EXPERT OPINION

Patents for Toll-like receptor ligands as radiation countermeasures for acute radiation syndrome

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Acute radiation exposure induces apoptosis of tissues in the hematopoietic, digestive, cutaneous, cardiovascular and nervous systems; extensive apoptosis of these tissues ultimately leads to acute radiation syndrome. A novel strategy for developing radiation countermeasures has been to imitate the genetic mechanisms acquired by radiation-resistant tumors. Two mechanisms that underlie this ability of tumor cells are the p53 and NF-κB pathways. The loss of p53 function results in the inactivation of pro-apoptotic control mechanisms, while constitutive activation of NF-κB results in the up-regulation of anti-apoptotic genes. Various Toll-like receptor ligands are capable of up regulating the NF-κB pathway, which increases radio-resistance and reduces radiation-induced apoptosis in various tissues. Several Toll-like receptor ligands have been patented and are currently under development as radiation countermeasures for acute radiation syndrome. Ongoing studies suggest that a few of these attractive agents are progressing well along the US FDA approval pathway to become radiation countermeasures.

Keywords: acute radiation syndrome, NF-κB, radiation, Toll-like receptor ligands

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1. Introduction

Due to the increasing prevalence of radioactive material, incidences involving radiation exposure, either intended or unintended, are an undeniable possibility with potentially catastrophic consequences [1]. Such concerns have justified substantial efforts to develop medically effective radiation countermeasures for ARS [2,3]. Although such efforts were initiated > 6 decades ago, only one radiation countermeasure, granulocyte colony-stimulating factor (G-CSF), has recently been approved by the US FDA for ARS [4,5]. Countermeasures can be categorized based on their administration in relation to the time of radiation exposure. Radioprotectors, also called prophylactic agents, must be administered before radiation exposure to prevent injury by acting prior to and during the initial radiochemical events. Radiation mitigators are administered after irradiation but before the appearance of overt evidence of injury to accelerate recovery. Radiation therapeutics or treatments are the agents given after symptoms manifest to stimulate repair or regeneration of organ and tissue functions [2].

High-dose whole-body irradiation induces inflammation, massive apoptosis, necrosis, and susceptibility to infection. All three leukocyte populations (lymphocytes, monocytes, and granulocytes) fall within a few days after exposure, reaching

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their respective nadirs in several days. Other blood cell populations (e.g., erythrocytes, platelets) decline slowly over a several-week-long period due to reduced hematopoietic activity with the exception of granulocytes. Cellular determinants of the adaptive immune response are significantly more sensitive than the majority of components of innate immunity (e.g., macrophage populations and natural killer cells) with the exception of granulocytes. However, significant decline in neutrophils (neutropenia) increases the risk and severity of infections [6,7].

Effective protection from irradiation requires the defense of existing mature immune cells and the rescue of precursor and stromal cells as both are essential for regeneration and recovery. Relative sensitivity also varies greatly among different tissue components [8]. Mature B- and T-cells are the most sensitive to irradiation and will undergo apoptosis after exposure to < 1 Gy, whereas hematopoietic precursors are significantly more resistant and can regenerate the immune system even after exposure to 10 Gy in mice. Bone marrow stroma is the most resistant component of immune system, which can survive exposure to level as high as 15 – 17 Gy, although functional alterations can be observed at lower doses.

2. Biological responses to radiation

Exposure to ionizing radiation produces breaks and cross-links in the DNA, and alters proteins, cell membranes, and other macromolecular structures. Ionizing radiation also induces secondary damage to the cellular components by creating free radicals and reactive oxygen species (ROS). Several pathways protect and restore the integrity of the DNA. For example, antioxidant chemicals and enzymes scavenge the free radicals and ROS in addition to reducing the levels of oxidized proteins and lipids. Cellular checkpoint systems detect DNA defects and delay cell cycle progression until the damage is repaired, the cell commits to permanent growth arrest, or the cell initiates programmed death (apoptosis).

