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Original Article

Subacute toxicity evaluation of KMRC011, a Toll-like receptor-5 agonist administered by intramuscular injection to rats

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ABSTRACT — The present study investigated the potential subacute toxicity of KMRC011, a Toll-like receptor-5 agonist, by a 4-week repeated intramuscular injection in Sprague-Dawley rats. The test article was administered once daily by intramuscular injection to rats at doses of 0, 0.06, 0.13, and 0.25 mg/kg/day for 4 weeks. At the end of the treatment period, 10 rats/sex/group were sacrificed. The study was continued for the remaining 5 rats/sex in the vehicle control and high dose groups without treatment for 2 weeks (recovery period). During the test period, clinical signs, mortality, body weight, food consumption, ophthalmoscopy, urinalysis, hematology, serum biochemistry, gross findings, organ weight, and histopathology were examined. Hematological investigations revealed a decrease in the hemoglobin, hematocrit, and mean corpuscular hemoglobin values and an increase in the absolute and relative reticulocyte counts. Histopathological evaluation indicated an increase in the incidence of inflammatory cell infiltration in the cecum, lymphocytes infiltration in the duodenum, and hemopoiesis in the femoro-tibial joint/marrow and sternum/marrow in male and female rats. These changes decreased or were no longer observed after the 2-week recovery period, indicating these were reversible changes. Otherwise, no adverse effects were observed in any treatment group. Based on these results, the no-observed-adverse-effect level was considered to be greater than 0.25 mg/kg/day in the rats.

Key words: KMRC011, Subacute toxicity, Toll-like receptor-5, No-observed-adverse-effect level

INTRODUCTION

Acute radiation syndrome (ARS), known as radiation sickness, is the clinical manifestation of pathologies that develop soon after a high dose of penetrating, ionizing radiation exposure over a short period of time (Donnelly *et al*., 2010). ARS involves hematopoietic, gastrointestinal, and cerebrovascular components and has four phases, including prodromal, latent, manifest, and recovery or death (Dörr and Meineke, 2011). It is also associated with injury to the radiosensitive organs caused by DNA damage, oxidative stress, inflammation, and the innate immune response (Atkinson *et al*., 2011; Hall *et al*., 2016). Therefore, a strategy to diminish the toxic effects of ARS is necessary.

Many compounds are being developed for use as agents

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to protect against radiation-induced toxicities. However, the development of radioprotectants has primarily focused on anti-oxidants such as amifostine, glutamine, and lycopene (Andic *et al*., 2009; Choi, 2003; Gu *et al*., 2014; Eda *et al*., 2016; Sayles *et al*., 2016) and anti-inflammatory agents (Cho *et al*., 2013; Sheibani *et al*., 2015). Recently, various Toll-like receptor (TLR) ligands have been under development as potential radioprotective agents because they are characteristic of large groups of pathogens and cannot be easily mutated (Singh and Pollard, 2015). The TLR pathway also plays a pivotal role in various diseases, including cancer, fibrosis, and infectious and inflammatory diseases (O'Neill *et al*., 2009; So and Ouchi, 2010; Huebener and Schwabe, 2013).

Among TLRs, TLR 5 recognizes flagellin and is the only protein-binding TLR that is conserved in vertebrates (Hayashi *et al*., 2001; Stockhammer *et al*., 2009). Entolimod, a TLR 5 agonist, is currently being developed under the FDA's animal efficacy rule to treat ARS (Snoy, 2010). A previous study indicated that entolimod activated NF-κB signalling, which inhibited apoptosis *in vitro* (Burdelya *et al*., 2008). They showed that entolimod had radioprotective activity in rodents and non-human primates (rhesus macaques). A single injection of entolimod before lethal total-body irradiation protected mice from both gastrointestinal and hematopoietic ARS and led to improved survival. In contrast to normal tissues, treatment with this TLR 5 ligand did not change the radiosensitivity of the mouse tumors, implying that TLR 5 ligand may be valuable adjuvants for cancer radiotherapy and relatively safe protectors of normal cells against high dose radiation during radiation treatment. Recently, Krivokrysenko *et al*. (2015) reported that a single dose intramuscular injection of entolimod enhanced the morphological recovery of hematopoietic and immune system organs, decreased the severity and duration of thrombocytopenia, anemia and neutropenia, and increased the clonogenic potential of the bone marrow. The study also indicated that there was a decrease in apoptosis and an increase in crypt regeneration in the gastrointestinal tract. Therefore, we focused on a TLR 5 agonist because these agent are leading candidates for use as radioprotectants.

Recently, the design of entolimod has been improved by the removal of the ancillary regions of entolimod to leave only the TLR 5-activating flagellin parts (Song and Yoon, 2016). Korea Medical Radiation Countermeasure 011 (KMRC011) is a new recombinant entolimod derivative TLR 5 agonist developed at the Korea Institute of Industrial Technology in Korea. As a part of the safety evaluation studies of KMRC011, a 4-week repeated intramuscular administration toxicity study was performed in Sprague-Dawley rats and the no-observed-adverse-effect level (NOAEL) was determined. The present study was conducted according to the test guidelines from the Ministry of Food and Drug Safety (MFDS) and Organisation for Economic Cooperation and Development (OECD) guidelines for the testing of test article under modern Good Laboratory Practice Regulations.

MATERIALS AND METHODS

Animal husbandry and maintenance

Fifty Sprague-Dawley rats of each sex were purchased from Orient Bio (Seoungnam, Korea) at 5 weeks of age and used after 1 week of quarantine and acclimatization. The animals were housed in a room maintained at a temperature of 23 ± 3°C and a relative humidity of 50 ± 10% with artificial lighting from 08:00 to 20:00 and with 13 to 18 air changes per hr. Only healthy animals were assigned to the study. Animal were housed in a stainless wire cage (255 mm W × 465 mm L × 200 mm H) with ≤ 5 animals/cage for the quarantine period and ≤ 3 animals/cage for the observation period. The animals were allowed sterilized tap water and commercial rodent chow (PMI Nutritional International Inc., Richmond, IN, USA) *ad libitum*. This experiment was conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All procedures were approved by the Institutional Animal Care and Use Committee, Korea Institute of Toxicology.

Test article and treatment

KMRC011 was provided by Advanced Protein Technologies Corp. (Suwon, Korea). The test article was dissolved in distilled water with 10 mM L-histidine and the solution was adjusted to pH 7.0. Dosing solutions were prepared daily before treatment. After the skin was cleaned with 70% isopropyl alcohol using cotton, the test article was administered daily by intramuscular injection in both thigh muscles of the rats for 4 weeks at doses of 0.06, 0.13, and 0.25 mg/kg/day (equivalent to 0.06, 0.13, and 0.25 mL/kg, respectively). The control group rats received the same volume (0.25 mL/kg) of vehicle alone as the highest dose group. The daily application volume of KMRC011 was calculated in advance based on the most recently recorded body weight of each individual animal. The intramuscular route is the clinically intended route for the test article. The recovery groups were kept for 2 weeks after the cessation of treatment.

Experimental groups

Healthy male and female rats were randomly assigned

to four experimental groups: three KMRC011 treatment groups receiving 0.06, 0.13, and 0.25 mg/kg/day and a vehicle control group. Each group consisted of 10 (low and middle dose groups) or 15 (vehicle control and high dose groups) rats of each sex. After 4 weeks of treatment, 10 rats/sex/group were sacrificed. The remaining male and female rats (recovery group) in the vehicle control and high dose groups were autopsied after a 2-week recovery period. The animals in the recovery groups were observed for reversibility, persistence, and delayed occurrence of toxic effects.

Selection of doses

In a previous 2-week repeated dose toxicity study (unpublished data), decreased body weight gain and food consumption and increased reticulocyte (RET) count and spleen weight were observed at a dose of 0.25 mg/kg/day. Therefore, a dose of 0.25 mg/kg/day was selected for the highest dose in this study. This dose is 500 times that of the clinical dosage (approximately 0.5 μg/kg/day). Doses of 0.06 and 0.13 mg/kg/day were selected as low and middle doses, respectively.

Clinical observation and mortality

Mortality and clinical observations were conducted twice a day at the start and end of the work day throughout the study period.

Body weight

The body weight of each rat was measured at the initiation of treatment, once a week during the treatment period, on the day of scheduled termination, and once a week during the recovery period.

Food consumption

Food consumption was measured at the start of treatment and once a week during the treatment and recovery periods. The amount of food was weighed before it was supplied to each cage and the remnants were weighed the next day to calculate the difference, which was regarded as the daily food consumption (g/rat/day).

Ophthalmoscopy

Ophthalmologic examination was carried out shortly before the start of treatment and shortly before the termination of treatment for all animals using a slit lamp (XL-1, Ohira Co. Ltd., Japan) and a binocular indirect ophthalmoscope (Vantage Plus Digital, Keeler Ltd., England) after the animals were treated with a mydriatic (Mydrin-P, Santen Pharmaceutical Co., Japan).

