



A PPAR-gamma agonist protects from radiation-induced intestinal toxicity

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Abstract

Objective: Because of its anti-inflammatory, anti-fibrotic, anti-apoptotic and anti-neoplastic properties, the PPAR- γ agonist rosiglitazone is an interesting drug for investigating for use in the prevention and treatment of radiation-induced intestinal damage. We aimed to evaluate the radioprotective effect of rosiglitazone in a murine model of acute intestinal damage, assessing whether radioprotection is selective for normal tissues or also occurs in tumour cells.

Methods: Mice were total-body irradiated (12 Gy), with or without rosiglitazone (5 mg/kg/day). After 24 and 72 hours, mice were sacrificed and the jejunum was collected. HT-29 human colon cancer cells were irradiated with a single dose of 2 (1000 cells), 4 (1500 cells) or 6 (2000 cells) Gy, with or without adding rosiglitazone (20 μ M) 1 hour before irradiation. HT-29-xenografted CD1 mice were irradiated (16 Gy) with or without rosiglitazone; tumour volumes were measured for 33 days.

Results: Rosiglitazone markedly reduced histological signs of altered bowel structures, that is, villi shortening, submucosal thickening, necrotic changes in crypts, oedema, apoptosis, and inflammatory infiltrate induced by irradiation. Rosiglitazone significantly decreased p-NF- κ B p65 phosphorylation and TGF β protein expression at 24 and 72 hours post-irradiation and significantly decreased gene expression of *Collagen1*, *Mmp13*, *Tnfa* and *Bax* at 24 hours and *p53* at 72 hours post-irradiation. Rosiglitazone reduced HT-29 clonogenic survival, but only produced a slight reduction of xenograft tumour growth.

Conclusion: Rosiglitazone exerts a protective effect on normal tissues and reduces alterations in bowel structures and inflammation in a radiation-induced bowel toxicity model, without interfering with the radiation effect on HT-29 cancer cells. PPAR- γ agonists should be further investigated for their application in abdominal and pelvic irradiation.

Keywords

Rosiglitazone, PPAR-gamma agonists, radiation-induced toxicity, bowel, radioprotection

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Introduction

Ionizing radiation is commonly used to treat a wide variety of cancers. Despite improvements in radiation technology, the delivery of radiotherapy to a tumour unavoidably affects the surrounding normal tissues. Normal tissue toxicity is often a dose-limiting factor, since the therapeutic ratio of radiotherapy is dictated by a balance between normal tissue toxicity and tumour control.^{1,2} Normal tissue radiation toxicity mainly affects rapidly renewing cell systems, such as bone marrow and gastrointestinal (GI)-tract mucosa. Radiation-induced GI damage is a complication of irradiation (IR) exposure, characterized by early effects, when observed in a few days, or delayed effects, when observed in a few months to years.^{3,4} Early effects

develop as a result of intestinal crypt cell death and disruption of the epithelial barrier.⁵ Damage to stem

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cells and decrease in the progenitor cell compartment plays a critical role in intestinal toxicity induced by radiation.⁶ Early effects are also characterized by a classical inflammatory response involving local leukocyte influx, proinflammatory cytokine production, and the induction of endothelial adhesion receptors.^{7–9} The delayed effects are characterized by progressive intestinal wall fibrosis and vascular sclerosis.¹⁰ Furthermore, in a number of experimental models, it has been shown that excessive inflammatory response after intestinal injury can also lead to multiple organ failure.¹¹ Therefore, early intestinal changes occurring after radiation exposure are particularly promising targets for interventions aimed at preventing or reducing radiation syndromes.¹²

Currently, effective agents to counteract radiation-induced normal tissue injury are lacking and there is a great need for new agents.

Peroxisome proliferator-activated receptor (PPAR)- γ is a member of the nuclear hormone receptor superfamily, which regulates the expression of several target genes and a multitude of metabolic processes.¹³ Numerous PPAR- γ synthetic activators have been developed, such as thiazolidinediones, including pioglitazone, rosiglitazone (RGZ), ciglitazone, and trioglitazone, which have shown various metabolic effects,¹⁴ including anti-inflammatory,¹⁵ anti-fibrotic¹⁶ and anti-neoplastic¹⁷ effects. Particularly in HT-29 human colorectal cancer cells, RGZ suppresses radiation-induced survival signals and DNA damage response, and enhances the radiation-induced apoptosis signalling cascade.¹⁸

Anti-inflammatory properties make PPAR- γ ligands very interesting drugs to investigate for the prevention and treatment of radiation-induced intestinal damage. Moreover, anti-neoplastic activity suggests a possible differential effect between normal and tumour tissues for thiazolidinediones.

