of novel radioprotectors and particularly mitigators (called "radiation countermeasures") is currently a high priority. As radioprotectors and mitigators can potentially improve the outcomes of radiation therapy for cancer treatment (*e.g*., by allowing higher doses of radiation and/or reduced damage to normal tissues), there is an interest in whether there is a role for some of these novel agents in the radiotherapy clinic. The applicability of these agents to the cancer clinic is critically dependent on whether or not they will also render tumors more resistant to radiation, which, in turn, relates to their biochemical and biological mechanism(s) of action. Here, we will review what has been learned during the development of these protectors and mitigators on the molecular mechanisms leading to radiation injury, the proposed radioprotective agents that have been developed, and the molecular pathways that can lead to radioprotection and mitigation.

KEYWORDS: radiation protector, radiation mitigator, medical countermeasure, prophylaxis

INTRODUCTION

Recently, there has been a great interest in developing agents that can protect against injury caused by ionizing radiation (IR) and improve survival, due to the possibility of accidental or intentional exposure of large populations to radiation. Accidental exposures may result from nuclear reactor disasters (*e.g*., Chernobyl and Fukushima reactor meltdowns). Intentional exposures could result from detonation of a nuclear weapon or a "dirty bomb", a radiological device containing conventional explosives and radioactive substances that is designed to disperse radioactive materials over a wide area consisting of a large number of civilians. "Medical countermeasure" (abbreviated MCM) is a term adopted by the Departments of Defense and of Health and Human Services to describe agents (usually but not necessarily pharmacologic) that can be used to prevent or treat radiation injury. Three general types of MCMs are recognized: 1) "radioprotectors" (also called radiation protectors or pre-irradiation

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prophylactics, defined as agents that can protect against radiation injury when given before exposure to radiation; 2) "mitigators", defined as agents that protect against radiation injury when administered after radiation exposure but prior to development of symptoms; and 3) "therapeutics" or agents that can ameliorate radiation sickness (see below) or injury when applied after the onset of symptoms. In this review, we will be concerned only with the first two categories of agents, radioprotectors and mitigators.

It is hoped that at least some of the radiation countermeasures originally intended for use during radiation emergencies of the type described above will also be useful in preventing radiation injury due to therapeutic radiation utilized for cancer treatment. Most advances in the field of radiation oncology (also called radiation therapy, therapeutic radiology or radiation medicine) are related to different methods of making the radiation beam better conform to the size and shape of the tumor, which reduces the volume of normal tissue within the path of the radiation beam and the dose delivered to normal tissues. These approaches include three-dimensional conformal radiation therapy, intensity modulated radiation therapy (IMRT), stereotactic radiosurgery (*e.g*., using the Gamma Knife or CyberKnife) and proton beam therapy. However, it is impossible to entirely exclude normal tissues from the radiation field; and treatment frequently requires significant exposure to radiosensitive tissues and organs. Thus, normal tissue radioprotection is a promising strategy. The requirements for clinical radioprotectors differ from those for use as MCMs in at least one major respect: it must be considered whether an agent used for normal tissue protection will also protect the tumor.

Acute radiation syndrome

While total body irradiation (TBI) affects multiple organ systems in an interactive manner, death in humans in the first 30 days is primarily due to two mechanisms: 1) gastrointestinal (GI) syndrome, which often leads to death within 10 days after exposure to 8-20 Gy of γ-rays, due to fluid and electrolyte imbalance and bacterial translocation (sepsis); and 2) hematopoietic syndrome, which leads to death within 30 days after exposure to 3-8 Gy, due to neutropenia and thrombocytopenia [1-6]. These effects of radiation within the first 30 days are commonly referred to as "acute radiation syndrome" (ARS) or "radiation sickness". ARS follows a generally similar pattern in humans and rodents (rats and mice), with the exception that the $LD_{50/30}$ values (dose of whole body exposure required to reduce survival to 50% by day 30, without medical support) are considerably lower in humans (*ca*. 3.5-4 Gy) than in rodents (*ca*. 7-9 Gy) [7]. Thus, initial studies of proposed whole body radioprotectors and mitigators are usually carried out in rodents in 30-day experiments, using survival as the primary end-point.

An effective radioprotector/mitigator should improve 30-day survival in rodents by protecting against GI syndrome, hematopoietic syndrome, or both. And if its intended use is for protection of large populations rather than the occasional individual who receives an accidental exposure, it should have little or no toxicity at doses required for bioactivity and it should have a convenient mode of delivery (*e.g*., orally, subcutaneously, or by intramuscular injection). In the case of hematopoietic syndrome, it is generally thought that death within the first 30 days is due to depletion of hematopoietic progenitor cells (HPCs) for white blood cells and megakaryocyte lineages, leading to neutropenia and thrombocytopenia [1, 2]. HPCs are thought to be more radiosensitive than pluripotent stem cells (HSCs) (hematopoietic stem cells) [8-10]. However, irradiated HSCs take a long time (30 days or so) to be recruited into the cell cycle and reconstitute neutrophils and platelets. Thus, if an individual survives for 30 days, HSCs will have sufficient time to reconstitute the various bone marrow lineages, and further hematological support may not be required.

Acute gastrointestinal (GI) syndrome is due to depletion of intestinal stem cells (ISCs) located at or near the base of the intestinal crypts of Lieberkuhn [11-13]. These cells usually die very rapidly after exposure to a high dose of radiation, due to apoptosis. It is thought that PUMA (p53 up-regulated modulator of apoptosis) is a critical mediator of apoptosis in ISCs [11-13]. Intestinal crypts become progressively more denuded of cells as apical cells are shed, and ISCs either die or undergo cell cycle arrest due to DNA damage.

In irradiated mice, the mean villus length, number of villi per circumference, and mitotic index decrease starting about four days after irradiation, and the effects become pronounced by day 8 [14]. Death due to GI syndrome in mice usually occurs within 10-15 days, depending on the mouse strain and radiation dose. However, in surviving animals (*e.g.*, due to treatment with a radioprotector), crypts begin to regenerate (indicated by BrdU uptake, indicating DNA synthesis) by day 15 or so.

A third syndrome associated with whole body radiation exposure is the neurovascular syndrome (or cardiovascular and central nervous system syndrome). This syndrome is relatively rare and is usually observed at doses greater than 50 Gy in a single exposure, but it can occur at lower doses too [15, 16]. Symptoms include headache, dizziness, nausea and confusion; and death usually occurs within three days due to increased intracranial pressure and cardiovascular collapse. It is doubtful whether any radioprotector or mitigator could enable survival following whole body radiation doses that high. Thus, current research has been focused on protecting against the hematopoietic and gastrointestinal syndromes rather than the neurovascular syndrome.

The syndromes described above are usually the result of radiation exposure to the whole body or most of the body. Although the GI system and bone marrow are rapidly reacting systems that contribute to ARS, high dose partial body radiation that includes the lungs can result in delayed toxicity that occurs 3-10 months after exposure. This syndrome appears to be related to repeated cycles of inflammation, eventually resulting in pulmonary fibrosis and death, depending on the dose and volume of lung tissue irradiated [17-20]. The skin and kidneys are also “radiosensitive” tissues in which severe effects can be observed in individuals who receive high dose partial body irradiation. ARS is the most studied and best understood consequence of whole body radiation exposure. However, much less is known about the later consequences of high dose partial body exposures and the late consequences of ARS. Much of what we know about the sensitivity of specific tissues and organs to radiation comes from early experience with radiation therapy, before the radiation tolerances of these tissues and organs had been established and before the introduction of skin sparing high energy (megavoltage) radiation was introduced.

Radiation-induced tissue injury in the radiotherapy clinic

Typically, radiation therapy is delivered as a course of fractionated treatments using relatively small dose increments (1.8-3 Gy) delivered five days per week to the tumor site and, in some cases, the draining regional lymph nodes. Total doses may vary from about 30 Gy to 80 Gy, depending upon the intent of treatment (curative or palliative) and the type and location of the tumor. Side effects of radiation have been well studied and are classified as acute, intermediate or late effects [21-29]. Acute effects occur during a course of radiotherapy and are usually resolved within a few weeks after the last treatment. Examples include epidermitis and mucositis due to injury to the skin and mucosal membranes. Intermediate effects are less common and typically occur within 8-12 weeks after the end of radiation. An example is pneumonitis, which reflects inflammation of the lung and is usually confined to within the radiation portals. Late effects occur at least 9 months after the end of radiation and are often a dose-limiting consideration in planning a course of radiotherapy. The type of late effects we are discussing are injury to specific tissue and organs located within the radiation field or in the entrance or exit paths of the radiation beam. Other types of late effects due to irradiation include carcinogenesis (second tumors caused by radiation), teratogenesis (malformation of fetus, which is very rare because pregnant women are rarely treated with radiation) and effects on growth and development due to irradiation in childhood.

