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Original Articles

Piperlongumine increases sensitivity of colorectal cancer cells to radiation: Involvement of ROS production via dual inhibition of glutathione and thioredoxin systems

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ABSTRACT

Piperlongumine (PL), naturally synthesized in long pepper, is known to selectively kill tumor cells via perturbation of reactive oxygen species (ROS) homeostasis. ROS are the primary effector molecules of radiation, and increase of ROS production by pharmacological modulation is known to enhance radioresponse. We therefore investigated the radiosensitizing effect of PL in colorectal cancer cells (CT26 and DLD-1) and CT26 tumor-bearing mice. Firstly, we found that PL induced excessive production of ROS due to depletion of glutathione and inhibition of thioredoxin reductase. Secondly, PL enhanced both the intrinsic and hypoxic radiosensitivity of tumor cells, linked to ROS-mediated increase of DNA damage, G2/M cell cycle arrest, and inhibition of cellular respiration. Finally, the radiosensitizing effect of PL was verified *in vivo*. PL improved the tumor response to both single and fractionated radiation, resulting in a significant increase of survival rate of tumor-bearing mice, while it was ineffective on its own. In line with *in vitro* findings, enhanced radioresponse is associated with inhibition of antioxidant systems. In conclusion, our results suggest that PL could be a potential radiosensitizer in colorectal cancer.

1. Introduction

Radiotherapy, particularly low linear energy transfer radiation such as X-rays, exerts its cytotoxic effects towards cancer cells primarily through generation of reactive oxygen species (ROS). About two-thirds of radiation-induced DNA damage is attributed to ROS in mammalian cells. Concordantly, ROS scavenger reduces the radiation induced biological effects by a factor close to 3 [1].

In cancer cells, ROS are continuously generated as a by-product of metabolism and possess multifaceted functions depending on the concentrations [2]. At low to moderate levels, ROS aid tumor development and aggressiveness through accelerating the rate of cancer-causing mutations and activating various signalling pathways. At high levels, ROS are toxic to tumor cells by causing oxidative damage to different components of the cell, such as DNA, proteins, and lipids. Compared with normal cells, cancer cells usually harbour elevated ROS as a result of their uncontrolled proliferation and high metabolic rate [3]. To restrict ROS levels below a threshold in order to avoid cytotoxicity, antioxidant enzymes are commonly dysregulated in cancer cells, which ascribes to their radioresistance [4]. Thus targeting redox proteins has become an attractive strategy to increase the therapeutic efficacy of radiotherapy with dozens of compounds examined in preclinical studies and several being tested in clinic trials [5–8].

Piperlongumine (PL), a biologically active alkaloid and naturally synthesized in long pepper, is widely used as a traditional medicine in Asia and the Pacific islands [9]. PL selectively kills cancer cells in diverse models and delays tumor growth in sarcoma, melanoma, pancreatic, bladder, breast, lung, gastric, and colon cancers [10–19]. Various mechanisms have been suggested to underlie the cytotoxic effect of PL, for example, induction of apoptosis and autophagy, restoration of mutant p53, and cell cycle arrest [19–21]. However, it appears that among the mechanisms, there is a common concordance that these cellular changes triggered by PL are frequently associated with increased production of ROS. As aforementioned, ROS are persistently generated in cells with both beneficial and detrimental effects. To keep the balance, cells evolve sophisticated antioxidant systems in which glutathione (GSH) and thioredoxin (Trx) systems represent major pathways [22–24]. PL proved to perturb GSH system by inhibition of

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glutathione S-transferase (GST) that catalyzes the conjugation of GSH with its substrate [25,26]. Recently, PL has been reported to target Trx system as well by inhibition of thioredoxin reductase (TrxR), an enzyme that sustains the reduced form of Trx, leading to apoptosis of cancer cells [27].

GSH and Trx systems are over activated in cancer cells, and enzymes implicated in these two systems such as GST and TrxR are commonly overexpressed in different types of cancer including colorectal cancer, which is correlated to poor outcome [28–30]. Pre-operative (chemo) radiation is standard of care in locally advanced rectal cancer. Patients demonstrating a complete response after (chemo)radiation can benefit further from an optional non-operative treatment, defined as "watch and wait" approach [31]. As a research focus to improve the radioresponse of rectal cancer, hereby, we examined the radiosensitizing effect of PL in colorectal cancer cells at both aerobic and hypoxic conditions. Next, to unveil the mechanisms, activity of GSH and Trx systems, ROS production, ROS-induced DNA damage, cell cycle arrest, and oxygen consumption rate of tumor cells were assessed. Importantly, the effects as well as the underlying mechanisms *in vitro* were validated *in vivo*.

2. Materials and methods

2.1. Cell lines and chemicals

Mouse colon adenocarcinoma cell line CT26 and human colon adenocarcinoma cell line DLD-1 were obtained from American Type Culture Collection (ATCC, Manassas, USA). All experiments were performed in RPMI 1640 medium (Thermo Fisher, Belgium) supplemented with 10% bovine calf serum (Greiner Bio-one, Belgium). PL was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Chemicals were obtained from Sigma-Aldrich (Antwerp, Belgium) unless otherwise stated.