Urinalysis

Before termination, all animals were restricted from food, but not water, and urine was collected overnight (approximately 16 hr) using metabolic cages. For urinalysis, volume, color, clarity, pH, specific gravity, bilirubin, protein, urobilinogen, nitrite, glucose (GLU), leukocyte, erythrocyte, and ketone were analyzed using Combur 10 TM urine sticks (Roche, Germany) and a Cobas U411 urine analyzer (Roche, Germany). In addition, microscopic examination was conducted for sediments such as epithelial cells, red blood cells (RBC), and white blood cells (WBC).

Hematology

Approximately 1.5 mL of blood was collected from the cauda vena cava of the animals under deep isoflurane anesthesia prior to necropsy. Blood samples were collected into complete blood count bottles containing EDTA-2K and analyzed using an ADVIA 2120i hematology analyzer (Siemens, USA). The following parameters were determined: RBC count, hemoglobin (HB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), RET, platelet (PLT) count, WBC count, and differential WBC count. In addition, prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined using an ACL 9000 coagulation analyzer (Instrumentation Laboratory, Italy).

Serum biochemistry

The animals were fasted for more than 16 hr prior to blood collection. Approximately 1.5 mL of blood was collected from the cauda vena cava of the animals under deep isoflurane anesthesia prior to necropsy. Blood samples were placed at ambient room temperature for at least 30 min and centrifuged (3000 rpm, 10 min, room temperature) to separate the serum. The following serum biochemistry parameters were evaluated using a Toshiba 120FR chemistry analyzer (Toshiba Co., Japan): aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRTN), albumin/globulin (A/G), GLU, total cholesterol (T-CHO), total bilirubin (T-BIL), total protein (TP), albumin (ALB), creatine phosphokinase (CPK), triglycerides (TG), calcium (Ca), inorganic phosphorus (IP), phospholipid (PL), gamma glutamyl transferase (GGT), chloride (Cl), sodium (Na), and potassium (K).

Gross findings

All surviving animals were anesthetized with isoflu-

rane to collect blood samples at the end of the experiment. The rats were sacrificed by exsanguination from the abdominal aorta. Complete gross postmortem examinations were performed on all terminated animals.

Organ weights

Absolute and relative (organ-to-body weight ratios) weights of the following organs were measured: brain, pituitary gland, liver, spleen, heart, thymus, salivary glands, prostate, seminal vesicles (with coagulation glands), adrenal glands, testes, epididymides, lung, thyroid (with parathyroids), uterus (with cervix), ovaries (with oviduct), and kidneys.

Histopathology

The following tissues were obtained from all animals: abnormal lesions, skin, mammary gland, spleen, pancreas, jejunum, stomach, duodenum, ileum, cecum, colon, mesenteric lymph node, salivary glands, mandibular lymph node, ovaries, uterus, vagina, urinary bladder, epididymides, prostate, seminal vesicles, rectum, kidneys, adrenal glands, liver, sternum, thymus, heart, lungs, trachea, esophagus, thyroid (including parathyroids), tongue, aorta, sciatic nerve, skeletal muscle, femur, spinal cord, Harderian glands, brain, pituitary gland, eyes, testes, and injection site. Eyes and testes were preserved in Davidson's fixative and Bouin's fixative, respectively. Other tissues were fixed with 10% neutral buffered formalin solution. The tissues were routinely processed, embedded in paraffin, and sectioned at 3-5 μm. The sections were stained with hematoxylin-eosin (H&E) for microscopic examination. All organs and tissues taken from animals in the vehicle control and high dose groups were examined microscopically. All gross lesions as defined by the study pathologist were also included in the examination.

Statistical analysis

Data are expressed as means ± S.D. Data collected during the study were examined for variance homogeneity using the Bartlett's test. Homogeneous data was analyzed using Analysis of Variance (ANOVA) and the significance of inter-group differences was analyzed using Dunnett's Test. Heterogeneous data were analyzed using the Kruskal-Wallis Test and the significance of inter-group differences between the vehicle control and treated groups was assessed using Dunn's Rank Sum Test. For comparing the vehicle control group and recovery group, the data was analyzed for homogeneity of variance using the F-test. Homogeneous data was analyzed using the T-test and significant differences between vehicle control and recovery groups was assessed using Dunnett's Test. Heterogeneous data was analyzed using the Kruskal-Wallis Test and significant differences between vehicle control and recovery groups was assessed using Dunn's Rank Sum Test. The statistical analyses were performed by comparing the dose groups to the vehicle control group using the Pristima system (Xybion Medical System Co., USA). The results of the comparisons are only indicated for *P*-values less than 0.05 or 0.01.

RESULTS

Clinical signs and mortality

There was no treatment-related mortality in any of the animals treated with KMRC011 during the study period (data not shown). Loss of fur was observed in one male rat in the vehicle control group and one male rat in the 0.13 mg/kg/day group. This symptom was not seen at the end of the recovery period.

Body weight changes

As shown in Fig. 1, the body weight of male rats was significantly suppressed in the 0.06, 0.13, and 0.25 mg/kg/day groups on days 8 and 15 compared with that in the vehicle control group. The significant suppression of body weight observed in the treatment groups was reversible during the 2-week recovery period. There were no significant differences in the body weight of the female rats between the vehicle control and treatment groups (Fig. 2).

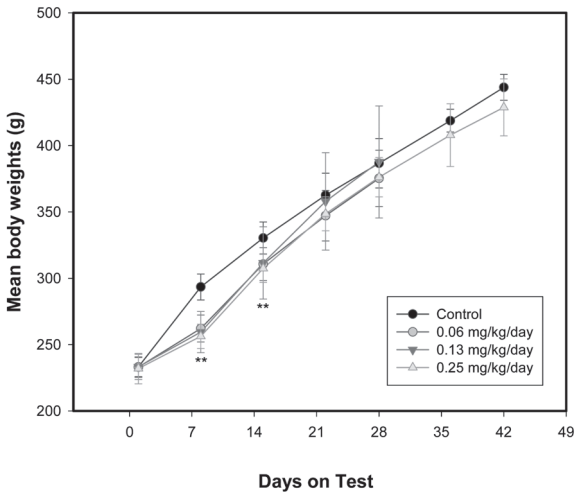

Fig. 1. Mean body weights for male rats treated with KMRC011. Values are presented as means ± S.D. ** Significant difference at $P < 0.01$ level compared with the control group.

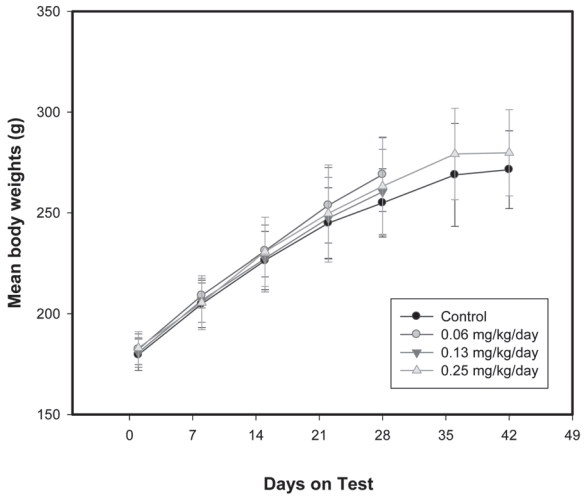

Fig. 2. Mean body weights for female rats treated with KMRC011. Values are presented as means ± S.D.

Food consumption

In male rats, food consumption was significantly less on days 2 and 9 in the treatment groups than in the vehicle control group (Table 1). A significant increase in food consumption was observed at days 16, 23, and 28 in the 0.06 and 0.13 mg/kg/day groups and at day 16 in the 0.25 mg/kg/day group compared to the vehicle control group. In female rats, food consumption was significantly less on day 2 in the treatment groups than that in the vehicle control group. A significant increase in food consumption was observed at day 28 in the 0.13 mg/kg/day group and at days 9, 16, 23, and 28 in the 0.25 mg/kg/day group compared to that in the vehicle control group. In the recovery group, no statistically significant change was observed in either gender.

Ophthalmoscopy

Ophthalmologic examination did not reveal treatment-related ocular lesions in any of the animals (data not shown).

Urinalysis

No significant differences between the vehicle control and treatment groups were observed for any of the urinary parameters examined (Table 2).

Hematology

In male rats, the relative neutrophils count was significantly decreased in the 0.06 mg/kg/day group and the relative lymphocytes count and PT value were significantly increased compared with those in the vehicle control group (Table 3). The MCH and APTT values and relative neutrophils and monocytes counts significantly decreased in the 0.13 mg/kg/day group and the relative lymphocytes count, absolute and relative RET counts, and PT value significantly increased compared with those in the vehi-

Table 1. Daily Food consumption in animals treated with KMRC011 for 4 weeks.