The likelihood of a late effect depends upon the total dose of radiation, the fraction size (*i.e.*, dose delivered during each treatment), the volume of tissue being treated, and other treatments, particularly cytotoxic chemotherapy, and also surgery. Other factors that may contribute to risk for late effects include genetic factors unique to the individual patient, pre-existing vascular damage (diabetes), hypertension, age, and other pre-existing conditions that can affect the tissue or

organ that was irradiated (*e.g.*, inflammatory bowel disease in patients who receive abdominal irradiation). There are often cases where the dose of radiation and/or volume of irradiated tissue is limited due to late effects: *e.g.*, tumors of the brain and spinal cord and locally advanced cancers of the lung, cervix, breast, head and neck, and other sites. These are examples where a selective normal tissue protector could allow a higher dose, a larger treatment volume, and/or reduced late normal tissue injury, thus increasing the therapeutic index. A reduction in the early effects (*e.g.*, epidermitis, mucositis of the oropharynx, cystitis, and proctitis) due to a normal tissue radioprotector could increase patient comfort. Although these effects usually resolve by themselves, they sometimes require a treatment break that delays the completion of a course of radiation. Concurrent chemotherapy and radiotherapy can cause much more severe acute effects (*e.g.*, debilitating mucositis and weight loss); and here a normal tissue protector may be beneficial [30-35]. Finally, normal tissue protection may be particularly beneficial in young children who are undergoing cranial or craniospinal irradiation by protecting a central nervous system that is not fully developed [36, 37]. The effects on the growth of bones (before epiphyseal closure) and the possibility of a second tumor due to radiation must be considered whenever children are treated with radiation therapy alone or in combination with chemotherapy drugs.

A relatively recently recognized late consequence of thoracic and chest wall irradiation (*e.g.*, treatment of Hodgkin's lymphoma or post-operative radiotherapy for breast cancer) is radiation-induced heart disease (RIHD), which is usually observed at least several years after treatment and is characterized by accelerated atherosclerosis, cardiac fibrosis, valvular damage, and a significantly increased risk of cardiac-related mortality [38, 39]. RIHD can occur when either part of the heart or all of it is included within the radiation field. This condition is usually progressive and its incidence increases with time after treatment. A significantly increased risk of neurovascular events (*e.g.*, stroke or transient ischemic attack (TIA)) has been observed following cranial irradiation for brain tumors in children [40]. Neurocognitive decline following whole brain irradiation in adults is fairly common, particularly in individuals who have also received cytotoxic chemotherapy. As there is no specific treatment for these complications, a novel prevention strategy is required.

Pathways of radiation injury

To provide a framework for understanding how radioprotectors and mitigators function, we have provided a schematic diagram illustrating some of the pathways at the molecular and tissue levels activated in response to IR (Fig. 1). Although IR can directly target critical cellular macromolecules such as DNA, water (H_2O) is by far the most abundant molecule within cells and is thus the most likely target for radiolysis by high-energy photons [41-44]. As indicated in Fig. 1, molecular oxygen (O_2) is a central component involved in the formation of highly reactive free radicals, and so it is not surprising that high concentrations of O_2 potentiate the effects of IR, while low concentrations of O_2 (hypoxia) protect cells and tissues from IR, a phenomenon called the "oxygen effect" [45-47]. While many types of these free radicals are produced, the most damaging species is probably the hydroxyl radical (OH.) [48, 49]. As indicated, DNA is the most critical target for cell survival, but significant damage can be imparted to other cellular molecules such as proteins and lipids [50, 51]. These oxidative radicals produce two major forms of DNA damage: double strand breaks (DSBs), which is the most lethal form of damage, and base lesions, which are normally repaired by the base excision repair (BER) pathway [52-55]. It should be noted that during the processing of base lesions, single-strand DNA breaks (SSBs) are generated, which are then repaired by one of several mechanisms that involves a scaffolding protein, DNA polymerase, and a DNA ligase. If two base lesions on opposite DNA strands are close enough, the result can be a DSB.

At this point, a DNA damaging signaling and repair complex accumulates at and surrounding the DSB site. The "MRN" complex of three proteins (MRE11-RAD50-NBS1) acts as a proximal sensor and binds to the broken ends of DNA [56]. Following MRN, ataxia telangiectasia mutated

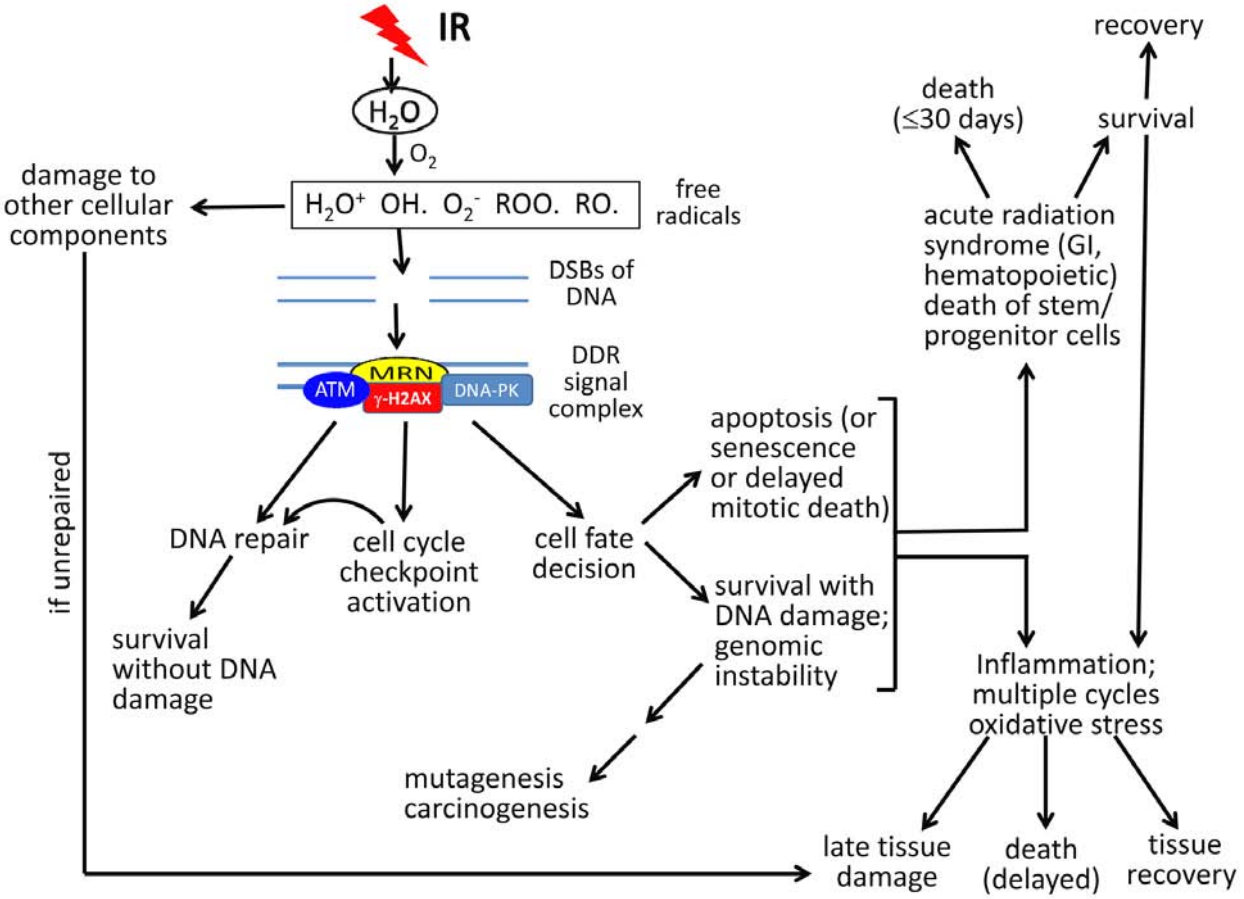

Fig. 1. **DNA damage response (DDR) to double-strand DNA breaks (DSBs) in relation to acute radiation syndrome and late effects**. DSBs caused by oxidative radicals are sensed by the MRN complex (MRE11-RAD50-NBS1), resulting in an ATM (ataxia-telangiectasia, mutated)-driven DDR. Gamma-H2AX (phosphorylated histone H2AX protein) is both a participant in the DDR and a marker of DSBs. Depending upon the dose of radiation, the type of radiation, the volume of tissue irradiated, and other factors, the DDR may lead to some combination of DNA repair, permanent cell cycle arrest (senescence), cell death, or survival with DNA damage. As a result of these processes, acute and late radiation effects may ensue, resulting in survival, death, or survival with late tissue damage. Note that "acute radiation syndrome" refers to the consequences of whole body radiation exposure. Acute effects of radiation may be limited to specific tissues or organs in the case of partial body radiation exposures or radiotherapy treatment to tumor-bearing tissue.