At the organismal level, the immediate effects of low- and moderate-intensity radiation are largely caused by cell death, necrosis, or by the bystander effect. These effects cause radiation-induced inflammation [9-11]. At high doses, lethality is caused by the hematopoietic and gastrointestinal radiation syndromes (H-ARS and GIS, respectively). H-ARS is the loss of blood cells and their progenitors, which makes it impossible to regenerate the blood and lymphoid systems. This process leads to death from hemorrhage, anemia and infection. GIS is caused by massive cell death in the intestinal epithelium, predominantly in the small intestine, followed by disintegration of the intestinal wall and death from bacteremia and sepsis. Another important syndrome of radiation exposure is cutaneous syndrome. Exposure of the human skin to ionizing radiation > 3 Gy results in a distinct clinical representation, characterized by a transient and faint erythema after a few hours, and then followed by severe erythema, blistering, and necrosis.

3. Role of NF-κB in radiation-induced apoptosis

The important radioprotective strategy is to activate the NF-κB pathway. The protective role of NF-κB is mediated by the transcriptional activation of genes coding for proteins that block major apoptotic pathways [12]. The NF-κB pathway makes an attractive target because it is activated by several naturally occurring factors that are considered radioprotectants. Sources of NF-κB activators include tumor cells and microbial parasites of mammals, since both employ inhibition of apoptosis for survival in the host. The idea of using NF-κB pathway induction to inhibit apoptosis was initially tested using *Salmonella typhimurium* flagellin, which activates NF-κB through Toll-like receptor 5 (TLR5) (**Figure 1**) [13]. NF-κB regulates anti-apoptotic genes especially the TNF receptor-associated factor 1 and 2 (TRAF1 and TRAF2) and, therefore, checks the activities of the caspase enzyme family, which are central to most apoptotic processes. Bacterial lipoproteins are ligands for TLR2 which also activate NF-κB. The immunomodulatory properties of lipoproteins and their effects on hematopoietic cytokines make small synthetic lipopeptides excellent candidates as radiation countermeasures. Consequently, several patents have been filed for different TLR ligands for development as candidate radiation countermeasures (**Table 1**) [14-26].

4. Toll-like receptors and activation of NF-κB

TLRs are the key sensor elements of innate immunity and are evolutionary conserved receptors. They are homologues of the Drosophila Toll protein which is important for the defense against microbial infection. TLRs recognize highly conserved structural motifs known as pathogen-associated molecular patterns (PAMPs) (exclusively expressed by microbial pathogens) or damage/danger-associated molecular patterns (DAMPs) (endogenous molecules released from necrotic or dying cells). Stimulation of TLRs, by PAMPs and DAMPs, initiates signaling cascades which lead to the activation of transcription factors, such as NF-κB (**Figure 1**). TLR signaling results in a variety of cellular responses including the production of interferons, pro-inflammatory cytokines, and effector cytokines that direct the adaptive immune response. A total of 10 human and 12 murine TLRs have been well characterized (**Table 2**).

Consistent with their function of immunocyte activation, TLRs are expressed in spleen and peripheral blood leukocytes. Other TLR-specific patterns are expressed in other lymphoid organs and subsets of leukocytes as well as other tissues and organs. TLR ligands are appealing as potential radioprotectors since they are characteristic of large groups of pathogens, and cannot be easily mutated. Unlike cytokines, many PAMPs have little effect besides activating TLRs and thus are unlikely to produce side effects. Many PAMPs (and DAMPs) are