Parameters	KMRC011 (mg/kg/day)			
	0	0.06	0.13	0.25
Male				
No. of rats	15	10	10	15
Food consumption (g)				
Day 2	29.0 ± 1.04[a]	13.4 ± 3.17*	10.4 ± 2.71**	8.9 ± 1.57**
Day 9	31.4 ± 1.22	26.1 ± 1.68**	26.9 ± 1.57**	26.2 ± 3.89**
Day 16	29.1 ± 0.85	35.7 ± 2.00**	38.0 ± 6.75**	33.4 ± 1.72**
Day 23	31.9 ± 2.72	34.4 ± 1.81*	37.4 ± 2.61**	32.0 ± 2.05
Day 28	31.6 ± 1.72	34.3 ± 1.52*	35.3 ± 3.25**	32.9 ± 2.52
Female				
No. of rats	15	10	10	15
Food consumption (g)				
Day 2	21.1 ± 1.01	10.5 ± 1.26**	11.6 ± 1.46**	10.4 ± 1.29**
Day 9	22.5 ± 1.99	20.5 ± 3.00	22.2 ± 2.11	24.7 ± 2.64*
Day 16	22.6 ± 1.92	23.0 ± 1.74	23.3 ± 2.57	25.5 ± 3.11**
Day 23	23.8 ± 2.97	25.5 ± 1.24	23.8 ± 2.79	27.2 ± 1.70**
Day 28	23.4 ± 1.50	25.0 ± 1.33	26.1 ± 3.35*	26.6 ± 1.47**

[a] Values are presented as means ± S.D.

* Significant difference at $P < 0.05$ level compared with the control group.

** Significant difference at $P < 0.01$ level compared with the control group.

Table 2. Urinalysis finding in animals treated with KMRC011 for 4 weeks.

Parameters		KMRC011 (mg/kg/day)							
		Male				Female			
		0	0.06	0.13	0.25	0	0.06	0.13	0.25
No. of rats		10	10	10	10	10	10	10	10
Urine volume	Mean	14.7	14.3	16.3	13.8	15.5	15.9	16.0	15.6
(ml)	SD	4.24	5.31	8.15	5.63	7.76	7.06	6.91	9.19
Specific gravity	1.005	0	0	3	0	0	0	0	0
	1.010	2	2	2	1	3	3	3	1
	1.015	7	4	3	7	4	6	7	8
	1.020	1	4	2	2	3	1	0	1
pH	6.0	0	2	0	4	4	2	1	2
	6.5	5	4	4	4	2	4	6	7
	7.0	5	4	6	2	4	4	3	1
Glucose	-	10	10	10	10	10	10	10	10
Bilirubin	-	10	9	9	10	10	10	10	10
	1+	0	1	1	0	0	0	0	0
Ketone	-	0	0	1	0	5	3	5	3
	1+	10	8	9	10	5	7	5	7
	2+	0	2	0	0	0	0	0	0
Protein	-	7	5	4	4	10	9	9	8
	1+	3	5	6	6	0	1	1	2
Urobilinogen	-	10	10	10	10	10	10	10	10
Nitrite	-	10	9	10	10	10	10	10	10
	+	0	1	0	0	0	0	0	0
Leukocyte	-	8	10	8	9	10	10	9	10
	1+	2	0	1	1	0	0	1	0
	2+	0	0	1	0	0	0	0	0
Erythrocyte	-	10	10	9	10	10	10	9	10
	1+	0	0	1	0	0	0	1	0
Clarity	Clear	10	10	10	10	10	10	10	10
Color	Pale yellow	0	0	1	0	0	0	0	1
	Amber	10	8	8	10	10	10	10	9
	Brown	0	2	1	0	0	0	0	0
Sediment: Cast	< 1	10	10	10	10	10	10	10	10
EPI	< 1	10	10	10	10	10	9	10	10
	1-4	0	0	0	0	0	1	0	0
WBC	< 1	10	10	10	10	9	8	10	10
	1-4	0	0	0	0	1	2	0	0
RBC	< 1	10	10	10	10	10	10	10	10

EPI, epithelial cells; WBC, white blood cells, RBC, red blood cells.

cle control group. The HB and MCH values and relative neutrophils and monocytes counts significantly decreased in the 0.25 mg/kg/day group and the large unstained cell counts, absolute RET count, and PT value significantly increased compared with those in the vehicle control group. In female rats, the MCH and MCHC values significantly decreased in the 0.06 mg/kg/day group and the relative basophils count significantly increased compared with that in the vehicle control group. The HB, MCH, and MCHC values significantly decreased in the 0.13 mg/kg/day group and the relative basophils count and absolute and relative RET counts significantly increased compared with those in the vehicle control group. The HB, HCT, MCH, and MCHC values significantly decreased and the relative and absolute RET counts significantly increased in the 0.25 mg/kg/day group compared with those in the vehicle control group. At the end of the recovery period, however, no significant differences were noted for any of these hematological parameters between the vehicle control and highest dose groups (data not shown).

Table 3. Hematological values in animals treated with KMRC011 for 4 weeks.

Parameters	KMRC011 (mg/kg/day)			
	0	0.06	0.13	0.25
Male				
No. of rats	10	10	10	10
RBC ($\times 10^{12}$/L)	8.35 ± 0.316[a]	8.46 ± 0.417	8.48 ± 0.484	8.30 ± 0.413
HB (g/dL)	16.3 ± 0.37	16.2 ± 0.64	15.9 ± 0.69	15.5 ± 0.73*
HCT (%)	50.1 ± 1.19	49.9 ± 2.28	49.4 ± 2.26	48.5 ± 2.36
MCV (fL)	60.0 ± 1.63	59.0 ± 1.76	58.3 ± 2.12	58.5 ± 1.89
MCH (pg)	19.6 ± 0.69	19.1 ± 0.58	18.7 ± 0.62*	18.7 ± 0.58**
MCHC (g/dL)	32.6 ± 0.63	32.4 ± 0.63	32.1 ± 0.46	31.9 ± 0.41
RET (%)	2.47 ± 0.344	2.55 ± 0.269	3.00 ± 0.570*	2.93 ± 0.468
Absolute RET ($\times 10^{9}$/L)	205.6 ± 28.42	215.3 ± 19.49	252.8 ± 42.77**	242.3 ± 37.13*
PLT ($\times 10^{12}$/L)	1.14 ± 0.280	1.21 ± 0.134	1.24 ± 0.129	1.34 ± 0.250
WBC ($\times 10^{9}$/L)	10.09 ± 2.746	11.40 ± 2.021	12.76 ± 5.134	10.66 ± 2.107
NEU (%)	13.9 ± 4.28	7.5 ± 1.85**	8.0 ± 2.45**	8.7 ± 3.97**
LYM (%)	80.8 ± 4.83	87.8 ± 2.49**	87.4 ± 2.46**	84.9 ± 5.24
MON (%)	2.5 ± 0.63	1.8 ± 0.48*	1.7 ± 0.54**	1.7 ± 0.58**
EOS (%)	0.8 ± 0.37	0.7 ± 0.36	0.7 ± 0.19	0.7 ± 0.34
BAS (%)	0.3 ± 0.11	0.4 ± 0.15	0.5 ± 0.16	0.5 ± 0.20
LUC (%)	1.6 ± 0.67	1.8 ± 0.91	1.7 ± 0.26	3.4 ± 1.93**
PT (sec)	12.4 ± 0.40	13.1 ± 0.67*	13.0 ± 0.38*	13.0 ± 0.45*
APTT (sec)	16.6 ± 0.85	16.2 ± 0.85	15.6 ± 0.72*	16.2 ± 0.89
Female				
No. of rats	10	10	10	10
RBC ($\times 10^{12}$/L)	8.30 ± 0.496	8.29 ± 0.363	8.14 ± 0.548	7.94 ± 0.543
HB (g/dL)	16.6 ± 0.74	15.9 ± 0.62	15.2 ± 1.47*	15.1 ± 0.73**
HCT (%)	49.0 ± 2.14	48.3 ± 1.73	47.3 ± 2.87	45.7 ± 2.22**
MCV (fL)	59.1 ± 1.73	58.2 ± 1.37	58.1 ± 1.47	57.6 ± 1.87
MCH (pg)	20.1 ± 0.53	19.2 ± 0.54*	18.7 ± 1.51**	19.0 ± 0.66**
MCHC (g/dL)	33.9 ± 0.46	33.0 ± 0.53**	32.2 ± 2.19**	33.0 ± 0.40**
RET (%)	2.11 ± 0.290	2.44 ± 0.544	3.06 ± 1.539*	2.78 ± 0.604**
Absolute RET ($\times 10^{9}$/L)	174.5 ± 20.15	201.7 ± 41.17	247.8 ± 121.85**	218.9 ± 35.05*
PLT ($\times 10^{12}$/L)	1.11 ± 0.131	1.28 ± 0.123	1.21 ± 0.251	1.23 ± 0.114
WBC ($\times 10^{9}$/L)	10.66 ± 3.611	11.51 ± 3.004	10.58 ± 3.701	11.98 ± 3.333
NEU (%)	9.5 ± 3.61	9.1 ± 3.93	7.8 ± 4.32	10.6 ± 5.19
LYM (%)	85.2 ± 4.51	84.8 ± 4.15	87.0 ± 4.18	83.4 ± 5.44
MON (%)	2.3 ± 0.80	2.1 ± 0.73	1.9 ± 0.86	2.1 ± 0.82
EOS (%)	0.9 ± 0.46	1.0 ± 0.37	0.7 ± 0.23	1.0 ± 0.33
BAS (%)	0.4 ± 0.07	0.6 ± 0.15**	0.5 ± 0.14*	0.5 ± 0.13
LUC (%)	1.8 ± 0.47	2.5 ± 1.55	2.1 ± 0.93	2.5 ± 1.39
PT (sec)	12.9 ± 0.43	12.9 ± 0.36	12.7 ± 0.22	13.1 ± 0.59
APTT (sec)	15.0 ± 1.12	15.2 ± 0.93	14.3 ± 2.04	14.9 ± 1.04

RBC, red blood cells; HB, hemoglobin, HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RET, reticulocyte; PLT, platelets; WBC, white blood cells; NEU, neutrophils; LYM, lymphocytes; MON, monocytes; EOS, eosinophils; BAS, basophils; and LUC, large unstained cells.