Our study aimed to investigate the protective effect of RGZ in a murine model of acute radiation-induced intestinal damage. In addition, we aimed to see if the radioprotective effect of RGZ was selective for normal tissues or if it also occurred in tumour cells.

Materials and methods

Reagents

RGZ was from Alexis Biochemicals (San Diego, CA, USA). Reagents for cells cultures and Western blot were from Sigma-Aldrich (St. Louis, MO, USA). TaqMan Reverse Transcription Reagents kit, primer/probe mix (TaqMan Gene Expression Assays), *Bax* (ID no. Mm00432050_m1), collagen type I (*Coll*, ID no. Mm00483888_m1), *Mmp13* (ID no. Mm00439491_m1),

Tnfa (ID no. Mm00443258_m1), *Trp53* (ID no. Mm01731287_m1), *Gapdh* (ID no. 4352339E) and 1 \times Universal Master Mix were from Applied Biosystems (Foster City, CA, USA). BCA protein assay kit was from Pierce Biotechnology (Rockford, IL, USA). Rabbit pAbs anti-GAPDH, anti-p-NF-kB p65 (S536) and anti-TGF β were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Peroxidase-secondary Ab, Immobilon-P PVDF membranes, Immobilon Western Chemiluminescent HRP Substrate, Protease/Phosphatase inhibitor cocktails were from Millipore (Milan, Italy), and DeadEnd(TM) Colorimetric TUNEL System was from Promega Italia Srl (Milan, Italy).

Cell cultures

HT-29 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

HT-29 (500, 1000, 1500 or 2000 cells/well) were seeded in six-well plates in growth medium. After 24 hours, a single dose of 2 (1000 cells), 4 (1500 cells) or 6 (2000 cells) Gy of IR was delivered, with or without adding RGZ (20 μ M) 1 hour before IR. Control cells were plated at 500 cells/well. At 24 hours after IR, growth medium was removed, and fresh drug-free medium was added. Cells were incubated for 12–15 days and then fixed and stained with crystal violet (0.5% in methanol). Only colonies exhibiting at least 50 cells were counted. The surviving fraction was calculated by the formula: (mean colony count/seeded cells) \times plating efficiency (PE: mean colony count/cells seeded for unirradiated controls). PE was normalized to the respective control value (RGZ control in combined treatments) for each single experiment. Linear-quadratic survival expression was fitted to the data by non-linear regression. The radiosensitization enhancement ratio for RGZ at 50% survival (ER50) was as follows: ER50 = dose at 50% survival without RGZ/dose at 50% survival with RGZ.

In vivo studies

Animals and experimental protocol

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Animal experiments were approved by the Animal Ethics Committee of the University of Florence (protocol n° 56/2009).

C57BL/6J mice and CD1 mice from Charles River Laboratories Italia s.r.l. (Milan, Italy) were housed in plastic cages at controlled temperature (22 \pm 2°C), lighting (12 hours) and humidity (60 \pm 10%).

Radiation-induced intestinal damage. Mice were exposed to total-body IR (TBI), with a single dose of 12 Gy delivered with Linac, with or without RGZ (5 mg/kg/day, oral gavage) started 24 hours before. Non-irradiated mice were used as control. After 24 and 72 hours, mice were sacrificed and jejunum segments collected.

Human intestinal cancer cells xenograft. HT-29 tumour xenografts were obtained by subcutaneous injection of 6×10^6 cells in the flank of CD1 mice. When the tumour reached a mean diameter of 5 mm, animals were randomly assigned to the following groups: IR, IR + RGZ, RGZ, and saline (control). IR consisted of a dose of 16 Gy selectively delivered to the tumour. The treatment with RGZ (5 mg/kg/day, oral gavage) was started 24 hours before IR and continued until the end of the experiment.