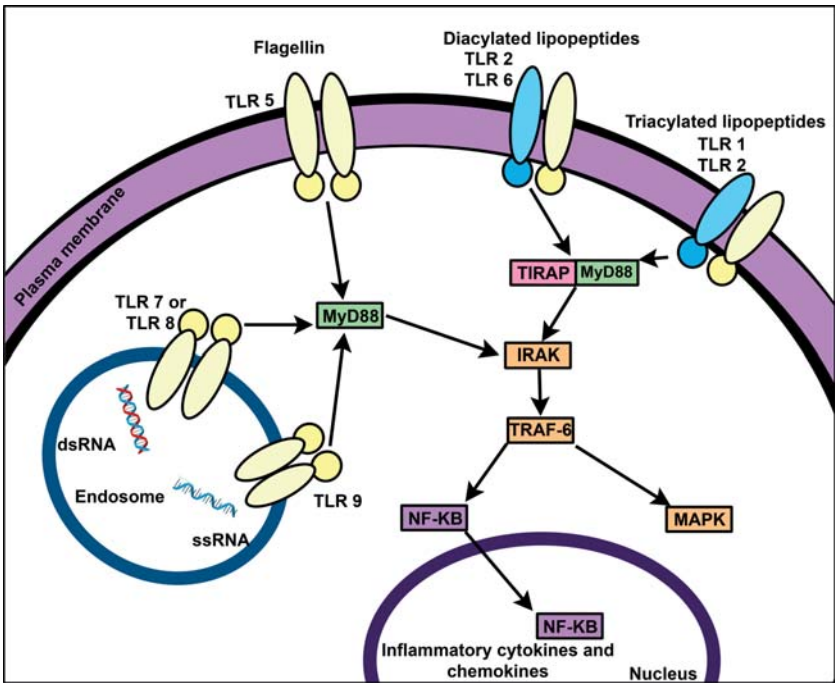

Figure 1. Schematic representation of TLR-ligand-mediated NF-κB activation. TLRs bind microbial PAMPs leading to the activation of NF-κB and IRF pathways. Activation of these pathways is mediated by the two key adaptor molecules MyD88 and TRIF. NF-κB enters the nucleus where it 'turns on' the expression of specific genes resulting in inflammatory, immune, or cell survival response. Key biological effects of NF-κB activation include the prevention of apoptosis. A set of endosomal TLRs recognize nucleic acids derived from viruses and endogenous nucleic acids.

IRAK: IL-1-receptor-associated kinase; IRF: IFN regulatory factor; MyD88: Myeloid differentiation primary-response protein 88; PAMPs: Pathogen-associated molecular patterns; TIR: Toll-IL-1 receptor: TIRAP: TIR domain-containing adaptor protein; TLR: Toll-like receptor; TRAF6: TNF-receptor-associated factor-6; TRIF: TIR-domain-containing adapter-inducing interferon-β.

constantly present in humans. Several TLR ligands are under different stages of the development as radiation countermeasures (**Table 3**).

5. Development of TLR ligands as radiation countermeasures

Flagellin of *Salmonella typhimurium* is a stable protein and natural activator of NF-κB which has been shown to protect mice from lethal doses of total-body irradiation. A truncated version of this protein has been developed (CBLB502) which retains the radioprotective efficacy and stability while lacking its immunogenic properties. CBLB502 has been found to be effective against both H-ARS and GIS in mice and nonhuman primates (NHPs), both as a radioprotector and also as a radiomitigator [27,28]. These attributes make CBLB502 uniquely useful as a radiation countermeasure for civilian and military applications. CBLB502 has received FDA investigational new drug status and is currently in clinical development. A human safety study indicated that it was well tolerated in humans and the biomarker results correspond to data from animal models [28].

A shortened version of a naturally occurring N-terminal lipopeptide from *Mycoplasma arginine*, R-Pam_2-CGETDK (*S*-[(2*R*)-2,3-bis(palmitoyloxy)propyl]-cysteinyl-GETDK) (CBLB613), is water soluble, activates TLR2/TLR6-dependent NF-κB production, and demonstrates significant radioprotective efficacy in murine model of ARS [29]. *Mycoplasma arginine* is a symbiotic asymptomatic microflora of mammals, commonly present in humans and is expected to be safe. A synthetic mimetic of diacylated mycoplasma lipopeptides (Pam_2-CSKKKK, agonists of TLR2, CBLB612) has also been shown to have radioprotective and radiomitigative efficacy in murine model of ARS [30]. The TLR agonists are activators of anti-apoptosis pathways and their role in carcinogenesis deserves investigation. Although the role of TLRs in cancer is not well understood, results suggest a dual role of TLRs in cancer: high doses of TLR agonists appear to have an anti-cancer effect whereas low doses of TLR agonists promote cancer growth [31].

6. Expert opinion

Currently, G-CSF is the only one FDA-approved countermeasure to mitigate ARS-related morbidity and mortality. It

Table 1. Promising TLR ligands under development as radiation countermeasures.