(ATM), a nuclear serine/threonine protein kinase, is recruited to the MRN complex and activated through autophosphorylation, after which it phosphorylates a number of substrate proteins on SQ/TQ motifs. The eventual result is the coating of DNA surrounding the break with a set of proteins that orchestrates the DNA repair process. These events are reviewed in detail elsewhere [57]. Fig. 1 is simplified in regard to the DNA damage response (DDR) signaling pathway, in that once the damage is recognized, DSB repair can proceed by two pathways: 1) homology-directed repair (HDR) (homologous recombination) (orchestrated by ATM/BRCA1/BRCA2 signaling), which is usually an error-free process; and 2) nonhomologous end joining (NHEJ), which can be accurate or can lead to significant sequence deletions and translocations (orchestrated by DNA-dependent protein kinase (DNA-PK) reviewed in [58, 59]). HDR occurs only in S-phase and G2, because it requires a sister chromatid as a template for DNA repair synthesis, while NHEJ can occur in any phase of the cell cycle, but preferably occurs during G1.

In addition to mediating DNA repair, ATM signaling also results in activation of DNA damage-dependent cell cycle checkpoints (*e.g.*, S and G2/M), which allows time for damaged cells to repair their damage, so that it is not passed on to daughter cells (Fig. 1). ATM also orchestrates the "cell fate decision" (the molecular events underlying the cell fate decision are reviewed in [60]). Here, cells that have too much damage to repair are pushed into rapid death by apoptosis (or alternatively permanent cell cycle arrest ("senescence") or delayed death through mitotic catastrophe). Alternatively, ATM can also stimulate cell survival pathways (*e.g.*, NF-κB signaling) [61, 62], If cells protected by the anti-apoptotic transcription factor NF-κB have not fully and accurately repaired their DNA damage, this can result in cells with genomic instability, which can result in the accumulation of mutations and eventually in carcinogenesis, a late effect that usually occurs at a minimum of 3-5 years after radiation exposure [63, 64].

Depending upon the radiation dose and proportion of the body exposed to radiation, the relative apoptotic *vs.* surviving GI and hematopoietic stem/progenitor cell populations may result in ARS (described above), which can lead to death or survival and recovery. In the case of partial body radiation exposure, high dose clinical radiotherapy, or even in survivors of ARS, late complications of radiation may ensue, the seriousness of which depends upon the specific tissue, the radiation dose, and the volume of tissue irradiated. The mechanism(s) of late tissue damage is not fully understood, but may result from damage to parenchymal stem/progenitor cells, blood vessels, inflammation, and/or ongoing oxidative stress due to generation of reactive oxygen species (ROS) (reviewed in [65]). Repeated cycles of inflammation may lead to fibrosis (*e.g.*, in lung [65-68]); and ROS can cause additional DNA damage by causing oxidation of DNA bases, creating a vicious cycle. The possible outcomes of these processes include death, survival with permanent late tissue damage of different degrees of severity, or tissue recovery with little or no functional deficit. The scheme in Fig. 1 provides many points that may be amenable to MCMs to improve the outcome, as will be discussed later in this review.

Amifostine as a radioprotector

To date, no radioprotectors or mitigators have been approved by the Food and Drug Administration (FDA) for general use in humans for the treatment of ARS. Although amifostine (Ethyol^{R}) is not a new or novel agent, to date, it is the only drug that has been approved by the FDA to reduce the toxicity of radiation therapy in the setting of cancer treatment [69]. This agent is also utilized to protect from renal toxicity due to cis-platinum, a DNA cross-linking agent that is also known to cause oxidative stress [70-72]. Amifostine (formerly called "WR2721") was originally developed by the U.S. Army Anti-Radiation Drug Development Program as an MCM. It is a thiol compound that acts as a scavenger for oxygen-related free radicals, to reduce the levels of oxidative radicals that would otherwise attack important cellular targets, such as DNA and other macromolecules [73]. Amifostine has been used successfully to prevent xerostomia (dry mouth) due to head and neck irradiation, which can otherwise cause permanent dry mouth due to inclusion of the salivary glands (particularly the parotid glands) within the radiation field [74, 75]. Initially, there were some concerns that the widespread usage of amifostine would also protect the tumor against radiation or chemotherapy drugs, but accumulated experience with its use has shown this not to be the case [76].

From a recent report that examined 30 studies utilizing amifostine, no conclusion could be made regarding the efficacy of amifostine in preventing or reducing oral mucositis, because of confusing and conflicting data [77]. In a recent meta-analysis that included multiple clinical trials in which amifostine was utilized to prevent cis-platinum toxicity, there was a trend toward a reduction in the incidence of platinum-induced ototoxicity (hearing loss due to cochlear damage), but the trend did not reach statistical significance [78]. In a study of locally advanced non-small cell lung cancers treated with chemoradiotherapy without or with amifostine, patients who received amifostine reported a significant reduction in pain and dysphagia (difficulty in swallowing). And in a study of patients who received post-mastectomy radiation with or without amifostine (at different dose levels), the authors reported that patients

who received high dose amifostine had a lower incidence of skin toxicity and that amifostine conferred a reduction in pulmonary and soft tissue fibrosis [79]. Of considerable importance, in a recent meta-analysis of cancer treatment trials that tested amifostine to reduce acute side effects, it was concluded that the use of amifostine did not reduce overall survival or progression-free survival in patients who received radiation therapy plus amifostine or chemoradiotherapy plus amifostine [76].

The most commonly accepted explanation for the lack of radioprotection of tumors by amifostine is that amifostine itself (WR-2721) is an inactive pro-drug which must be converted to an active drug compound (WR-1065) by dephosphorylation. This conversion is usually due to alkaline phosphatase which is present in the cell membrane of normal endothelium. Tumors, which have abnormal vasculature that is sparser than in normal tissues and contains lower levels of alkaline phosphatase, appear to be much less efficient at activating amifostine than normal tissue (reviewed in [69]). Several other mechanisms have been proposed to explain the selective radiation protection by amifostine, including protection of DNA by certain metabolites of amifostine, causing hypoxia in normal tissues by increasing oxygen consumption, and accelerated recovery of normal endothelial cells (reviewed in [69]).

However, amifostine has certain clinically relevant limitations including: 1) the need to administer it within a narrow time window (15-30 min) before each radiation dose; 2) its approval only for intravenous use, although other routes of administration (*e.g.*, subcutaneous injection) are under investigation and appear to be viable [79, 80]; and 3) its toxicity profile, including nausea, vomiting, somnolence and hypotension.

3,3'-Diindolylmethane (DIM)

DIM is a small molecule compound formed in the stomach by acid hydrolysis of indole-3-carbinol (I3C), a component of cruciferous vegetables (*e.g.*, cabbage, cauliflower, and broccoli) [81]. DIM is a proposed cancer prevention agent that is available as a nutritional supplement and has been administered safely by oral route to humans in repeated doses in phase I/II clinical trials [82-85]. In a recent study, it was found that administration of DIM in a multidose schedule protected rodents against lethal doses of TBI up to 13 Gy, whether DIM dosing was initiated 24 hr before or up to 24 hr after irradiation [86]. The dose reduction factor (DRF) (*i.e.*, ratio of $LD_{50/30}$ values in the presence *vs*. absence of DIM) was 1.6 when DIM treatment was initiated 24 hr after irradiation. Low, physiologically relevant submicromolar concentrations of DIM protected cultured cells against radiation by a novel mechanism. DIM caused rapid activation of ATM and phosphorylation of various ATM substrates, suggesting that DIM induces an ATM-dependent DDR-like response, and DIM enhances radiation-induced ATM signaling and NF-κB activation. Similarly, DIM induced ATM activation and signaling in normal tissues in rodents. However, DIM did not protect human breast cancer xenograft tumors against radiation under the conditions tested. In the tumors, ATM signaling appeared to be defective. Although the results appear promising, further research will be required to determine whether DIM may be a useful radioprotector and/or mitigator.

