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The LPA₂ receptor agonist Radioprotectin-1 spares Lgr5-positive intestinal stem cells from radiation injury in murine enteroids

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Abstract

Rapidly proliferating cells are highly sensitive to ionizing radiation and can undergo apoptosis if the oxidative and genotoxic injury exceed the defensive and regenerative capacity of the cell. Our earlier work has established the antiapoptotic action of the growth factor-like lipid mediator lysophosphatidic acid (LPA). Activation of the LPA₂ GPCR has been hypothesized to elicit antiapoptotic and regenerative actions of LPA. Based on this hypothesis we developed a novel nonlipid agonist of LPA₂, which we designated Radioprotectin-1 (RP-1). We tested RP-1 at the six murine LPA GPCR subtypes using the transforming growth factor alpha shedding assay and found that it had a 25 nM EC₅₀ that is similar to that of LPA18:1 at 32 nM. RP-1 effectively reduced apoptosis induced by γ -irradiation and the radiomimetic drug Adriamycin only in cells that expressed LPA₂ either endogenously or after transfection. RP-1 reduced γ -H2AX levels in irradiated mouse embryonic fibroblasts transduced with the human LPA₂ GPCR but was ineffective in vector transduced MEF control cells and significantly increased clonogenic survival after γ -irradiation. γ -Irradiation induced the expression of *lpar2* transcripts that was further enhanced by RP-1 exposure within 30 min after irradiation. RP-1 decreased the mortality of C57BL/6 mice in models of the hematopoietic and gastrointestinal acute radiation syndromes. Using Lgr5-EGFP-CreER;Tdtomato^{flox} transgenic mice, we found that RP-1 increased the survival and growth of intestinal enteroids via the enhanced survival of Lgr5⁺ intestinal stem cells. Taken together, our results suggest that the LPA₂-specific agonist RP-1 exerts its radioprotective and radiomitigative action through specific activation of the upregulated LPA₂ GPCR in Lgr5⁺ stem cells.

Graphical abstract

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Keywords

enteroid; intestinal stem cells; Lgr5; LPA; octadecyl thiophosphate; radiation; radioprotection; radiomitigation

1. Introduction:

Lysophosphatidic acid (LPA) is a growth factor-like lipid mediator generated from lysophosphatidylcholine (LPC) by the lysophospholipase autotaxin [1–3]. The effects of LPA are mediated through six G protein-coupled receptors (GPCR, LPA₁₋₆) and include stimulation of wound healing, cell proliferation, cell survival, cell motility, and DNA repair [2, 4–9]. LPA GPCR are coupled to multiple signaling pathways including the extracellular signal—regulated kinases (ERK 1,2), protein kinase (PKC), and Protein kinase B (PKB a.k.a. AKT)[9–14]. In addition to G protein-coupled signaling pathways, LPA₂ mediates its antiapoptotic and prosurvival effect via the recruitment of supra-macromolecular signaling complex containing SIVA-1, TRP-6, and NHERF2 proteins to unique Zn-finger and PDZ-binding motifs in its C-terminus [15, 16].

The intestinal epithelium is not only essential for digestion and absorption of nutrients but it also must maintain the integrity of the intestinal mucosal barrier to prevent the loss of body fluids and protect against microbial invasion. Because of the harsh digestive milieu of the intestinal lumen, intestinal epithelial cells undergo rapid and constant renewal, relying on the crypt base columnar intestinal stem cells (ISC) located at the bottom of the crypts. A subpopulation of murine ISC expresses the Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) as described by Barker et al., [17]. As new cells are generated from the crypt, they differentiate into secretory cells enteroendocrine or Goblet cells, and absorptive enterocytes as they migrate apically until they reach the tip of the villi where in mice they undergo spontaneous apoptosis and are shed into the lumen every 3–4 days [18–20]. Even though the ISC have extensive DNA repair ability compared with other cell types

within the epithelium they remain highly sensitive to ionizing radiation [21]. Consequently, if ISC are lost due to radiation-induced cell death, an alternative stem cell population referred to as the “reserve” stem cell population expressing the polycomb complex protein Bmi1 [22]. Hopx [23] and Lrig1 [24] markers can promote the regeneration of the ISC, including the Lgr5 subpopulation, and thus maintain a functional epithelium and intestinal barrier (for a review see [25]).

Cancer treatment typically involves chemotherapy and radiation. Radiation is an effective method to kill cancer cells because it induces DNA damage which if unrepaired leads to programmed-cell death (apoptosis) or mitotic catastrophe. Although apoptosis is the objective in cancer treatment, extensive damage of the normal ISC leads to intestinal barrier deterioration, due to epithelial damage and breakdown of the lamina propria, further leading to exposure to luminal pathogens, sepsis and even death [26–28]. Therefore, it is imperative to combat the deleterious effects of radiation on the gastrointestinal (GI) tract by protecting the ISC during radiotherapy. Equally challenging are the consequences of radiation injury in the context of nuclear accidents like in Fukushima or Chernobyl, and the potential risk of radiation exposure due to belligerent explosion of a nuclear device. In these latter two cases, radiation-induced loss of ISC is the primary cause of the ensuing gastrointestinal acute radiation syndrome (GI-ARS) with fatal outcome.

The activation of LPA GPCR with the metabolically stabilized LPA analog octadecyl thiophosphate (OTP), a LPA receptor subtype non-selective agonist, showed reduction in radiation-induced lethality [29, 30]. Our earlier studies established that LPA and OTP via the LPA₂ GPCR reduced radiation-induced apoptosis in the stem cell region of the intestinal crypt [29]. Recently, using an *in silico* drug discovery approach, we have identified 2-((3-(1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)propyl)thio)-benzoic acid (GRI 977143, [31]) that is a specific agonist of LPA₂ with the undesired side effect of inhibiting LPA₃. Nevertheless, GRI 977143 was effective in reducing GI-ARS mortality in mice as a radiation mitigator when administered 24 h after irradiation [31]. Further medicinal chemistry explorations have led to the synthesis of 5-chloro-2-(*N*-(4-(1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)butyl)sulfamoyl)benzoic acid, designated as Radioprotectin-1 (RP-1), which is a high-potency (EC₅₀ value of 5 pM) specific agonist of the human LPA₂ with no detectable effect on the other LPA_{1/3/4/5} GPCR or the type 1 sphingosine-1-phosphate GPCR [32]. 2-[4-(1,3-Dioxo-1*H*,3*H*-benzoisoquinolin-2-yl)butylsulfamoyl]benzoic acid, a less potent derivative of this sulfamoyl benzoic acid (SBA) family of compounds showed protection against the GI-ARS when applied up to 72 h after acute radiation exposure [3].