Tumour response was defined as the relative change in tumour volume versus volume before therapy. Tumour volume (mm^3) was measured twice a week for 33 days ($\text{tumour volume} = \text{length (mm)} \times \text{width}^2 (\text{mm}^2)/2$).

RNA extraction and real-time PCR

Tissue samples were homogenized using stainless steel beads and a TissueLyser. Total RNA from tissues was extracted with the RNeasy Mini kit according to the manufacturer's recommendations. Total RNA (400 ng) was reverse transcribed in a final volume of 80 μl containing 500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl (pH 8.3), 5.5 mM MgCl₂, 500 mM of each dNTP, 2.5 mM random examers, 0.4 U/ml RNase inhibitor, and 1.25 U/ml Multiscribe Reverse Transcriptase. The reverse transcription reaction was performed at 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 3 minutes. Gene expression measurement was performed by quantitative real-time PCR (TaqMan). The amount of target, normalized to an endogenous reference (*Gapdh*, pre-developed TaqMan assay reagents) and relative to a calibrator (quantitative PCR human reference total RNA), was determined by $2^{-\Delta\Delta\text{Ct}}$ calculation.¹⁹ For each sample, 12.5 ng cDNA were added to 10 μl of PCR mix containing each primer/probe mix and 1 \times Universal Master Mix. The samples were then subjected to 40 cycles of amplification at 95°C for 15 seconds and 60°C for 60 seconds in an ABI Prism 7900 Sequence Detector (Applied Biosystems).

Histological analysis

For each sample, representative sections of 5 μm in thickness were cut from tissue blocks of formalin-fixed, paraffin-embedded tissues and placed on glass slides for morphological evaluation. Sections were stained with hematoxylin and eosin. Measurement of

villus height was performed by light microscopy using a calibrated micrometre (100 \times). The inflammatory cell infiltration was quantified by counting the number of neutrophils in 10 different areas of histological sections from each sample. The average number of neutrophils was divided by the average number in non-irradiated control mice for normalization. The degree of regeneration of jejunal crypts was assessed by counting the number of surviving crypts per circumference using microscopy, as previously described.²⁰

TUNEL assay

The degree of apoptosis was assessed in jejunum sections by TUNEL assay, according to the manufacturer's instructions. Briefly, the sections were deparaffinized with xylene, dehydrated through a graded alcohol series and treated with permeabilization solution. The labelling reaction was performed using a solution containing terminal deoxynucleotidyl transferase (TdT). The number of TUNEL-positive cells (cells with brown nuclei) per field was counted in three randomly selected fields of each section (at least three sections/animal) at 100 \times magnification.

Western blot analysis

Tissues were lysed in 200 μl of ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS) supplemented with protease/phosphatase inhibitor cocktail. Tissues were centrifuged for 10 minutes at 700 $\times g$ at 4°C, and the supernatant was then collected and protein concentration measured. Protein aliquots (30 μg) were processed and separated by 12% SDS-PAGE; the proteins were then transferred onto PVDF membranes, which were incubated for 2 hours at room temperature with primary Abs diluted in Tween Tris-buffered saline (anti-p-NF-kB p65 (S536), anti-TGF β 1:400, anti-GAPDH 1:1500), followed by peroxidase-conjugated secondary IgG (1:5000). Proteins were revealed using the Immobilon Western Chemiluminescent HRP substrate. The chemiluminescent signal was analysed with Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories). GAPDH was used as loading control. The densitometric analysis of Western blot bands was performed using the Quantity One software (Bio-Rad Laboratories, Inc., Milan, Italy), normalized to GAPDH, and reported as percentage relative to the respective control, set at 100.

Statistics

The statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA,

USA). One-way analysis of variance (ANOVA) was used, and a p -value less than 0.05 was considered statistically significant. Data are expressed as mean \pm SEM.

Results

Histopathological features

Histopathological analysis of jejunal specimens (Figure 1) showed that the untreated control mice

exhibited normal features, with long villi lined with a continuous columnar epithelium and well-developed tubular gland.

Radiotherapy caused time-dependent injurious effects on the jejunal mucosa, with loss of normal histological structure, as shown by the significant shortening of villi (Table 1), hypoplasia of the tubular glands, and submucosal thickening. A marked inflammatory infiltrate was also observed in irradiated mice (Table 1).

