Supporting Online Material for

An Agonist of Toll-Like Receptor 5 Has Radioprotective Activity in Mouse and Primate Models

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This PDF file includes

- Materials and Methods
- Figs. S1 to S8
- Tables S1 and S2
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Materials and Methods

Flagellin and its derivative, CBLB502

Full-length recombinant flagellin was purified from Salmonella enterica serovar dublin as previously described (S1) or isolated as a recombinant protein produced in E. coli. In order to optimize flagellin as a radioprotectant, we generated a number of constructs utilizing the conserved N- and C-terminal domains of flagellins known to be required for TLR5 activation (S2), but eliminating the highly immunogenic hyper-variable region (see Fig. 1B). Various combinations of DNA fragments encoding three types of N-termini (A, B, C) and two types of C-termini (A’, B’) connected by a flexible linker derived from the pGEX-KG cloning vector (Invitrogen) were cloned into an expression construct to produce flagellin derivatives for functional testing. In addition, constructs encoding isolated N-termini (A, B, and C) and glutathione-S-transferase (GST)-fusions of C-termini (GST-A’, GST-B’) were prepared. All constructs were cloned in the pRSETb bacterial expression vector (Invitrogen) in frame with a 6xHis-tag N-terminal fragment for affinity purification. Construct AA’ encodes CBLB502 (Fig. S1).

Expression and purification of CBLB502 protein

CBLB502 was expressed as a fusion protein with an N-terminal His6-tag from the vector pRSETb in the E. coli BL21/DE3 expression strain using standard techniques. Briefly, the cells were grown in LB containing 100 µg/mL Ampicillin in 2L shaking flasks at 37°C to an OD600 of ~1.0. IPTG was added to a final concentration of 1 mM to induce protein expression and cells were incubated with shaking for an additional 4-5 hrs. Cells were harvested by centrifugation and frozen at -70°C. The frozen cell paste (from ~15L of culture) was suspended in 400 mL of 20 mM Hepes pH 7 containing 100 mM NaCl, 0.03% Brij35 and 0.1 mM PMSF. Lysozyme was added to 1 mg/mL and the cell suspension was subjected to a freeze-thaw cycle and sonication. Cell debris was removed by centrifugation and the supernatant, containing the soluble fraction of CBLB502 (~20% of total), was incubated at 90°C for 20 min leading to denaturation and precipitation of the bulk of E. coli proteins. CBLB502 (as well as full-length flagellin) is stable to thermal denaturation under these conditions. CBLB502 was further purified using Ni-NTA column chromatography in a gradient of Imidazole (0-250 mM) followed by size-exclusion chromatography in 1xPBS buffer on Superdex 200 (16/60) using AKTA-FPLC (GE). We found that ~ 80% of the expressed CBLB502 protein initially present in the cell pellet (likely due to the natural propensity of flagellin to form oligomers) can be readily solubilized in 2M Urea, 50 mM Tris-HCl pH 8. The solubilized protein was further purified using the same 2-step column chromatographic procedure. After initial solubilization, CBLB502 remained stable in solution in the absence of urea, even when concentrated up to 20 mg/mL (prior to loading on the size-exclusion column). The overall yield of purified CBLB502 (>90% pure by SDS-PAGE) was ~20 mg per liter of culture. Both protein samples, purified (i) from the soluble fraction with thermal treatment, and (ii) from the pellet with 2M urea treatment, eluted at the same position during size-exclusion chromatography as a symmetrical narrow peak corresponding to a possible dimer (with an apparent Mₚ of ~ 72 kDa). Trace amounts of endotoxin were removed from the purified protein using Detoxigel (Pierce) according to the manufacturer’s protocol. Purified CBLB502 (2 mg/mL in PBS) was frozen in liquid nitrogen and was stable for at least 1 year of storage in aliquots at -80°C. Similar protocol is being used for production, isolation and purification of a full-length flagellin cloned with His-tag-containing sequence on its N-terminus.
**Other reagents**

For comparison of CBLB502 to other radioprotectants (Fig. 1C), amifostine (Sigma, St. Louis, MO, Cat. # A5922) was used at a dose of 150 mg/kg, injected i.p. 30 min prior to irradiation (3) and 5-AED (Sigma, St. Louis, MO, Cat. # A7830) was used at a dose of 30 mg/kg, injected s.c. 24 hrs prior to irradiation (S4).