2.2. Treatments

CT26 and DLD-1 cultures were grown to subconfluence and treated with PL for 3 h at indicated concentrations [27]. The ROS scavenger N-acetyl cysteine (NAC) was added at 10 mM to cultures both 1 h prior and during treatment with PL. Afterwards, cultures were used for further analysis as described below.

2.3. Cytotoxicity and MTT assay

A number of 50,000 cells were plated in 200 µl medium in 96-well plates and allowed to adhere overnight. Afterwards, PL was added at indicated concentrations for 3 h, and cultures were rinsed with fresh medium and re-incubated for additional 24 h. Next, 50 µl of the MTT reagent (5 mg/ml) was added for 3 h, and then 150 µl of DMSO was admixed to dissolve the formazan crystals. Absorbance was measured at a wavelength of 540 nm by using a spectrophotometer (Biorad, Temse, Belgium). Cell viability was determined by dividing the absorbance values of treated cells with untreated cells.

2.4. Radiation and clonogenic assays

Radiation and clonogenic assays were performed, and hypoxia was induced as reported in detail elsewhere [32]. To induce hypoxia, 0.5×10^6 cells were collected into a 15 ml conical tube and centrifuged into micropellet at 2000 rpm with a volume of 100 µl [33]. Prior to radiation, the micropellets were placed in a 37 °C water bath for 5 min in order to metabolically consume oxygen and induce hypoxia. Micropellets (hypoxic radioresponse) and monolayer cell cultures (aerobic radioresponse) were then irradiated at indicated doses with a Linac (Elekta SLi, Crowley UK) using energy of 6 MV at a dose rate of 400 MU/min and reseeded for colony. After 8–12 days, cultures were fixed with crystal violet, and colonies (> 50 cells) were counted. Survival curves were fitted to the linear quadratic model using GraphPad Prism 6 software (GraphPad Prism Software Inc., La Jolla, CA, USA). Radiosensitization was expressed as an enhancement ratio determined at a survival fraction (SF) of 0.1.

2.5. ROS production

The intracellular level of ROS was detected using 5-(6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA), an oxidation-sensitive fluorescent probe (Abcam, Cambridge, UK). Briefly, cells were treated with PL for 3 h, and then stained with 5 µM CM-H_2DCFDA at 37 °C for 30 min. The mean fluorescence intensity was measured by a FACSCanto flow cytometer (BD Biosciences) and calculated by Flowjo software (TreeStar, Ashland, OR, USA).

2.6. GSH assay

GSH levels were measured by using a commercial GSH assay kit (Cayman, US). Briefly, after treatment, cells were washed twice with PBS and resuspended in cold MES buffer. Next, cells were lysed by sonication for 1 min and then centrifuged at 10000 rpm for 15 min at 4 °C, followed by deproteinization by phosphoric acid. Afterwards, 50 µl of the collected supernatant was added to 150 µl assay cocktail, and absorbance was measured at 405 nm during 30 min with 5-min intervals using a spectrophotometer (Biorad, Temse, Belgium).

2.7. TrxR activity

TrxR activity was measured by using a commercial kit (Sigma-Aldrich, Antwerp, Belgium) according to the manufacturer's instructions. In this assay, TrxR catalyzes the reduction of 5, 5-dithiobis (2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid, which generates a strong yellow color as the indication of the activity of TrxR. Briefly, cells lysed by CelLytic Buffer (Sigma-Aldrich, Antwerp, Belgium) were disrupted by sonication for 1 min. Afterwards, 180 µl of TrxR assay buffer containing DTNB and NADPH was added to 10 µl of supernatant and then measured by a spectrophotometer at 412 nm for 30 min. TrxR activity was presented as percentage of control.

2.8. Cell cycle assay

Cell cycle was analysed by using propidium iodide (PI). Briefly, cells were harvested by trypsinization and then washed with FACS buffer. Afterwards, 1×10^6 cells were resuspended in 300 µl 50 µg/ml PI staining solution and incubated for 4 h at 4 °C in the dark. Cell cycle was then measured within 30 min by a flow cytometer and analysed by Flowjo software.

2.9. Double-strand DNA breaks

DNA damage was determined by measuring the extent of phosphorylation of the histone protein γH2AX. Briefly, cells collected after treatment were fixed in fixation buffer (Miltenyi biotec, Leiden, Netherlands) for 15 min at room temperature and then permeabilized for another 20 min in 90% methanol at −20 °C. Afterwards, cells were incubated with 0.1 µg γH2AX antibody (Abcam, Cambridge, UK) for 40 min at 4 °C. The mean fluorescence intensity was measured by FACS and analysed by Flowjo software.