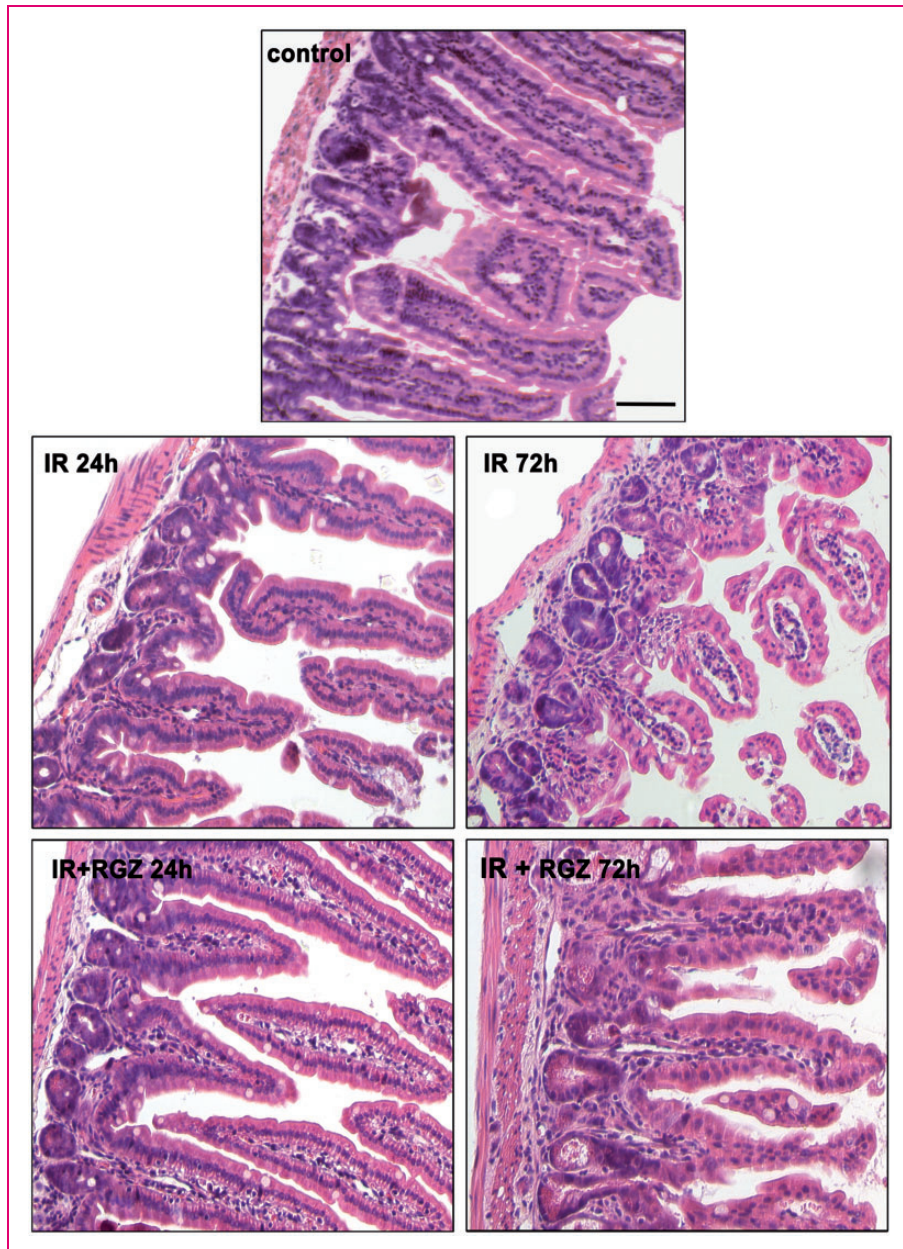


Figure 1. Rosiglitazone reduced histological signs of radiation-induced intestinal damage.

Representative full-thickness images of jejunal wall of mice treated with saline (control), irradiation (IR, 12 Gy) or IR + RGZ (5 mg/kg) after 24 and 72 hours. Bars = 100 μ m.