For comparison of CBLB502 to bacterial lipopolysacharide (LPS) (Fig. S5), NIH-Swiss mice were injected s.c. with CBLB502 (5 µg/mouse) or LPS (50 µg/mouse, LPS K-235 from Escherichia coli, Sigma) one hr prior to TBI with 13 Gy.

To test for involvement of COX2 signaling in radioprotection by CBLB502 or LPS, the COX2 inhibitor NS398 (Biomol, Cat. # EL-261) was injected (i.p., 25 µg/mouse) 15 min before injection of PBS, LPS or CBLB502 and subsequent TBI. (Fig. S5).

**Irradiation and radioprotection of mice**

NIH-Swiss and ICR mice were obtained from Harlan (Indianapolis, IN). Female mice, 10 weeks old (NIH-Swiss) or 12-15 weeks old (ICR) were used unless otherwise noted. TLR5-deficient MOLF/Ei (12-15 weeks old) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Total body gamma irradiation (TBI) was accomplished using 4000 Ci Cesium-137 source (J.L. Shepherd and Associates, San Fernando, CA with a dose rate of 2.33 Gy/min. Mice were irradiated on a rotating platform to ensure even dose delivery to all tissues. All injections of mice with flagellin or CBLB502 used a dose of 0.2 mg/kg unless otherwise noted. Flagellin and CBLB502 were injected subcutaneously (s.c.) unless otherwise noted. Intramuscular (i.m.) injection was found to be equally effective and was used in some experiments.

**Irradiation and radioprotection of non-human primates**

Rhesus macaques (Macaca mulatta), a commonly used non-human primate model for studies of potential human drugs, were housed and treated in a dedicated facility at the National Chengdu Center for Safety Evaluation of Drugs (NCCSED), Chengdu, China - a contract research laboratory specializing in drug safety evaluation. The monkeys were 2-4 years old, weighing 3.5-7.0 kg. PBS (n=8, 4 males, 4 females) or CBLB502 (0.04 mg/kg, n=11, 6 males, 5 females) was injected i.m. into the quadriceps muscle 45 minutes prior to 6.5 Gy (LD70/40) bilateral TBI from a 60Co source with a dose rate of 1.08 Gy/min (Fig. 3). The dose of CBLB502 used was found to result in blood levels of the compound equivalent to those in mice treated with the effective dose of 0.2mg/kg CBLB502. Behavior and survival of monkeys was observed for 40 days, with monitoring of multiple physiological parameters, blood counts, serum chemistry variables and cytokine levels in plasma. To prevent suffering, animals were euthanized if moribund or seriously injured (according to strict pre-defined IACUC-approved criteria established according to NIH guidelines). No supportive care other than rehydration and application of topical antibiotics to ulcerated skin lesions was given. All monkeys that were euthanized or found dead were subjected to gross pathological evaluation by a certified pathologist and to histopathological analysis of the GI tract and HP system (Fig. S6). For determination of platelet counts, anticoagulated peripheral blood was obtained from the femoral vein and subjected to complete blood count and differential leukocyte analysis (Fig. 3B). A detailed report of the monkey study will be published elsewhere.
Analysis of NF-\(\kappa\)B DNA binding activity

NF-\(\kappa\)B DNA binding activity (reflecting its activation and nuclear translocation) was assessed using an electrophoretic mobility shift assay (EMSA) with a labeled synthetic NF-\(\kappa\)B-binding oligonucleotide probe \((S1)\). Nuclear extracts from flagellin- and flagellin derivative-treated A549 lung cancer cells were incubated with the probe and DNA-protein complexes were resolved by SDS-PAGE and visualized by autoradiography (Fig. 1B).

TUNEL staining of apoptotic cells

Apoptosis in the small intestine of NIH-Swiss mice injected with CBLB502 (s.c., 0.2 mg/kg) or PBS 30 min before 15 Gy TBI was detected five hours after irradiation in paraffin-embedded specimens. Apoptotic cells were stained by the indirect terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method with a Fluorescein conjugated reporter (green, Millipore, Cat # S7110). In addition, the TUNEL-stained specimens were incubated with rat antibodies against CD31 (PECAM-1, a specific endothelial cell surface marker, PharMingen, Cat. #1951D) and developed with anti-rat-Cy3 antibodies (Rockland, Cat #612-104-120). Thus, apoptotic endothelial cells were detected by yellow fluorescence resulting from overlap of green TUNEL staining and red anti-CD31/Cy3 staining. Nuclei were visualized by DAPI staining (blue) (Fig. 2A and Fig. S3A).