2.10. Oxygen consumption rate

Oxygen consumption rate was determined using a Seahorse XF96 analyzer (Seahorse Biosciences, North Billerica, MA, USA) as previously reported [34]. Briefly, 2×10^5 CT26 cells or 3×10^5 DLD-1 cells were seeded in 96-well plates overnight and then treated with PL for 3 h.

Afterwards, cultures were equilibrated in unbuffered DMEM medium with 2 mM glutamine and 10 mM glucose at 37 °C in a CO_2-free incubator, and then measured by Seahorse analyzer. To extract detailed information on the electron transport chain in mitochondria, specific inhibitors consisting of oligomycin, FCCP, rotenone, and antimycin A were added sequentially.

2.11. Mouse tumor model

CT26 cells (0.5×10^6) were inoculated intramuscularly into the left hind limb of BALB/c mice (Charles River Laboratories, L'Arbresle Cedex, France). Once the tumors become palpable, mice were randomized into 6 mice/group. PL (2.4 mg/kg) was administered intraperitoneally (i.p.) from day 6–19 after inoculation of tumor cells (day 0). Tumors were irradiated either with a single fraction of 8 Gy on day 9 or with three-fractionated radiation of 3 Gy on days 9, 11 and 13 with a Linac (Elekta SLi, Crowley UK) using energy of 6 MV at a dose rate of 400 MU/min. The tumor volume was calculated using the formula: volume = (length * width^2)*0.5. All the experiments were performed according to directive 2010/63/EU of the European Parliament for the protection of animals used for scientific purposes and approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (No. 18-552-1).

2.12. Oxidative stress in vivo

GSH content, TrxR enzymatic activity and malonydialdehyde (MDA) assays were conducted to assess the oxidative state in CT26 tumor. Tumor bearing mice were administered with either PBS (control group) or PL i.p. for 3 consecutive days from day 6–8 after inoculation of tumor cells (day 0). One hour after the last administration, tumors were resected and cut into pieces of 40 mg each. Fragments of tumor were then homogenized by TissueLyser (Qiagen, Antwerp, Belgium), and lysed by MES buffer (GSH assay), TrxR assay buffer or RIPA buffer (MDA assay), and followed by sonication for 1 min. The supernatants were then collected respectively for GSH, TrxR, and MDA assays according to the manufacturer's instructions.

2.13. Statistics

All assays were repeated at least three times. A one-way ANOVA followed by a Bonferonni's multiple comparison tests was performed using GraphPad Prism 6. In cell survival curves, two-way ANOVA was performed for statistical comparison of the radiosensitivity among groups, and P-values < 0.05 were considered significant. Data are expressed as mean with corresponding standard deviations. The number of asterisks in the figures indicates the level of statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

3. Results

3.1. Cytotoxic profile of PL in colorectal cancer cells

The cytotoxic profile of PL was tested in murine and human colorectal cancer cell lines CT26 and DLD-1, which have been extensively studied in our lab. CT26 and DLD-1 cell cultures were exposed to different concentration of PL for 3 h, and cell viability was determined by MTT assay. As shown in Fig. 1A, PL decreased the cell viability in a dose-dependent manner with an IC_{50} of 15.98 μM and 11.20 μM in CT26 and DLD-1 respectively. To determine whether apoptosis and necrosis were involved in PL-induced cytotoxicity, cancer cells exposed to PL were stained with Annexin-V/7-AAD followed by flow cytometry analysis (Supplementary Fig. 1). We observed an increase of the apoptosis and necrosis to 6.2% and 9.9%, and 12.9% and 18.7% at 15 μM in CT26 and DLD-L cells respectively. While the percentage of apoptotic cells are much lower as compared with the findings from others at same concentrations [27,35], which is most probably due to the shorter exposure time to PL (3 h vs 24 h) and different cell types. In addition, the impact of PL on cell proliferation was assessed by colony formation assay. PL impaired the proliferation capacity of both cell lines as evidenced by the reduction in the number of colonies (Fig. 1C–E). Based upon the data from MTT and colony formation assays, maximal doses of PL at 15 μM and 10 μM with acceptable toxicity towards CT26 and DLD-1 respectively were used for radiation experiments.

3.2. PL induced ROS production via dual targeting of GSH and Trx systems

Cytotoxicity of PL was commonly reported to be associated with upregulation of intracellular ROS [25,36], a phenomenon leading to radiosensitization as well [5–7]. We first examined the ROS levels in tumor cells by using CM-H_2DCFDA probe. As shown in Fig. 2A–C, PL triggered ROS production in both CT26 and DLD-1 cells in a dose-dependent manner ($*p < 0.1$ and $**p < 0.01$), reaching 3-fold augmentation at 15 μM and 10 μM respectively. These effects were counteracted by addition of ROS scavenger NAC.