Table 1. Effect of RGZ on histological changes induced by irradiation in mouse small intestine

	control	IR	IR + RGZ
Villus height (μm)	202.90 \pm 3.78	91.24 \pm 2.21	137.19 \pm 4.28*#
Number of crypts/circumference	94.40 \pm 1.05	34.55 \pm 1.14*	55.80 \pm 1 \pm 43*#
Inflammatory cell relative number (fold increase vs. control)	1 \pm 0.17	8.42 \pm 0.39*	4.26 \pm 0.29*#

Data are shown as mean \pm SEM (* p < 0.05 vs control, # p < 0.05 vs. IR).

Moreover, IR reduced the number of regenerating crypts in the jejunum compared with healthy controls (Table 1).

RGZ-treated mice showed a dampened radiation-induced jejunal damage, at both 24 and 72 hours after IR (Figure 1), with increased villi length, improved crypt survival, and a marked reduction of oedema and inflammatory response (Table 1), but preservation of submucosal thickness.

TUNEL findings

TUNEL staining revealed that few apoptotic cells were visible in the tip of villi in control mice (Figure 2). Obvious apoptotic cells were observed in the tip of villi, in the villous stroma and in the crypts of irradiated mice 24 hours after IR (111.25 \pm 1.37, p < 0.05 vs. control). Apoptosis was still significant in jejunum of irradiated mice 72 hours after IR (35.26 \pm 0.61, p < 0.05 vs. control).

Treatment with RGZ significantly reduced the number of TUNEL-positive cells at both time points (RGZ + IR at 24 hours = 72.1 \pm 3.21, p < 0.05 vs. IR, p < 0.05 vs. control; RGZ + IR at 72 hours = 26.97 \pm 1.63, p < 0.05 vs. IR, p < 0.05 vs. control).

Molecular analysis

To evaluate the anti-inflammatory, anti-fibrotic and anti-apoptotic effects of RGZ, we assessed the changes in several markers by Western blot analysis and real-time RT-PCR in jejunal specimens.

Bowel tissues of irradiated mice exhibited significantly increased protein expression of the fibrosis marker TGF β and phosphorylation of the p65 subunit of the stress response marker NF- κ B, at both 24 and 72 hours, as compared with untreated control (Figure 3). RGZ significantly inhibited the overexpression of radiation-induced p-NF- κ B p65 and TGF β protein expression, at both time points.

In jejunal specimens, IR significantly increased gene expression of *Bax*, *Coll1*, *Mmp13*, *Tnfa* at 24 hours post-TBI, and of *Trp53* at 72 hours post-TBI (Figure 4). RGZ treatment significantly reduced these increases in mRNA expression induced by IR (p < 0.05).

Effect on irradiated human intestinal cancer cells

To evaluate the effect of RGZ on irradiated cancer cells in vitro, clonogenic assays were performed on HT-29 cells, pre-treated or not with RGZ (20 μM), and irradiated with increasing ionizing radiation doses. According to previous works,¹⁸ the addition of RGZ reduced the clonogenic survival of the cancer cells as compared with IR alone (Figure 5): cells treated with RGZ had significantly fewer colonies vs. control cells at each radiation dose (p < 0.05), with an ER50 of 1.93.

RGZ effect on cancer cells was also evaluated in a murine HT-29 xenograft model (Figure 6). RGZ alone was not able to significantly reduce tumour growth. A significant control of tumour growth was achieved with IR and the combination of RGZ with IR (p < 0.05 vs. control and vs. RGZ alone), without significant differences between the two groups. Thus, when combined with IR, RGZ did not protect the tumour from the therapeutic effect of radiotherapy.

Discussion

The GI tract has a high radiosensitivity, and bowel constraints represent an important factor in treatment planning. Acute radiation-induced bowel toxicities range from symptoms such as pain, diarrhoea and bloating to obstruction, perforation, fistula and bleeding. Late toxicities include bowel obstruction, diarrhoea, fibrosis, and malabsorption.