Histological assessment of small intestine morphology

Small intestine specimens were collected from NIH-Swiss and MOLF/Ei mice five days after 15 Gy TBI with or without pre-treatment with CBLB502 (0.2 mg/kg) 1 hour before TBI. Mice that were not treated or irradiated (“untreated”) were used as controls. Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with Hematoxylin-Eosin (H&E) (Fig. 2B).

Assessment of the viability of crypts in the small intestine by BrdU labeling

Crypt stem cell survival was determined 3 days after irradiation by 5-bromo-2'-deoxyuridine (BrdU) incorporation into proliferating crypt cells, using a modification of the microcolony assay (S5). S phase cells were labeled in vivo by administering BrdU (i.p., 120 mg/kg) to each mouse 2 hours before euthanasia. Mice were euthanized 3 days after irradiation and the small intestine was rapidly dissected, fixed in 10% neutral buffered formalin, and embedded in paraffin. Paraffin sections (5 \(\mu\)m) were cut perpendicular to the long axis of the intestine. Cells incorporating BrdU were visualized by immunohistochemistry using rat monoclonal anti-BrdU antibodies (Abcam, Cambridge, MA; Cat. # ab6326, dil.1:100) and secondary FITC-conjugated donkey anti-rat IgG antibody (Jackson ImmunoResearch Laboratories, cat.No.712-165-153; dil.1:100). The BrdU-stained specimens were incubated with Cy3-conjugated mouse monoclonal antibodies against smooth muscle actin (Sigma, St. Louis, Mo, Cat. # C6198; dil.1:500). (Fig. 2C and Fig. S4).

Quantitation of granulocyte-macrophage colony-forming units (CFU-GM)

CFU-GM were assayed in semisolid methylcellulose culture (R&D Systems, MN, Cat. #HSC001-005). Mononuclear bone marrow cells (BMC) were obtained from NIH-Swiss mice (3 mice per group) 3 hours after 10 or 13 Gy TBI with or without CBLB502 pretreatment (0.2 mg/kg CBLB502 or PBS injected 30 min before TBI). BMC were also obtained from non-irradiated mice pretreated with PBS or CBLB502. BMC were flushed from femora and tibiae into Hanks’ balanced salt solution containing 2% FBS. BMC from the three mice in each group were pooled. Red blood cells were removed using RBC lysis buffer (eBioscience, San Diego,
BMC were suspended in Iscove’s modified Dulbecco’s medium (IMDM) containing 0.8% MethoCult M3231 for mouse bone marrow (StemCell Technologies, Canada, Cat. # 03231), 30% fetal calf serum, 1% bovine serum albumin, 10^-4 M β-mercaptoethanol, and 10 ng/ml recombinant mouse granulocyte monocyte-colony stimulating factor (mGM-CSF; StemCell Technologies, Canada, Cat.# 02735). One-milliliter aliquots of the BMC suspension were plated in triplicate in 35-mm tissue culture dishes and incubated for 7 days in a humidified incubator at 37°C with 5% CO2. Colonies were counted under a light microscope. (Fig. 2D)

Detection of superoxide dismutase 2 (SOD2) expression
NIH-Swiss mice were injected with CBLB502 (s.c., 0.2 mg/kg) or PBS 30 min before exposure to 15 Gy TBI. SOD2 expression was detected using immunofluorescence on sections of paraffin-embedded small intestine samples collected five hours after irradiation. SOD2 was visualized (green staining) by incubating tissue sections with goat anti-SOD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA Cat. # sc-18503; dil.1:100) followed by FITC-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, Cat. # 705-095-003; dil.1:100). Tissue sections were also incubated with a Cy3-conjugated mouse monoclonal antibody against smooth muscle actin (Sigma, St. Louis, MO, Cat. # C6198; dil. 1:500, red staining) (Fig. 2E).