Since antioxidant GSH system is the primary target of PL [25,27], we next measured GSH level in cells after exposure to PL at indicated concentrations. In line with the increased ROS production, PL decreased GSH levels in both cancer cell lines, reaching 45% and 40% inhibition at 15 μM and 10 μM respectively for CT26 and DLD-1 (Fig. 2D and E). GSH and Trx systems back up each other to detoxify endogenous and exogenous ROS [37]. Simultaneous inhibition of both systems leads to synergistic cytotoxic and radiosensitizing effects [38,39]. Next to GSH system, PL is reported to target Trx system via inhibition of TrxR as well [27]. In our settings, PL inhibited the activity of TrxR by 35% and 30% at 15 μM and 10 μM respectively for CT26 and DLD-1 (Fig. 2F and G). Thus, excessive production of ROS induced by PL in colorectal cancer cells is caused by inhibition of both GSH and TrxR systems.

3.3. PL triggered DNA damage and cell cycle arrest via ROS

ROS as super active chemical species can cause detrimental effect to cells through reaction with various cellular components. Among ROS-induced damages, DNA lesions have profound effects on cell viability, particularly double-strand DNA (ds-DNA) damages that are known as the primary cause of radiation-induced cell death [40]. We therefore examined the ds-DNA damage by quantifying the phosphorylation status of γH2AX. As shown in Fig. 3A and B and Supplementary Fig. 2, PL increased the formation of ds-DNA damages in both CT26 and DLD-1, which was abolished by NAC, indicating that this effect is mediated by ROS.

In response to DNA damage insults, cell cycle checkpoints are normally activated, followed by arresting cells in certain cell stage to initiate the process of DNA repair [41]. We then asked whether PL could induce cell cycle arrest, more appealing, G2/M arrest that confers enhanced radiosensitivity. As illustrated in Fig. 3C and D, PL modulated the cell cycle distribution and arrested CT26 and DLD-1 cells in G2/M phase at a dose of 15 and 10 μM respectively. To ensure this effect was underpinned by ROS, cells were treated with both NAC and PL, wherein the effect was completely counteracted.

3.4. PL inhibited oxygen consumption via ROS

Mitochondria as the primary site of ROS production, accounting for about 90% of the total cellular ROS generation, heavily rely on antioxidants to sustain homeostasis [42]. Depletion of GSH or inhibition of TrxR has been shown to impair the normal function of mitochondria, leading to decreased oxygen consumption rate that currently is considered as an attractive approach to overcome hypoxic radioresistance [38,43]. We therefore assessed oxygen consumption rate of colorectal

