Teaser Entolimod is a potentially useful radiation medical countermeasure and, based on its efficacy and relatively good safety profiles, it received investigation new drug (IND) status from US FDA and its pre-emergency use authorization application is currently under review.

Entolimod as a radiation countermeasure for acute radiation syndrome

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High doses of total-body or partial-body radiation exposure can result in a life-threatening acute radiation syndrome as manifested by severe morbidity. Entolimod (CBLB502) is effective in protecting against, and mitigating the development of, the hematopoietic and gastrointestinal subsyndromes of the acute radiation syndrome in rodents and nonhuman primates. Entolimod treatment reduces radiation-induced apoptosis and accelerates the regeneration of progenitors in radiation-damaged tissues. The drug has been evaluated clinically for its pharmacokinetics (PK), toxicity, and biomarkers. The US Food and Drug Administration (FDA) has granted investigational new drug, fast-track, and orphan drug statuses to entolimod. Its safety, efficacy, and animal-to-human dose conversion data allowed its progression with a pre-emergency use authorization application submission.

Introduction

Radiation exposure from radiological/nuclear incidents, intentional or unintentional, can result in various types of injury. Such radiation exposure-induced injuries require appropriate diagnoses and treatments [1]. Irradiation can be either total-body irradiation (TBI) or partial-body irradiation (PBI). Such accidental and/or nuclear exposures are more than likely to be non-uniform (heterogeneous) by nature. Exposure of the whole-body or the partial-body to high, intense doses of sparsely ionizing, deeply penetrating radiation can lead to acute illnesses known in aggregate as the acute radiation syndrome (ARS). The progression of ARS depends on the absorbed radiation dose, the intensity of exposure, and its distribution within bodily tissues [2]. Clinical manifestations of ARS are recognized by several subsyndromes, namely: (i) hematopoietic ARS (H-ARS); (ii) gastrointestinal ARS (GI-ARS); and (iii) neurovascular ARS [3,4]. The ranges of radiation doses that are estimated to elicit these subsyndromes in humans following uniform TBI (a less than likely exposure scenario) and in the absence of supportive care are as follows: 2–6 Gy for H-ARS, 6–8 Gy

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for GI-ARS, and >10 Gy for neurovascular syndrome. The neurovascular subsyndrome is believed to be untreatable because of the requisite supralethal doses of radiation that initiate pathogenic process(es) that are quickly and highly fatal as a result of systemic vascular complications and multiorgan failure.

Medical radiation countermeasures for ARS are categorized into three major classes; (i) radioprotectors are administered before radiation exposure; (ii) radiomitigators are used shortly after radiation exposure but before the development of symptoms of ARS; and (iii) radiotherapeutics are used as ARS manifests [5]. Numerous medical radiation countermeasures are currently being developed by academic institutions, government laboratories, and corporations [6–8]. Radioprotectors and radiomitigators are mostly small-molecule chemicals or biologics, and most of the radiotherapeutics are cell-replacement therapies [9,10]. Radioprotectants are useful for planned and predictable radiation exposure events and can be deployed largely by military personnel, first responders, and perhaps civilians involved in emergency responses. However, such agents might not be as valuable for unpredictable radiation mass casualty scenarios for which preparations cannot be made. The use of radiomitigators, or agents effective when administered after radiation exposure, can serve to reduce the overall number of casualties. For radiomitigation, agents promoting tissue regeneration that enable the organism to survive coagulopathy, immunosuppression, and loss of gastrointestinal (GI) tract integrity are valuable, especially compared with antioxidant-based agents or agents that selectively limit free radical-mediated cell injury and/or inhibit subsequent cell death (i.e., apoptosis) [11]. An agent having a combination of radioprotective and radiomitigative attributes would have added medicinal benefit and, thus, utilitarian value.

There are several promising radiation countermeasures under advanced development that appear to be efficacious and safe for use as radioprotectors for radiological/nuclear scenarios. However, such agents have not yet been approved by the FDA and require additional investigations [7,8,12–16]. Nevertheless, there are three radiomitigative agents that have been approved by the FDA: Neupogen®, Neulasta®, and Leukine®. These countermeasures are all recombinant biologics and have been approved solely for H-ARS [17–25]. Currently, there are no radioprotectors for H-ARS or for GI-ARS that have been approved by the FDA [6]. Entolimod is another promising agent under development following the Animal Rule of the FDA [26]. It is effective in animal models with single-dose administration and does not need full supportive care. However, there is difficulty in comparing the relative efficacy of entolimod, with the previously listed, FDA-approved recombinant growth factors (Neupogen, Neulasta, and Leukine), because no direct head-to-head comparative testing has been reported. The three FDA-approved radiomitigators have been in clinical use for decades and have well-established efficacy and safety profiles based on the large number of patients treated for other indications. The maximum efficacy of Neupogen is achieved with daily dosing until neutropenia is improved for more than 3 days (~16 injections total). The use of Neulasta reduces the need for frequent dosing but is hard to remove if patients develop intolerable adverse effects. It is also notable that, in a 2014 study, granulocyte-colony stimulating factor (G-CSF) failed to demonstrate radiomitigative efficacy in a nonhuman primate (NHP) model in the absence of full supportive care. including blood transfusion [6]. Furthermore, both Neupogen and Neulasta result in delayed acute respiratory distress syndrome (ARDS). It is important to recognize the limitations of approved growth factors while acknowledging their effectiveness. Ideal medical countermeasures (MCM) for radiation would be those that can both mitigate and protect injuries without supportive care because infrastructure might not be available for the treatment of all the victims under a large-scale mass causality scenario. Another limitation of these three already FDA-approved radiomitigators is that they do not protect against radiation injuries when administered before radiation exposure.

Role of Toll-like receptors and their ligands in NF-κB stimulation and subsequent induction of radioprotection

Acute radiation-induced injury within radiosensitive tissues, such as the hematopoietic, GI, and neurovascular systems, occurs mainly through programmed cell death process(es), commonly called 'apoptosis.' Such cell death is controlled largely by the p53 pathway and its activation. Neoplastic cells, as part of their survival strategy, frequently lose apoptotic mechanisms during malignant progression [27]. Fundamentally important mechanisms for tumor resistance to select types of chemo/radiotherapy protocols and associated induced apoptosis involve the dysregulation of the p53 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways. Tumors lose p53 function by the inactivation of the proapoptotic pathway, while activating NF-κB by the upregulation of antiapoptotic genes [28–30]. The strategic development of entolimod, also known as CBLB502, was based on a well-founded, basic biological concept that select genetic mechanisms acquired by malignant cells serve to avoid apoptotic cell death through inhibition of p53 and activation of NF-κB. As such, these processes can effectively increase resistance within sensitive cells, tissues, and organ systems of the body resulting in a decline of induced injuries to normal tissues.

Radiation-induced apoptosis within select radiosensitive tissues is thought to be mediated by the activation of proapoptotic p53, whereas the pharmacological inhibition of p53 serves to limit this inhibition, thus promoting radioprotection of sensitive cells [31]. This hypothesis was tested through the use of pifithrin-α, an inhibitor of p53 that protected mice exposed to γ-radiation [32]. The inhibition of apoptosis proved to be helpful for protection from radiation exposure, but the suppression of p53 solely for the purpose of radioprotection had significant limitations. Mice deficient in p53 were resistant to the radiation-induced hematopoietic syndrome, but were more sensitive to the GI syndrome because growth arrest in continuously dividing crypt epithelium led to mitotic catastrophe [33]. Given such limitations, activation of NF-κB was used as an alternate tumor-specific antiapoptotic strategy. The protective role of NF-κB is mediated by the activation of multiple genes and their expressed gene products: (i) antiapoptotic proteins inhibit major apoptotic pathways [34]; (ii) growth factors and cytokines induce the proliferation and survival of hematopoietic and other stem cells; and (iii) free radical-scavenging antioxidant proteins [e.g., manganese superoxide dismutase (MnSOD) and superoxide dismutase 2 (SOD2)] scavenge reactive oxygen species (ROS) [35].