Measurement of cytokine levels in mouse plasma
ICR mice were injected with 0.2 mg/kg CBLB502 in the absence of irradiation. K2EDTA plasma was collected before injection and 0.5, 1, 2, 4, 8 and 24 hrs after injection. Plasma was collected from 3 mice at each time point. The levels of G-CSF, IL-6, KC, TNFα and IP-10 were measured in mouse plasma using Linco Research Mouse Cytokine/Chemokine LINCOplex assays and Luminex-100 analyzer according to the manufacturer's directions. All cytokine measurements were performed at the University of Minnesota Hospital Cytokine Reference Laboratory (Fig. 2F).

Experimental radiotherapy of tumor-bearing mice
The effect of CBLB502 on the radiosensitivity of tumors was analyzed using two models: 1) NIH3T3-derived sarcoma (S6) grown s.c. in NIH-Swiss mice, and 2) B16 mouse melanoma grown in syngeneic C57BL6 mice. 1-3x10^6 in vitro-grown tumor cells were injected s.c. into each flank of mice of the appropriate strain (n=20 for sarcoma model, n=40 for melanoma model). When the tumors reached about 5 mm in diameter, the mice were randomly divided into four equal groups and treatment was initiated. The untreated group (U/t) received no injections or irradiation. The irradiated groups (IR and IR+CBLB502) received PBS or CBLB502 (0.2 mg/kg) s.c injections followed by 4 Gy TBI one hr later. The CBLB502 group was injected with the drug (0.2 mg/kg) without accompanying TBI. Treatment was applied three times daily on days 6, 7, and 8 following tumor cell injection for the sarcoma model and on days 8, 9, and 10 for the melanoma model. Tumor volumes were measured every second day using calipers (Fig. 4A and S7A) and survival of irradiated mice from control and CBLB502-treated groups was followed for 30 days (Fig. 4B and S7B).

Radiation-induced tumorigenesis in p53^+/− mice
Tumor development was followed for 49 weeks in two groups of age- and sex-matched p53^+/− mice (S7) (8-10 weeks of age, C57BL/6 background) that received a non-lethal tumor-inducing single dose of 4 Gy TBI (S8, S9) with (n=31, 15 males, 16 females) or without (n=32, 16 males, 16 females) injection of CBLB502 (i.m., 0.2 mg/kg) 30 minutes prior to irradiation. Mice were
observed daily; tumor palpation was performed biweekly, and body weight measurement was done weekly. Mice were euthanized according to IACUC criteria, e.g. signs of severe distress, 20% weight loss, appearance of a tumor >17 mm in size on the longest axis, or signs of tumor ulceration or spreading. A complete gross pathological examination and histopathological evaluation of affected organs was performed to confirm the cause of death. A single experiment was performed (Fig. 4C).

**NF-κB-dependent luciferase reporter assays**
Induction of NF-κB transcriptional activity was measured in HCT116 human colon cancer cells carrying the luciferase gene under the control of a synthetic NF-κB-responsive promoter consisting of three NF-κB binding sites from the E-selectin promoter combined with the Hsp70 minimal promoter (S10). Luciferase activity was measured in whole cell lysates six hours after addition of flagellin or its derivatives to the culture medium using a luciferase reporter assay system (Promega, Madison, WI, Cat. # E1501) (Fig. S1A).

**Detection of anti-flagellin antibodies in the serum of flagellin- or CBLB502-treated mice**
NIH-Swiss mice (2 per group) were injected s.c. with PBS, 5µg flagellin or 5 µg CBLB502. The presence of anti-flagellin antibodies in mouse serum was determined on day 28 post-immunization using an Enzyme-Linked ImmunoSorbent Assay (ELISA). 96-well plates were coated with flagellin (20 µg/ml in PBS, 50 µl/well) by incubation overnight at +4°C. Blood serum samples collected from immunized mice were diluted into 1% BSA diluting/blocking solution (KPL, Gaithersburg, Maryland) to 2.5%, 0.5%, 0.1% and 0.02% and added to wells of the ELISA plate in triplicate. Plates were incubated with serum overnight at +4°C. The next day, serum was removed and plates were washed and then incubated with secondary goat anti-mouse IgG horseradish peroxidase-conjugate antibodies for 6 hours. The intensity of the peroxidase reaction with one-component ABTS (2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate)) substrate (KPL, Gaithersburg, Maryland) was measured using a Multiscan Ascent spectrophotometer (Thermo Labsystems, Finland) with a 414 nm filter. (Fig. S2).