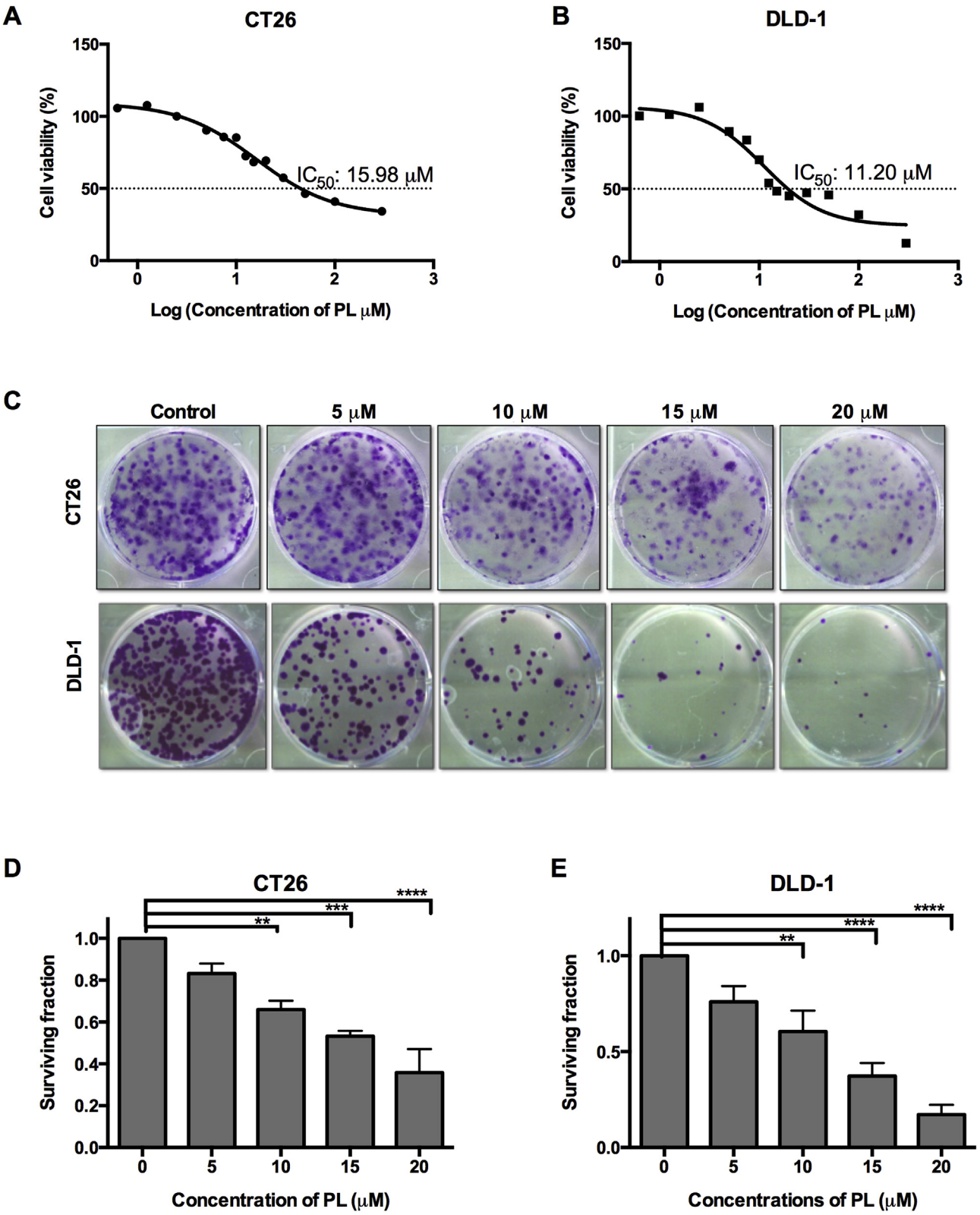

Fig. 1. Cytotoxic profile of PL in colorectal cancer cells. CT26 and DLD-1 cells were treated with PL for 3 h at indicated concentrations. (A–B) Cell viability was assessed by MTT assay at 24 h after treatment, and IC_{50} was calculated accordingly. (C–E) Cell proliferation capacity was evaluated by colony formation assay, wherein cells after treatment were plated with a density of 1000 cells/well and grown for 8–12 days followed by staining. (C) Representative colonies of CT26. (D–E) Quantitative results of colony formation assay. Data are shown as mean ± SD (n = 3). One-way ANOVA with multiple comparison test was used to calculate statistics: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