Agents (examples)	Receptors	Document title	Pending applications	Patent number	Ref.
Flagellin or flagellin derivatives (CBLB502)	TLR5	Modulating apoptosis		US7638485B2, US8106005B2	[14]
		Method for screening modulators of apoptosis		US8784840B2	[14]
		Method for reducing the effects of chemotherapy using flagellin-related polypeptides	US 20130324462A1, WO2009102818A1	US8580321B2	[15]
		Methods of protecting against radiation using flagellin	WO2005056042A2		[17]
		Flagellin related polypeptides and uses thereof	WO2006069198A1	US8007812B2, US8287882B2, US8932609B2, US8871215B2, JP5285278B2	[16]
Lipopeptides of muco-plasma origin (CBLB612 and CBLB613)	TLR2 or TLR2/6	Methods of protecting against apoptosis using lipopeptides	NZ565063A, MX2007015834A, KR20140041874A, KR20080030566A, WO2008111585A1, WO2006138238A3, IL188091A, HK1123495A1, CA2612102A1, BRPI0611586A2, EA200702510A1	US8008260B2, US8524668B2, EP1904084B1, EA014644 B1, CN101242852B, AU2006259630B2, JP5000644B2, ES2421447T3, US9006183B2	[23,24]
		Compositions and methods comprising TLR stimulating agents for prophylaxis and therapy for damage to dermal epithelium	EP2833876 A1, WO2013151994A1		[18]
		Use of TLR agonist for treating cancer	US20140248260A1, SG191830A1, MX2013007967A, KR20140030132A, JP2014502973A, EP2663367A4, EA201390843A1, CO6781506A2, CN103476458A, CL2013002001A1, CA2824438A1, AU2012205681A1, WO2012097012A1		[25,26]
		Methods for increasing and mobilizing hematopoietic stem cells	US20140045747A1, ZA200905378A, SG177959A1, NZ603805A, NZ578928A, MX2009007391A, KR20090108703A,IL199766A, HK1134835A1,EP2115124 A2, EA200900806A1, CA2675032A1, BRPI0806557A2, AU2008204836A1, WO2008086426A3	JP5389666B2, EA018983B1, CN101631850B	[21,22]
α-1-antitrypsin	TLR2	Peptides and methods for using the same	US20130274187A1, MX2014007799A, EP2802338A2, CA2859777A1, AU2013208293A1, WO2013106273A3		[19,20]

One citation has been provided for each 'simple patent family' or patent that has been published at different times or with multiple countries.
TLR: Toll-like receptor.

Table 2. Well characterized TLRs of mouse and human.

TLRs	Ligand	Expressing cells	Location	Adapter(s)
TLR1	Bacterial lipoprotein: triacyl lipopeptides	Monocytes, macrophages, dendritic cells, B-cells	Cell surface	TIRAP, MyD88
TLR2	Bacterial peptidoglycans: lipoproteins, lipoteichoic acid	Monocytes, macrophages, mast cells, B-cells	Cell surface	TIRAP, MyD88
TLR3	Viruses: double stranded RNA (poly I: C), tRNA, siRNA	B-cells, T-cells, natural killer cells	Endosomes	TRIF
TLR4	Bacteria and host cells: LPS	B-cells, T-cells, natural killer cells, dendritic cells	Cell surface/ endosomes	TRAM, TRIF, TIRAP, MyD88
TLR5	Bacteria: bacterial flagellin	Monocytes, macrophages, dendritic cells, intestinal epithelium	Cell surface	MyD88
TLR6	Mycoplasma: lipoprotein	Monocytes, macrophages, mast cells, B-cells	Cell surface	TIRAP, MyD88
TLR7	RNA viruses: ssRNA	Endosomes, monocytes, macrophages, dendritic cells, B cells	Endosomes	MyD88
TLR8	Small synthestic compounds: ssRNA	Monocytes, macrophages, dendritic cells, mast cells	Endosomes	MyD88
TLR9	Bacteria and DNA viruses: CpG DNA (CpG ODNs)	Monocytes, macrophages, dendritic cells, B-cells, T-cells	Endosomes	MyD88
TLR10	Profiling-like proteins	Monocytes, macrophages, dendritic cells	Endosomes	MyD88