Interestingly, DIM has also been shown to have cardioprotective properties. Thus, subcutaneous administration of DIM decreased the extent of fibrosis due to adriamycin, a DNA-damaging chemotherapy agent, by a mechanism that involves up-regulation of BRCA1 expression and activation of the antioxidant transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) [87]. DIM mediated cardioprotection against other stressors including aortic banding, which causes cardiac hypertrophy, due to a mechanism involving 5'-adenosine monophosphate-activated protein kinase-α2 (AMPK- α2) and mammalian target of rapamycin (mTOR) [88]. Whether DIM would also protect the heart against ionizing radiation has not been reported at this time.

Genistein

Genistein (4',5,7-trihydroxyisoflavone) is a soy isoflavone with a variety of cellular activities, including selective estrogen receptor activation, protein tyrosine kinase inhibition, antioxidant activity and free radical scavenging activity [89-92]. Genistein has been established as an anti-cancer

agent, and has additionally been demonstrated to have anti-microbial and anti-inflammatory activity *in vivo* [93-97]. Genistein was reported in clinical trials to reduce the adverse effects of chemotherapy and radiotherapy [98, 99].

The protective effects of genistein for radiation-induced injury to the bone marrow was observed in a murine model of ARS [100]. A single subcutaneous injection of genistein administered 24 hr prior to radiation exposure provides increased thirty-day survival from total body irradiation [100]. Genistein protection of the hematopoietic system was associated with improved recovery of neutrophils and platelets [101]. Genistein was observed to specifically protect bone marrow progenitor cell populations, thus preventing hematopoietic stem cell pool exhaustion [101, 102]. Genistein reduced micronuclei in Lin^- bone marrow cells, a population of spared hematopoietic cytokine responsive bone marrow cells enriched for hematopoietic stem/progenitor cells, suggesting a direct reduction of radiation-induced DNA damage [103]. Several mechanisms have been proposed for both direct and indirect protection of DNA by genistein. Genistein was demonstrated to increase the activity of the DNA repair enzyme Gadd45 [104-106]. Additionally, genistein was demonstrated to cause transient quiescence of the cell cycle of Lin^- cells in the G_0/G_1 phase of the cell cycle *in vivo* [102]. Genistein also prevented radiation-induced entry of Lin^- bone marrow cells into the cell cycle *in vivo*, also transiently maintaining these cells in the G_0/G_1 phase of the cell cycle [102]. As the G_0/G_1 phase is associated with the activation of DNA repair enzymes, a pause in this phase would thereby allow an extended period of time for DNA repair activities [107].

Genistein administration reduced radiation-induced injury in the lung and increased survival from thoracic irradiation in mice [108]. During the entire course of this study, genistein was provided in a specially formulated diet containing 750 mg/kg genistein that yielded serum levels of genistein at ~1 μmol/L. Protection of the lung from radiation damage was associated with improved lung function, reduced activation of alveolar macrophages, and reduced collagen deposition [108]. Genistein administration in the food or administered in a single subcutaneous injection (200 mg/kg) 24 hr prior to irradiation significantly reduced radiation-induced micronuclei in primary lung fibroblasts in mice exposed to thoracic irradiation [108-110]. Several mechanisms have been proposed for the reduction of radiation damage to normal tissues. In cell cultures, genistein was demonstrated to increase DNA repair activities, especially through the upregulation of Gadd45 expression and the activation of p53 [104, 105, 111]. Radiation-induced lung injury is believed to proceed in part through the induction of inflammation [17]. Thus, a primary mechanism for genistein mitigation of lung injury may lie in its ability to reduce radiation-induced inflammation, including the reduction of proinflammatory factors IL-1β, IL-6, and cyclooxygenase-2 as well as through the modulation of transforming growth factor β signaling [97, 112, 113]. This suppression of inflammation is believed to contribute to the protection of a number of organs, including the lung, against radiation injuries [112].

Several epidemiological studies indicated that a diet high in genistein was associated with low incidences of some cancers, including breast cancer [114, 115]. In cell culture, genistein was demonstrated to block proliferation and induce apoptosis in a variety of cancer cell types including renal carcinomas, non-small cell lung cancers, prostate cancer cells, gastrointestinal, leukemia, cervical cancer, and specific breast cancer cell lines [106, 116-125]. Growth inhibition by genistein in some cells was associated with altered gene expression. In a study of two breast cancer cell lines, genistein was shown to increase the expression of anti-oncogenes BRCA2 mRNA and BARD1 mRNA [118]. Genistein was also demonstrated in cell culture to increase the expression of both cell the cycle check point protein p21/waf1 and the pro-apoptotic protein BAX, resulting in increased apoptosis [96, 106, 124]. Increased apoptosis in cancer cells has been associated with reduced signaling by a variety of proteins, including p42/p44 MAPK, PI3K/AKT/PKB, and/or phospholipase C [125, 126]. In esophageal cancer cell lines, wild type p53 was required for blockade of the cell cycle [126]; however, in non-small cell lung cancer cell lines, genistein inhibited the cell cycle in cells harboring either wild type or mutant

p53 [96]. The cell culture effects of genistein have been supported by animal model studies of cancer. *In vivo*, genistein was demonstrated to suppress cancer cell proliferation. Genistein suppressed angiogenesis of human renal carcinoma cells in a murine allograft model [116].

Although genistein protects normal tissue from radiation, it was demonstrated to radiosensitize a variety of cancer cells in cell culture and *in vivo* [96, 99, 119, 127-130]. In studies of human esophageal cancer cells and prostate carcinoma cells in culture, genistein (15-30 μM) enhanced the effects of radiation, especially increasing the percentage of apoptotic cells induced by radiation [119, 126]. Treatment of non-small cell lung cancer cell lines in culture with genistein increased the amount of DNA damage by radiation, as indicated by increased γ-H2AX focus formation [131]. Interestingly, although genistein was demonstrated to pause hematopoietic Lin^- cells in the G1/G0 phase of the cell cycle (a phase associated with increased DNA repair), it was demonstrated to pause human prostate, lung, leukemic and gastric cancer cells in culture in the G2/M phase of the cell cycle (a phase not associated with DNA repair activity) [96, 105, 123, 125, 129, 132].

Genistein potentiated radiation-induced inhibition of tumor growth in several animal models of human cancers. Genistein (5 mg/d for 2 days) provided significant improvement in tumor growth inhibition in a murine orthotopic prostate carcinoma model [133]. Genistein increased the apoptosis of prostate cancer cells, inhibiting the activation of NF-kappaB, and promoting apoptosis and G2/M cell cycle arrest [134-137]. Administration of genistein together with radiation significantly increased blockade of lung and kidney tumor growth compared with radiation alone [138, 139]. These studies additionally provided evidence for genistein protection of normal kidney and lung tissue from radiation damage [138, 139].

Captopril

Captopril, a sulfhydryl-containing analog of proline, is a competitive inhibitor of the angiotensin converting enzyme (ACE) protease, and reduces systemic blood pressure by blocking both the activation of the vasoconstrictor angiotensin II (Ang II) and the inactivation of the vasodilator bradykinin. Although captopril was initially developed for the treatment of hypertension and heart failure, it was found that captopril was also useful in animal models of radiation-induced renal dysfunction, for the increase of renal plasma flow and improved glomerular filtration [140, 141]. Captopril was first tested clinically for the ability to reduce hypertension in patients with progressive radiation-induced nephropathy [142]. Further clinical investigations demonstrated that captopril reduced pulmonary-related mortality and chronic renal failure in oncology patients receiving radiation for hematopoietic stem cell transplantation [143-145].