Toll-like receptors (TLRs) serve as potent immunostimulants by activating immunocytes and inducing NF-κB [36–38]. They mediate NF-κB signaling and activate both the innate and adaptive immune systems [36,39–41]. The immunostimulatory effect of NF-

κB activation can be achieved by triggering TLRs. The activation of NF-κB by TLR ligands makes these molecules interesting as potential radioprotectors. Among these ligands are multiple pathogen-associated molecular patterns (PAMPs). PAMPs are molecules that are not generally present in the host, cannot be easily mutated, and are characteristic of large groups of pathogens [42,43]. However, several select types of PAMP appear to be ubiquitous in humans, but they are unlikely to produce serious adverse effects because they have limited effects besides activating TLRs [44].

Several TLR ligands are under development as radiation medical countermeasures [13,45,46]. There are several patents and publications with different TLR ligands for development as candidate radiation countermeasures (patents listed in earlier publication [46]). Lipopeptides from *Mycoplasma* have also been shown to have radioprotective activity [47,48]. There are several reports describing direct stimulation of natural killer cells and T lymphocytes by flagellin from *Salmonella enterica* serovar *Dublin* [39–41]. This protein is a strong activator of NF-κB via its interaction with TLR5 [49,50]. Flagellin of *Salmonella typhimurium* origin is also a known activator of NF-κB. A truncated form of this protein, known as CBLB502/entolimod, retains the radioprotective ability and stability of flagellin, but largely lacks immunogenicity [51]. Entolimod has demonstrated promise as a radiation countermeasure based on its efficacy in countering the development of both H-ARS and GI-ARS in mice as well as in NHPs [51–53]. This agent has shown efficacy as both a radioprotector and radiomitigator (Fig. 1) and could be an effective MCM in acutely irradiated humans.

Entolimod/CBLB502 as a radiation countermeasure

The bacterial protein flagellin has been shown to have radioprotective-promoting attributes; however, it is clear that the native bacterial protein is not an optimal candidate for development as a radiation countermeasure because of its antigenicity and toxicity [51,54]. To better understand the toxicity and immunogenicity of bacterial flagellin, its TLR5-activating domains were mapped using recombinant technology to its evolutionarily conserved N and C termini [55]. The most potent of these domain NF-κB activators, namely the N- and C-terminal domains of flagellin separated by a flexible linker, was designated as CBLB502. Recombinant CBLB502 retained the NF-κB-stimulating efficacy and stability of flagellin, but was both less immunogenic and less toxic [51]. Its maximum tolerated dose (MTD) in mice was 25 mg/kg compared with 12 mg/kg for flagellin [56]. Additional derivatives of flagellin lacking NF-κB-activating capacity failed to provide radioprotection in mice, thus suggesting that TLR5-mediated NF-κB activation is necessary for achieving radioprotection. CBLB502 significantly protected mice exposed to TBI from both H-ARS and GI-ARS when administered before irradiation [51]. When administered shortly after (up to 48 h) radiation exposure, entolimod still increased the survival of irradiated animals. Interestingly, administration of this agent

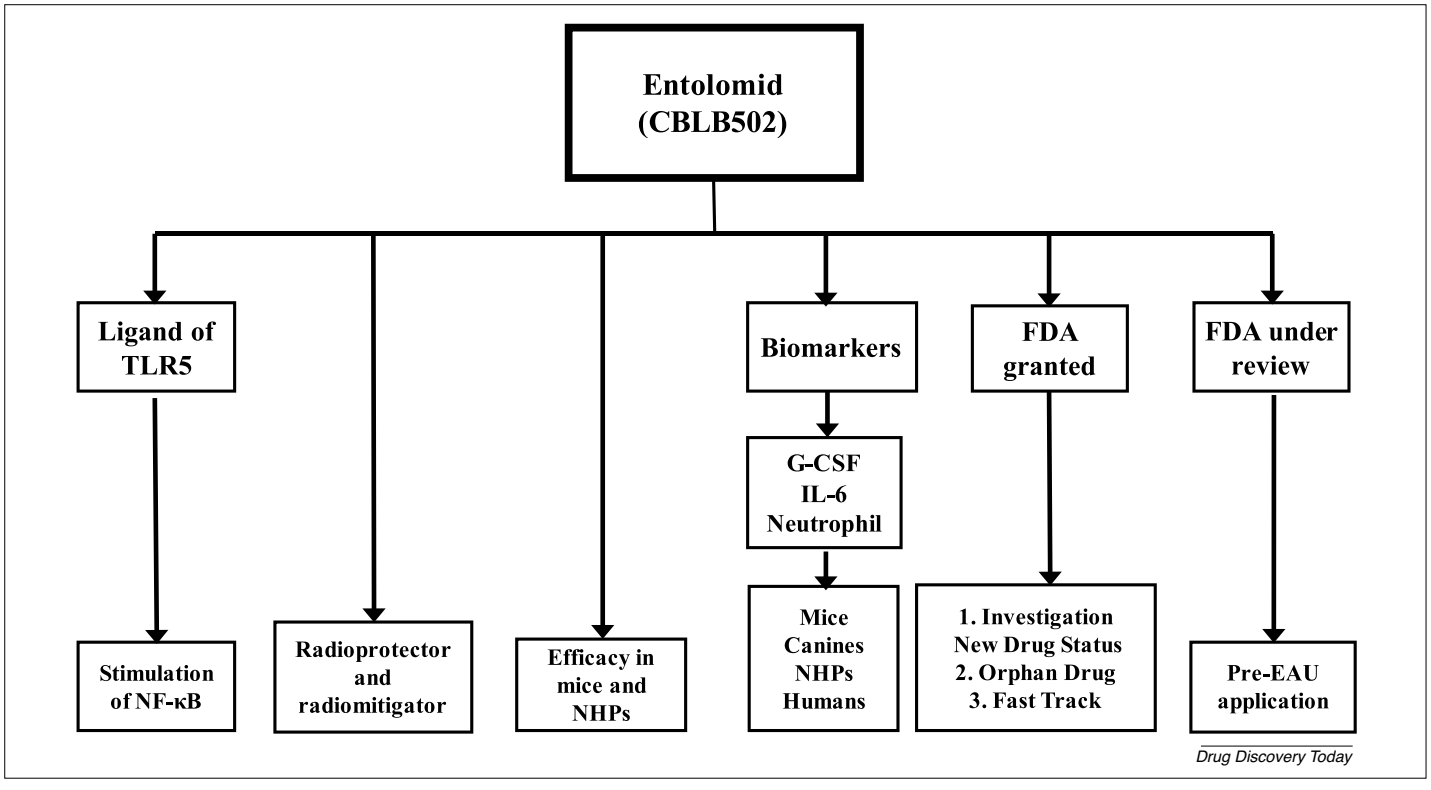

FIGURE 1

Schematic representation of entolimod development. Entolimod is a toll-like receptor 5 (TLR5) receptor ligand that acts via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling mechanisms. It is under development as a radioprotective and radiomitigative countermeasure. The efficacy of entolimod as a radiation countermeasure has been assessed in animal models, such as mice and nonhuman primates (NHPs). All animal (mouse, canine, and NHP) and human studies have established efficacy biomarkers, such as granulocyte-colony stimulating factor (G-CSF), interleukin 6 (IL-6), and neutrophils. The US Food and Drug Administration (FDA) has granted entolimod an Investigational New Drug (IND) status as well as Fast Track and Orphan Drug status. Additionally, a pre-Emergency Use Authorization (EUA) application has been submitted to the FDA.

did not decrease tumor radiosensitivity in the murine model. In addition, entolimod also demonstrated radioprotective efficacy in lethally irradiated NHPs.