**Measurement of small intestine crypt size**
NIH-Swiss mice were injected with PBS or CBLB502 (s.c., 0.2 mg/kg) 30 min before 15 Gy TBI. Specimens of small intestine were collected 24 hrs after irradiation. Samples of small intestine from a non-irradiated mouse (U/t) served as a control. Sections of paraffin-embedded tissue were stained with H&E and analyzed under a microscope using Image Pro analysis software. Mean size (M±SE) of well oriented crypts was calculated by multiplying length times width for 5 crypts from the untreated mouse, 20 crypts from PBS-treated irradiated mice (n=3) and 15 crypts from CBLB502-treated irradiated mice (n=3) (Fig. S3B).

**Analysis of mouse bone marrow cell populations**
Bone marrow cells (BMC) were harvested on day 6 post-irradiation from femora, tibiae and humeri of euthanized NIH-Swiss mice treated with PBS or CBLB502 (0.2 mg/kg) 30 min before 13 Gy TBI. Pooled BMC (from 3 mice per group) were incubated in flow cytometry staining buffer (eBioscience, San-Diego, Ca) with biotin-conjugated mouse hematopoietic lineage panel antibodies specific for CD3, CD5, CD45R/B220, CD11b, Erythroid marker and Ly6G (eBioscience, San Diego, Ca). The labeled cells were incubated with Streptavidin-conjugated MagnaBind paramagnetic beads (10 µl/10^7 cells, Pierce, Rockford, IL), and lineage marker positive cells were removed from BMC populations by exposure to a magnetic field (Dynal.
MPC-15 magnetic Particle Concentrator, Dynal Biotech LLC, Lake Shearer, NY). The resulting lineage-depleted cells were incubated with blocking anti-CD16/32 antibodies and then stained with phycoerythrin (PE)-conjugated anti-Ly-6A/E (Sca1, clone D7) and Fluorescein isothiocyanate (FITC)-conjugated anti-CD119 (c-kit, clone 2B8) rat monoclonal antibodies (eBioscience, San Diego, Ca). 7-AAD (BD Biosciences, San Diego, CA) was added to stained BMC 10 minutes before FACS analysis, only viable cells were calculated. BMC that were stained with appropriate fluorochrome-conjugated isotype control monoclonal antibodies (eBioscience, San Diego, Ca) were used as background controls. Flow cytometry was performed using a single-laser FACSCalibur instrument and the data was analyzed on CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). (Table S1).
Supporting Figures

![Graph](image)

**Figure S1.** **A.** Induction of NF-κB-responsive transcription by full-length flagellin and derivatives A’ and AA’ (CBLB502). HCT116 cells carrying an NF-κB-dependent luciferase reporter construct were incubated with the indicated concentrations of flagellin (FL) or its derivatives (A’ or AA’, see Figure 1B; AA’ is CBLB502). Luciferase activity was measured in whole cell lysates (in triplicate) six hours later. The results of a representative experiment are shown. Error bars indicate standard deviations (S.D.). **B.** The amino acid sequence of CBLB502 (encoded by construct AA’). Sequence derived from the FliC flagellin protein of Salmonella enterica serovar dublin (GenBank accession# AAA27081) is shown in bold blue type (amino acids 1-176 and 402-505 of the full-length protein). The 33 amino acids N-terminal to the first residue from FliC are from the pRSET B vector (Invitrogen, cat# V351-20) and include a His6-tag (green underlined type) and an enterokinase cleavage site (red type). The sequence between FliC residues 176 and 402 is from pGEX-KG and encodes a flexible linker domain.
Figure S2. Comparison of the antigenic properties of flagellin and CBLB502. NIH-Swiss mice were injected with full-length flagellin (Fl), CBLB502, or PBS (2 animals per group, designated in the Figure as (1) and (2)). Antibody titers were determined using a flagellin Enzyme-Linked ImmunoSorbent Assay (ELISA) producing quantitative spectrophotometric readings at 414 nm. Serum samples were diluted as indicated on the x-axis for use in the ELISA and tested in triplicate. Error bars indicate S.D.
Supporting Figures (contd)