Here we extended the pharmacological characterization of RP-1 to include all six murine LPA GPCR subtypes using the transforming growth factor alpha (TGFα)-shedding assay [33] and found that it had a 25 nM EC₅₀. We found that RP-1 effectively reduced apoptosis induced by γ-irradiation and the radiomimetic drug Adriamycin only in cells that expressed LPA₂ either endogenously or after transfection of this receptor subtype. RP-1 reduced γ-H2AX levels in irradiated mouse embryonic fibroblasts (MEF) transduced with the human LPA₂ GPCR but was ineffective in vector-DKOMEF control cells. RP-1 significantly increased clonogenic survival of IEC-6 intestinal epithelial cells after γ-irradiation.

Although RP-1 increased the expression of LPA_{1/2/3} GPCR in unirradiated IEC-6 intestinal epithelial cells, we also found that γ -irradiation only increased *lpar2* transcript abundance that was further enhanced by RP-1 exposure 30 min after radiation exposure. RP-1 decreased mortality of C57BL/6 mice in models of the hematopoietic ARS (HE-ARS) and the GI-ARS. Subsequently, we sought direct evidence for whether RP-1 protects the Lgr5⁺ ISC subpopulation when administered to small intestinal enteroid cultures derived from Lgr5-EGFP-IRES-CreERT2^{+/+}; Rosa26-flox-STOP-flox-Tdtomato transgenic mice (hereafter referred to as Lgr5-EGFP-CreER;Tdtomato^{flox})[34]. RP-1 showed radioprotective properties when administered to intestinal enteroids 1 day prior to radiation, and a positive effect on Lgr5⁺ cells survival. Taken together, our results suggest that the LPA₂-specific agonist RP-1 exerts its radioprotective and radiomitigative action through specific activation of the upregulated LPA₂ GPCR in Lgr5⁺ISC.

2. Materials and Methods:

2.1. Receptor pharmacology determination using the TGF α -shedding assay.

LPA receptor activation was measured using the TGF α -shedding assay as described previously Inoue et al[33]. Briefly, 4×10^5 HEK293 cells were seeded in each well of a 6-well plate and incubated for 24 h. Using polyethylenimine, cells were transiently transfected with the individual LPA receptor plasmid (200ng), alkaline-phosphate conjugated -TGF α (AP-TGF α , 500ng) and the respective G α chimeric proteins (100ng) per well in 2mL of Opti-MEM medium. Transfected cells were detached 24 h later and plated at 90 μ L per well in 96-well plates in HBSS medium containing 5mM HEPES (pH7.4). Cells were incubated at 37°C with 5% CO₂ for 30 min, followed by addition of 10 μ L of 10 $\times$ stock concentration of 18:1 LPA or RP-1 per well as indicated. After a 1-h incubation with the agonist plates were centrifuged at $190 \times g$ for 2 min and 80 μ L per well of conditioned medium were transferred into a new 96-well plate. Subsequently, 80 μ L of 10 mM para-nitro phenylphosphate (p-NPP) substrate were added per well into both the plates containing either cells or conditioned medium, respectively. Absorbance at 405nm was read using a VersaMax microplate reader (Molecular devices) at the 0-time point (immediately after p-NPP addition) and again after 1h incubation at room temperature. LPA receptor activation is measured as a % of AP-TGF α released by ligand stimulation subtracted from vehicle treated condition. Further details into the precise calculations of % of AP-TGF α release have been described in our previous report. [33]

2.2. Induction of apoptosis, apoptosis- and phospho-H2AX (γ -H2AX)-assays.

Mouse embryonic fibroblast (MEF) cells were isolated from embryonic day 13.5 LPA_{1/2} double knockout (LPA₂ DKO) embryos. These MEF cells express LPA_{4/5/6} receptors endogenously at very low levels but completely lack the LPA_{1/2/3} receptor subtypes. The human LPA₂ receptor was transduced into these MEF cells using lentiviruses, selected with 1.5 μ g/ml puromycin, and will be designated here in this study LPA₂-DKOMEF. Empty vector-transduced MEF cells in this study will be designated vector DKOMEF. The non-transformed rat intestinal epithelial cell line IEC-6 was purchased from ATCC (Bethesda, MD) and was cultured as previously described [3].

The irradiation induced-apoptosis experiments using the MEF cells were performed on 48-well plates. MEF cells were plated at 2×10^4 cells/well density and cultured overnight in a complete growth medium. Next day before the irradiation the cells were serum starved in the growth medium supplemented with 0.1% (w/v) BSA in place of serum for one hour before irradiation. One hour before irradiation, the medium was replaced with fresh serum-free starvation medium, and the cell cultures were exposed to a dose of 15 Gy γ -irradiation (dose rate 4.3 Gy/min, from a J.L. Shepherd & Assoc. Mark I, Model 25 irradiator (San Fernando, CA). After the irradiation the cells were immediately placed back into the incubator (37 °C, 5% CO₂). One hour postirradiation the cells were treated with either vehicle or RP-1 and radiation-induced apoptosis was measured using the Caspase-Glow^R 3/7 activation assay from Promega (Madison, WI) 6 h after the irradiation. Apoptosis was also induced by 1.7 μ M Adriamycin and was also measured by the Caspase-Glow^R 3/7 assay 6 or 24 h after drug challenge in MEF cells and IEC-6 cells, respectively. Luminescence was measured using a BioTek (Winooski, VT) plate reader using quadruplicate wells for each treatment.