Radiation-injury of the GI tract is considered to be one of the critical causes for systemic complications following radiation exposure, and may mediate some effects that lead to multiple organ failure.^{11,21} Exposure to IR leads to increased oxidative stress, DNA damage, genomic instability and inflammation,² that induce increased apoptosis and cell loss. When DNA is damaged due to IR exposure, the transcription factor p53 is activated and initiates its signal cascade along the apoptosis pathway,²² in turn activating Bax, a member of the pro-apoptotic Bcl-2 family.²³ IR induces proinflammatory cytokines including TNF- α , TGF- β 1, IL-1 β and IL-6, which have been shown to contribute significantly to the complications associated with radiotherapy.²⁴⁻²⁷

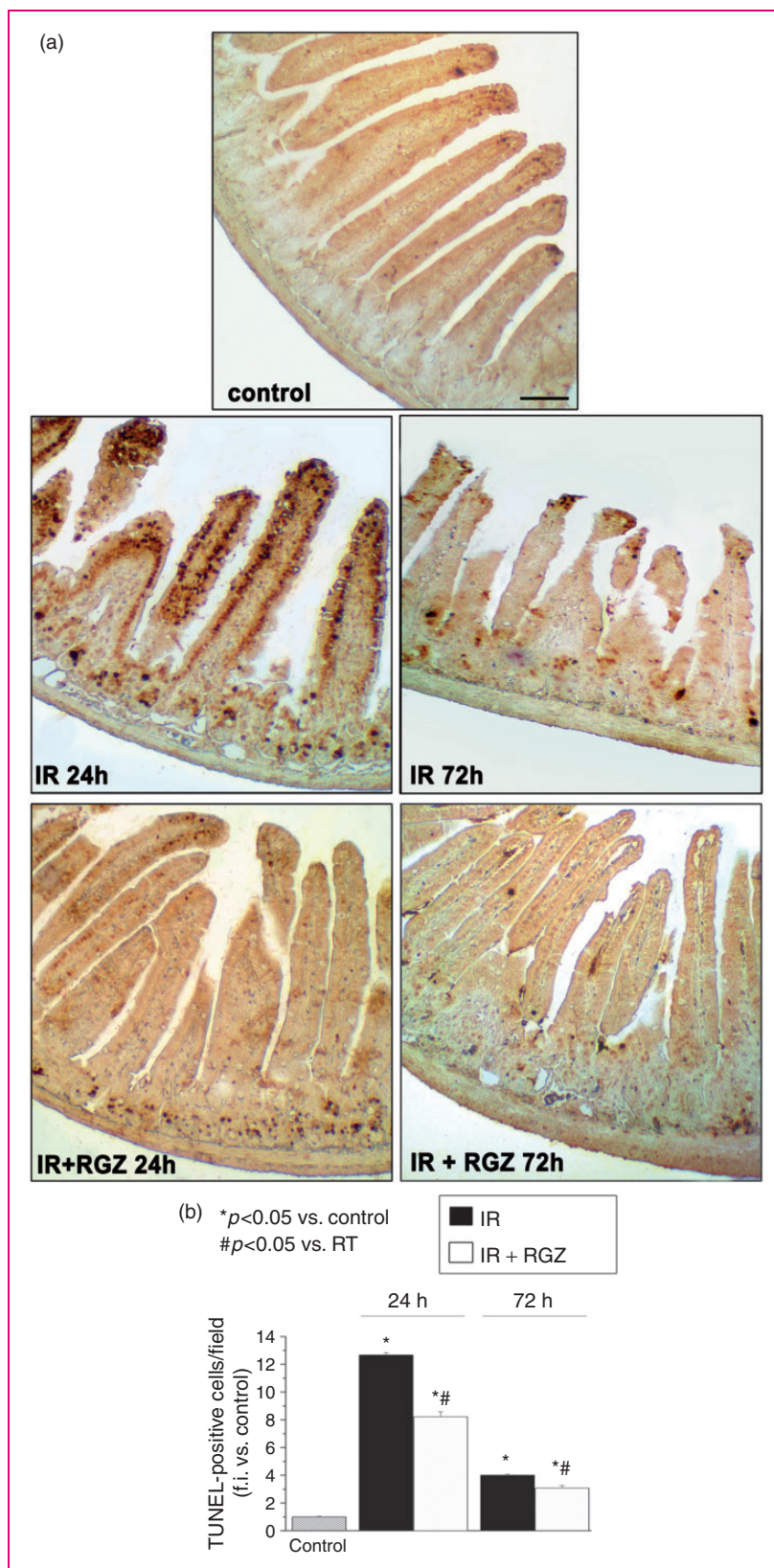


Figure 2. Rosiglitazone decreased radiation-induced apoptosis in bowel tissues.

a. Representative images of TUNEL-stained jejenum sections from mice treated with saline (control), irradiation (IR, 12 Gy) or IR + RGZ (5 mg/kg), after 24 and 72 hours. Bars = 100 μ m.

b. Average number of TUNEL-positive cells per field at 100 \times magnification. Data, measured as fold increase in expression compared to control (taken as 1), are shown as the mean \pm SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. IR.