[a] Values are presented as means ± S.D.

* Significant difference at $P < 0.05$ level compared with the control group.

** Significant difference at $P < 0.01$ level compared with the control group.

Serum biochemistry

In male rats, a significant decrease in T-BIL, ALB, and A/G levels was observed in the 0.06 mg/kg/day group compared with that in the vehicle control group (Table 4). A significant increase in AST level and a significant decrease in T-BIL and A/G levels were observed in the

Table 4. Serum biochemical values in animals treated with KMRC011 for 4 weeks.

Parameters	KMRC011 (mg/kg/day)			
	0	0.06	0.13	0.25
Male				
No. of rats	10	10	10	10
AST (IU/L)	130.4 ± 16.41[a]	148.6 ± 26.23	166.4 ± 26.44**	157.3 ± 44.56
ALT (IU/L)	33.9 ± 4.31	51.4 ± 33.30	40.2 ± 11.25	45.5 ± 9.54*
ALP (IU/L)	653.1 ± 127.73	543.3 ± 89.70	585.2 ± 78.59	616.9 ± 79.24
BUN (mg/dL)	16.4 ± 2.08	16.4 ± 2.21	16.8 ± 2.29	15.7 ± 2.07
CRTN (mg/dL)	0.43 ± 0.026	0.45 ± 0.055	0.45 ± 0.056	0.45 ± 0.043
GLU (mg/dL)	108.0 ± 18.13	87.1 ± 20.58	89.0 ± 28.20	91.0 ± 46.10*
T-CHO (mg/dL)	51.4 ± 11.67	49.4 ± 8.68	48.7 ± 10.27	51.4 ± 8.49
T-BIL (mg/dL)	0.110 ± 0.0157	0.076 ± 0.0127**	0.073 ± 0.0298**	0.075 ± 0.0192**
TP (g/dL)	6.25 ± 0.285	6.22 ± 0.229	6.24 ± 0.327	6.40 ± 0.267
ALB (g/dL)	4.11 ± 0.146	3.97 ± 0.144*	3.98 ± 0.144	3.95 ± 0.071*
A/G (ratio)	1.94 ± 0.153	1.77 ± 0.087*	1.77 ± 0.143*	1.62 ± 0.180**
CPK (IU/L)	537.9 ± 149.55	710.2 ± 357.37	751.1 ± 275.84	797.1 ± 602.91
TG (mg/dL)	12.7 ± 4.14	18.6 ± 6.42	17.6 ± 6.89	17.3 ± 5.60
Ca (mg/dL)	10.94 ± 0.342	10.57 ± 0.474	10.68 ± 0.527	10.78 ± 0.408
IP (mg/dL)	11.00 ± 0.842	11.00 ± 1.192	11.42 ± 1.026	11.75 ± 0.914
PL (mg/dL)	83.8 ± 11.97	78.4 ± 11.35	77.6 ± 11.95	81.7 ± 10.33
GGT (IU/L)	0.40 ± 0.128	0.43 ± 0.175	0.54 ± 0.188	0.48 ± 0.187
K (mmol/L)	8.26 ± 0.916	8.58 ± 1.997	9.03 ± 2.023	9.25 ± 1.806
Na (mmol/L)	143.5 ± 1.27	144.3 ± 1.16	144.9 ± 1.20	146.6 ± 2.41**
Cl (mmol/L)	101.1 ± 1.85	101.4 ± 0.97	102.2 ± 1.81	103.4 ± 1.26**
Female				
No. of rats	10	10	10	10
AST (IU/L)	143.3 ± 25.65	143.5 ± 17.71	147.2 ± 28.04	146.2 ± 27.44
ALT (IU/L)	27.9 ± 3.14	46.9 ± 40.29*	37.9 ± 20.80	35.7 ± 7.93*
ALP (IU/L)	381.0 ± 64.80	362.4 ± 52.75	301.3 ± 67.08*	316.1 ± 47.80*
BUN (mg/dL)	20.7 ± 3.51	20.4 ± 3.16	18.6 ± 2.64	19.4 ± 2.89
CRTN (mg/dL)	0.54 ± 0.082	0.52 ± 0.062	0.50 ± 0.057	0.51 ± 0.069
GLU (mg/dL)	79.6 ± 17.31	92.6 ± 17.25	81.1 ± 22.79	94.9 ± 22.96
T-CHO (mg/dL)	65.8 ± 12.07	65.1 ± 18.91	63.3 ± 11.58	68.1 ± 17.36
T-BIL (mg/dL)	0.131 ± 0.0104	0.106 ± 0.0269**	0.114 ± 0.0174	0.118 ± 0.0331
TP (g/dL)	6.84 ± 0.276	6.79 ± 0.237	6.72 ± 0.377	6.92 ± 0.164
ALB (g/dL)	4.48 ± 0.198	4.27 ± 0.139	4.25 ± 0.248*	4.26 ± 0.150*
A/G (ratio)	1.90 ± 0.134	1.71 ± 0.130**	1.73 ± 0.084*	1.61 ± 0.145**
CPK (IU/L)	691.8 ± 198.74	621.8 ± 162.58	608.3 ± 232.18	580.0 ± 215.82
TG (mg/dL)	12.9 ± 3.77	17.0 ± 6.53	15.0 ± 8.43	15.6 ± 10.67
Ca (mg/dL)	11.13 ± 0.231	11.20 ± 0.522	11.10 ± 0.400	11.21 ± 0.402
IP (mg/dL)	9.93 ± 0.671	10.24 ± 0.641	10.29 ± 1.110	10.60 ± 0.755
PL (mg/dL)	124.8 ± 13.42	120.2 ± 29.93	116.1 ± 21.88	120.9 ± 28.03
GGT (IU/L)	1.10 ± 0.451	0.84 ± 0.311	0.73 ± 0.271	0.84 ± 0.257
K (mmol/L)	8.09 ± 1.396	8.38 ± 0.613	8.74 ± 0.900	8.43 ± 0.754
Na (mmol/L)	145.6 ± 1.65	146.0 ± 1.49	146.6 ± 0.97	147.4 ± 0.70**
Cl (mmol/L)	104.0 ± 1.33	103.4 ± 1.43	105.4 ± 1.51	105.4 ± 1.17

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRTN, creatinine; GLU, glucose; T-CHO, total cholesterol; T-BIL, total bilirubin; TP, total protein; ALB, albumin; A/G, albumin/globulin; CPK, creatine phosphokinase; TG, triglycerides; Ca, calcium; IP, inorganic phosphate; PL, phospholipid; GGT, gamma glutamyl transferase; K, potassium; Na, sodium; and Cl, chlorine.

[a] Values are presented as means ± S.D.

* Significant difference at $P < 0.05$ level compared with the control group.

** Significant difference at $P < 0.01$ level compared with the control group.

0.13 mg/kg/day group compared with those in the vehicle control group. A significant increase in ALT, Na, and Cl levels and a significant decrease in GLU, T-BIL, ALB, and A/G levels were observed in the 0.25 mg/kg/day group compared with those in the vehicle control group. In female rats, a significant increase in ALT level and a significant decrease in T-BIL and A/G levels were observed in the 0.06 mg/kg/day group compared with those in the vehicle control group. A significant decrease in ALP, ALB, and A/G levels was observed in the 0.13 mg/kg/day group compared with that in the vehicle control group. A significant increase in ALT and Na levels and a significant decrease in ALP, ALB, and A/G levels were observed in the 0.25 mg/kg/day group compared with that in the vehicle control group. At the end of the recovery period, the male 0.25 mg/kg/day group exhibited a significant decrease in A/G level (data not shown) and the female 0.25 mg/kg/day group exhibited a significant decrease in ALP level.

Gross findings

Gross findings at male rat necropsy included cream color changes in the lung (1/10) and small thyroid and parathyroid glands (2/10) in the 0.06 mg/kg/day group, small thyroid and parathyroid glands (1/10) in the 0.13 mg/kg/day group, and enlarged spleen (1/10) in the 0.25 mg/kg/day group. In female rats, gross findings included enlarged spleen (1/10) in the 0.06 mg/kg/day group, red color change in the lung (1/10) and enlarged spleen (1/10) in the 0.13 mg/kg/day group, and enlarged spleen (1/10) in the 0.25 mg/kg/day group (data not shown). At the end of the recovery period, however, no significant differences were noted for any of these hematological parameters between the vehicle control and highest dose groups.