Immunofluorescence staining for the phosphorylated histone in LPA₂-DKOMEF and vector MEF cells was done as described previously by Balogh et al.[9]. Briefly, LPA₂ and LPA₂-DKO MEFs cells were plated in 6 well plates (0.15×10^6 cells/well in 1.5 ml DMEM supplemented with 2mM L-Glutamine and 10% FBS). Next day, the cells were serum starved for 2.5 hours and irradiated with 15 Gy. Prior to irradiation cells were pretreated for 15 minutes with either 10 μ M RP-1, 10 μ M LPA, or vehicle (0.1 % DMSO or 0.1% BSA). After irradiation with 15 Gy at a dose rate of ~4.3 Gy/min, the medium was replaced with fresh serum-free medium containing the compounds. Four hours postirradiation the cells were trypsinized and stained with anti-human/mouse phospho-H2AX (residue S139, γ -H2AX) labeled with eFluor660 (eBioscience, San Diego, CA) using the Fopx3/Transcription Factor Staining Buffer Set and protocol from Biolegend Inc. (San Diego, CA). Fluorescence was measured using a LSR II instrument (Becton Dickinson Inc., Franklin Lakes, NJ) and analyzed with the FACSDiva software from Becton Dickinson Inc.

2.3. Clonogenic survival assay in γ -irradiated IEC-6 cells.

The rat intestinal epithelial cell line IEC-6 was used for assessment of the antiapoptotic effect of RP-1 following exposure to 10 Gy γ -irradiation. IEC-6 cells were plated at a density 250 cells/well to 6—well plates in triplicates using complete growth medium (DMEM with 10% fetal bovine serum supplemented with 0.1 U/ml bovine insulin). Next day, the cells were washed twice with HBSS without phenol red with 2 mM Ca and Mg and the growth medium was replaced with starvation medium (DMEM supplemented with 10 μ g/ml of insulin) for 18 – 24 h. On day 3, the serum free medium was replaced with fresh serum-free medium and the cultures were irradiated from a ¹³⁷Cs γ -radiation source with 2-10 Gy doses at a dose rate of ~4.3 Gy/min. Two hours after irradiation the medium was replaced with fresh serum-free medium containing 10 μ M RP-1 or vehicle (3 μ M charcoal-stripped bovine serum albumin, 0.1% DMSO in DMEM) and the cells were cultured for 3 more days with daily replenishment of the appropriate treatment medium. Colonies were stained with 0.5% crystal violet after fixation with ice-cold methanol for 10 minutes. After 15 min, the crystal violet solution was removed and the plates were rinsed several times with

distilled water and allowed to dry. Colony survival was determined using the following formula: Surviving fraction (SF) = (colonies counted/cell seeded $\times$ plating efficiency)/100.

2.4. Quantitative RT-PCR for LPA₂ transcripts.

Five hundred crypts were plated in a 24-well plate in IntestiCult (STEMCELL Technology) media and cultured for 6 days prior to treatment with 1 μ M RP-1 for one day and then returned to regular medium. On the same day, cultures received 5Gy dose of γ -irradiation from a ^{137}Cs source at a dose rate of 0.126 Gy/min. RNA from the enteroids was isolated 30 min after irradiation using RNeasy Micro extraction kit (Qiagen). QPCR analysis was performed on the Quantstudio 6 Flex real-time PCR system (ThermoFisher) using Taqman gene expression assays with *gapdh* as the house keeping gene. Reactions were run in triplicate, and the C_T cutoff value was 40. The difference in C_T between the assayed gene (X) and *gapdh* for any given sample was defined as $C_{T(X)}$. The difference in $C_{T(X)}$ between two samples was defined as $C_{T(X)}$, which represents a relative difference in expression of the *lpar2* gene. The fold change *lpar2* gene relative to *gapdh* was defined as 2^{-C_T} described by Livak and Schmittgen [35].

2.5. HE-ARS & GI-ARS murine animal models.

Analog of LPA have shown radiomitigative efficacy in animal models of HE- and GI-ARS. [29] Experimental procedures were reviewed and approved by the IACUC of the University of Tennessee Health Science Center. Eight to ten-week-old C57BL/6 female mice (Jackson Laboratories, Bar Harbor, ME) were acclimated for at least one week prior to irradiation using a Cs^{137} γ -source. Mice were housed in Allentown Biocontainment cages (Allentown Inc., Allentown, NJ) in the Regional Biocontainment Laboratory of the University of Tennessee Health Science Center, Memphis.

The mice received minimal supportive care by being provided gel-food packs (Diet Gel 76A, Clear H₂O, Inc., Westbrook, ME) on post-irradiation day 4 and were fed sterilized chow (Harlan Teklad LM-485 mouse/rat diet) throughout the study, ad libitum with autoclaved tap water. Animals were group housed with a maximum of 4 animals per cage using autoclaved bedding, enrichment blocks, and nestlets.

We applied a total body irradiation (TBI) to model the HE-ARS described by Patil et al.[3] In this TBI model, the 8–10 week old C57BL/6 mice were exposed to 9.0 Gy γ -irradiation ($\sim$ LD_{80/30}) from the Cs^{137} γ -source at a dose-rate of $\sim$ 0.82 Gy/min in a rotating chamber. This radiation dose yielded an $\sim$ LD_{80/30} mortality in this model with the minimal supportive care applied.

The partial body irradiation (PBI) model of the GI-ARS with 15% bone marrow sparing we applied in these experiment has been described earlier[3]. In our PBI model, mice were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg, IP) and placed in a plexiglass restrainer such that the legs below the knee were shielded. The shielding protected both tibiae, fibulae, and the paws from radiation that contain $\sim$ 15 % of the bone marrow. Mice placed into the isodose field of the irradiator were irradiated in groups of four with a 16.0 Gy dose. The mice were housed and maintained under identical conditions as described above for the TBI model with minimal supportive care. Dosimetry was done using TLD

dosimeter in every irradiation session. Twenty-six $\pm$ two hours after the irradiation the animals were treated with either vehicle, 0.1 or 0.3 mg/kg RP-1 via subcutaneous injection every 12 h for 3 days. Animal survival was recorded twice daily up to 30 days, which was the endpoint of these experiments. Statistical testing of the survival data was done by Fisher's exact test.