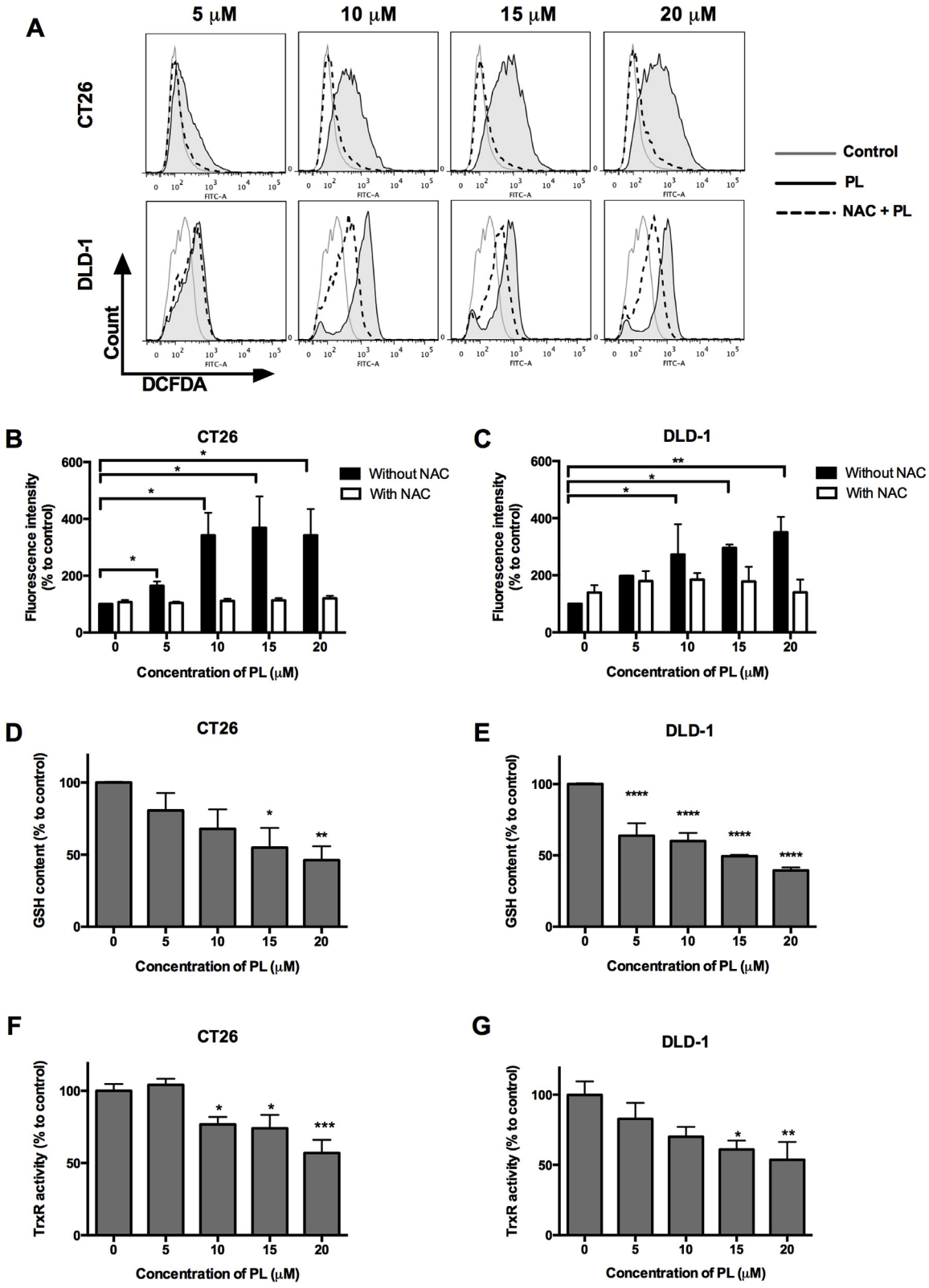

Fig. 2. PL induced ROS production in colorectal cancer cells via depletion of GSH content and inhibition of TrxR activity. CT26 and DLD-1 cells were treated with PL for 3 h at indicated concentrations, while NAC (10 mM) was added 1 h prior and during treatment. (A–C) ROS generation was measured by flow cytometry using CM-H_2DCFDA probe. (A) Representative histogram of intracellular ROS. (B–C) Summarized data on ROS production in CT26 and DLD-1 cells with or without the pretreatment of ROS scavenger NAC. (D–E) GSH levels and (F–G) TrxR activity were determined by commercial kits and represented as percentage of that of untreated control. Data are shown as mean ± SD (n ≥ 3). One-way ANOVA with multiple comparison test was used to calculate statistics: $^{*}p < 0.05$, $^{***}p < 0.001$.