Captopril has been investigated as a radiation countermeasure for the pulmonary, renal and hematopoietic systems as well as for the brain and skin [146-151]. Early studies indicated that ACE inhibitors mitigated pulmonary vascular structural alterations in rats in response to hypoxia [152]. Later investigations demonstrated that radiation-induced pulmonary endothelial dysfunction in rats was also mitigated by captopril [153]. Captopril reduced both radiation-induced pneumonitis and fibrosis in rats [154]. This protection was associated with reductions in mast cell accumulation and collagen deposition [154]. The dose-modifying factor for captopril mitigation of radiation-induced lung injury was reported to be 1.07-1.17 for morbidity up to 80 days postirradiation (survival) and 1.21-1.35 for tachypnea at 42 days postirradiation [155].

Findings of radiation protection of the lung from radiation-induced injuries led scientists to explore the effects of captopril on radiation-induced nephropathy. Prophylactic administration of captopril (500 mg/L in the drinking water) resulted in lower systemic blood pressure and improved renal function following total body irradiation in rats [141, 156, 157]. In fact, clinical studies of patients undergoing radiation treatment for hematopoietic stem cell transplantation revealed a trend toward reduced chronic renal failure with captopril treatment [158].

Later studies investigated the effects of captopril on radiation-induced hematopoietic injuries. Captopril and another ACE inhibitor, perindopril, were demonstrated to block radiation-induced

hematopoietic syndrome when administered [146, 159]. Captopril increased survival from radiation hematopoietic injury through accelerated recovery of erythrocytes, reticulocytes, leukocytes and platelets [146]. The improved blood cell recovery was associated with improved survival of specific hematopoietic progenitor populations CFU-GM, CFU-M, and total CFC [146].

The mechanism of captopril-induced amelioration of radiation-induced injury has not been established. Initial studies for captopril mitigation of radiation injuries in the lung were based on the hypothesis that the renin-angiotensin system was involved in inflammation and/or fibrotic remodeling of the lung [152]. Angiotensin II has been demonstrated to play a key role in fibrotic remodeling in the lung, kidney, heart, liver and other organs [160-162]. In fact, subsequent studies demonstrated that other inhibitors of ACE and inhibitors of the angiotensin type 1 receptor had similar radioprotective properties, suggesting the renin-angiotensin system is the critical target [147, 159, 163]. The protective effects of captopril have also been attributed to the antioxidant capacities of captopril, especially as a free radical scavenger [164]. Studies on captopril protection of DNA have provided mixed findings. Captopril (4 mM) was demonstrated to reduce the formation of γ-H2AX foci in microvascular endothelial cell cultures exposed to 8 Gy radiation [165]. At this high concentration, it is possible that captopril acts through its thiol group to suppress reactive oxygen species formation, not through its function as an ACE inhibitor. However, in these studies captopril did not reduce radiation-associated cell death, suggesting that suppression of γ-H2AX foci was not sufficient to protect cells from critical damage [165, 166]. In addition, mice injected intraperitoneally with 10-50 mg/kg captopril 1 hr before 2 Gy ^{60}Co exhibited reduced radiation-induced micronuclei in bone marrow cells, although the effect was not demonstrated to be dose-dependent [164]. In a later study of radiation-induced micronuclei in bone marrow progenitor cells *in vivo* in response to 7.5 Gy total body irradiation, captopril (110 mg/kg/day administered in the water, initiated 1 hr postirradiation) had no effect on micronucleus levels on radiation-induced DNA damage in bone marrow Lin^- cells [103]. The observed reduction of radiation-induced micronuclei may depend on captopril dose, route of administration, time relative to radiation exposure, and/or radiation levels.

An alternative mechanism of captopril for the reduction of hematopoietic injury may occur through cell cycle regulation. ACE inhibitors and Ang II have been reported to have a variety of effects on hematopoietic cells, including the direct and indirect regulation of hematopoiesis [167-175]. Captopril was demonstrated *in vitro* and *in vivo* to induce a transient pause of these cells in the G_1/G_0 phase of the cell cycle in hematopoietic progenitors, and to prevent radiation-induced entry of these cells into the cell cycle [146, 173]. Interestingly, captopril was also demonstrated to suppress basal EPO levels in mice and in healthy human volunteers [176], and to inhibit the induction of EPO by radiation-associated hypoxia [176]. Thus, the effects of captopril on the hematopoietic system may occur through the combined reduction of Ang II and suppression of radiation-induced EPO, to transiently reduce cycling in hematopoietic cells responsive to these factors. Thus, captopril prevented the rapid recruitment and depletion of specific hematopoietic progenitors, with the long-term effect of delaying recovery of hematopoietic cells dependent upon these factors for differentiation/maturation [103, 146].

Captopril was demonstrated to enhance tumor cell apoptosis by radiation while simultaneously protecting the underlying normal tissue. In a study of the incidence of skin tumors in irradiated rats, captopril treatment was found to reduce radiation-induced epilation and to prevent the appearance of moist desquamation [154]. It was found that captopril also inhibited tumor formation in rats following radiation exposure [154].

Inhibitors of radiation-induced accelerated senescence

Loss of cellular clonogenic potential following exposure to radiation can be caused by apoptosis, necrosis, autophagy and accelerated cellular senescence. The type of cellular injury induced by radiation depends upon many variables including the transformed status of the cell, the cell type, the rate of proliferation, etc. [107, 177-179].

Accelerated cellular senescence, a primary effect of radiation on normal (non-transformed, non-immortalized) epithelial and endothelial cells and fibroblasts, results in a range of aberrant biological activities and can influence overall tissue dysfunction [178, 180-185]. Senescence is associated with alterations in cellular morphology and polarity, changes in protein expression, and abnormalities in cell-cell contacts [180, 186]. Radiation-induced changes in endothelial barrier function are believed to play a critical role in tissue edema and inflammation in cycles that occur long after the initial radiation insult [187]. Following radiation exposure, endothelial cells exhibit enhanced adhesiveness for monocytes, decreased production of nitric oxide, and increased secretion of pro-inflammatory cytokines [180, 184, 188-191]. It is postulated that vascular leakage to the extravascular space may play a role in the development of pneumonitis and fibrotic remodeling [187, 192, 193]. Of particular concern, senescence of adult stem cells results in the loss of tissue repair activities due to the inability of this key cell population to proliferate and migrate in response to injury [194]. The combination of production of such pro-inflammatory agents together with loss of normal repair function are fundamental characteristics of delayed radiation-induced tissue damage, especially in the lung [17].

Recent studies suggest that accelerated senescence occurs as the result of proliferative signaling in the presence of a cell cycle blockade, often p21/waf1 [195]. mTOR, a cytoplasmic kinase, is a central integration point for a number of cell signaling pathways, regulating cell proliferation and homeostasis [196]. mTOR was identified as a central molecular target for the inhibition of aging-associated senescence and for stress-induced cellular senescence [197-199]. Treatment with rapamycin, an mTOR binding protein that inhibits mTOR complex 1 (TORC1) activity, prevents accelerated senescence in cells exposed to DNA-damaging agents [199, 200]. A recent study demonstrated that inhibition of radiation-induced cellular senescence by inhibition of mTOR prevented mucositis in mice following irradiation of the head and neck area [182]. In this study it was demonstrated that rapamycin blocked radiation-induced senescence, but not apoptosis, in primary keratinocytes in cell cultures and *in vivo* in a murine model of head/neck irradiation injury.

Insulin like growth factor-1 receptor (IGF-1R) is a single transmembrane tyrosine kinase receptor whose ligands include IGF-1 and IGF-2 [201]. The activation of IGF-1R involves autophosphorylation of its intracellular domain, followed by recruitment of docking intermediates including the insulin-receptor substrate-1 (IRS-1), which in many cell types leads to activation of PI3K/Akt, MAPK and mTOR [202-205]. As a growth factor receptor, IGF-1R plays a role in cell growth and proliferation under normal conditions and is widely expressed in most transformed cells, conferring pro-survival properties upon stress application [204, 206-208]. Increased IGF-1R phosphorylation following radiation exposure was demonstrated in cancer cells in the absence of detectable IGF-1 or IGF-2 ligands, with the subsequent activation of cytoprotective signaling cascades [209, 210]. Besides direct activation in the absence of ligand, IGF-1R gene expression was shown to be upregulated in tumor cell cultures in response to ionizing radiation via the ATM pathway [211]. IGF-1 conferred radioprotection from apoptosis in hematopoietic progenitor cells and crypt and intestinal stem cells [13, 212].