Efficacy in murine model

As indicated previously, ionizing radiation-induced injury is associated with significant apoptosis within radiosensitive organs. Entolimod can provide a relatively high degree of protection to normal cells from radiation-induced toxicity. The latter point was demonstrated clearly by laboratory results and the use of small animal/radiobiological testing protocols [51]. Specifically, the radioprotective efficacy of CBLB502 was evaluated as follows [51]: single subcutaneous [sc, i.m. (intramuscular) equally effective] injections at doses of 0.2 mg/kg were administered to NIH-Swiss mice 30 min before 13 Gy irradiation. Mice were exposed to total-body γ-radiation on a rotating platform using ^{137}Cs source at a dose rate of 2.33 Gy/min. The treated/irradiated mice were observed over a 30-day period and daily rates of survival were recorded for the duration. Results showed that this treatment protected 87% of mice; a survival rate considerably better than the survival rates afforded by two other radioprotective agents, namely amifostine [150 mg/kg, intraperitoneally (i.p.), 30 min before irradiation] and 5-androstenediol (5-AED) or Neumune (30 mg/kg, sc, 24 h before irradiation). Flagellin (0.2 mg/kg) was also tested for comparison in a similar experiment using 10, 13, and 17 Gy. Flagellin protected mice against 10 and 13 Gy but failed to demonstrate efficacy against 17 Gy [51]. CBLB502 protected mice against doses of radiation inducing H-ARS or GI-ARS, utilizing 10 Gy and 13 Gy, respectively, when administered 15– 60 min before irradiation. There was no survival benefit if the drug was administered before or after the aforementioned time window. Against a lower, but still highly lethal radiation dose of 9 Gy ($LD_{90/30}$), CBLB502 demonstrated efficacy when administered up to 24 h before or up to 1 h after irradiation. With an injection of CBLB502 1 h post irradiation, 40% of the CBLB502-treated mice survived compared with 7% of control animals [51].

To find the drug dose–response for the radioprotective efficacy of entolimod, ICR mice were injected i.m. with the drug over a wide range, from 0.6 to 60 mg/kg 30 min before 10 Gy TBI in mice. Mouse survival was recorded for 30 days post irradiation. The lowest dose of entolimod that demonstrated significant survival benefit over the control was 6 mg/kg, whereas 20 and 60 mg/kg doses provided maximal survival of ~83–92% (increase of ~75–83% over the control). Based on these studies, the drug dose at the beginning of the radioprotective efficacy plateau was estimated at ~20 mg/kg, whereas the ED_{50} value was determined as ~7 mg/kg [51].

To investigate the radiomitigative potential of entolimod, the drug was administered after irradiation. A full range (0.6–600 mg/kg) was tested, with single doses injected s.c. into C57BL57/6 J mice that had been exposed to 9.5 Gy radiation 24 h before the administration of the drug. The s.c. route was used instead of i.m. because the i.m. injection resulted in high variability/poor reproducibility as a result of the small muscle mass in mice, and the radiomitigative efficacy of CBLB502 was modest compared with the radioprotective schedule. The observed CBLB502-induced survival benefits were significant for doses ≥6 mg/kg. Significant benefit in mouse survival was also shown with administration of entolimod 24 h after 8.5 and 9.0 Gy doses of TBI. The entolimod dose at the beginning of its radiomitigative efficacy plateau was estimated to be in the range of ~10–20 mg/kg, whereas the ED_{50} was determined to be ~2–4 mg/kg. Administration of CBLB502 intravenously (i.v.) 24 h after irradiation resulted in better protection compared with s.c. administration at 8.5–9.5 Gy. There were no differences in radioprotective efficacy of entolimod when assessed within comparably radiosensitive male and female mice of either the ICR or C57BL/6 J strains. The radiation sensitivity was the same between the sexes. TLR5 dependence of radioprotection by entolimod and flagellin, or the parental protein of entolimod, was shown by their failure to protect TLR5-deficient irradiated mice [51,52,54].

Radiation injury to the hematopoietic system as a result of acute, intense irradiation is the major cause of mortality. Thus, it is important to investigate the countermeasure effects of the drug on different cell types within the peripheral blood and bone marrow of irradiated animals [57]. Entolimod treatment reduced the duration and severity of neutropenia, thrombocytopenia, and anemia when administered as a single dose at various time points within 1–48 h post irradiation [51,53].

Treatment with CBLB502 30 min before 15 Gy irradiation reduced the prevalence of apoptotic cells in the lamina propria of the small intestine, including vascular endothelial cells of irradiated mice. This observation supports the suggestion that endothelial apoptosis has a role in GI-ARS [58]. Furthermore, it was shown that CBLB502 ameliorated radiation-induced reduction in crypt size and in crypt cell density within the small intestine when mice were administered 0.2 mg/kg of drug 1 h before 15 Gy irradiation. As judged by an assay using 5-bromo-2'-deoxyuridine (BrdU) labeling, unprotected control animals exposed to such supralethal doses of radiation (e.g., 13 Gy) demonstrated almost complete loss of crypt cells, whereas CBLB502-treated mice retained normal levels of proliferative cells [51]. CBLB502 administration (0.2 mg/kg, 30 min before 15 Gy irradiation) resulted in heightened expression of SOD2 in the small intestine lamina propria. In unirradiated mice administered CBLB502, G-CSF, interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) were all elevated compared with control animals [51].

Efficacy in large animal models of NHPs

In an initial study that used NHPs (rhesus macaque - *Macaca mulatta*), 11 animals (six males and five females) were administered a single dose of 0.04 mg/kg entolimod i.m. 45 min before TBI. The control group had 8 animals: 4 males and 4 females. These NHPs were irradiated with ^{60}Co source with a dose of 6.5 Gy ($LD_{70/40}$) at a dose rate of 1.08 Gy/min and were observed for 40 days. No additional supportive care was administered, except for required fluids and topical antibiotics used to treat cutaneous lesions that occasionally arose. This dose of drug (0.04 mg/kg) represented an equivalent blood concentration of ~0.2 mg/kg entolimod in the blood of mice; these drug dosing concentrations did not demonstrate any apparent signs of toxicity in either species. Survival and behavior of animals were observed for 40 days post irradiation with examination of physiological parameters, serum chemistry, blood counts, and cytokines in blood plasma. This drug dose (0.04 mg/kg) of entolimod when used prophylactically before whole-body irradiation not only delayed the onset of radiation-induced mor-

bidity and mortality, but also increased overall survival rates from 25% in the control group to 64% at 40 days post-irradiation ($P < 0.03$) [51]. As reported earlier, radiation-induced thrombocytopenia is a better predictor of mortality in acutely irradiated primates [59]. In this regard, it is interesting that the entolimod-treated NHPs had less severe and less protracted courses of thrombocytopenia compared with the placebo-treated control animals [51]. Entolimod treatment was associated with accelerated recovery of hematopoietic and immune system tissues, decreased severity and duration of thrombocytopenia, neutropenia, as well as lessened anemia. Clonogenic potential of selected progenitors of the bone marrow was also enhanced [53]. although entolimod did not change the incidence of Grade 4 neutropenia, it decreased the incidence of Grade 4 thrombocytopenia. It accelerated recovery of erythropoiesis and also led to decreased incidence of Grade 4 anemia [53]. The severity of bone marrow, thymus, and spleen damage as revealed by histological examination was reduced in surviving CBLB502-treated NHPs compared with surviving control animals [51]. Drug-treated animals had significantly better bone marrow regeneration and accelerated lymphoid organ (thymus, spleen, and lymph nodes) recovery. The bone marrow of treated animals displayed morphologically accelerated recovery [53]. Gross necropsy and histopathological investigations of entolimod-treated surviving animals demonstrated minor injury to hematopoietic and lymphoid organs (thymus, spleen, and bone marrow), whereas surviving control animals showed moderate to severe injury in these organs [51].

Several additional studies were conducted with different doses of entolimod administered at different times in relation to irradiation using NHPs (Table 1) [53]. Data from studies in lethally irradiated NHPs treated with a single i.m. administration of Entolimod 1–48 h after irradiation were collected, assembled, and analyzed. In brief, these results indicate that, as late as 48 h after acute irradiation, entolimod treatment effectively decreased morbidity while reducing the overall risk of mortality. In all cases, improved survival was associated with accelerated recovery of hematopoietic and immune system organs, reduced severity and duration of anemia, neutropenia, and thrombocytopenia (Table 2), and enhanced clonogenic potential of select progenitor compartments within the bone marrow compared with control irradiated NHPs [53]. The connection between entolimod treatment and platelet recovery has been well established [53]. Furthermore, entolimod administration led to reduced apoptosis and accelerated crypt regeneration in the GI tract [53]. The above NHP studies further support the suggestion of the potential utility of TLR5 agonists as medical countermeasures for nuclear/radiological contingencies.

Biomarkers of entolimod efficacy as a medical radiation countermeasure

The Animal Rule of the FDA directs the development of countermeasures for which clinical trials for efficacy in humans would be unethical [26]. Without human efficacy data, the projection of a human efficacious dose would need to be estimated based on the determination of the efficacious doses in relevant and appropriate animal models of the target disease under study. The estimates would be supported by PK and/or pharmacodynamic (PD) effects data, along with biomarker response information of the drug in animals and healthy human volunteers [60,61].