TLR1 through TLR10 are human and TLR1 through TLR9, TLR11, TLR12, and TLR13 are murine (homologue of TLR 10 being a pseudogene). Murine TLR11, TLR12, and TLR13 are still being characterization and therefore not displayed in this table.
CpG ODN: CpG oligodeoxynucleotides; MyD88: Myeloid differentiation primary-response protein 88; LPS: Lipopolysaccharides; poly I/C: Polyinosinic/polycytidylic acid; ssRNA: Single-stranded RNA; siRNA: Small interfering RNA; TIR: Toll-IL-1 receptor; TIRAP: TIR domain-containing adaptor protein; TLR: Toll-like receptor; TRAM: TRIF-related adaptor molecule; TRIF: TIR-domain-containing adapter-inducing interferon-β; tRNA: Transfer RNA.

Table 3. Promising TLR ligands under development as radiation countermeasures for ARS.

Agents	Origin	Receptors	Efficacy	Animal model of evaluation	Comments
CBLB502	Truncated flagellin of *Salmonella typhimurium*	TLR5	Radioprotector and radiomitigator	Murine and nonhuman primates (cytokines also evaluated in canines)	Has US FDA investigational new drug status, pre-emergency use authorization application may be submitted soon
CBLB612	Mycoplasma origin, small lipopeptide	TLR2	Radioprotector, marginal radiomitigative potential	Murine	A radioprotector and is also a mobilizer of hematopoietic stem cells
CBLB613	Mycoplasma origin, small lipopeptide	TLR2/6	Radioprotector, marginal radiomitigative potential	Murine	Radioprotective dose reduction factor in mouse is 1.25
SP16	α-1-antitrypsin	TLR2	Not yet tested for acute radiation syndrome in animal model	Not applicable	Being investigated for several indications, including endotoxemia, following acute radiation exposure

TLR: Toll-like receptor.

has been procured for Strategic National Stockpile for use in a radiological emergency. However, the adverse consequences of G-CSF administration need to be taken into consideration. For examples, G-CSF administration after cytotoxic agents that damage bone marrow stem cells exacerbates long-term stem cell damage through excessive differentiation stimulation [5]. Additional concerns stem from its role in exacerbating delayed lung damage in an animal model of ARS. Finally, in a recent study, G-CSF failed to demonstrate radiomitigative efficacy in the NHP model. This failure may be due to the lack of supportive care [5]. It is important to note that the European standard for the evaluation and treatment of ARS recommends the use of cytokines 14 – 21 d post-exposure to promote hematological reconstruction, and there are several studies in which G-CSF has been used for the treatment of radiation accident victims with beneficial effects [5,32,33].

Fortunately, there are several promising radiation countermeasures for ARS at different stages of development [2]. In the recent past, several patents have been filed for the use of TLR ligands as radiation countermeasures, specifically as anti-apoptotic agents. Among these products, CBLB502 (truncated flagellin) and lipopeptides of mycoplasma origin

are promising agents. CBLB502 is the leading candidate currently under development, as this agent has been evaluated as a radioprotector as well as a radiomitigator with promising results in NHPs. A good laboratory practice compliant study with CBLB502 has also been conducted in NHPs. In addition, this agent has undergone clinical trial for toxicity, pharmacokinetics, and biomarkers in healthy volunteers. Its existing efficacy, safety data, and animal-to-human dose conversion are enough to proceed with a pre-emergency use authorization (EUA) application to reduce the risk of death following radiation exposure. In our opinion, CBLB502 holds the most promise for the future due to its limited side effects and studies indicate it is likely to be a safe and effective agent when approved.

CBLB612 and CBLB613 are at early stages of development and their promising efficacy in a murine model has been reported [29,30]. However these peptides, along with CBLB502, are derived from human pathogens/symbionts (Salmonella and Mycoplasma), pre-existing immunity in the host may be an issue. Efforts are, therefore, being made to identify hyperthermophilic flagellin derivatives to which the human immune system has not yet been exposed. These derivatives are also stable, have a relatively low molecular weight and can be incorporated with de-immunizing deletions and mutations.

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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 employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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