2.6. Transgenic mouse models.

All animal work conducted during the course of this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tennessee Health Science Center (UTHSC) and the VA Medical Center Memphis, and was performed according to National Institutes of Health guidelines. Lgr5-EGFP-IRES-CreERT2^{+/+} (stock #008875) and Rosa26-flox-STOP-flox-Tdtomato (stock #007908) mouse strains were purchased from the Jackson Laboratory. The offspring from the crosses of the transgenic mice were genotyped as previously described by Sato et al. [34].

2.7. Small intestinal crypt epithelial enteroid cultures.

Small intestinal crypt cells were isolated and cultured according to Mahe et al., [36]. Briefly, small intestines were isolated from 4-6-week-old animals, flushed with phosphate buffered saline (PBS) and dissected into smaller pieces. EDTA (2.5 mM, Sigma) in PBS was then used to dissociate the intestinal crypts, and the suspension was filtered through a 70 μ m filter. The pelleted crypts were resuspended in IntestiCult medium (STEMCELL Technologies) and counted. Subsequently, five hundred crypts were taken up in 150 μ L IntestiCult and mixed with 150 μ L Matrigel (Corning). For each pre-warmed 24-well plate (Corning), 50 μ L of the mixture was pipetted in a single well. The plates were placed in a 37°C incubator with 5 % CO₂ atmosphere for 30 minutes prior to addition of 750 μ L of IntestiCult per well. The enteroids were cultured for 6 days with fresh medium replacement every 2-3 days. For pre-treatment with LPA (1 μ M) or RP-1 (1 μ M), fresh drug solutions were prepared in IntestiCult in the morning of culture day 6 and added to the treatment wells for 24 h. Non-treated wells received fresh IntestiCult medium with vehicle. The next day, the medium was replaced with fresh medium prior to irradiation using 5Gy γ -irradiation from a ¹³⁷Cs source at a dose rate of 0.126 Gy/min. After radiation exposure, the cultures were maintained for the 3–6 days depending on the experimental design. For lineage tracing, 4-hydroxytamoxifen (4-OHT, 1 μ M; Sigma-Aldrich) in IntestiCult was added to the enteroid cultures for 24 hours on post-irradiation day 2 or 3.

2.8. Immunofluorescence staining of cultured enteroids.

Cultures were fixed in 4% paraformaldehyde for 30 min and rinsed with PBS. Cells were permeabilized using a 0.1% Triton X100 solution in PBS 30 min at room temperature and blocked with immersion in 5% (W/V) bovine serum albumin in PBS for 60 min at room temperature. Anti-GFP (chicken, 1:1000, ab13970, Abcam, Cambridge, MA), and goat-anti-chicken Alexa-Fluor 488 (Invitrogen, Carlsbad, CA) were diluted in PBS and incubated at 4°C overnight. Hoechst dye (1:1000 dilution in PBS, Invitrogen) was used to label the cell nuclei for 30min at room temperature. Tdtomato⁺ cells were counted from the bottom of the crypt up to 50 μ m in the direction of the villus tip. At least 9 crypts were counted per biological replicate.

2.9. Statistical analysis.

Statistical analysis was performed using GraphPad Prism 5.0 (Graphpad, San Diego, CA). ANOVA with Bonferroni correction was used to determine statistical significance. Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Radioprotectin-1 is a high-potency specific agonist of the murine LPA₂ GPCR

Our earlier in silico drug discovery work has identified a non-lipid compound GRI-977143 that showed agonist selectivity for LPA₂ but had low potency with an EC₅₀ of 3.3 μ M and was also an antagonist of LPA₃. Subsequent medicinal chemistry structure-activity (SAR) studies led us to the SBA family of compounds that lacked antagonist activity at LPA_{1/2/3/4/5} and activated on the LPA₂ GPCR subtype. [32] RP-1 was the most potent agonist compound of this series with an EC₅₀ 5 pM and functioned as a full agonist at the human ortholog of LPA₂ in Ca²⁺ mobilization assays. Here we utilized the TGF α -shedding assay platform that allows the testing of ligands at all LPA GPCR including LPA₆ that we have not previously tested the SBA compounds at. Using the mouse orthologs of the six LPA GPCR with cotransfection of the appropriate heterotrimeric G protein α -subunits, we have determined the agonist potency of RP-1 in this assay platform (Figure 1). These results confirmed our previous findings at the human orthologs using Ca²⁺ mobilization assays, showing that RP-1 was a specific agonist of the murine LPA₂ subtype with an EC₅₀ value of 25 nM. This is comparable to the 32 nM EC₅₀ value of LPA_{18:1} in this same assay. This EC₅₀ value of RP-1 at the murine LPA₂ ortholog is ~5000-fold higher than that at the human ortholog determined using both the Ca²⁺ mobilization and TGF α -shedding assays. Thus, RP-1 is a specific and approximately equipotent agonist of the murine LPA₂ GPCR as LPA_{18:1}.

RP-1 protects cells from radiation- and radiomimetic chemotherapeutic-induced apoptosis—LPA and its analogs protect cells that express the LPA₂ GPCR from apoptosis induced by exposure to γ -irradiation- and radiomimetic chemotherapeutics [13, 15, 16, 29–31]. We examined whether RP-1 protects MEF cells transduced with the human LPA₂ and the rat IEC-6 intestinal crypt-derived non-transformed cell line from radiation- and Adriamycin-induced apoptosis. Post-irradiation application of RP-1 dose-dependently reduced the activation of the main executioner caspases 3/7 in LPA₂-DKOMEF exposed to 15 Gy γ -irradiation but was completely ineffective in vector-DKOMEF derived from LPA_{1/2} double KO mice (Figure 2A). RP-1 applied at 1.0 or 3.0 μ M concentration 30 min after exposure to 1.7 μ M concentration of the radiomimetic cytostatic Adriamycin decreased caspase 3/7 activity by ~ 50% ($p < 0.01$) in the IEC-6 cell line (Figure 2B). When applied to LPA₂-DKOMEF cells treated with Adriamycin, RP-1 (post-Adriamycin) dose-dependently reduced apoptosis measured by caspase 3/7 activity and was completely ineffective in vector-DKOMEF (Figure 2C).