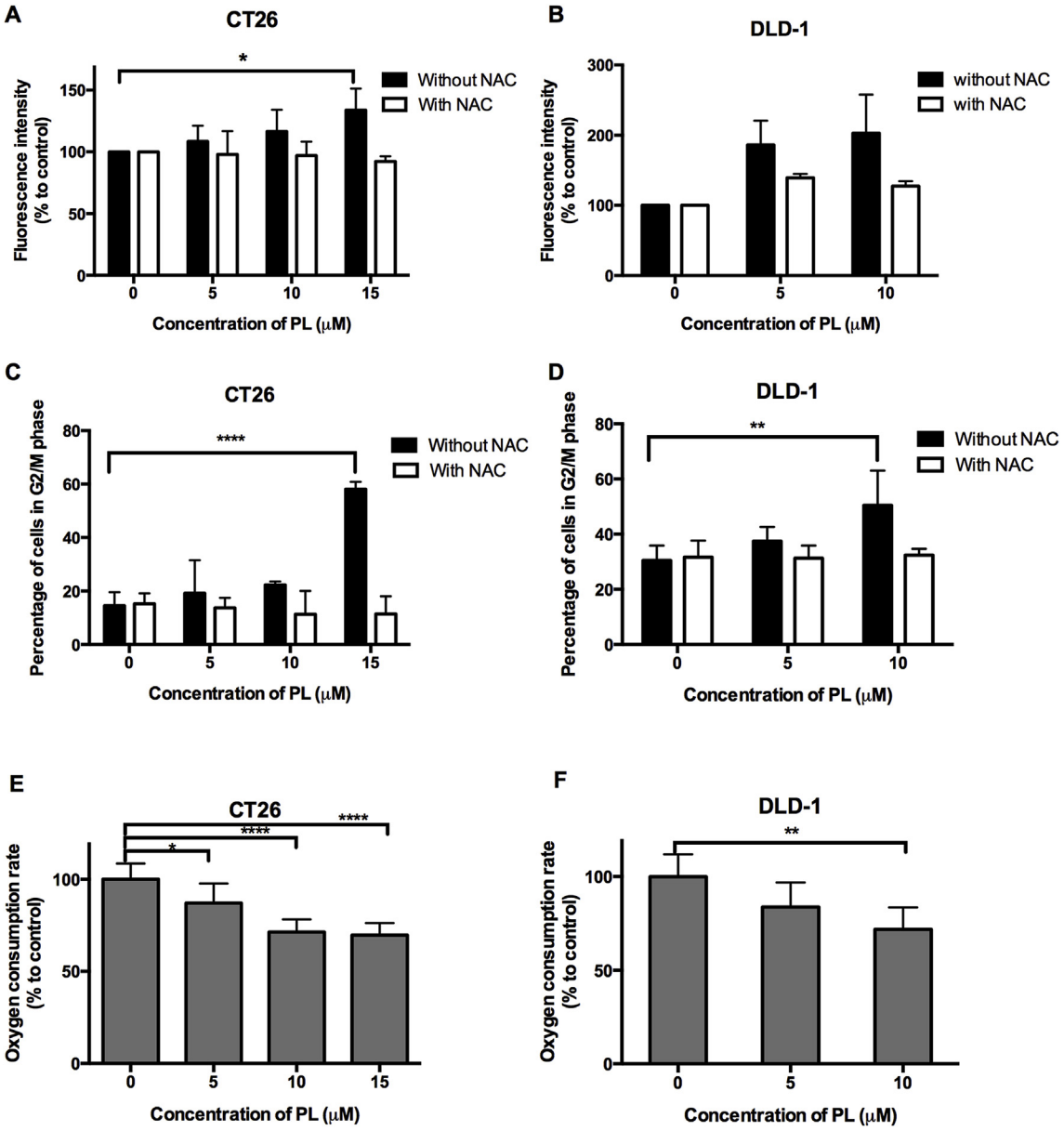

Fig. 3. PL induced DNA damage, G2/M arrest, and cellular respiration inhibition in colorectal cancer cells, which were associated with ROS production. CT26 and DLD-1 cells were treated with PL for 3 h at indicated concentrations, while NAC (10 mM) was added 1 h prior and during treatment. (A–B) Double-strand DNA breaks were analysed by flow cytometry using the γH2AX-based foci measurements. (C–D) G2/M cell cycle arrest was analysed by flow cytometry using PI staining. (E–F) Oxygen consumption rate was measured by Seahorse Analyzer, and data on the baseline respiratory rate was represented as percentage of that of untreated control. Data are shown as mean ± SD (n ≥ 3). One-way ANOVA with multiple comparison test was used to calculate statistics: $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

cancer cells after exposure to PL by using Seahorse analyzer. As shown in Fig. 3E and F, PL significantly decreased basal oxygen consumption rate of both CT26 and DLD-1 cells at doses above 5 μM and 10 μM respectively. Furthermore, in CT26, ATP output as well as the maximal respiratory capacity was reduced, whereas these were not observed in DLD-1 (data not shown).

3.5. PL radiosensitized both aerobic and hypoxic tumor cells mediated by ROS

Our data so far demonstrated that PL inhibited both GSH and Trx systems in colorectal cancer cells resulting in excessive production of ROS, subsequently leading to augmented ds-DNA damage and G2/M arrest as well as declined cellular respiration. All these effects triggered by ROS are contributable to enhance radiosensitization [8,38]. We then came to the key question: can PL radiosensitize colorectal cancer cells? To answer this question, the radiosensitivity of CT26 and DLD-1 cells were assessed in both aerobic and hypoxic conditions by colony formation assay. In line with increased ROS production, PL enhanced the radioresponse of both CT26 and DLD-1 in a dose-dependent manner in aerobic conditions. At the dose of 15 μM and 10 μM, the enhancement ratio reached to 1.58 and 1.74 respectively for CT26 and DLD-1 (Fig. 4A and B). This effect was completely reversed by NAC, examined in indicated doses of PL at 4 Gy (Fig. 4C and D). ROS generated by radiation and PL can trigger apoptosis and necrosis [35]; therefore, we speculated that the combination might further accelerate cell death. We found that PL at 10 μM integrated with 4 Gy increased the apoptotic and necrotic rate to 6.7% and 13.9%, and 15.9% and 22.3% respectively in CT26 and DLD-L cells (Supplementary Fig. 3).