Investigation into receptor signaling pathways that contribute to aging-associated cellular senescence revealed the involvement of IGF-1R [213, 214]. IGF-1 enhances senescence in primary cell cultures via a mechanism that involves increase in reactive oxygen species (ROS) leading to induction of the p53/p21 pathway [215]. In mouse embryonic fibroblasts, IGF-1 inhibits the deacetylase activity of sirtuin 1 (SIRT1) and promotes stability of p53, ultimately leading to induction of senescence [216]. IGF-1R expression levels increase during the development of replicative *in vitro* senescence in primary cortical neurons [217]. UVB-induced premature senescence was found to require functional IGF-1R in human keratinocytes [214]. In agreement with these findings, a recent study demonstrated that inhibition of IGF-1R, PI3K and mTor blocked radiation-induced accelerated senescence in primary lung endothelial cells [178].

Given the significance of IGF signaling for cancer cell survival and proliferation, inhibitors of IGF-1R

and mTor have been investigated as anticancer agents [218-223]. Methods for inhibition of IGF-1 signaling include blockade of IGF-I signaling and IGF-1 receptor antibodies or small molecule blockers [219, 220]. Three common IGF-1R inhibitors are tyrophostins including AG1024 [224], monoclonal antibodies, and pyrrolo-(2,3-d)-pyrimidine derivatives. A variety of IGF-1R inhibitors, including AG1024, have been used preclinically to study the role of IGF-1R in cancer cell proliferation and tissue injury [218, 225, 226]. AG1024 has been shown to be effective in blocking the propagation of neuronal damage even when administered 24 or 48 hr after traumatic brain injury [226]. The IGF-1R inhibitor INSM-18 (Insmed and UCSF), an orally available drug, passed preclinical trials for the treatment of breast, lung, pancreatic and prostate tumors, and was shown to be safe in Phase I clinical trials. INSM-18 Phase II clinical trials for prostate cancer treatment were completed in 2008 [222]. However, the use of IGF-1R tyrosine kinase inhibitors as adjuncts to radiation for cancer treatment remains to be investigated.

CBLB502/Entolimod™

Acute radiation-induced damage in humans occurs via programmed cell death (apoptosis) of radiosensitive tissues of the hematopoietic, gastrointestinal and nervous systems. This cell death is largely determined by activation of the p53 pathway [227]. Tumors frequently lose apoptotic mechanisms during their progression as part of their survival strategy. Among the mechanisms underlying tumor resistance to apoptosis are the deregulation of two important stress response pathways, p53 and NF-κB [228]. Tumors frequently lose p53 function (inactivation of pro-apoptotic control mechanism) and acquire constitutive activation of NF-κB (upregulation of anti-apoptotic genes). One can try imitating genetic mechanisms acquired by tumors to avoid apoptosis using pharmacological inhibitors of p53 and activators of NF-κB, presumably increasing overall radioresistance of the organism and reducing radiation-induced damage of normal tissue.

Radiation-induced apoptosis in some radiosensitive tissues is mediated by the activation of p53. The temporary and reversible pharmacological inhibition of p53 could be radioprotective [229]. This hypothesis was validated by the isolation and administration of the small molecule inhibitor of p53, pifithrin-α that was capable of protecting mice from lethal doses of γ-radiation [230]. However, suppression of p53 as a radioprotective strategy has limitations. While activation of p53 induces massive apoptosis in the hematopoietic system, it causes growth arrest that affect tissue recovery in other tissues such as various epithelial cells. It was found that p53-deficient mice are resistant to radiation-induced hematopoietic syndrome but are more sensitive to gastrointestinal syndrome due to the lack of growth arrest in crypt epithelial cells that continue dividing and undergo mitotic catastrophe [231].

Studies have also focused on alternative tumor-specific anti-apoptotic strategies such as the activation of the NF-κB pathway. The protective role of NF-κB includes induction of: (a) anti-apoptotic proteins that inhibit major apoptotic pathways [232]; (b) cytokines and growth factors which induce proliferation and survival of hematopoietic and other stem cells; and (c) potent reactive oxygen species-scavenging antioxidant proteins, such as manganese superoxide dismutase (Mn-SOD) [233].

Toll-like receptor (TLR) mediated NF-κB signaling is known to activate both the innate and the adaptive immune response, including anti-tumor immunity [234]. Thus, by temporary activation of NF-κB, it will be theoretically possible not only to confer radioprotection but also to reduce the incidence of cancers due to the simultaneous immunostimulatory effect of NF-κB activation. It was hypothesized that the latter effect may be optimally achieved if activation of NF-κB is reached via the triggering of TLRs, the key sensor elements of innate immunity. The activation of the NF-κB by TLR ligands makes these ligands appealing as potential radioprotectors. Among these are multiple pathogen-associated molecular patterns (PAMPs). Unlike cytokines, many PAMPs have minor effect besides activating TLRs and thus are unlikely to produce side effects if used as radiation countermeasures. Moreover, many PAMPs are present in humans at all times [235].

TLR5 is expressed in the tissues damaged the most by radiation exposure, hematopoietic cells

and the small intestine. Flagellin of *Salmonella enterica serovar Dublin* is a ligand for TLR5 and is an extremely stable protein. Preliminary studies indicated that an injection of purified flagellin protected mice from lethal doses of total body gamma radiation with better efficacy than other known radioprotectants [236]. A series of flagellin derivatives were generated and screened for NF-κB-inducing and radioprotective activities. A significantly improved product, CBLB502 (now known as Entolimod™, Cleveland BioLabs, Inc., Buffalo, NY), was created, which retained the radioprotective efficacy of flagellin while having reduced toxicity and immunogenicity [237, 238].

A single injection of CBLB502 before lethal total-body irradiation (24 hr prior or up to 48 hr post-irradiation) protected mice from both gastrointestinal and hematopoietic subsyndromes with higher survival [239]. CBLB502 also demonstrated radioprotective and radiomitigative potential in lethally irradiated non-human primates [237]. A single intramuscular (im) injection of CBLB502 significantly increased the survival of rhesus non-human primates exposed to 6.5 Gy total-body irradiation (LD_{50} dose) and promoted the regeneration of their small intestine, spleen, thymus and bone marrow when administered from 1 to 48 hr after irradiation [239]. The severity and duration of irradiation-induced thrombocytopenia and neutropenia also were decreased significantly by CBLB502 treatment.

Recent studies identified two cytokines, granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6), as candidate biomarkers for the radioprotective and radiomitigative efficacy of CBLB502. Induction of both G-CSF and IL-6 by CBLB502 is TLR5-dependent, occurs in a CBLB502 dose-dependent manner, and is critically important for the ability of CBLB502 to rescue irradiated animals from death [240]. Administration of either G-CSF or IL-6 neutralizing antibody abrogated the radiomitigation by CBLB502. These biomarkers are likely to be useful for the accurate prediction of the CBLB502 dose for radioprotection or radiomitigation in humans. Further, CBLB502 has been demonstrated to significantly reduce the severity of dermatitis and oral mucositis caused by local radiation exposure [241]. CBLB502 has also been shown to alleviate the occurrence of pneumonitis, radiation-induced pulmonary fibrosis and skin injury [242].

The FDA has granted investigational new drug (IND) status to CBLB502 as a radiation countermeasure for ARS, and currently, it is in clinical development [243]. Data from a human safety study indicates that CBLB502 is well tolerated and biomarker results correspond to previously demonstrated biomarkers in animal models for ARS [240]. Cleveland BioLabs anticipates filing a Biologic License Application for FDA approval in the near future. Like other radiation countermeasures for ARS, CBLB502 has been granted fast-track status by the FDA.

In addition to its evaluation as a radiation countermeasure, CBLB502 has also been investigated for its use as an anti-cancer drug. Mechanistic analyses showed that CBLB502 stimulates $CD8^+$ T-cell proliferation and enhances their tumor killing activity through a mechanism that involves the IL-12 signaling pathways [244]. Activation of TLR5/Myd88 (myeloid differentiation factor 88) signaling upregulates the secretion of cytokines, which in turn regulates the neutrophil infiltration in tumor xenografts. The infiltrating neutrophils inhibit the growth of tumor xenografts demonstrating that microflora in the respiratory tract (which can initiate innate immunity) might be helpful in tumor regression [245].

Additionally, TLR5 is widely expressed in breast carcinoma and other cancer cells. Activation of TLR5 by flagellin modulates the production of proinflammatory cytokines to elicit a potent antitumor activity in breast cancer [246]. This approach may serve as a novel therapeutic target for human breast cancer therapy. Administration of CBLB502 results in rapid activation of STAT3 (signal transducer and activator of transcription 3) pathway in addition to induction of NF-κB in the liver and rescues mice from lethal doses of hepatotoxic Fas-agonistic antibodies. Thus, TLR5 agonists can be considered for the prevention and treatment of liver metastasis and hepatoprotective applications [247].