We showed earlier that LPA₂ KO mice showed delayed resolution of DNA damage monitored by staining for γ -H2AX [9]. LPA₂-DKOMEF and vector-DKOMEF cells were exposed to 15 Gy γ -irradiation and stained for γ -H2AX and analyzed using flow cytometry (Figure 2D).

Post-irradiation RP-1 treatment of LPA₂-DKOMEF showed significantly ($p < 0.001$) fewer γ -H2AX positive cells 4 h post-irradiation compared with RP-1 treated vector MEF cells. To ascertain whether cells that were protected from apoptosis maintain their ability to grow, we determined clonogenic survival of irradiated IEC-6 cells with or without 10 μ M RP-1 treatment (Figure 3). IEC-6 cells treated with RP-1 showed significantly higher survival fractions compared with vehicle treated cells ($p < 0.01$). These results collectively indicate that RP-1 is effective in reducing apoptosis induced by γ -irradiation and Adriamycin.

3.2. RP-1 post-irradiation treatment mitigates HE-ARS and GI-ARS mortality

The HE-ARS causes severe mortality in mice due to the rapid death of leukocytes and the ensuing sepsis. We have developed a TBI model in the C57BL/6 mouse strain that is suitable for testing of radiation mitigator compounds which when applied at least 24 h after radiation exposure decrease mortality. In this model, mice were exposed to an $\sim$ LD₈₀ dose of 9.0 Gy γ -irradiation with or without 0.1 or 0.3 mg/kg RP-1 treatment starting at 26 ± 2 h post-irradiation applied $\sim$ every 12 h via subcutaneous injection for the duration of 3 days. The control group received vehicle (0.1% Ethanol, 2% Propanediol in PBS) injections with the same regimen. Out of groups of 20 mice, 12 mice survived in the 0.1 mg/kg dose group (65%), and 11 mice (55%) survived in the 0.3 mg/kg dose group whereas, only 5 mice survived in the vehicle control group (Figure 4A). In the PBI model of the GI-ARS mice were exposed to a 16 Gy dose with partial bone marrow shielding. In this study out of 20 mice per group only 9 mice survived in the vehicle group (45%), in contrast to 17 (86%) and 14 (73%) mice in the 0.1 mg/kg and 0.3 mg/kg RP-1 treated groups, respectively (Figure 4B). These results extend our *in vitro* observations to murine models of the HE- and GI-ARS confirming the radiomitigative and radioprotective role of RP-1. Furthermore, the increase in clonogenic survival indicates that the cells protected by RP-1 treatment maintain their ability to proliferate.

3.3. LPA and RP-1 protects intestinal enteroid cultures from radiation injury

To explore the cellular targets of the radioprotective effect of LPA on ISC-derived enteroids, we pre-treated the cultures with 1 μ M LPA 24 hours pre-irradiation. We followed the irradiation model originally described by Wang et al.[37] applying a 5 Gy dose of γ -irradiation, in which $\sim$ 30% of the cultures survived to 6 days post-irradiation. We found that LPA-treated organoids showed a significant increase in survival compared with non-treated controls throughout the 6-day course of the experiment (Figure 5A, Table 1, and Supplemental Figure 1).

On day 6, the survival of the pre-treated irradiated cultures reached 77 ± 5 % (SEM) compared with 45 ± 3 % in the untreated cultures. LPA pretreatment of non-irradiated control cultures resulted in a 114 ± 13 % survival compared with 67 ± 4 % in the untreated non-irradiated cultures at day 6.

Next, we analyzed the radioprotective effect of RP-1 in similar enteroid cultures. Here, we observed an increased survival in the RP-1 pre-treated irradiated cultures which became significant at day 5 and 6. At day 6, the survival reached 64 ± 6 % in the RP-1 treated group compared with 50 ± 3 % in the non-treated group (Figure 5B, Table 1, and Supplemental

Figure 2). Altogether, the results showed that LPA and RP-1 act as radioprotective agents in irradiated enteroid cultures.

3.4. RP-1 protects Lgr5⁺ ISC

The Lgr5⁺ intestinal stem cells described by Barker et al. have been shown to drive crypt-villus homeostasis and regeneration [17, 25, 38, 39]. In order to address whether these Lgr5⁺ cells are protected from radiation by RP-1, we cultured enteroids derived from Lgr5-EGFP-CreER; Tdtomato^{flox} mice. We subsequently performed lineage tracing experiments of the LGR5⁺ cells by the addition of 4-OHT to induce the expression of the Tdtomato reporter which we reasoned will identify those healthy enteroids that contain Lgr5⁺ cells capable of synthesizing the red fluorescent marker protein. We induced the expression of the reporter at day 2 and 3 post-irradiation and analyzed Tdtomato expression with confocal fluorescence microscopy on days 3 and 4, respectively. The 24-h delay for the analysis was necessary for achieving detectable expression of the Tdtomato reporter. Our results showed that Lgr5-EGFP⁺/Tdtomato⁺ cells were still present in RP-1 pre-treated irradiated enteroids (arrows in Figure 6A, 5Gy RP-1 irradiation panel) on day 3 post-irradiation, whereas no such cells were present in the non-treated irradiated cultures (Figure 6A, 5Gy Vehicle panel). When we quantified the number of Tdtomato⁺ cells within the crypt up to a distance of 50 μ m from the bottom of the crypt, we found an average of 11.6 ± 0.6 cells/50 μ m in RP-1 pre-treated irradiated (5 Gy RP-1) cultures, whereas no Tdtomato positive cells were detectable in non-RP-1 pre-treated irradiated (5 Gy Vehicle) cultures (Figure 6B). Furthermore, the average number of Tdtomato positive cells found in the non-irradiated control cultures (0Gy Vehicle) were 11.4 ± 0.8 cells/50 μ m, and 11.9 ± 0.9 cells/50 μ m in RP-1 non-irradiated (0Gy RP-1) pre-treated cultures.