Organ weights

The absolute and relative organ weights of the male rats are shown in Table 5. The relative weight of the spleen was significantly greater in the 0.06 mg/kg/day group compared with that in the vehicle control group. In the 0.13 mg/kg/day group, the absolute and relative weights of the salivary glands were significantly less, whereas the absolute and relative weights of the spleen were significantly greater, compared with those in the vehicle control group. A significant increase in the absolute and relative weights of the spleen was observed in the 0.25 mg/kg/kg group compared with that in the vehicle control group. In female rats (Table 6), the absolute and relative weights of the spleen were significantly greater in the 0.06 mg/kg/day and 0.13 mg/kg/day groups compared with those in the vehicle control group. A significant increase in the absolute and relative uterus/cervix weights was also found in the 0.13 mg/kg/day group. A significant increase in the absolute and relative weights of the kidneys and spleen was observed in the 0.25 mg/kg/kg group compared with those in the vehicle control group. At the end of recovery period, the absolute and relative weights of the spleen were significantly increased in the female 0.25 mg/kg/day group (data not shown).

Histopathological findings

The results of histopathological examination are shown in Tables 7 and 8. In male rats, inflammatory cell infiltration of the cecum was found in 8 rats in the 0.06 mg/kg/day group, 7 rats in the 0.13 mg/kg/day group, and 5 rats in the 0.25 mg/kg/day group (Fig. 3). Lymphocytes infiltration of the duodenum was observed in 1 rat in the vehicle control group, 1 rat in the 0.06 mg/kg/day group, 1 rat in the 0.13 mg/kg/day group, and 6 rats in the 0.25 mg/kg/day group (Fig. 4). Increased hemopoiesis in the femorotibial joint/marrow was found in 2 rats in the 0.06 mg/kg/day group, 4 rats in the 0.13 mg/kg/day group, and 6 rats in the 0.25 mg/kg/day group (Fig. 5). Increased hemopoiesis in the sternum/marrow was also observed in 2 rats in the 0.06 mg/kg/day group, 4 rats in the 0.13 mg/kg/day group, and 6 rats in the 0.25 mg/kg/day group. In female rats, inflammatory cell infiltration in the cecum was found in 2 rats in the vehicle control group, 7 rats in the 0.06 mg/kg/day group, 6 rats in the 0.13 mg/kg/day group, and 10 rats in the 0.25 mg/kg/day group. Lymphocytes infiltration in the duodenum was observed in 2 rats in the 0.06 mg/kg/day group and 6 rats in the 0.25 mg/kg/day group. Increased hemopoiesis in the femoro-tibial joint/marrow was found in 3 rats in the 0.06 mg/kg/day group, 3 rats in the 0.13 mg/kg/day group, and 6 rats in the 0.25 mg/kg/day group. Increased hemopoiesis in the sternum/marrow was also observed in 3 rats in the 0.06 mg/kg/day group, 3 rats in the 0.13 mg/kg/day group, and 6 rats in the 0.25 mg/kg/day group. The other findings observed in the treatment groups were also found in the vehicle control group or were accidental changes without any dose-response relationship. At the end of the recovery period, no significant differences were observed for any of these histopathological changes between the vehicle control and highest dose groups.

DISCUSSION

The present study investigated the potential subacute toxicity of KMRC011, a TLR 5 agonist, by a 4-week repeated intramuscular injection to Sprague-Dawley rats

Table 5. Absolute and relative organ weights in male rats treated with KMRC011 for 4 weeks.

Parameters	KMRC011 (mg/kg/day)			
	0	0.06	0.13	0.25
No. of male rats	10	10	10	10
Body weight	357.6 ± 21.60[a]	341.7 ± 19.28	352.1 ± 35.85	345.7 ± 29.54
Salivary glands (g)	0.671 ± 0.0540	0.620 ± 0.0433	0.584 ± 0.0958*	0.650 ± 0.0707
Per body weight (%)	0.1876 ± 0.01179	0.1815 ± 0.01017	0.1657 ± 0.01935**	0.1882 ± 0.01321
Adrenal glands (g)	0.064 ± 0.0077	0.063 ± 0.0052	0.065 ± 0.0141	0.068 ± 0.0113
Per body weight (%)	0.0179 ± 0.00218	0.0186 ± 0.00183	0.0185 ± 0.00316	0.0196 ± 0.00219
Brain (g)	1.994 ± 0.0614	1.980 ± 0.0862	1.924 ± 0.0599	1.961 ± 0.1157
Per body weight (%)	0.5595 ± 0.03875	0.5807 ± 0.03102	0.5512 ± 0.05255	0.5697 ± 0.04174
Heart (g)	1.202 ± 0.0855	1.183 ± 0.0894	1.255 ± 0.1701	1.204 ± 0.1385
Per body weight (%)	0.3365 ± 0.01917	0.3470 ± 0.03035	0.3557 ± 0.01860	0.3481 ± 0.02542
Kidneys (g)	3.064 ± 0.2198	2.918 ± 0.2610	3.049 ± 0.3745	3.079 ± 0.3258
Per body weight (%)	0.8576 ± 0.04801	0.8534 ± 0.05013	0.8683 ± 0.09358	0.8901 ± 0.04100
Liver (g)	11.595 ± 0.8127	11.142 ± 0.9477	11.665 ± 1.5547	11.327 ± 1.3155
Per body weight (%)	3.2447 ± 0.16721	3.2618 ± 0.21411	3.3100 ± 0.24560	3.2761 ± 0.24897
Pituitary gland (g)	0.012 ± 0.0016	0.011 ± 0.0016	0.012 ± 0.0022	0.012 ± 0.0023
Per body weight (%)	0.0034 ± 0.00041	0.0033 ± 0.00045	0.0034 ± 0.00035	0.0036 ± 0.00069
Spleen (g)	0.667 ± 0.1062	0.860 ± 0.1455	1.003 ± 0.1363**	0.985 ± 0.2815**
Per body weight (%)	0.1861 ± 0.02389	0.2512 ± 0.03711*	0.2846 ± 0.02283**	0.2836 ± 0.06796**
Testes (g)	3.133 ± 0.1941	3.117 ± 0.2174	3.235 ± 0.3117	3.176 ± 0.3627
Per body weight (%)	0.8782 ± 0.06311	0.9134 ± 0.05783	0.9214 ± 0.06636	0.9188 ± 0.07935
Thymus (g)	0.491 ± 0.0916	0.451 ± 0.1258	0.468 ± 0.1338	0.428 ± 0.0955
Per body weight (%)	0.1371 ± 0.02182	0.1320 ± 0.03565	0.1310 ± 0.02883	0.1240 ± 0.02605
Epididymis (g)	1.085 ± 0.0967	1.024 ± 0.0811	1.078 ± 0.1036	1.067 ± 0.0719
Per body weight (%)	0.3041 ± 0.02879	0.3007 ± 0.03036	0.3079 ± 0.03304	0.3096 ± 0.02154
Lung (g)	1.472 ± 0.1223	1.354 ± 0.1018	1.445 ± 0.1689	1.408 ± 0.1567
Per body weight (%)	0.4120 ± 0.03139	0.3966 ± 0.02465	0.4106 ± 0.02735	0.4066 ± 0.01976
Thyroid glands (g)	0.021 ± 0.0034	0.018 ± 0.0041	0.018 ± 0.0036	0.019 ± 0.0046
Per body weight (%)	0.0060 ± 0.00115	0.0053 ± 0.00121	0.0051 ± 0.00100	0.0055 ± 0.00110
Seminal vesicles	1.227 ± 0.1768	1.203 ± 0.0998	1.109 ± 0.1699	1.107 ± 0.1014
Per body weight (%)	0.3427 ± 0.04224	0.3533 ± 0.03847	0.3162 ± 0.04697	0.3229 ± 0.04661
Prostate	0.474 ± 0.0518	0.479 ± 0.0486	0.453 ± 0.0670	0.464 ± 0.0940
Per body weight (%)	0.1330 ± 0.01748	0.1409 ± 0.01765	0.1290 ± 0.01716	0.1339 ± 0.02412

[a] Values are presented as means ± S.D.

* Significant difference at $P < 0.05$ level compared with the control group.

** Significant difference at $P < 0.01$ level compared with the control group.

at doses of 0, 0.06, 0.13, and 0.25 mg/kg/day. After the end of the dosing period, reversibility was assessed following a 2-week recovery period.

Loss of fur was found in a few male rats, but was not considered related to treatment with KMRC011 because it occurred in a low incidence and did not exhibit a dose-response relationship. The suppression of body weight observed in male rats in the treatment groups was considered related to the KMRC011 treatment and was consistent with the decreased food consumption observed in the groups. However, these changes were recovered at the end of the experimental period. The decreased food consumption observed in females of the treatment groups was transient and did not show a dose-response relationship, therefore it was not related to the test article administration. Because the decreased food consumption was largely observed in the early stages of the treatment period, this finding may have been related to physical stress from handling and intramuscular injection rather than to KMRC011 itself. According to a developmental toxicity study of the TLR 5 agonist, entolimod, in female Wistar rats (Chow and Faqi, 2014), there was no treatment-related mortality or clinical signs of toxicity, but reduced body weight and food consumption were observed at subcutaneous doses of 0.1 mg/kg/day or greater. This apparent discrepancy between their results and ours may be

Table 6. Absolute and relative organ weights in female rats treated with KMRC011 for 4 weeks.