ON01210/Ex-RAD®

ON01210 (a chlorobenzylsulfone derivative known as Ex-RAD®) is a novel, small-molecule

kinase inhibitor under development as a radiation countermeasure. Ex-RAD® provided significant protection against cobalt-60 γ–irradiation when administered subcutaneously (sc) (500 mg/kg) to C3H/HeN mice 24 hr and 15 min before radiation exposure. The estimated dose reduction factor (DRF) for Ex-RAD® is 1.16 [248]. A significant survival benefit was observed as well after prophylactic oral (po) administration of the drug (comparable levels of survival compared to sc administration) [249]. In a radiation mitigation experiment, when Ex-RAD® was administered 24 and 36 hr after radiation exposure (7.5 Gy cesium-137), it protected 90% of C3H/HeN mice compared to 50% survivors in a vehicle-treated control group [250]. Despite the promising nature of these observations and in order to more critically examine the efficacy of this radiation countermeasure, additional studies are clearly needed using higher, whole-body radiation doses rather than those that are at or near the LD_{50} radiation dose level for this strain of laboratory mice. Further, experimental work on Ex-RAD®'s radioprotective/injury mitigative effects (i.e., drug efficacy) needs to be extended to more relevant, large animal models, such as the nonhuman primate of acute radiation injury.

Ex-RAD® accelerated the recovery of peripheral blood elements in irradiated mice when administered either by sc or oral (po) routes [249, 251]. Similarly, Ex-RAD®-treated mice (either through po or sc route) contained a higher number of granulocyte macrophage-colony forming units (GM-CFUs) than in vehicle-injected mice. Ex-RAD®-treated mice had a higher number of surviving intestinal crypts in acutely ionizing radiation-exposed mice as compared to untreated, ionizing radiation-exposed controls [251]. Bone marrow obtained from irradiated mice indicated that Ex-RAD® protected cells from radiation-induced apoptosis after exposure to cobalt-60 γ-irradiation [251]. It has also been demonstrated that attenuation of an ATM-p53 mediated DNA damage response by Ex-RAD® contributed the mitigation of radiation-induced hematopoietic toxicity [252]. Recently, Kang *et al.* showed that Ex-RAD® manifests its protective effects through the up-regulation of PI3-kinase/AKT pathways in cells exposed to radiation [253].

In brief, Ex-RAD® is a radiation countermeasure that has been granted US FDA IND status and has demonstrated oral efficacy. Oral administration holds better clinical promise as an effective countermeasure for first responders as well as for at-risk civilian populations in a nuclear accident. Onconova Therapeutics, *Inc*. (Newtown, PA), the pharmaceutical drug developer, has completed two phase-I clinical studies using Ex-RAD® in healthy volunteers and reported no evidence of systemic side effects [254]. Recently, a study using nonhuman primates has been initiated at the Armed Forces Radiobiology Research Institute in collaboration with Onconova Therapeutics with research support from the Defense Medical Research and Development Program (DMRDP), US Department of Defense.

Vitamin E isomers

Vitamin E represents a family of fat-soluble compounds that act as important antioxidants in the human body. It is an essential vitamin that must be obtained from outside sources like food and supplements, as the human body cannot manufacture it on its own. As an antioxidant, Vitamin E acts to regulate peroxidation reactions and controls free radical production within the body [255]. This family of compounds has eight different isoforms that belong to two categories: four saturated analogues (α, β, γ and δ) called tocopherols and four unsaturated analogues referred to as tocotrienols. These 8 components are collectively known as tocols. Tocotrienols differ structurally from tocopherols in the presence of three trans-double bonds in the hydrocarbon tail. The isomeric forms of tocopherol and tocotrienol are distinguished by the number and location of methyl groups on the chromanol rings. Although tocotrienols were discovered five decades ago, the majority of their biological properties have been revealed only in the last decade due to substantial increase in research interest. The anti-inflammatory, antioxidant and cholesterol-lowering properties of tocotrienols can prevent cancer and diabetes, as well as cardiovascular and neurodegenerative diseases [256]. While several investigations have suggested that α-tocotrienol is highly neuroprotective [257], it has been demonstrated that δ-tocotrienol is

effective in targeting prostate cancer stem cell-like population and was found to be effective against pancreatic carcinoma [258, 259]. δ-tocotrienol and γ-tocotrienol are comparable and appear to be better than other tocols [260, 261]. Here, we will briefly summarize recent progress with δ- and γ-tocotrienol).

δ-tocotrienol

δ-tocotrienol has demonstrated antioxidant activity greater than that of γ- and α-tocotrienol in the membrane system while protecting primary neuronal cells against glutamate toxicity [262, 263]. Such powerful antioxidant activity made δ-tocotrienol another promising candidate for evaluation as a radiation countermeasure. A single sc injection of δ-tocotrienol before or after cobalt-60 γ-irradiation significantly protected mice in a 30-day survival experiment. δ-tocotrienol was effective at a wide dose range of 19 to 400 mg/kg [264, 265]. The DRF values for radioprotective treatment (24 hr before irradiation) with 150 and 300 mg/kg were 1.19 and 1.27, respectively. For radiomitigation treatment with 150 mg/kg of δ-tocotrienol administered 2 hr after irradiation, the DRF was 1.1. When δ-tocotrienol was administered at 300 mg/kg dose 24 hr before irradiation, it significantly reduced radiation-induced cytopenia, suggesting its stimulatory effects on hematopoietic recovery [265]. Recently, it was demonstrated that δ-tocotrienol reduces activation of caspases 3, 7 and 8 while increasing autophagy-related beclin-1 expression in irradiated bone marrow cells [266]. δ-tocotrienol has been reported to increase cell survival and regeneration of hematopoietic microfoci and lineage$^-$/Sca-1$^+$/c-Kit$^+$ stem and progenitor cells in irradiated mouse bone marrow cells. δ-tocotrienol also protected CD34$^+$ cells from radiation-induced damage [264]. δ-tocotrienol activated extracellular signal-related kinase (Erk) phosphorylation and inhibited γ-H2AX foci. Further, δ-tocotrienol up-regulated mTOR and phosphorylation of its downstream effector 4EBP-1. These changes were associated with activation of mRNA translation regulator eIF4E and ribosomal protein S6. These findings suggest that δ-tocotrienol protects mouse bone marrow and human CD34$^+$ cells from radiation-induced injury through Erk activation associated with the mTOR survival pathway.

γ-tocotrienol

γ-tocotrienol has received attention in recent years due to its antioxidant activity. Several important studies have been reported looking into its radioprotective efficacy. At a dose of 100 and 200 mg/kg administered 24 hr before cobalt-60 γ-irradiation, γ-tocotrienol significantly protected mice against radiation doses as high as 11.5 Gy. Its dose reduction factor as a radioprotector (24 hr before irradiation, 200 mg/kg dose) was 1.29. γ-tocotrienol treatment accelerated hematopoietic recovery as judged by higher numbers of total white blood cells, neutrophils, monocytes, platelets, and reticulocytes in peripheral blood [267], and enhanced hematopoietic progenitors in bone marrow of irradiated mice [268]. γ-tocotrienol-treated irradiated mice had higher numbers of colony-forming cells, more regenerative microfoci for myeloid and megakaryocytes, higher cellularity in bone marrow, and reduced frequency of micronucleated erythrocytes compared to vehicle-treated irradiated mice [268]. Prophylactic γ-tocotrienol administration demonstrated upregulation of anti-apoptotic genes and downregulation of pro-apoptotic genes (both at transcription and protein levels) at 4 and 24 hr after irradiation [269]. Results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and jejunal crypt analysis showed protection of gastrointestinal tissue by prophylactic treatment with γ-tocotrienol.