**Figure S3.** CBLB502 treatment prevents radiation-induced cell death in small intestine crypts. NIH-Swiss mice were exposed to 15 Gy TBI 30 min after injection of PBS (ionizing radiation, IR) or CBLB502 (CBLB502+IR). Untreated non-irradiated mice served as controls (U/t). **A.** TUNEL staining of apoptotic cells was performed on sections of small intestine collected 5 hrs after TBI. The number of apoptotic cells in the lamina propria (predominantly endothelial cells as judged by simultaneous CD31 antibody staining) per villus was quantitated. The mean value for 30 villi (10 villi per mouse, 3 mice per group) is shown; error bars indicate S.D. **B.** Crypt size (length X width) was determined in Hematoxylin-Eosin (H&E) stained sections of small intestine collected 24 hrs post-irradiation. The mean crypt size (±SD) is shown for 15 crypts from untreated mice (n=3), 20 crypts from irradiated mice injected with PBS (n=3) and 15 crypts from irradiated mice injected with CBLB502 (n=3). Here and above (A.): *, P<0.05 for the difference between the IR and CBLB502+IR groups, by two-tailed t-test.
Supporting Figures (contd)

**Figure S4.** CBLB502 protects against radiation-induced loss of crypt stem cells. Proliferation of stem cells in the crypts of the small intestine was assessed by immunohistochemical detection of in vivo 5-bromo-2'-deoxyuridine (BrdU) incorporation. Small intestine samples were collected three days post-irradiation from NIH Swiss mice treated with PBS (control) or 0.2 mg/kg CBLB502 30 min before 13 Gy TBI. Representative photomicrographs taken at 20X magnification show BrdU-stained cells (green fluorescence, indicated by arrows) and actin filaments (red fluorescence).
**Supporting Figures (contd)**

**Figure S5.** Protection of mice from lethal irradiation by CBLB502 (left panel) as compared to LPS (right panel). NIH-Swiss mice were injected s.c. with CBLB502 (5 µg/mouse, n=10) or LPS (50 µg/mouse, n=10) one hr prior to receiving 13 Gy TBI. Four other groups of mice were injected i.p. with the COX-2 inhibitor NS-398 (25 µg/mouse) 15 min before injection of PBS (2 groups of 5 mice), LPS (50 µg/mouse, n=5), or CBLB502 (5 µg/mouse, n=5) and irradiated (13 Gy TBI) 30 min later. The same control group “PBS+NS398” is shown on both graphs. P<0.05 by two-tailed Fisher’s test for any comparison of high-survival groups (CBLB502, CBLB502+NS398, or LPS) with zero-survival groups (LPS+NS398, PBS+NS398).
**Supporting Figures (contd)**

**Figure S6.** Highlights of gross pathology/histopathology evaluation of monkeys surviving to the end of the study (40 days after 6.5 Gy TBI). The incidence and severity of thymus, spleen and bone marrow damage was reduced in surviving CBLB502-treated monkeys (n=7) as compared to surviving controls (n=2). Two-tailed Fisher’s exact test was used to calculate P-values for the incidence of “moderate or strong” vs. “minor or none” abnormalities for comparison of the control group against the CBLB502-treated group.

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| P value  | 0.03 | 0.03 | 0.25 | 0.44 | 0.08 | 0.03 |

**Figure S6.** Highlights of gross pathology/histopathology evaluation of monkeys surviving to the end of the study (40 days after 6.5 Gy TBI). The incidence and severity of thymus, spleen and bone marrow damage was reduced in surviving CBLB502-treated monkeys (n=7) as compared to surviving controls (n=2). Two-tailed Fisher’s exact test was used to calculate P-values for the incidence of “moderate or strong” vs. “minor or none” abnormalities for comparison of the control group against the CBLB502-treated group.
Supporting Figures (contd)