Parameters	KMRC011 (mg/kg/day)			
	0	0.06	0.13	0.25
No. of female rats	10	10	10	10
Body weight	239.4 ± 16.58a	248.5 ± 18.13	236.7 ± 20.03	248.1 ± 24.24
Salivary glands (g)	0.449 ± 0.0343	0.435 ± 0.0254	0.426 ± 0.0308	0.447 ± 0.0428
Per body weight (%)	0.1881 ± 0.01524	0.1758 ± 0.01307	0.1805 ± 0.01093	0.1812 ± 0.01811
Adrenal glands (g)	0.073 ± 0.0093	0.078 ± 0.0127	0.075 ± 0.0052	0.079 ± 0.0079
Per body weight (%)	0.0307 ± 0.00358	0.0315 ± 0.00421	0.0318 ± 0.00333	0.0319 ± 0.00414
Brain (g)	1.922 ± 0.0679	1.920 ± 0.0902	1.856 ± 0.0849	1.896 ± 0.0550
Per body weight (%)	0.8061 ± 0.05497	0.7747 ± 0.03826	0.7881 ± 0.06428	0.7698 ± 0.06568
Heart (g)	0.832 ± 0.0865	0.889 ± 0.0698	0.839 ± 0.0709	0.904 ± 0.0822
Per body weight (%)	0.3473 ± 0.02171	0.3580 ± 0.01677	0.3549 ± 0.01818	0.3650 ± 0.02034
Kidneys (g)	2.000 ± 0.2182	2.097 ± 0.1918	2.024 ± 0.2131	2.272 ± 0.2358*
Per body weight (%)	0.8354 ± 0.06902	0.8444 ± 0.05246	0.8546 ± 0.04831	0.9196 ± 0.09030*
Liver (g)	7.997 ± 0.7967	8.569 ± 0.9491	8.241 ± 0.9880	8.579 ± 0.9489
Per body weight (%)	3.3373 ± 0.17521	3.4461 ± 0.23903	3.4773 ± 0.24911	3.4592 ± 0.19593
Pituitary gland (g)	0.015 ± 0.0018	0.014 ± 0.023	0.015 ± 0.0022	0.015 ± 0.0030
Per body weight (%)	0.0062 ± 0.00087	0.0056 ± 0.00099	0.0062 ± 0.00088	0.0060 ± 0.00107
Spleen (g)	0.565 ± 0.1178	0.772 ± 0.1805*	0.780 ± 0.2366*	0.839 ± 0.3557*
Per body weight (%)	0.2358 ± 0.04608	0.3091 ± 0.05618*	0.3293 ± 0.09968**	0.3347 ± 0.11688**
Thymus (g)	0.511 ± 0.1804	0.515 ± 0.1041	0.479 ± 0.0750	0.565 ± 0.1413
Per body weight (%)	0.2130 ± 0.06801	0.2074 ± 0.03904	0.2027 ± 0.02592	0.2264 ± 0.04580
Lung (g)	1.132 ± 0.0814	1.146 ± 0.0626	1.154 ± 0.1228	1.182 ± 0.0693
Per body weight (%)	0.4734 ± 0.02069	0.4622 ± 0.01524	0.4878 ± 0.03593	0.4785 ± 0.03033
Thyroid glands (g)	0.017 ± 0.0029	0.016 ± 0.0036	0.015 ± 0.0037	0.018 ± 0.0028
Per body weight (%)	0.0073 ± 0.00138	0.0065 ± 0.00143	0.0065 ± 0.00155	0.0071 ± 0.00121
Uterus/cervix	0.465 ± 0.1400	0.554 ± 0.1924	0.731 ± 0.2958*	0.508 ± 0.1627
Per body weight (%)	0.1932 ± 0.04853	0.2258 ± 0.08523	0.3090 ± 0.11964*	0.2050 ± 0.06270
Ovaries/Oviduct	0.126 ± 0.0189	0.124 ± 0.0200	0.117 ± 0.0102	0.128 ± 0.0177
Per body weight (%)	0.0528 ± 0.00739	0.0499 ± 0.00612	0.0498 ± 0.00594	0.0518 ± 0.00498

[a] Values are presented as means ± S.D.

[*] Significant difference at $P < 0.05$ level compared with the control group.

[**] Significant difference at $P < 0.01$ level compared with the control group.

explained by the differences in test article structure, test system, and duration and route of exposure to the test article.

There were no treatment-related changes in ophthalmology and urinalysis. The significant decreases in HB, HCT, and MCH values in male and/or female rats in the treatment groups were closely related to the administration of the test article, but were considered to have a low toxicological significance. Conversely, the significant decrease in MCHC value in the female 0.13 and 0.25 mg/kg/day groups was of doubtful toxicological significance because it was slight, within the limits of normal biological variations (Wolford *et al*., 1986; Kang *et al*., 1995), and observed in females only. The significant increases in absolute and relative RET counts in both sexes in the 0.13 and 0.25 mg/kg/day groups indicated that these findings were closely related to the administration of KMRC011 because correlated histopathological changes, such as increased hemopoiesis of the femoro-tibial joint/marrow and sternum/marrow, were detected in the groups at high frequencies. These changes may have been related to the release of reticulocytes from the bone marrow as a compensatory response to a reduction in HB, HCT, and MCH values owing to the effect of KMRC011. Previous studies also indicated that an increase in RET count was observed when the bone marrow was highly active in an attempt to replace RBC loss, such as in hemolytic anemia and hemorrhage (Macdougall, 2014; Moll and Davis, 2017). However, the change was slight and no longer observed after the 2-week recovery period. Therefore, the increase in RET count was considered of low toxicological significance. The other hematological changes found

Table 7. Histopathological findings in male rats treated with KMRC011 for 4 weeks.

Items	Dose (mg/kg/day)			
	0	0.06	0.13	0.25
No. of male animals	10	10	10	10
Adrenal glands				
Cortical vacuolation	4	0	0	0
Cecum				
Infiltration, Inflammatory cell, Interstitial	0	8	7	5
Colon				
Infiltration, Mononuclear cell	0	0	0	1
Duodenum				
Infiltration, lymphocytes, Villous tip	1	1	1	6
Epididymis				
Infiltration, Mononuclear cell	1	0	0	0
Eye and optic nerve				
Retinal fold	1	0	0	0
Femoro-tibial joint/marrow				
Increased hemopoiesis	0	2	4	6
Heart				
Cardiomyopathy	0	0	0	1
Infiltration, Mononuclear cell	0	0	0	1
Kidney				
Basophilia, tubules	4	0	0	4
Casts, hyaline	3	0	0	3
Granuloma	1	0	0	0
Infiltration, Mononuclear cell, interstitial	3	0	0	4
Nephroblastosis	1	0	0	0
Liver				
Basophilic focus	0	0	0	1
Fibrosis	0	1	0	0
Focal necrosis	2	0	0	0
Focal necrosis Infiltration, Mononuclear cell	9	1	0	8
Periportal vacuolation	1	0	0	0
Vacuolated area	3	0	0	2
Vasculopathy	0	0	0	1
Lung with bronchi				
Eosinophilic crystal	0	0	0	1
Infiltration, mixed cell	0	0	0	1
Prostate				
Infiltration, mononuclear, interstitial	2	0	0	0
Skeletal muscle				
Infiltration, mononuclear cell, interstitial	0	0	0	1
Sternum/Marrow				
Increased hemopoiesis	0	2	4	6
Thyroid and parathyroid glands				
Hypertrophy, follicular cell	9	0	1	9

in the treated groups were of no toxicological significance because they were also within normal ranges (Wolford *et al*., 1986; Kang *et al*., 1995) and were not associated with gross or microscopic pathological changes.

The increase in the Na level that was found in both sexes in the 0.25 mg/kg/day groups was of doubtful toxicological significance because it was a very slight increase (1-2%) and within historical control range for the rat strain (Wolford *et al*., 1986; Kang *et al*., 1995). Significant decreases in the ALB and A/G levels were observed in both sexes in the treatment groups. Because these alterations were very small in their fluctuation range and there

Table 8. Histopathological findings in female rats treated with KMRC011 for 4 weeks.

Items	Dose (mg/kg/day)			
	0	0.06	0.13	0.25
No. of female animals	10	10	10	10
Cecum				
Infiltration, inflammatory cell, interstitial	2	7	6	10
Duodenum				
Infiltration, lymphocytes, villous tip	0	2	0	6
Femoro-tibial joint/marrow				
Increased hemopoiesis	0	3	3	6
Harderian glands				
Infiltration, mononuclear cell	1	0	0	2
Heart				
Infiltration, m ononuclear cell	1	0	0	1
Injection site(s)				
Infiltration, mononuclear cell, interstitial	0	0	0	1
Kidney				
Basophilia, tubules	2	0	0	2
Dilation, tubules	0	0	0	2
Infiltration, mononuclear cell, interstitial	4	0	0	3
Mineralization, corticomedullary junction	2	0	0	6
Liver				
Hypertrophy, Kupffer cell	0	0	0	3
Infiltration, mononuclear cell	9	0	0	10
Periportal vacuolation	4	0	0	2
Lung with bronchi				
Alveolar macrophage	0	0	1	0
Eosinophilic crystal	0	0	1	1
Hemorrhage	0	0	1	0
Infiltration, mixed cell	0	0	0	1
Lymph node, mesenteric				
Mastocytosis	0	0	0	1
Pancreas				
Infiltration, mononuclear cell	0	0	0	1
Skin, inguinal				
Infiltration, mononuclear cell, dermis	1	0	0	0
Spleen				
Extramedullary hemopoiesis	0	0	0	1
Sternum/Marrow				
Increased hemopoiesis	0	3	3	6
Thyroid and parathyroid glands				
Ectopia, thymus	1	0	0	0
Hypertrophy, follicular cell	5	0	0	2

were no changes in other related parameters, these changes were considered of no toxicological significance. The other serum biochemical changes found in the treated groups were of no toxicological significance because these changes were not associated with the finding of histopathological examination, were observed in only one sex, and did not indicate a dose-response relationship. At scheduled autopsy, some abnormal gross findings were found in both sexes in the treatment groups at a low frequency. Because they occurred infrequently and were not dose related, they were considered spontaneous findings.