Kulkarni *et al*. measured various cytokines and growth factors by cytokine array and Luminex in a mouse model [270]. γ-tocotrienol treatment resulted in significant induction of G-CSF in mice. G-CSF levels increased markedly within 12-24 hr after γ-tocotrienol injection. Time course analysis demonstrated that G-CSF was induced transiently after γ-tocotrienol administration, and returned to background levels by 48 hr after γ-tocotrienol administration. IL-6 followed a similar stimulation pattern in response to γ-tocotrienol administration, however, the peak of IL-6 was observed at an earlier time point compared to G-CSF. Survival studies with γ-tocotrienol suggested

the most efficacious time for drug administration was 24 hr prior to irradiation. This may be due to induction of key hematopoietic cytokines in that time frame. These results also suggest a possible role of γ-tocotrienol-induced G-CSF stimulation in protection from radiation-induced neutropenia and cytopenia. Using various radiation countermeasures (including γ-tocotrienol), we have demonstrated that the use of G-CSF antibody abrogates radioprotective efficacy of countermeasures [240, 271-275]. Using different animal models (mice, nonhuman primates and canines), it was demonstrated that G-CSF and IL-6 may serve as biomarkers for selected radiation countermeasures [240].

γ-tocotrienol is also an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Study was conducted to evaluate whether HMG-CoA reductase inhibition plays a role in the radioprotection afforded by γ-tocotrienol. Results demonstrate that γ-tocotrienol decreases radiation-induced vascular oxidative stress, an effect that was reversible by mevalonate (the product of reaction catalyzed by HMG-CoA) [276]. γ-tocotrienol also reduces intestinal radiation injury and accelerates the recovery of soluble markers of endothelial function [276]. HMG-CoA reductase inhibitors mediate their pleiotropic effects via endothelial nitric oxide synthase that needs the cofactor 5,6,7,8-tetrahydrobiopterin. Radiation exposure decreased tetrahydrobiopterin in lungs, which was reversed by γ-tocotrienol administration. Both γ-tocotrienol and tetrahydrobiopterin supplementation reduced post-irradiation vascular peroxynitrite production [277]. γ-tocotrienol also ameliorated endothelial cell apoptosis and reduced endothelial cell guanosine triphosphate cyclohydrolase 1 (GTPCH) feedback regulatory protein (GFRP) levels and GFRP-GTPCH binding by decreasing transcription of the GFRP gene.

Combined treatment with tocols and the methylxanthine derivative pentoxyfylline (phosphodiesterase inhibitor) is effective in reducing and even reversing radiation-induced cardiac, lung, intestinal, and dermal injury [278, 279]. A majority of these reports studied the effects of the tocol/pentoxyfylline combination on radiation-induced fibrosis, a late effect of irradiation, and there is very little known about the effects of this combination on acute radiation injury. Berbee *et al.* reported improval in survival of mice against cobalt-60 γ-irradiation by combined treatment with γ-tocotrienol and pentoxyfylline compared with either γ-tocotrienol or pentoxyfylline administered alone [280]. The γ-tocotrienol and pentoxyfylline combination protected all mice against radiation doses as high as 12-Gy. γ-tocotrienol plus pentoxyfylline also improved bone marrow colony-forming units, spleen colony counts and platelet recovery compared to γ-tocotrienol alone. There was no benefit of the combination in ameliorating intestinal injury and vascular peroxynitrite production [280]. Based on such encouraging findings, γ-tocotrienol has been selected by AFRRI as the most promising agent of tocols for development as a radiation countermeasure. Currently, it is being investigated for its pharmacokinetics, pharmacodynamics and efficacy against cobalt-60 γ-irradiation using nonhuman primates.

There is great interest in identifying the potential of vitamin E analogues as anticancer drugs and adjuvants in the past decades. α-tocopherol exhibits the highest vitamin E bioactivity among the eight isoforms of vitamin E. Although the best understood function of vitamin E is its antioxidant propensity, recent studies demonstrate that certain vitamin E forms do exhibit antitumor properties. α-tocopherol succinate (TS), the most studied apoptogenic vitamin E analogue, has rationally been the optimal choice. Several studies have clearly demonstrated TS to be a promising anticancer agent [281]. Many chemotherapeutic drugs kill not only tumor cells but also normal cells, resulting in side effects. However, TS shows unique selectivity in killing tumor cells, while not affecting normal cells. TS has shown high levels of apoptosis induction in a variety of cancer cells from different species and various organs. Although mitochondria are central to apoptosis induction by vitamin E analogues in cancer cells, there are other pathways modulated by these agents that may run parallel to the major mitochondrial apoptotic signaling [282]. The downstream signals originating from mitochondria are initiated by cytosolic translocation of the mediators such as cytochrome c, apoptosis

inducing factor (AIF) or Smac/Diablo, all of which may relocate as a response to exposure of cells to vitamin E analogues. These pathways result in either caspase-dependent (cytochrome c) or caspase-independent apoptosis (AIF) or in secondary modulation of other signaling pathways (Smac/Diablo) [281].

There are several promising radiation countermeasures under development such as CBLB613 [283], CBLB612 [284], IL-12 [285, 286], epidermal growth factor [287], fibroblast growth factor-2 [288], fibroblast growth factor-peptide [289], insulin-like growth factor-1 [290], tempol [291], tocopherol succinate [14], TPO (thrombopoietin) receptor agonist (ALXN4100TPO) [292], 5-Androstenediol (5-AED)/Neumune® [261], BDP/OrbeShield™ (Soligenix) [293], AEOL-10150 (Aeolus Pharmaceuticals) [294], growth factors [3] etc. Since it was not possible to discuss all the agents under development in this review, we selected some of those agents which are at advanced stages of the development. Information sharing and full cooperation among health care providers, scientists, and research organizations, both public and private alike, are key elements to that future success in this area of public concern.

CONCLUSION

Herein, we have reviewed the basic principles of radiation and mitigation and described some of the more recent research into the radiobiology and potential clinical applications of these agents. These agents represent a wide variety of molecule types, including small molecule drugs and drug-like compounds (captopril, Ex-RAD), phytochemicals (plant-derived agents) (DIM, genistein), vitamins (tocols), peptides (flagellin, CBLB502), growth factors, and other agents. Our goal was to provide the reader with a framework for understanding the types of agents under development and the molecular pathways that they may target, rather than presenting a compendium. Examples of agents that radioprotect normal tissues but not tumors have been provided. Some such agents may exhibit antitumor activity, particularly at higher concentrations (*e.g.*, DIM and genistein). Due to the complexity of the responses of different cell types and tissues to radiation, opportunities for rational drug design have been limited, whereas most examples of radioprotectants are based upon empirical observations. Many questions remain, such as why some compounds are strong protectants but weak mitigators (*e,g.*, tocopherols) and why protectants often selectively target normal tissues and not tumors.

Mitigation of radiation injury is a more stringent requirement than radioprotection *per se*, although there are situations where radiation exposure is likely and an effective protectant would be useful. Examples include individuals responding to a nuclear disaster (*e.g.*, reactor meltdown or astronauts who will be exposed to cosmic radiation). In the setting of mitigation, the longer after an acute radiation exposure that a prospective mitigant can be first administered, the more useful it would be for situations where there is no advanced warning or knowledge of exposure. Here, a mitigant that works when administered 48 hr after exposure will be more useful than one that works only within a few hours of exposure depending upon the time it takes to deliver the agent to exposed individuals; but the requirements of such an agent becomes more stringent as the time after exposure increases.

Typically, agents under consideration are tested in rodents using a 30 day time interval to determine survival; while non-human primates (monkeys) are tested using a 60 day interval. These time intervals were chosen to reflect the ability of the agent to protect against or mitigate acute radiation syndrome following whole body exposure to nuclear radiation (*e.g.*, ^{60}Co). However, they do not reflect the possibility of serious injury to other radiosensitive organs besides the gastrointestinal and hematopoietic systems that might occur at later time points (*e.g.*, lungs, skin, and kidneys). Later effects of whole body, near whole body, or partial body exposures are understudied areas of research in the field. Another is the use of combinations of agents for radioprotection/mitigation analogous to the way combination chemotherapy with agents of differing toxicity and mechanisms of action has been utilized to obtain superior results to single agents. Finally, it would be interesting to know if there are other FDA-approved drugs (see section on captopril) or food additives (see sections on DIM and genistein)

that exert radioprotective or mitigative activity and could be "repurposed" for these indications.

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CONFLICT OF INTEREST STATEMENT

Dr. Rosen has an intellectual property interest in the usage of diindolylmethane (DIM) as a radioprotector and mitigator.

DISCLAIMER

The opinions or assertions contained herein are the private views of the authors and are not necessarily those of the Armed Forces Radiobiology Research Institute, the Uniformed Services University of the Health Sciences, or the Department of Defense.

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