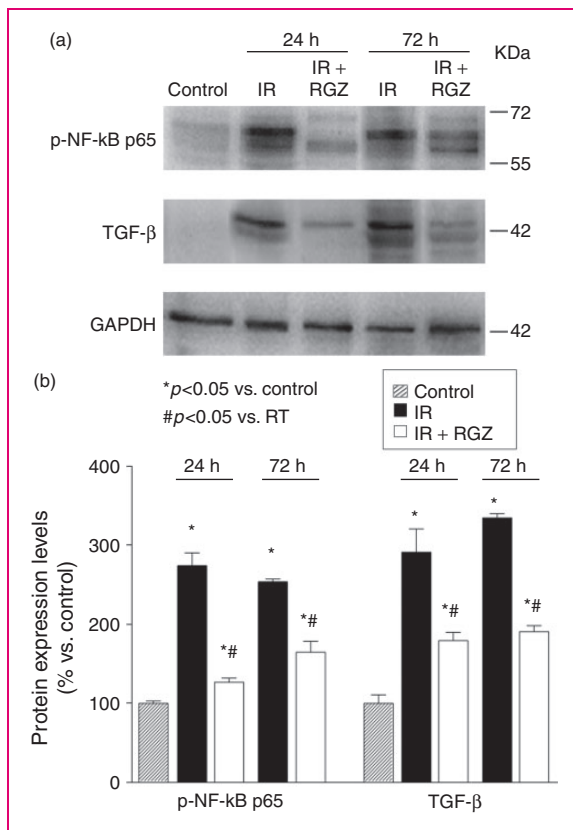


Figure 3. Rosiglitazone decreased overexpression of radiation-induced fibrosis/inflammatory marker proteins in bowel tissues. a. Representative Western blot analysis of p-NF-kB p65 and TGF-β proteins in jejunum tissue homogenates from mice treated with saline (control), irradiation (IR, 12 Gy) or IR + RGZ (5 mg/kg), after 24 and 72 hours. GAPDH was used as a loading control. b. Densitometric analysis of Western blot bands, normalized for GAPDH and reported as percentage versus the respective control, set at 100. Data are shown as the mean \pm SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. IR.

There is a major need for more effective radioprotective agents, but despite many compounds having been studied,^{28,29} none has so far been introduced in common clinical practice. An ideal radioprotector must be able to protect healthy tissue from the radiation toxicity without interfering with the therapeutic effect of ionizing radiation on the tumour tissue, and should be well tolerated.

In this study, we evaluated the radioprotective properties of RGZ (5 mg/kg/day started 24 hours before RT) in normal bowel tissue in a murine model of intestinal injury induced by 12-Gy TBI, as previously described.^{30,31} Histological analysis revealed that IR caused noticeable intestinal damage, with villi shortening, submucosal thickening, crypt necrotic changes, oedema and inflammatory infiltrate, and increased numbers of TUNEL-positive (apoptotic) cells. RGZ

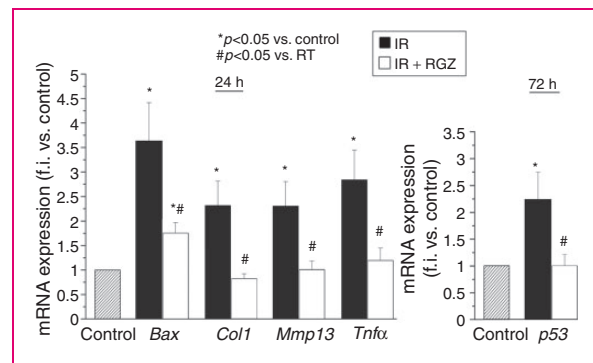


Figure 4. Rosiglitazone decreased the radiation-induced gene expression of fibrosis, inflammatory and apoptosis markers in bowel tissues.

mRNA expression of *Bax*, *Col1*, *Mmp13* and *Tnfa*, *p53* in jejunum tissues of mice treated with saline (control), irradiation (IR, 12 Gy) or IR + RGZ (5 mg/kg) after 24 and 72 hours. Data, measured as fold increase in expression compared with control (taken as 1), are shown as mean \pm SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. IR.