Identification of G-CSF and IL-6 as biomarkers: dose-dependent responses to entolimod in unirradiated mice

Several cytokines/growth factors were analyzed in peripheral blood (plasma or serum) of ICR and C57BL/6 J at different time points after single administration of Entolimod or vehicle; these included G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, interferon-γ-induced protein-10 kDa (IP-10), keratinocyte chemoattractant (KC), TNF-α, thrombopoietin (TPO), stem cell factor, monochemoattractant protein 1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and monokine induced by γ-interferon (MIG). Results of these assays showed no significant changes occurred in the blood levels of GM-CSF, IL-1β, IL-10, IL-12p70, and TNF-α 0

TABLE 1

Efficacy of entolimod in lethally irradiated NHPs (40-day survival)

Study	Irradiation dose	Entolimod dose mg/kg	Injection time post irradiation (h)	Group size (*N*=)	Survival (%)	*P* value
1	~$LD_{75/40}$ (6.5 Gy)	Vehicle	+1	10	20	–
		40	+1	10	70	0.07
2	~$LD_{75/40}$ (6.5 Gy)	Vehicle	+16	8	25	–
		40	+16	12	67	0.17
		40	+25	10	70	0.15
		40	+48	12	67	0.17
3	~$LD_{50/40}$ (6.75 Gy)	Vehicle	+1	18	50	–
		0.3	+1	18	67	0.50
		3	+1	18	78	0.16
		10	+1	18	94	0.007
4	~$LD_{50/40}$ (6.75 Gy)	Vehicle	+25	10	40	–
		10	+25	10	100	0.01
		40	+25	10	80	0.17
Pooled vehicle vs ≥10 μg/kg entolimod, +25 h	~$LD_{50–75/40}$ (6.5 –6.75 Gy)	Vehicle	+1–25	46	37	–
		≥10	+25	30	83	0.0001

[a]Data from [53].

TABLE 2

Values of neutrophils, platelets, and hemoglobin in peripheral blood in irradiated and entolimod-treated NHPs[a]

Study	Irradiation dose	Entolimod dose (mg/kg)	Injection time post Irradiation (h)	Group Size (*N*=)	Neutrophils		Platelets		Hemoglobin	
					Mean nadir ± SE ($\times 10^3$/mL)	P-value	Mean nadir ± SE ($\times 10^3$/mL)	*P* value	Mean nadir ± SE (g/l)	*P* value
1	~$LD_{75/40}$ (6.5 Gy)	Vehicle	+1	10	0.01 ± 0.005	–	31 ± 27.9	–	59.8 ± 7.4	–
		40	+1	10	0.18 ± 0.128	0.2	59.6 ± 26.2	0.46	77.8 ± 6.9	0.09
2	~$LD_{75/40}$ (6.5 Gy)	Vehicle	+16	8	0.01 ± 0.011	–	3.2 ± 1.7	–	72.5 ± 5.4	–
		40	+16	12	0.06 ± 0.017	0.05	34 ± 16.4	0.09	92.9 ± 7.4	0.04
		40	+25	10	0.02 ± 0.007	0.51	15.8 ± 5.9	0.07	83.3 ± 5.4	0.18
		40	+48	12	0.02 ± 0.005	0.85	12.5 ± 4.6	0.08	93.1 ± 3.2	0.01
3	~$LD_{50/40}$ (6.75 Gy)	Vehicle	+1	18	0.01 ± 0.001	–	8 ± 1.4	–	66.1 ± 3.7	–
		0.3	+1	18	0.01 ± 0.003	0.44	7.2 ± 1.2	0.66	69.1 ± 3.5	0.56
		3	+1	18	0.02 ± 0.006	0.02	16.6 ± 3.7	0.04	78.9 ± 4.1	0.03
		10	+1	18	0.03 ± 0.009	0.01	22.4 ± 3.9	0.002	76.7 ± 3.7	0.05
4	~$LD_{50/40}$ (6.75 Gy)	Vehicle	+25	10	0.01 ± 0.006	–	6.8 ± 2.4	–	60.2 ± 7.6	–
		10	+25	10	0.04 ± 0.011	0.11	21.8 ± 5.4	0.03	77.5 ± 3.8	0.06
		40	+25	10	0.07 ± 0.029	0.09	39.9 ± 12.4	0.03	89.6 ± 4.4	0.005
Pooled vehicle vs ≥10 μg/kg entolimod, +25 h	~$LD_{50–75/40}$ (6.5–6.75 Gy)	Vehicle	+1–25	46	0.01 ± 0.003	–	11.9 ± 6.1	–	64.6 ± 2.9	–
		$\geq 10^E$	+25	30	0.04 ± 0.011	0.006	25.9 ± 5.1	0.08	83.5 ± 2.7	<0.0001

[a] Data from [53].

and 24 h following various doses (1–160 mg/kg) of entolimod. However, KC demonstrated a moderately strong dose-dependent response to entolimod, whereas IL-12p40, MCP-1, MIP-2, and MIG showed a modest dose-dependent response to the drug [52]. Two cytokines, G-CSF and IL-6, demonstrated significant, dose-dependent responses to entolimod.

Within drug-treated (i.m. injected) ICR mice, G-CSF levels peaked 2–4 h after drug administration and returned to the baseline level by 8–24 h post injection. The duration of G-CSF elevation increased with increasing doses of entolimod. None of the investigated cytokines were stimulated in response to administration of entolimod or to flagellin, the parent protein of entolimod, to TLR5-knockout mice [62,63].

Dose-dependent responses to entolimod in unirradiated NHPs and canines

To investigate whether entolimod-elicited cytokine responses are conserved across different species, studies were conducted in NHPs and canines, as well as in mice. Canines were administered entolimod (0.3–100 mg/kg) i.m. and blood plasma cytokines were investigated over a period of 0.5–120 h post drug administration. G-CSF was not tested because of the lack of appropriate reagents for canines. IL-6 and IL-8 demonstrated strong and entolimod dose-dependent stimulation, whereas IL-10 and TNF-α showed modest stimulation with weaker dose responses. IL-6 peaked 2 h post injection and returned to its background level 4–24 h after drug injection [52]. Entolimod-stimulated cytokine/growth factor responses were investigated in NHPs: animals were administered single doses of entolimod (0.3–40 mg/kg) i.m. and levels of G-CSF, GM-CSF, IL-6, IL-1β, IL-8, IL-10, IL-12p70, and IP-10 were assayed in blood plasma over an extended time course (i.e., pre drug administration and at 0.5, 1, 2, 4, 8, 24, and 48 h post drug administration). Levels of both G-CSF and IL-6 demonstrated strong dose dependence, whereas the stimulation of IL-8 was moderate. Low stimulation was found for IL-10 and there was no stimulation of GM-CSF, IL-1β, IL-12p70, or IP-10 by entolimod.

Entolimod dose-dependent induction kinetics of G-CSF and IL-6 were comparable in both NHPs and mice, although the effect was somewhat delayed in NHPs. The levels of IL-6 and G-CSF peaked at ~2 and ~4 h after injection, respectively, and the levels of these cytokines returned to background levels by 24 h post drug administration [52]. There was no major difference between males and females, in either NHPs or canines, for entolimod-elicited cytokine production.

Dose-dependent responses to entolimod in irradiated animals (mouse and NHP)

After assessing the entolimod dose dependence of G-CSF and IL-6 in unirradiated animals, these cytokines were investigated in irradiated ICR mice and NHPs [52]. Mice exposed to 9 Gy of radiation were administered single doses of entolimod ranging from 1.2 to 160 mg/kg 1 h after irradiation. Cytokines were measured in blood plasma collected at 0.25, 0.5, 1, 2, 4, 8, and 24 h after entolimod administration. These tests demonstrated that, when entolimod was administered closer to the time of irradiation (i.e., 1 h post irradiation), higher levels of cytokines resulted relative to those cytokine responses of unirradiated control mice. This might be because of the additive effects of drug and irradiation. Similar additive effects of entolimod and irradiation were reported for IL-6 and G-CSF in irradiated NHPs when entolimod was administered either 45 min before or 1 h after irradiation. These NHPs were exposed to a sublethal dose of irradiation (<$LD_{20/40}$), with single doses of entolimod (1–100 mg/kg) administered i.m. either 45 min before or 1 h after irradiation. Plasma samples were collected both at pre-injection and post-injections times (i.e., at 1, 2, 4, 8, 24, and 48 h). Plasma levels of G-CSF and IL-6 within the irradiated, drug-treated animals were significantly higher (~15–100 fold) than those observed in the NHPs that were either

irradiated without treatment or those not irradiated but administered comparable doses of entolimod [52].