These results indicate similar average number of Tdtomato⁺ Lgr5-derived cells in enteroids of RP-1 pre-treated irradiated (5Gy RP-1) cultures compared with untreated non-irradiated control (0Gy Vehicle), and RP-1 pre-treated non-irradiated control (0Gy RP-1) groups. However, when we analyzed the RP-1 pre-treated cultures on post-irradiation day 4 (induction of the reporter at day 3 post-irradiation), we did not find sustained protection as reflected by the absence of Tdtomato⁺ cells (data not shown) similarly to the nontreated irradiated cultures. These results indicate that RP-1 selectively protected the Lgr5⁺ subpopulation of ISC.

We showed earlier that exposure of IEC-6 cells to γ -irradiation caused an increase in the abundance of LPA₂ transcripts and also increased the responsiveness to RP-1 [9]. We have expanded this line of investigation to the LPA₂ transcripts by analyzing the expression of LPA₁ and LPA₃ GPCR transcripts with or without exposure to 5Gy γ -irradiation in combination with RP-1 treatment of the cultured enteroids. Treatment of the non-irradiated enteroids caused increases in the abundance of transcripts for each LPA GPCR (Figure 7 A/B/C).

In contrast, we detected an additional significant increase in LPA₂ transcripts in RP-1 treated enteroids within 30 min post-exposure γ -irradiation and drug treatment. These observations extend our earlier findings from the IEC-6 cell line and suggest that LPA₂ GPCR is a radiation-induced immediate-early gene product.

4. Discussion

The main objective of the present study was to test the hypothesis that a new LPA₂ specific nonlipid agonist RP-1 attenuates the ARS by protecting Lgr5⁺ ISC against radiation-induced apoptosis. Our data presented herein collectively support that RP-1 is a highly potent specific agonist of only the LPA₂ receptor subtype among the six LPA GPCR, which reduces apoptosis induced by exposure to ionizing radiation and the radiomimetic cytostatic drug Adriamycin *in vitro*. Furthermore, lineage tracing studies conducted with enteroids isolated from Lgr5-EGFP-CreER; Tdtomato^{flox} mice indicate that RP-1 and LPA enhance enteroid survival after radiation exposure via selectively enhancing the survival of the Lgr5⁺ marker-bearing subpopulation of ISC. RP-1-mediated activation of the LPA₂ GPCR is consistent with and likely to contribute to the reduced mortality of mice in models of the HE- and GI-ARS. LPA and RP-1 increased the survival of enteroids when administered prior to radiation exposure but were ineffective when applied postirradiation. We do not know what is the reason for the lack of a radiomitigative effect in this culture system but mouse experiments with RP-1 administered at 24 h postexposure demonstrated significant survival advantage compared with vehicle control treated mice. The delicate nature of the enteroid culture might be one of the technical reasons why we could only demonstrate the radioprotective action of LPA and RP-1 but failed to show radiation mitigation. The causes of the mismatch between *in vitro* and *in vivo* findings will have to be addressed in future experiments. Combined with results we reported earlier [9, 13, 29, 30, 40, 41], LPA₂ appears to be a major regulator of cell regeneration after radiation injury. We showed that LPA was ineffective as a radiation protective/mitigative agent in cells and mice lacking LPA₂ GPCR and that LPA₂ was upregulated by radiation-induced activation of NFκB-mediated gene transcription leading to enhanced sensitivity of the cells to LPA and RP-1 (see Graphic abstract). RP-1 has been developed from the non-lipid scaffold of the Genome Research Institute compound GRI977143. The medicinal chemistry guided by SAR studies identified the RP-1 chlorinated analog to be the most potent among bromine and fluorine substituted halogenated analogs at position 5 of the headgroup. However, the scope was limited to SAR studies using only human orthologs of the LPA GPCR and these SBA compounds have not been assessed at the mouse orthologs. Here we applied the comprehensive TGFα-shedding assay platform as described previously by Inoue et al [33] to comparatively determine the pharmacological selectivity of RP-1 among the six LPA GPCR known to date. For the first time, this assay technique allowed us to determine the ligand properties of RP-1 at all LPA GPCR including the LPA₆, which does not couple to Ca²⁺ mobilization and was not available previously to establish the selectivity of RP-1 at this receptor subtype. Our results have firmly established that RP-1 is a high-potency and exclusively specific ligand of the LPA₂ subtype without activating or inhibiting any other LPA GPCR subtype. Comparison of the potency of RP-1 at the human and mouse orthologs of the LPA₂ subtype surprisingly showed that this compound is much more potent to the human ortholog (EC₅₀~ 5 pM) compared with the murine ortholog (EC₅₀ ~25 nM), even though both have higher potencies compared with that of LPA 18:1 (1.4 nM and 32 nM, respectively). Given the very high sequence identity between the two LPA₂ orthologs, the differences in potency of RP-1 is surprising and difficult to explain at the present time. Nonetheless, these results underline that every new ligand must be experimentally tested at multiple orthologs of the LPA GPCR before it is to

be tested using cells from different species or in animal models. Our present data identify RP-1 as the highest potency specific nonlipid agonist of LPA₂ of the human and murine LPA₂ GPCR. The high potency and chemical structure of RP-1 make it a potential drug candidate for the treatment of conditions where LPA₂ activation has beneficial effects such as the prevention of apoptosis [40], prevention of stomach erosions caused by the NSAID family of drugs [42], attenuation of asthmatic reactions [43], and the inhibition of secretory diarrhea [44, 45].