The significant increases in absolute and relative spleen weights observed in the both sexes in the treatment groups were closely related to the administration of the test article. TLR 5 is expressed in internal tissues, such as the spleen, kidney, and heart, suggesting a wide role for TLR 5 in host defense (Gewirtz *et al*., 2001). According to previous studies, TLR 5 agonist treatment in irradiated

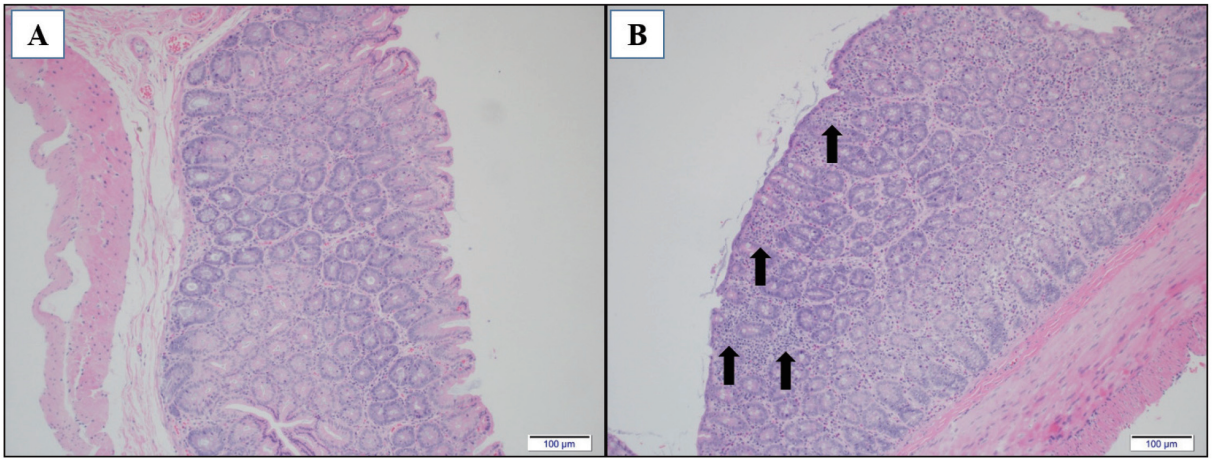

Fig. 3. Representative photographs of cecum sections from the vehicle control and high dose groups stained with hematoxylin & eosin. (A) a control male rat, showing normal appearance. (B) KMRC011 treated male rat, showing inflammatory cell infiltration in the interstitial tissue (arrow). Bar = 100 μm.

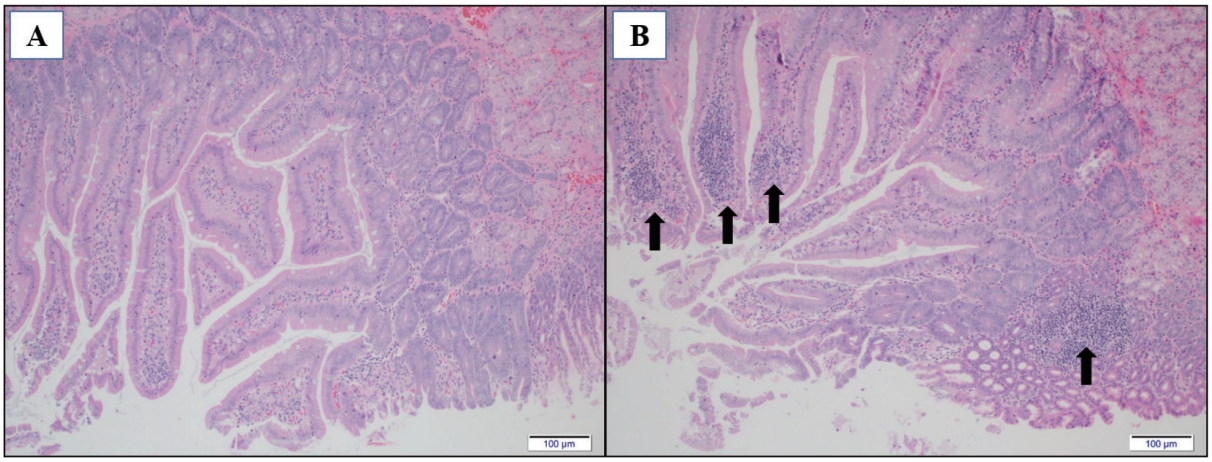

Fig. 4. Representative photographs of duodenum sections from the vehicle control and high dose groups stained with hematoxylin & eosin. (A) a control male rat, showing normal appearance. (B) KMRC011 treated male rat, showing lymphocytes infiltration in the villous tips (arrow). Bar = 100 μm.

non-human primates resulted in less hematopoietic injury owing to an increase in the proliferation of spleen cells (Burdelya *et al*., 2008; Krivokrysenko *et al*., 2015). In this study, the change was not associated with histopathological observations. Therefore, the increased spleen weight was not considered an adverse effect, but rather a beneficial pharmacological effect that enhanced the recovery capacity of the organism. A significant decrease in absolute and relative salivary glands weight was observed in male rats in the treatment groups. A significant increase in absolute and relative kidneys and uterus/cervix weight was found in female rats in the treatment groups. However, the weight change in the above organs was of doubtful toxicological significance because there was no corresponding pathology that accompanied these differences in organ weights.

In histopathological examination, the increased hemopoiesis in the femoro-tibial joint/marrow and sternum/marrow in both sexes in the treatment groups indicated that these findings were closely related to the admin-

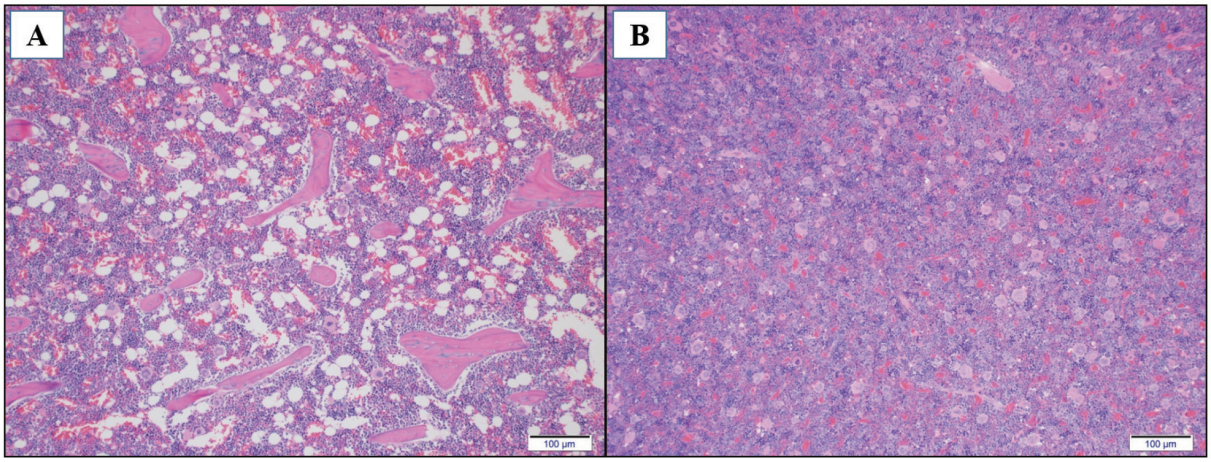

Fig. 5. Representative photographs of bone marrow (femur and sternum) sections from the vehicle control and high dose groups stained with hematoxylin & eosin. (A) a control female rat, showing normal appearance. (B) KMRC011 treated female rat, showing increased hemopoiesis. Bar = 100 μm.

istration of KMRC011 because there was a high incidence of these changes, they exhibited a dose–response relationship, and were accompanied by correlated hematological changes. A previous study demonstrated that TLR 5 agonist enhanced tumor killing activity indirectly through a mechanism that involved $CD11c^+$ and $CD11b^+$ populations in the bone marrow cells (Ding *et al*., 2012). Kojouharov *et al*. (2014) also reported that TLR 5 agonist promotes the recovery of hematopoiesis in the bone marrow after 5-fluorouracil-induced damage. These observations indicated that the increased hemopoiesis in the bone marrow may have been related to the pharmacological effects of KMRC011. An increase in the incidence of lymphocyte infiltration in the duodenum in both sexes of the 0.25 mg/kg/day group was closely related to the administration of the test article because there was a high incidence of this change compared with the vehicle control group. Although an increase in the incidence of inflammatory cell infiltration in the cecum in both sexes of the treatment groups did not exhibit a dose-response relationship, the change was closely related to the administration of the test article because this change was observed only in the treatment groups. However, the severity of these changes was slight and no longer observed after the 2-week recovery period; therefore, it was considered of low toxicological significance. The other histopathological changes observed in the treatment groups were not considered treatment-related effects because they occurred in a low incidence and did not exhibit a dose-response relationship. Moreover, these findings are well known to occur commonly in normal Sprague-Dawley rats (Greaves, 1990; Haschek and Rousseaux, 1998; Sugimoto *et al*., 2000).