In another study, entolimod (0.3–10 mg/kg) was administered 25 h after 5.75 Gy (~$LD_{30/40}$) of irradiation. In this study, cytokines were analyzed in samples collected at 0, 0.5, 1, 2, 4, 8, 24, and 48 h after treatment. Based on prior work, it was recognized that the markedly elevated irradiation-elicited cytokine responses had passed by the time entolimod was administered to the irradiated animals. Interestingly, levels of IL-6 and G-CSF induced by entolimod at this much delayed time point were very close to cytokine levels observed in unirradiated animals. The latter observations provided for comparisons of the cytokine responses between the same entolimod doses administered to either unirradiated or irradiated NHPs receiving the test drug in a 'delayed fashion' [52].

Role of entolimod-induced biomarkers, G-CSF and IL-6, in its radioprotective efficacy

The importance of a biomarker as a predictor of the efficacy of a countermeasure depends on whether the biomarker is a participant in its therapeutic effect. An optimal biomarker is a component of the mechanism of action of the countermeasure or a mediator of its effect. Monoclonal antibodies to G-CSF and IL-6 were used to neutralize these cytokines in entolimod-treated and irradiated mice to investigate the role of these cytokines in radioprotective efficacy of this countermeasure. C57BL/6 J mice were irradiated with 9.5 Gy and 22 h after irradiation, mice were treated with rat anti-mouse G-CSF antibodies, rat anti-mouse anti-IL-6 antibodies, or a control rat anti-mouse non-specific IgG1 i.p. (500 μg/mouse). Two h after antibody administration (i.e., 24 h after irradiation), all mice were administered 60 μg/kg entolimod i.v. Vehicle-injected mice served as the control. Neutralization of either IL-6 or G-CSF reduced the radiomitigative efficacy of entolimod [52] demonstrating that these cytokines have an important role in its radiomitigative actions.

Pharmacokinetics and pharmacodynamics of entolimod in unirradiated and lethally irradiated NHPs

PK studies have been reported for entolimod using NHPs [53]. The PK was similar in unirradiated and irradiated NHPs with doses of 0.3, 3, and 10 mg/kg. The values for maximum concentration observed (C_{max}) and area under curve for 24 h ($AUC_{0–24}$) were very close at same doses in two experimental conditions. In both unirradiated and irradiated NHPs, there was good dose response with the above three doses of drug [53].

Plasma levels of several cytokines at various time points following different doses of entolimod administration to unirradiated and irradiated NHPs have been reported [52]. G-CSF and IL-6 demonstrated consistent dose-dependent responses to entolimod when administered to NHPs after exposure to potentially lethal doses ($LD_{50–75/40}$) of radiation. Levels of both cytokines peaked 2–4 h after drug administration [52]. The results with unirradiated animals or animals exposed to near lethal doses ($LD_{20\text{-}30/40}$) of radiation were similar. Both cytokines were also induced by irradiation. Treating with entolimod close to the time of irradiation (e.g., at 1 h after exposure) resulted in a combined effect (i.e., irradiation, along with drug treatment) with respect to the levels of cytokines. If entolimod was administered 25 h post-irradiation, at the time radiation-induced G-CSF and IL-6 levels had leveled off and had returned to base-line values, induced cytokine levels were again comparable to those observed in unirradiated NHPs [52].

In addition to G-CSF and IL-6, a few other cytokines, such as IL-8, having mobilizing potential for neutrophils and IL-10 having anti-inflammatory activity, were induced by entolimod in both unirradiated and irradiated animals [52,53]. However, the induction and dose response patterns following entolimod administration were less consistent in both irradiated and non-irradiated animals.

Clinical studies with entolimod

Entolimod has also been investigated in five clinical studies (one active non-recruiting, one unknown status, one completed but no results published, one recruiting, and one withdrawn) using i.m. or s.c. routes in support of its development as a medical radiation countermeasure for ARS [64]. Safety profile and dose-dependent effects on efficacy biomarkers of entolimod have also been investigated in 150 healthy volunteers [64]. Entolimod administration to healthy individuals resulted in low level flu-like symptoms along with temporary decreases in blood pressure and a boost in liver enzymes. Such effects are common and consistent with the increase in cytokine levels expected after entolimod administration. A Phase I study for entolimod was conducted in patients with advanced solid tumors at Roswell Park Cancer Institute [64]. A second Phase I study for entolimod in patients with advanced cancer is ongoing in the Russian Federation [64]. In these above-mentioned clinical trials, entolimod was reported to be well-tolerated and to have a good safety profile [64]. Nevertheless, these reported findings reveal that, under select treatment conditions, entolimod has the capacity to elicit several significant, albeit transient, adverse effects, most prominently hypotension and a battery of flu-like symptoms [64]. Results of these clinical studies have not yet been published.

Mechanism of action of entolimod for radioprotection/radiomitigation

Cells of two vital, radiosensitive organ systems of the body, namely the hematopoietic (bone marrow cells) and GI systems (epithelial cells and dendritic cells located in the lamina propria) are known to express TLR5, thus prompting investigators at Cleveland BioLabs, Inc (CBLI) to explore the utility of TLR5 ligands as a potential medical radiation countermeasure [65]. Genetic polymorphisms of TLRs are well recognized, with some carrying documented effects on biological functions [66–68]. Similar to flagellin, entolimod binds/stimulates TLR5 with the same specificity [51] and biological activities. Furthermore, the binding capacity of flagellin has been attributed to the same key binding domains as found in entolimod (Fig. 2) [53]. The binding of entolimod to TLR5 results in the stimulation of several downstream signaling pathways, including those regulated by the TLR5-activated transcription factor, NF-κB, which is involved in multiple mechanisms of action that counter cell damage following sufficiently intense, ionizing irradiation [53].

Irradiation induces ROS [69]; in turn, entolimod stimulates SOD2 and serves to neutralize these radiation-generated ROS [51,53,70]. Furthermore, as discussed earlier, acute, intense irradiation induces massive apoptosis within sensitive cells, leading to tissue injury and associated cytopenias during ARS. The efficacy of