Characterization of the cellular responses elicited by RP-1 revealed that it was only effective in LPA₂-DKOMEF cells and lacked antiapoptotic actions in MEF cells that do not express transcripts of the LPA_{1/2/3} receptors. Post-irradiation administration of RP-1 mitigated apoptosis elicited by γ -irradiation and Adriamycin in IEC-6 cells that are of rat origin and express LPA₂ endogenously [29, 30]. RP-1 was found to enhance the resolution of γ -H2AX foci 4 h post-irradiation in LPA₂-DKOMEF cells compared with vector-DKOMEF cells. Finding fewer γ -H2AX positive cells is an important finding because γ -H2AX is an indicator of double-strand DNA breaks caused by radiation exposure. Thus, RP-1 not only prevents apoptosis caused by DNA damage but presumably enhances the DNA repair process thus augmenting the preservation of genetic integrity of the radiation-damaged cell. Our qRT-PCR examination of the effect of radiation on LPA_{1/2/3} GPCR indicated that RP-1 pre-treatment of the intestinal enteroids rapidly, within 30 min increased the abundance of these receptor transcripts in the non-irradiated intestinal enteroids. RP-1 treatment of the irradiated enteroids further increased the abundance of LPA₂ transcripts but not of other LPA GPCR members in the EDG gene family. Interestingly, only LPA₂ transcripts increased in irradiated enteroids, in line with earlier findings we reported using IEC-6 cells. We showed that radiation-elicited reactive oxygen species-mediated activation together with DNA damage-activated ATM kinase activation-coupled TNF α production via the transcription factor NF κ B upregulate *Ipar2* expression that is translated to higher responsiveness to RP-1 [9]. Taken together, these results confirm that LPA₂ is a radiation-induced early gene product and its expression is further enhanced by ligand-activation. The increased expression by radiation exposure and the enhancement of DNA repair mechanisms might have far reaching consequences if RP-1 is chosen to be developed as a radioprotective drug.

Using well-characterized murine models of the HE- [40, 41] and GI-ARS [3, 13, 29] we have obtained evidence that RP-1 mimics the action of LPA and the LPA-mimic ligand OTP in reducing radiation-induced mortality of mice [29, 40]. We point out that RP-1 was tested as a radiation mitigator because its administration was delayed by 24 ± 2 h after radiation exposure. Its significant efficacy in these models elevate RP-1 to the level of a potential radiation mitigator drug candidate.

Our previous histological examination of irradiated intestinal crypts showed that the highest level of protection exerted by LPA and also OTP in the crypt-villus axis coincided with the position of ISC [29]. Therefore, we hypothesized that LPA₂ expressed in Lgr5 ISC is the potential molecular target of RP-1 and LPA. Using the Lgr5-EGFP-CreER;Tdtomato^{flox} transgenic mice, we performed lineage-tracing of Lgr5⁺ cells. The double labeling allowed the detection of those EGFP-expressing cells that remained capable of protein and RNA synthesis after radiation exposure to express the red Tdtomato protein. These experiments

yielded several important new pieces of information about the effect of RP-1. First, we found that RP-1 increased the growth and proliferation of Lgr5 cells in unirradiated cultures. Next, pre-irradiation RP-1 treatment spared the Lgr5 positive cells from radiation exposure at a level seen in the unirradiated control enteroids. Third, we found that irradiation combined with RP-1 treatment upregulated the abundance of LPA₂ transcripts in the enteroids as we have shown it previously in IEC-6 cells [9]. Therefore, these results support the hypothesis that the molecular target of RP-1 is the LPA₂ receptor expressed in Lgr5⁺ ISC. This finding provides the foundation of our working hypothesis that RP-1 and LPA via the activation of LPA₂ can rescue cells from radiation injury. Of note, Bmi1 has been described as a marker for a “reserve” intestinal stem cell population [22]. As such, these cells have been described as an alternate stem cell pool that are not only less radiosensitive, but that can also give rise to Lgr5⁺ cells [22]. It will be of interest to determine the effect of RP-1 on Bmi1 cells in future studies.

It is important to recognize that ISC are not the only cells that express the Lgr5 maker. For instance, hematopoietic precursor cells in the bone marrow and other organs also express LGR5 [46]. Thus, it is feasible that the protective action of RP-1 observed in our HE-ARS model is mediated in part by all Lgr5⁺ stem/progenitor cells regardless what tissue they reside in. However, this hypothesis will have to be experimentally validated in future studies.

5. Conclusions:

Our results show that LPA and a new nonlipid drug-like LPA₂ agonist designated as RP-1, have radioprotective properties in irradiated Lgr5⁺ intestinal stem cells present in enteroid cultures. In addition, the lineage tracing experiments show that RP-1 specifically promotes the survival of Lgr5⁺ intestinal stem cells for at least 2 days after irradiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

6. Acknowledgements

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Abbreviations

LPA	Lysophosphatidic acid
ARS	Acute Radiation Syndrome (ARS)
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5

OTP	Octadecyl thiophosphate
TGFα	Transforming growth factor α
GPCR	G protein-coupled receptor
SBA	Sulfamoyl benzoic acid
MEF	mouse embryonic fibroblast
p-NPP	para-nitro phenylphosphate
AP-TGFα	Alkaline phosphatase conjugated transforming growth factor α
TBI	Total body irradiation
PBI	Partial body irradiation
HE-ARS	Hematopoietic acute radiation syndrome
GI-ARS	Gastrointestinal acute radiation syndrome
4-OHT	4-hydroxy tamoxifen

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Highlights

1. Radioprotectin-1 is a new nonlipid high-potency agonist of the murine LPA₂ GPCR.
2. Radioprotectin-1 only protects cultured cells that express LPA₂ from apoptosis induced by γ -irradiation or the radiomimetic cytostatic drug Adriamycin.
3. Radioprotectin-1 decreases mortality in mouse models of the hematopoietic and gastrointestinal acute radiation syndromes.
4. Radioprotectin-1 enhances the survival of cultured small intestine-derived enteroids and upregulates LPA₂ expression after radiation exposure.
5. Radioprotectin-1 protects Lgr5 positive intestinal stem cells from radiation injury.

Figure 1.