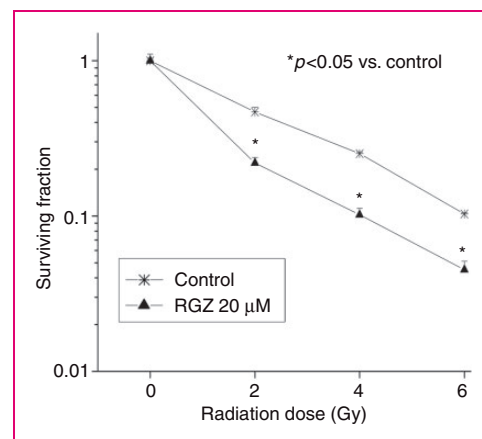


Figure 5. Rosiglitazone significantly reduced clonogenic survival of HT-29 cancer cells following irradiation with increasing radiation doses.

Cells, pre-treated or not with RGZ (20 μM), were irradiated with 0–6 Gy. After 12–15 days, cells were stained and colonies over 50 cells counted. Data are shown as mean \pm SEM, corrected by PE. PE was normalized to the respective control value (RGZ control in combined treatments) for each single experiment. * $p < 0.05$ vs. control.

produced histological improvement of tissue structure, with normalization of villi and crypts and oedema and reduction of inflammatory infiltrate and apoptotic cell death.

Gene expression analysis revealed that RGZ negatively modulated the radiation-induced expression of several tissue markers of fibrosis (*Collagen 1* and *Mmp13*), inflammation (*Tnfa*) and apoptosis (*Bax*, *p53*) in jejunum. Furthermore, Western blot analysis

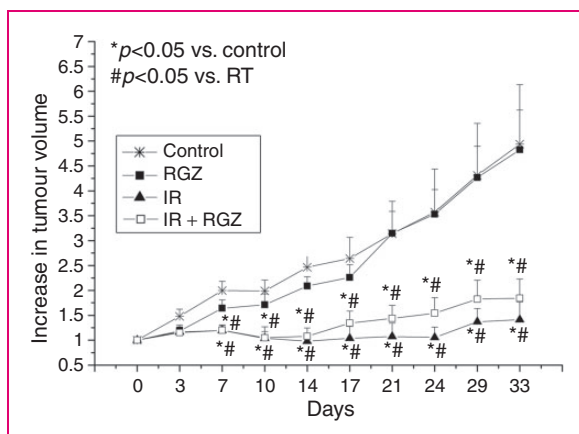


Figure 6. Rosiglitazone did not protect the tumour from irradiation.

When HT-29 tumour xenograft diameter reached 5 mm, mice were treated with vehicle (asterisks), RGZ (5 mg/kg, black squares), 16 Gy irradiation (IR, black triangles) or IR + RGZ (white squares). Tumour volume was measured twice a week for 33 days. Data are mean \pm SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. RGZ.

showed that RGZ reduced protein expression of the fibrosis marker TGF- β and phosphorylation of the p65 subunit of NF- κ B – the signalling pathway triggered by the proinflammatory cytokine TNF α .

On the basis of these data, we demonstrated that RGZ reversed the inflammatory and apoptotic changes induced by radiation, and that it had a radioprotective effect on irradiated healthy bowel.

In addition, we wanted to determine if the radioprotective effect of RGZ was selective for normal tissues or if it also occurred in tumour cells. A differential activity between normal and cancer tissues is critical for a putative radioprotective agent. Our *in vitro* clonogenic survival studies demonstrated a significant decrease in surviving cells irradiated in presence of RGZ, as compared with IR alone. According to previous works,¹⁸ RGZ has a radiosensitizing effect on human bowel cancer cells. *In vivo* studies in a HT-29 xenograft model failed to demonstrate a radiosensitizing effect but showed that RGZ did not protect the tumour from IR.

The anti-inflammatory effect of RGZ on the irradiated bowel and the lack of effect on the therapeutic activity of radiotherapy on irradiated cancer cells suggest that PPAR- γ agonists should be further investigated as a possible therapeutic option for the prevention and treatment of acute radiation-induced bowel injury.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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