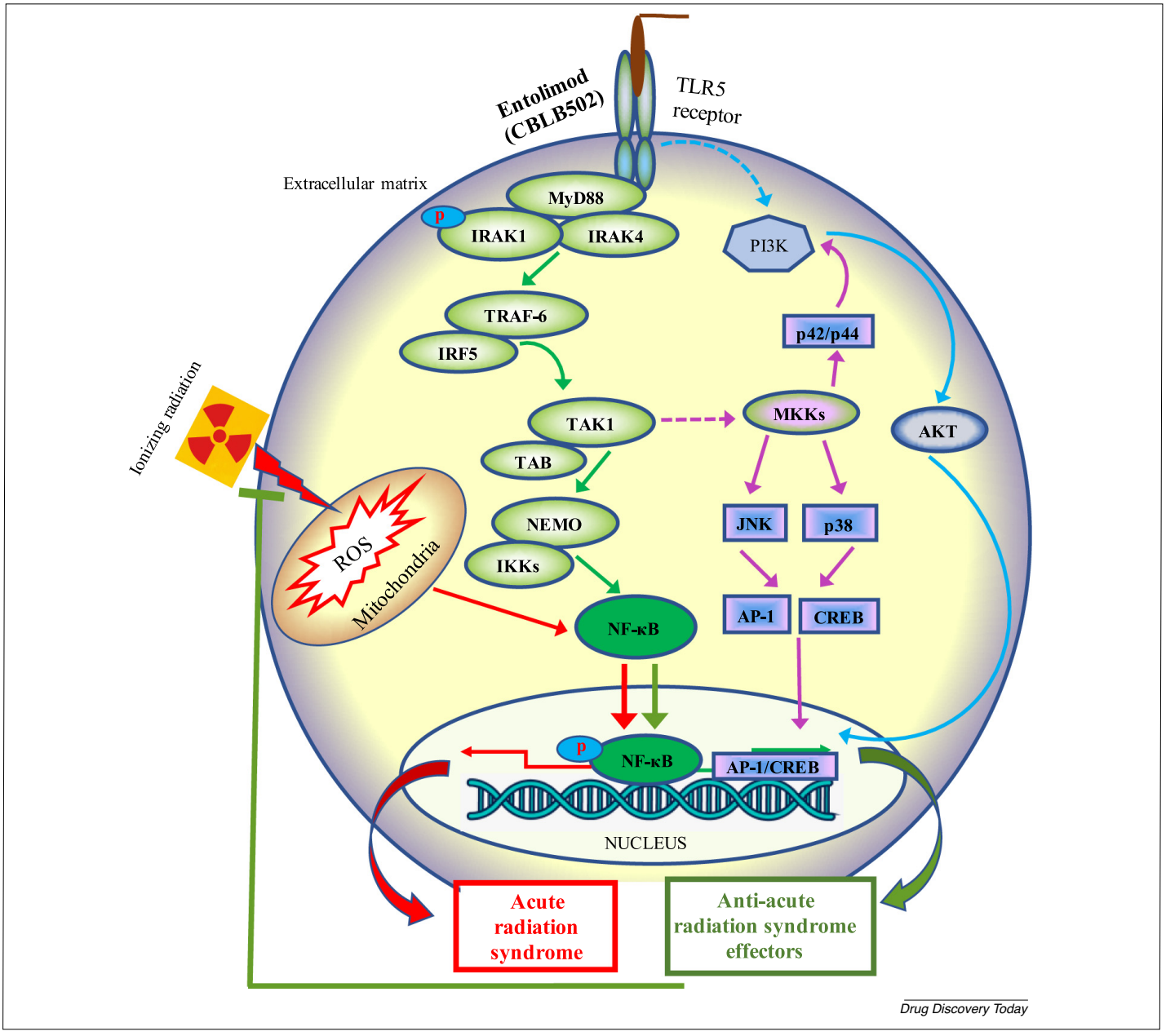

FIGURE 2

Schematic representation of entolimod signaling mechanisms in radioprotection. Entolimod binding to toll-like receptor 5 (TLR5) initiates a cascade of downstream signaling pathways. This involves **(a)** a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent pathway that is controlled by the adopter proteins myeloid differentiation primary-response protein 88 (MyD88) and TRIF and **(b)** NF-κB independent pathways [i.e., phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT) and mitogen-activated protein kinase (MKK) pathways]. The dotted lines represent signaling mechanisms mediated by TLR5, but that are not directly known to be initiated by entolimod. The downstream transcriptional factors induced by the signaling molecules trigger specific genes to release various molecules. These TLR5-dependent effectors counteract the damage to DNA and major pathological processes of acute radiation syndrome (ARS; involving both hematopoietic and gastrointestinal systems) initiated by ionizing radiation. Abbreviations: AP-1, Activator protein 1; CREB, cAMP response element-binding protein; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factors; JNK, c-Jun N-terminal kinase; NEMO, NF-κB essential modulator; p, phosphate; p38, p38 mitogen-activated protein kinases; p42/p44, p42/p44 mitogen-activated protein kinase; ROS, reactive oxygen species; TAB, transforming growth factor-β-activated kinase; TAK, transforming growth factor-β-activated kinase; TRAF, tumor necrosis factor receptor associated factors.

entolimod as a radioprotective/radiomitigative agent is, to a large extent, based on its capacity to limit radiation-induced apoptosis; with the latter being orchestrated by the induction of NF-κB and its downstream antiapoptotic effectors, IAP and Bcl-2 [71–73]. Other antiapoptotic mechanisms of entolimod through TLR5 stimulation might include the activation of the PI3K pathway and the antiapoptotic phosphatase, MKP7 [74,75]. Entolimod was shown to reduce radiation-induced apoptosis not only within hematopoietic tissues, but also within GI tissues in both mice and NHPs [52,53]. In addition, flagellin has been reported to inhibit neutrophil apoptosis [76]. The stimulation of TLR5 inhibits radiation-induced aseptic inflammation involved in secondary

TABLE 3

Investigation of entolimod in various models for other indications[a]

Serial #	Indications	Model used	Treatment details	Result/outcome	Refs
1.	Renal ischemia-reperfusion injury/acute renal ischemic failure	Mouse (acute renal ischemic injury)	0.5 or 1 mg in 200 ml PBS, i.v. 30 min before imposition of ischemia or within 30 min after ischemic kidney reperfusion	Before and after entolimod treatment regimens provided protection from acute renal ischemic failure, attenuated renal dysfunction and inflammation caused by reperfusion of ischemic kidney via TLR5-mediated inhibition of neutrophil and leukocyte infiltration, proinflammatory cytokine production and tubular injury	[95]
2.	Dermatitis and oral mucositis associated with head-and-neck cancer radiotherapy	Mouse (syngeneic and xenograft tumor models)	100 ml of entolimod, 1 mg/mouse for fractioned and 2.5 mg per mouse for single radiation treatment, s.c. 30 min before irradiation	Significantly reduced severity of dermatitis and mucositis developed as a result of single dose (15 or 20 Gy but not 25 Gy) or fractioned [cumulative doses of 25 (10 + 15 Gy) and 30 Gy (3X10 Gy)] local irradiation, radiation-induced weight loss, accelerated tissue recovery, protected normal epithelia without affecting radiosensitivity of squamous cell carcinoma, and elicited radiation-independent and TLR5-dependent tumor-suppressive effect in A549 lung cancer xenografts	[81]
3.	Head and neck cancer radiotherapy toxicity in normal tissues	Mouse	0.3 or 1 μg/mouse/injection/100 μl, s.c., all regimens of entolimod treatment of five injections (at 24 h intervals) starting either 30 min before or 1 h after irradiation	Whereas administration of entolimod 1 h after each irradiation was identified as optimal treatment, both 30 min before or 1 h after irradiation regimens significantly reduced radiation-induced damage to epithelial tissues (tongue, lips, esophagus, skin, and salivary glands), accelerated restoration of normal structure, but no significant improvement in weight loss	[96]
4.	Testicular injuries in cancer patients undergoing radiotherapy	Mouse	0.2 mg/kg, i.p., 30 min before 5 Gy irradiation	Alleviated radiation-induced oxidative stress and distorted architecture of seminiferous tubules, reversed decline of sperm quantity and quality, helped recover male mouse fertility, serum testosterone and superoxide dismutase, decreased levels of malondialdehyde, DNA damage and chromosomal aberrations in irradiated mice and their offspring, and reduced apoptosis via NF-κB	[97]
5.	LPS and TNF-induced adverse toxicity towards normal tissues	Mouse (hepatocellular carcinoma and colorectal cancer models)	1 μg/mouse, s.c., entolimod 30 min before LPS/TNF treatment; 1 μg/mouse, s.c., 30 min – 48 h before LPS/ D-GalN and 30 min – 24 h before TNF/ D-GalN treatments	Entolimod protected liver and lung from LPS/TNF-induced toxicity without interfering with antitumor activity of TNF, upregulated genes for tissue protection, increased resistance of vascular endothelium to TNF toxicity, reduced indicators of toxicity, apoptotic caspase 3/7, lipid peroxidation and serum ALT, and prevented mortality caused by LPS/TNF combined with sensitizer D-GalN	[98]
6.	Hepatic metastases associated with uveal melanoma	Mouse (TLR5-positive B16LS9 mouse model of ocular melanoma)	Seven s.c. injections of entolimod (1 μg/100 ml) 72 h apart, 1 day before, on the day or 3 days after intraocular injection of B16LS9 cells	Treatment before tumor cell inoculation most effective, reduced B16LS9 metastasis, elicited NK cell response by mobilizing and promoting maturation, differentiation, and activation of NK cells in liver, antibody-mediated depletion of NK cells before entolimod treatment abrogated anti-metastatic effect *in vivo* and antitumor cytotoxic activity from hepatic lymphocyte populations *in vitro*	[99]
7.	Genotoxicity (chemotherapy-induced side effects associated with myelo-suppression and gastrointestinal damage)	Mouse: 5-FU	1 mg/100 ml, s.c., entolimod after first 5-FU injection, when given three i.p. injections of 5-FU (100 mg/kg/day), entolimod (1 mg/mouse) injected 24 and 48 h after last 5-FU injection	Reduced toxic effects of 5-FU on intestinal and hematopoietic tissues, stimulated restoration of hematopoiesis and rescued mice against hematopoietic tissue damage in a IL-6-dependent manner, improved integrity of intestinal tissue, and decreased mortality rates	[80]
		Mouse: syngeneic mouse CT26 colon adeno-carcinoma model)	1 mg/ 100 ml, s.c., 1, 48 and 96 h after 5-FU	Entolimod protective effect selective for normal tissues as shown by the reduced systemic toxicity of 5-FU with no changes in its antitumor efficacy	
8.	Ulcerative colitis (UC)	Mouse (TNBS-induced UC model)	3.2 mg/kg, s.c., after 2 h TNBS administration	TLR-IL and dose-dependent therapeutic effects on TNBS-induced colitis, inhibited inflammation, and TLR expression via TLR and NF-κB signaling, reduced mucosal damage	[100]