Figure S7. CBLB502 does not reduce the radiosensitivity of mouse B16 melanoma. A. C57BL/6 mice carrying B16 melanoma tumors (10 mice per group, two tumors per mouse) were treated with PBS alone (U/t, untreated control), radiation alone (IR, 4 Gy TBI per fraction), CBLB502 alone (0.2 mg/kg i.m. per injection) or radiation together with CBLB502 (IR+CBLB502). All treatments were applied on days 8, 9 and 10 after tumor cell injection (red arrows). Tumor volume was measured every two days (graph shows medians of tumor volumes normalized by the initial size with error bars indicating S.D.) Differences in tumor growth rate among groups were analyzed by two-way (time and treatment) repeated measures ANOVA and by Student’s t-test (two-tailed, unequal variances). B. Thirty-day survival curves for the IR and IR+CBLB502 groups of mice described in A. *, P<0.05, by two-tailed Fisher’s test.
**Figure S8.** Effect of repeated administration of CBLB502 on its radioprotective efficacy. ICR mice (n=20 in each group) were subjected to five different schedules of pre-irradiation CBLB502 administration (i.m., 0.2 mg/kg) involving 1, 4, 6, 8 and 11 daily injections according to the schedule indicated by the short arrows. All groups received 11 Gy TBI 30 min after the last CBLB502 injection. The bar graph shows the proportion of surviving animals in each group on day 30 post-irradiation (u/t, untreated control mice).
Table S1. CBLB502 treatment alleviates radiation-induced loss of HP stem cells and early progenitors. FACS analysis of hematopoietic stem cells and early progenitors in bone marrow from control (PBS-treated) and CBLB502-treated NIH-Swiss mice six days after 13 Gy TBI. PBS or CBLB502 was injected 1 hr before TBI. Lineage-minus cells were isolated from pooled bone marrow cells (BMC) from three mice for each treatment. Cells were stained for FACS analysis (see Materials and Methods). The total number of cells of each type is indicated and the corresponding percentage of total Lineage-minus cells is given in parentheses. c-kit, receptor for stem cell factor; Sca1, stem cell antigen, Ly-6 Ag family. c-kit⁺ Sca1⁺Lin⁻ = hematopoietic stem cells; c-kit⁺ Sca1⁻Lin⁻ = myeloid progenitors; c-kit⁻ Sca1⁺Lin⁻ = heterogeneous, some lymphoid progenitors.

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<td>PBS</td>
<td>$8.27 \times 10^7$</td>
<td>8167</td>
<td>0 (0.00)</td>
<td>58 (0.67)</td>
<td>815 (9.98)</td>
</tr>
<tr>
<td>CBLB502</td>
<td>$8.05 \times 10^7$</td>
<td>13680</td>
<td>27 (0.2)</td>
<td>662 (4.84)</td>
<td>1980(18.82)</td>
</tr>
</tbody>
</table>
Supporting Tables (contnd)

Table S2. Long-term pathologies appearing in mice rescued from lethal irradiation by CBLB502. Histopathological analysis of female NIH-Swiss mice was conducted 6 months after the mice were treated with 0.2 mg/kg CBLB502 one hour prior to 13 Gy TBI (n=4). Since there were no survivors in the group of mice that were irradiated without CBLB502, the CBLB502-treated irradiated mice were compared only to age-matched untreated and non-irradiated female NIH-Swiss control mice (n=2).

<table>
<thead>
<tr>
<th>Age- and strain-related pathologies seen to a comparable extent in unirradiated control and CBLB502-rescued mice</th>
<th>Pathologies that were more severe in CBLB502-rescued versus unirradiated control mice</th>
<th>Pathologies that were seen only in CBLB502-rescued mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Kidney – interstitial nephritis, lymphocytic</td>
<td>• Lung – perivasculitis/peribronchialitis, lymphocytic infiltration</td>
<td>• Uterus – endometrial atrophy</td>
</tr>
<tr>
<td>• Salivary gland – sialoadenitis, lymphocytic</td>
<td>• Ovary - atrophy</td>
<td>• Cervix, vagina – epithelial atrophy</td>
</tr>
<tr>
<td>• Pancreas – pancreatitis, lymphocytic</td>
<td>• Spleen – extramedullar hematopoiesis</td>
<td>• Sternum, femur – marrow hypoplasia; granulocytic hyperplasia</td>
</tr>
<tr>
<td>• Harderian gland – adenitis, lymphocytic</td>
<td>• Mammary gland – duct ectasia</td>
<td>• Eye - cataracts</td>
</tr>
<tr>
<td>• Urinary bladder – cystitis, lymphocytic</td>
<td>• Liver - cholangiohepatitis</td>
<td>• Intestine – enteritis; proctitis</td>
</tr>
<tr>
<td>• Liver - cholangiohepatitis</td>
<td>• Adrenal – lipofuscinosis</td>
<td></td>
</tr>
</tbody>
</table>
Supporting references