Pharmacological characterization of RP-1 using the TGF α -shedding assay. Panel A. Structural formula of RP-1. Panel B. Dose-response curves for LPA 18:1 (upper rows) and RP-1 (lower rows). Yellow dots represent the response in mock-transfected cells, blue dots in the LPA GPCR transfected HEK293 cells without an antagonist to block the endogenous LPA response, purple dots represent the receptor-specific activation defined by the difference between response in receptor expressing cells minus the mock cells. The LPA_{1/3} antagonist, Ki16425 was applied at 10 μ M to cells transfected with LPA₄, LPA₅, or LPA₆,

respectively, to inhibit the endogenous response from LPA_{1/3} receptors. Data are the means of four biological replicates $\pm$ SEM, each performed in triplicates.

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Figure 2.

RP-1 protects cells from apoptosis by γ -irradiation- and the radiomimetic chemotherapeutic Adriamycin. **Panel A.** RP-1 treatment administered 30 min postirradiation with 15 Gy dose-dependently reduced caspase 3/7 activation in LPA2-DKOMEF but not in vector DKOMEF cells exposed γ -irradiation. **Panel B.** IEC-6 cells were exposed to 1.7 μ M Adriamycin and caspase 3/7 activity was measured 6 h later. RP-1 was administered 30 min after Adriamycin challenge and it dose-dependently reduce caspase 3/7 activity. ** denote $P < 0.001$ relative to Adriamycin alone. **Panel C.** LPA2-DKOMEF were treated with 1.7 μ M Adriamycin to induce apoptosis. RP-1 treatment 30 min after Adriamycin exposure dose-dependently reduced caspase 3/7 activity 6 h later. RP-1 was ineffective in vector-DKOMEF cells. **Panel D.** μ -H2AX^{hi} LPA2-DKOMEF or vector cells were monitored using flow cytometry 4 h after γ -irradiation with 15 Gy. RP-1 (10 μ M) or LPA (10 μ M) treatment administered 30 min after irradiation reduced the % of μ -H2AX^{hi} positive LPA2-DKOMEF but not vector-DKOMEF. ** $p < 0.01$, *** $p < 0.001$ relative to vector-DKOMEF exposed to the same treatment. Note the significant reduction in μ -H2AX^{hi} LPA2-DKOMEF relative to vector -DKOMEF cells.

Figure 3. Effect of RP-1 and radiation on clonogenic survival of IEC-6 cells.

The cells were treated exposed to 2-10 Gy γ -irradiation and treated with 10 μ M RP-1 or vehicle 2 h postirradiation. Colony growth was determined 3 days later using crystal violet staining of the colonies. **P, 0.001 relative to vehicle treated controls.

Figure 4. RP-1 reduces mortality in HE-ARS and GI-ARS models.

Groups of 20 C57BL/6 mice were exposed to either 9 Gy of TBI or 16 Gy PBI γ -irradiation from a ^{137}Cs source to elicit the HE-ARS (panel A) and GI-ARS (panel B). Mice were treated intraperitoneally with either 0.1 or 0.3 mg/kg/day doses of RP-1 or vehicle for 3 days starting at +24 h postirradiation and survival was monitored for 30 days. The survival benefit was significant relative to the vehicle controls at $p < 0.05$ in both models.

Figure 5. Effect of LPA and RP-1 on the survival of small intestinal enteroids after 5 Gy γ -irradiation.

Panel A. Enteroid cultures were established for 6 days before treatment with 1 μ M LPA, 1 μ M RP-1, or vehicle. On day 7, the cultures were exposed to 5 Gy γ -irradiation and allowed to grow for 2 more days before the surviving enteroids were counted. Data points are the means of triplicate wells and the results are representative of at least three biological replicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 6. Effect of pre-irradiation RP-1 treatment on the survival of Lgr5⁺ ISC in irradiated enteroid cultures at day3 post-irradiation (day10).

Panel A. Treatment regimen and confocal fluorescence images of EGFP and TdTomato expressing ISC in Lgr5-derived enteroid cultures. Quantification of Lgr5 (B) TdTomato⁺ cells within the bottom 50 μ m of the enteroid crypts (mean $\pm$ SD, representative of two biological replicates).

Figure 7.

Effect of RP-1 pretreatment on the expression of *Lpar* in enteroids with or without 5 Gy γ -irradiation. RNA was collected at 30 min postirradiation and 24 h after 1 μ M RP-1 or vehicle treatment (* p < 0.05, ** p < 0.01 relative to vehicle). Data are means of n=3 experiments $\pm$ SD

Table 1.

LPA and RP-1 radioprotective effect on irradiated intestinal enteroid cultures

	0Gy Vehicle			0Gy + LPA			5Gy Vehicle			5Gy + LPA		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
day0	100	0	4	100	0	8	100	0	10	100	0	10
day1	99.2	5.2	4	128.5	8.8	8	75.3	3.5	10	89.6	2.3	10
day2	91.6	6.0	4	146.7	13.0	8	62.8	2.5	10	84.9	3.5	10
day3	89.9	7.1	4	146.5	16.4	8	58.0	2.9	10	76.7	3.6	10
day4	84.9	8.0	4	139.4	17.3	8	48.4	2.7	10	70.7	2.8	10
day5	74.2	6.1	4	122.9	14.9	8	44.4	3.0	10	76.6	4.3	10
day6	67.2	4.0	4	114	13.2	8	45.0	3.3	10	77.1	4.6	10

	0Gy Vehicle			0Gy + RP-1			5Gy Vehicle			5Gy + RP-1		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
day0	100	0	10	100	0	12	100	0	12	100	0	12
day1	96.9	2.3	10	103	4.2	12	78.1	3.6	12	83.7	2.8	12
day2	90.9	4.3	10	100.8	5.7	12	67.8	3.1	12	72.8	2.1	12
day3	87.9	3.6	10	97.4	6.6	12	57.3	2.3	12	67.6	2.5	12
day4	81.1	4.5	10	91.4	7.9	12	50.4	2.0	12	60.8	1.6	12
day5	67.4	3.0	10	81.4	9.1	12	48.2	2.6	12	65.5	4.3	12
day6	64.2	2.2	10	79.4	9.1	12	50.0	2.6	12	64.3	5.6	12

Quantification of surviving irradiated enteroid cultures pre-treated wit LPA or RP-1. Samples (n) listed in the table correspond to the number of enteroids