TABLE 3 (*Continued*)

Serial #	Indications	Model used	Treatment details	Result/outcome	Refs
9.	Fas-mediated hepatotoxicity/ liver metastasis	Mouse (colon carcinoma CT26, lymphoma A20, and breast carcinoma 4T1)	1 μg entolimod on days 5 and 6 or on days 5–9 or with 5 μg on days 5–9 after intrasplenic A20 cell inoculation, for CT26 metastases, two daily, s.c. injections of entolimod (1 μg/mouse)	Significantly suppressed liver metastases of different types of cancer regardless of TLR5 status, protected against Fas-mediated hepatotoxic insults via NF-κB and STAT3 signaling pathways, and protected against *Salmonella* infection	[101]
10.	Dose-limiting toxicities with systemic delivery and metastasis stimulation in cancer therapy	Mouse (CT26 CRC and 4T1 mammary tumor models)	1 μg per mouse/100 μl PBS, s.c.	Suppressed liver metastases and tumor development in lung by mechanisms involving NK and T cell responses, CXCR3-dependent mobilization of NK and other components of immunity to liver, development of $CD8^+$ T cell-dependent long-term antitumor immune memory	[102]
11.	Pneumonitis, pulmonary fibrosis and skin injury of radiotherapy for thoracic neoplasms.	Mouse	30 min before irradiation (20 Gy localized to thoracic area), s.c., as follows: 0.05 mg/kg, 0.2 mg/kg, 0.5 mg/kg	TLR5/MyD88 pathway-dependent activation of NF-□B, stimulation of antiapoptotic cytokines and chemokines, inhibited apoptosis of pulmonary cells, alleviated pneumonitis, pulmonary fibrosis and dermatitis	[103]
12.	GvHD and opportunistic infections in immune-compromised patients	Mouse (mCMV infection)	25 mg/200 ml PBS, i.p. 48 h before mCMV infection	Provided significant protection from mCMV lethality, decreased viral load associated with increased numbers of mature, activated cytotoxic NK cells via TLR5-dependent mechanisms, with no concomitant increase in proinflammatory cytokines	[104]
13.	GVT and GvHD effects during allogeneic bone marrow transplantation	Mouse (allo-BMT A20 B cell lymphoma)	1.0 mg/0.1 ml PBS/ mouse, s.c., 3 d after irradiation and for five times at 1, 3, 5, 7, and 9 d after allo-BMT	Significantly improved GVT immunity without exacerbating GvHD, TLR5-mediated immune modulation stimulated $CD8^+$ T cell response mainly through donor-derived immune and bone marrow cells, enhanced tumor killing via IL-12 and improved host survival	[105]
14.	Tumor immunity/vaccine-based tumor immunotherapy	Mouse (RMAS T cell lymphoma and A20 B cell lymphoma)	100 ml (10 mg/mL), s.c., 4 h after tumor inoculation, and then after every 48 h through day 8	Activated TLR5-expressing accessory immune cells and cytotoxic lymphocytes, upregulated co-stimulatory molecules, improved tumor immunity (innate and adaptive immune cells), induced potent antitumor responses without targeting known tumor antigens, promoted tumor clearance in multiple mouse strains and against different syngeneic tumor types via $CD8^+$ T cell, NK cell-mediated and perforin-dependent mechanisms, and improved survival	[106]
15.	Concanavalin A (Con A)-induced hepatic injury/ immune-mediated hepatitis	Murine Con A-induced acute liver injury	0.2 mg/kg, i.p., 0.5 h after administration of Con A; for D-Gal/LPS-induced fulminant hepatitis: 0.2 mg/kg, i.p., 12 h before i.p. D-Gal/LPS	Attenuated Con A-mediated hepatitis via TLR5/NF-κB-dependent signaling, elevated levels of IL-6, improved survival, reduced T and NK cell activity, proinflammatory cytokine release, Con A-induced increase in total MNCs, number of intrahepatic CD31, CD41, CD81, T and B cells; impaired infiltration of neutrophils, serum ALT, IL-4, TNF-α, IFNs and lymphocytes; ameliorated hepatocyte necrosis/ apoptosis, and suppressed α-GalCer-induced NK cell-dependent inflammatory liver injury	[107]
16.	Radiation-induced carcinogenicity	Mouse ($TLR5^+$ mouse sarcoma and B16 melanoma)	0.2 mg/kg, s.c., 1 h before each radiation treatment (three daily treatments of 4 Gy TBI)	Suppressed carcinogenicity of irradiation without diminishing therapeutic antitumor effects via tumor-specific antiapoptotic mechanisms, protected from lethal cumulative damage, reduced mortality rates, rescued mice from lethal irradiation (13 Gy) by a 6 month post-irradiation treatment with only signs of radiation-induced tissue damage and no evidence of cancer or massive fibrosis	[51]
17.	Lung adenocarcinoma therapy	Mouse [human lung adenocarcinoma (A549) xenograft]	10 μg/kg, s.c., in saline, around tumor, every 2 days	Via TLR5/MyD88 signaling, upregulated secretion of cytokines and neutrophil infiltration in tumor xenografts inhibited growth of tumor xenografts with no effect on radiosensitivity of A549 xenograft *in vivo*	[108]

[a]Abbreviations: α-GalCer, α-galactosylceramide; ALT, alanine aminotransferase; Allo-BMT, Allogenic bone marrow transplantation; CD, cluster of differentiation; CXCR3, chemokine receptor; D-GalN, D- galactosamine; 5-FU, 5-fluorouracil; GvHD, graft versus host disease; GVT, graft-versus-tumor; LPS, lipopolysaccharide; mCMV, mouse Cytomegalovirus; MNC, mononuclear cell; MyD88, myeloid differentiation primary response 88; NIH, National Institutes of Health; NK, natural killer; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline/Tween; STAT3, signal transducer and activator of transcription 3; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