In conclusion, the 4-week repeated intramuscular injection of KMRC011 to rats resulted in decreases in HB, HCT, and MCV values and increases in RET count and histopathological lesions, such as increased hemopoiesis in the femoro-tibial joint/marrow and sternum/marrow, lymphocytes infiltration in the duodenum, and inflammatory cell infiltration in the cecum. However, these changes were reversible, and therefore we determined they had no toxicological significance. Based on these results, the NOAEL of KMRC011 was considered to be greater than 0.25 mg/kg/day in male and female rats.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Andic, F., Garipagaoglu, M., Yurdakonar, E., Tuncel, N. and Kucuk, O. (2009): Lycopene in the prevention of gastrointestinal toxicity of radiotherapy. Nutr. Cancer, **61**, 784-788.

Atkinson, J., Kapralov, A.A., Yanamala, N., Tyurina, Y.Y.,

Amoscato, A.A., Pearce, L., Peterson, J., Huang, Z., Jiang, J., Samhan-Arias, A.K., Maeda, A., Feng, W., Wasserloos, K., Belikova, N.A., Tyurin, V.A., Wang, H., Fletcher, J., Wang, Y., Vlasova, I.I., Klein-Seetharaman, J., Stoyanovsky, D.A., Bayîr, H., Pitt, B.R., Epperly, M.W., Greenberger, J.S. and Kagan, V.E. (2011): A mitochondria-targeted inhibitor of cytochrome c peroxidase mitigates radiation-induced death. Nat. Commun., **2**, 497.

Burdelya, L.G., Krivokrysenko, V.I., Tallant, T.C., Strom, E., Gleiberman, A.S., Gupta, D., Kurnasov, O.V., Fort, F.L., Osterman, A.L., Didonato, J.A., Feinstein, E. and Gudkov, A.V. (2008): An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. Science, **320**, 226-230.

Cho, Y.J., Yi, C.O., Jeon, B.T., Jeong, Y.Y., Kang, G.M., Lee, J.E., Roh, G.S. and Lee, J.D. (2013): Curcumin attenuates radiation-induced inflammation and fibrosis in rat lungs. Korean J. Physiol. Pharmacol., **17**, 267-274.

Choi, N.C. (2003): Radioprotective effect of amifostine in radiation pneumonitis. Semin. Oncol., **30**, 10-17.

Chow, C.P. and Faqi, A.S. (2014): Developmental toxicity study of CBLB502 in Wistar rats. Reprod. Toxicol., **46**, 12-19.

Ding, X., Bian, G., Leigh, N.D., Qiu, J., McCarthy, P.L., Liu, H., Aygun-Sunar, S., Burdelya, L.G., Gudkov, A.V. and Cao, X. (2012): A TLR5 agonist enhances CD8(+) T cell-mediated graft-versus-tumor effect without exacerbating graft-versus-host disease. J. Immunol., **189**, 4719-4727.

Donnelly, E.H., Nemhauser, J.B., Smith, J.M., Kazzi, Z.N., Farfán, E.B., Chang, A.S. and Naeem, S.F. (2010): Acute radiation syndrome: assessment and management. South. Med. J., **103**, 541-546.

Dörr, H. and Meineke, V. (2011): Acute radiation syndrome caused by accidental radiation exposure - therapeutic principles. BMC Med., **9**, 126.

Eda, K., Uzer, K., Murat, T. and Cenk, U. (2016): The effects of enteral glutamine on radiotherapy induced dermatitis in breast cancer. Clin. Nutr., **35**, 436-439.

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J. and Madara, J.L. (2001): Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J. Immunol., **167**, 1882-1885.

Greaves, P. (1990): Histopathology of Preclinical Studies, Interpretation and Relevance in Drug Evaluation. Elsevier, New York.

Gu, J., Zhu, S., Li, X., Wu, H., Li, Y. and Hua, F. (2014): Effect of amifostine in head and neck cancer patients treated with radiotherapy: a systematic review and meta-analysis based on randomized controlled trials. PLoS One, **9**, e95968.

Hall, S., Rudrawar, S., Zunk, M., Bernaitis, N., Arora, D., McDermott, C.M. and Anoopkumar-Dukie, S. (2016): Protection against Radiotherapy-Induced Toxicity. Antioxidants, **5**, E22.

Haschek, W.M. and Rousseaux, C.G. (1998): Fundamentals of Toxicologic Pathology. Academic Press, San Diego.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M. and Aderem, A. (2001): The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature, **410**, 1099-1103.

Huebener, P. and Schwabe, R.F. (2013): Regulation of wound healing and organ fibrosis by toll-like receptors. Biochim. Biophys. Acta, **1832**, 1005-1017.

Kang, B.H., Son, H.Y., Ha, C.S., Lee, H.S. and Song, S.W. (1995): Reference values of hematology and serum chemistry in Ktc: Sprague–Dawley rats. Kor. J. Lab. Anim. Sci., **11**, 141-145.

Kojouharov, B.M., Brackett, C.M., Veith, J.M., Johnson, C.P., Gitlin, I.I., Toshkov, I.A., Gleiberman, A.S., Gudkov, A.V. and Burdelya, L.G. (2014): Toll-like receptor-5 agonist Entolimod broadens the therapeutic window of 5-fluorouracil by reducing its toxicity to normal tissues in mice. Oncotarget, **5**, 802-814.

Krivokrysenko, V.I., Toshkov, I.A., Gleiberman, A.S., Krasnov, P., Shyshynova, I., Bespalov, I., Maitra, R.K., Narizhneva, N.V., Singh, V.K., Whitnall, M.H., Purmal, A.A., Shakhov, A.N., Gudkov, A.V. and Feinstein, E. (2015): The Toll-like receptor 5 agonist Entolimod mitigates lethal acute radiation syndrome in non-human primates. PLoS One, **10**, e0135388.

Macdougall, I.C. (2014): Anaemia Management in Chronic Kidney Disease. In: Harber, M. (Eds.), Practical Nephrology, pp. 569-579, Springer London, London.

Moll, R. and Davis, B. (2017): Iron, vitamin B 12 and folate. Medicine (Baltimore), **45**, 198-203.

O'Neill, L.A., Bryant, C.E. and Doyle, S.L. (2009): Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol. Rev., **61**, 177-197.

Sayles, C., Hickerson, S.C., Bhat, R.R., Hall, J., Garey, K.W. and Trivedi, M.V. (2016): Oral glutamine in preventing treatment-related mucositis in adult patients with cancer: A systematic review. Nutr. Clin. Pract., **31**, 171-179.

Sheibani, K.M., Mafi, A.R., Moghaddam, S., Taslimi, F., Amiran, A. and Ameri, A. (2015): Efficacy of benzydamine oral rinse in prevention and management of radiation-induced oral mucositis: A double-blind placebo-controlled randomized clinical trial. Asia Pac. J. Clin. Oncol., **11**, 22-27.

Singh, V.K. and Pollard, H.B. (2015): Patents for Toll-like receptor ligands as radiation countermeasures for acute radiation syndrome. Expert Opin. Ther. Pat., **25**, 1085-1092.

Snoy, P.J. (2010): Establishing efficacy of human products using animals: the US food and drug administration's "animal rule". Vet. Pathol., **47**, 774-778.

So, E.Y. and Ouchi, T. (2010): The application of Toll like receptors for cancer therapy. Int. J. Biol. Sci., **6**, 675-681.

Song, W.S. and Yoon, S.I. (2016): Functional role of flagellin in bacterial flagellar assembly and immune receptor activation: structure and application. Biodesign, **4**, 98-107.

Stockhammer, O.W., Zakrzewska, A., Hegedûs, Z., Spaink, H.P. and Meijer, A.H. (2009): Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to Salmonella infection. J. Immunol., **182**, 5641-5653.

Sugimoto, K., Shibuya, K., Ihara, M., Saitoh, T., Itabashi, M. and Nunoya, T. (2000): Background data on organ weights and histopathological lesions in Crj:CD(SD)IGS rats for 4-, 13- and 26-week repeated-dose toxicity studies. CD(SD), **IGS-2000**, 79-87.

Wolford, S.T., Schroer, R.A., Gohs, F.X., Gallo, P.P., Brodeck, M., Falk, H.B. and Ruhren, F.R. (1986): Reference range data base for serum chemistry and hematology values in laboratory animals. J. Toxicol. Environ. Health, **18**, 161-188.