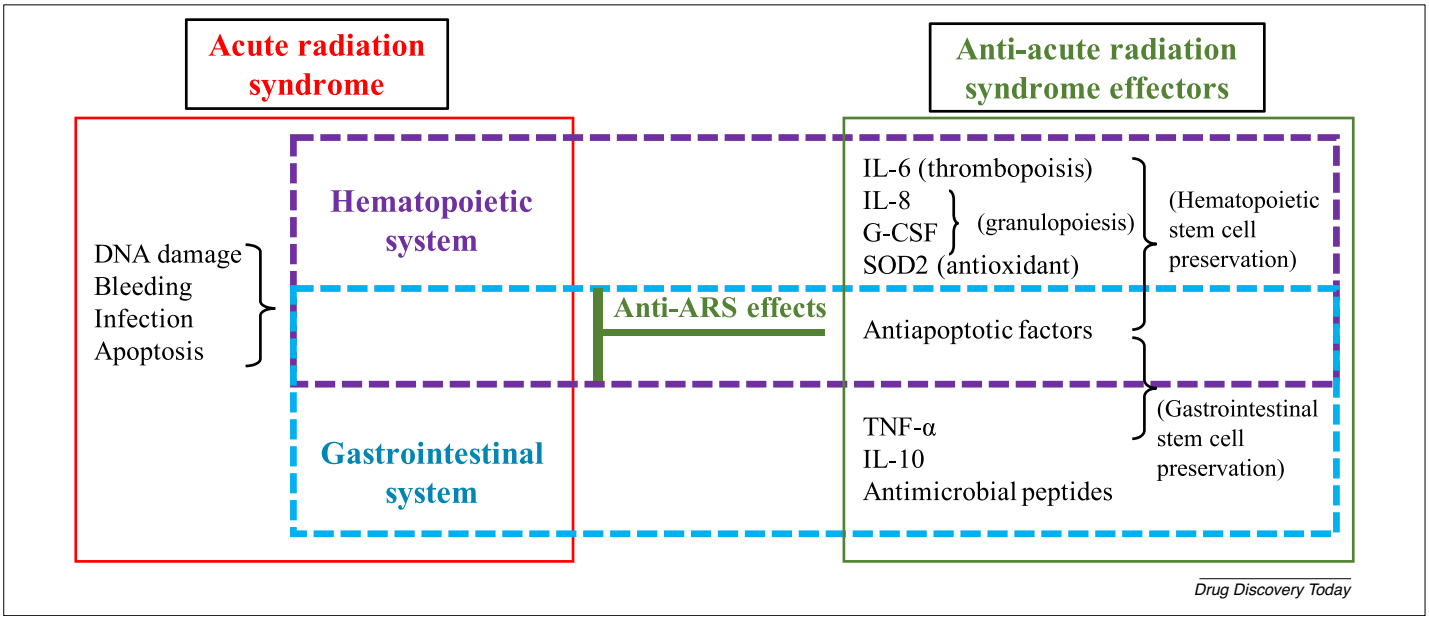

FIGURE 3

Schematic representation of attenuation of acute radiation syndrome by entolimod. Entolimod triggers the transcription of several downstream toll-like receptor 5 (TLR5)-dependent effectors, which includes hematopoietic cytokines, anti-inflammatory cytokines, antioxidants, antiapoptotic factors, and antimicrobial peptides. These effectors contribute to radioprotection by attenuating damage of DNA in the hematopoietic and gastrointestinal (GI) systems. Abbreviations: G-CSF, granulocyte-colony stimulating factor; IL, interleukin; SOD2, superoxide dismutase 2; TNF-α, tumor necrosis factor-α.

apoptotic tissue injury through the induction of the anti-inflammatory cytokines IL-1β antagonist (IL-1βa) and IL-10, in addition to the stimulation of mesenchymal stem cells (MSC) with TLR5 and anti-inflammatory properties [54,77–79].

There are studies that demonstrated the protective and stimulatory effects of entolimod on progenitor cells of both hematopoietic and GI tissues in irradiated or chemotherapy-treated mice and irradiated NHPs [52,53,80]. Entolimod increased the clonogenic potential of progenitors in bone marrow and also improved the survival of intestinal crypt stem cells, as shown by crypt cell proliferation. The beneficial effects of entolimod on hematopoietic and GI progenitors are translated into recovery (i.e., based on morphological features) of the corresponding tissues [51,80,81]. The stimulatory effects of entolimod on these progenitor cell compartments might be mediated by the cytokines induced. Some of these cytokines are known to have such activity [82–85]. G-CSF and IL-6 were consistently induced by entolimod across species. Their effect is consistent with expected biological activities as stimulators of both granulopoiesis and thrombopoiesis [86–88]. There is no published report demonstrating any adverse late effects as a result of entolimod treatment.

Concluding remarks

There is an increasing risk for military, emergency responders, and civilians to be exposed to acute radiation arising from nuclear/radiological exposure-related contingencies [89]. Lethality of high-dose TBI or PBI is generally the direct result of the onset of ARS that, in turn, is caused by extensive cell death and dysfunction of radiosensitive elements within vital organs of the body; namely and most prominently, within the hematopoietic and GI systems [6,90].

The availability of suitable FDA-approved medical radiation countermeasure(s) will make a considerable difference in developing clinical strategies to effectively protect and/or manage individuals at risk of developing ARS. An ideal candidate for mitigative purposes should have the following attributes: (i) to be effective when administered 24–48 h after radiation exposure as a single agent without the need for intensive supportive care, including transfusion of blood products and treatment with individualized antibiotics available in hospital settings; (ii) be easily administered by untrained medical personnel; and (iii) be effective against H-ARS as well as GI-ARS given that these two subsyndromes overlap.

The *Salmonella* protein, flagellin, is a natural ligand of TLR5, bearing innate immunity eliciting radioprotective attributes. Entolimod is a recombinant derivative of flagellin with comparable radioprotectiveness, but lacks significant toxic immunogenicity [50,51]. Entolimod has many of the most desirable attributes needed for full development, regulatory approval, and subsequent field deployment as a medical radiation countermeasure. Advanced preclinical studies using a large NHP animal model have clearly demonstrated that entolimod treatments are effective by virtue of increased survival, reduced injury, and enhanced recovery of both the hematopoietic and GI systems within acutely irradiated animals [51–53,91–94].

Entolimod is being developed following the Animal Rule of the FDA and needs the identification of biomarkers for its efficacy, which will allow the prediction of its efficacious dose for humans based on animal efficacy study data. This agent has also been tested in animal models of various other indications with positive outcomes (Table 3) [51,80,81,95–108]. There are two cytokines induced by entolimod, IL-6 and G-CSF, which have been identified as possible biomarkers of the efficacy of the drug. Their induction is TLR5 dependent, as are the radioprotective features of entolimod. Furthermore, these cytokines are not only pharmacodynamic readouts of Entolimod efficacy, but also intimately linked to its

mechanism of action through TLR5 induction [52]. This is supported by the fact that neutralization of these cytokines by specific antibodies reduced the radiomitigative efficacy of CBLB502 in irradiated mice [52]. G-CSF and IL-6 induction by entolimod is dose dependent in both unirradiated and irradiated animals [52,53]. Furthermore, radiation exposure also appears to potentiate the induction of the above two cytokines, but does not affect the dose proportionality of the noted cytokine responses. Entolimod-induced cytokine expression patterns, 25 h after radiation exposure, are similar to those of unirradiated NHPs. Based on observations from murine-based experiments, both G-CSF and IL-6 are induced over a wide dose range of entolimod that encompasses the radioprotective and radiomitigative activities of the drug (Fig. 3) [52,53]. Entolimod-stimulated cytokine levels were correlated with survival in irradiated mice and NHPs. These biomarkers are responsive across four animal species, namely mice, canines, NHPs, and humans [52,53,94]. In addition, entolimod induced similar dose-dependent increases in neutrophils in healthy humans, healthy NHPs, or irradiated NHPs. Thus, to calculate the efficacious dose for humans based on animal efficacy data, CBLI has developed a dose conversion paradigm and a statistical model based on these biomarker responses across species [94]. This work suggested that the effective dose of entolimod to counter significant, potentially lethal radiation injury in humans falls within a dosing range of ~0.4–0.6 ug/kg of body weight [94].

The effects of exogenously administered cytokines on the host response to radiation are different from those endogenously induced by entolimod. This is similar to the previous discussions and debates over the relative efficacy of exogenously administered interferon versus endogenously administered interferon inducers for the treatment of viral infections. The latter involves immune response/immunomodulation in addition to the effect of the involved agent for treatment. Although G-CSF and IL-6 have important roles in the radioprotective action of entolimod, there are additional entolimod-regulated factors involved. The PK and concentrations of tissue-associated exogenous cytokines differ from endogenous cytokines produced by entolimod by particular cells in certain tissues.

Entolimod is very promising in terms of its efficiency to minimize acute, potentially fatal radiation injuries within both small rodents and in large NHPs. Considering the extensive and carefully detailed analyses conducted by the company, it is more than likely that humans with similar irradiation-related injuries would also benefit from entolimod treatment.

The Emergency Use Authorization (EUA) authority under section 564 allows the FDA to facilitate availability and unapproved uses of MCMs needed to prepare for and respond to chemical, biological, radiological, and nuclear emergencies, while the Biologics License Application (BLA) regulated under 21 CFR 600–680 is a request to distribute a biologic across states. BLA is submitted after an investigational new drug (IND) status is received and after the appropriate studies have been conducted. A pre-EUA application has been submitted to the FDA. We are unaware of the status of the review of that application or more recent product development activities.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes current employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. T.M.S. has served previously as a consultant for Cleveland BioLabs, Inc.

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