To evaluate the hypoxic radioresponse, we used a simplified

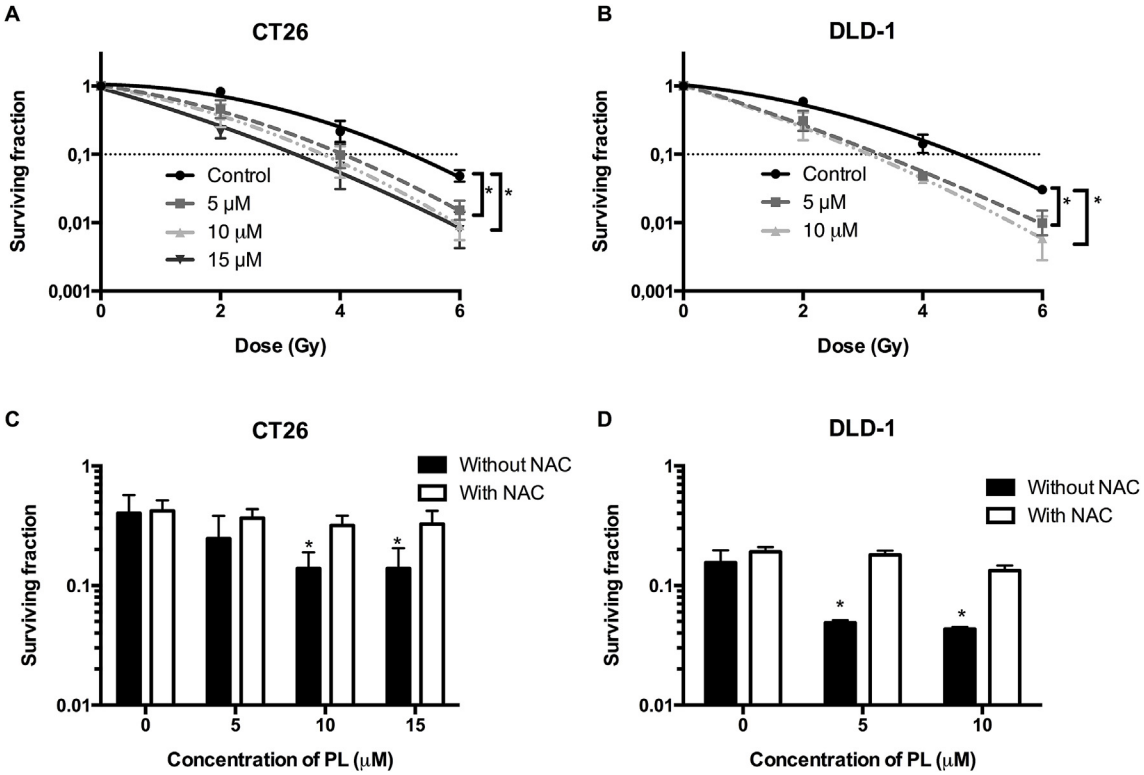

Fig. 4. PL sensitized aerobic colorectal cancer cells to radiation. CT26 and DLD-1 cells were treated with PL for 3 h at indicated concentrations, while NAC (10 mM) was added 1 h prior and during treatment. Afterwards, cells were radiated at aerobic conditions and reseeded for colony formation assay. (A–B) Dose-response curves of tumor cells upon exposure to different concentrations of PL. (C–D) Pretreatment of NAC reversed radiosensitizing effect of different concentrations of PL at 6 Gy. Data are shown as mean ± SD (n ≥ 3). Two-way ANOVA with multiple comparison test was used to calculate statistics: $*p < 0.05$.

metabolic micropellet model to prove the concept in which altered oxygen consumption rate could change the extent of hypoxia unlike widely used hypoxia chamber with fixed oxygen levels. Herein, an up to 3-fold radioresistance was observed as compared with aerobic conditions at 0.1 survival rate (Fig. 5A and B; Fig. 4A and B), indicating the existence of a deep hypoxia in micropellets. In this model, PL overcame hypoxic radioresistance and enhanced the radiosensitivity of CT26 and DLD-1 cells with an enhancement ratio up to 1.62 and 1.78 at 15 and 10 μM respectively (Fig. 5A and B). Likewise to aerobic conditions, addition of NAC fully abolished radiosensitizing effects induced by PL (Fig. 5C and D). In sum, these data suggest that PL sensitizes both aerobic and hypoxic colorectal cancer cells to radiation owing to ROS overproduction. ROS are universal radiosensitizers; so theoretically this effect could extrapolate to other type of cancer cells. To confirm it, we further examined the radiosensitizing effect of PL in breast cancer cell line 4T1. Similarly, the radiosensitivity of 4T1 was enhanced after exposure to PL in both aerobic and hypoxic conditions (Supplementary Fig. 4).

3.6. PL enhanced radioresponse of CT26 tumor associated with oxidative stress

With the promising *in vitro* data, we then examined the combinational effect of PL and radiation in CT26 tumor bearing mice. The experimental scheme was depicted in Fig. 6A. PL at a dose of 2.4 kg/mg/day for consecutive 13 days was adapted based upon results from other research groups [44]. Single (8 Gy) and fractionated radiations (3 Gy X 3) were chosen after a preliminary study, as these doses can induce substantial CT26 tumor growth delay but not eradicate the tumor (unpublished data). We found that radiation alone at 8 Gy or 3 Gy X 3 delayed the tumor growth by 24 and 22 days respectively as compared with control at a tumor volume of 1000 mm^3. PL alone did not exhibit any inhibitory effect on tumor growth; however, when combined with radiation, tumor growth was delayed by extra 6 and 9 days as compared with single and fractionated radiation alone at tumor volume of 1000 mm^3 (Fig. 6B and C). The combination improved the survival rate of mice as well, importantly, without causing obvious toxicity measured by body weight loss (Supplementary Fig. 5).

In vitro, both aerobic and hypoxic radiosensitization induced by PL were correlated with overproduction of ROS. We then explored whether similar mechanism underlies the effect *in vivo*. To do so, GSH content, TrxR activity, and lipid peroxidation were examined in resected tumor after PL administration. In line with the *in vitro* findings, PL significantly reduced GSH content and TrxR activity as compared with control (Fig. 6D and E). Furthermore, PL increased the level of lipid peroxidation, an indicator of oxidative stress and measured by MDA assay (Fig. 6F). Altogether, both *in vitro* and *in vivo* data point out that disruption of antioxidant defence systems ascribes to PL enhanced response of colorectal cancer to radiation.

4. Discussion

We demonstrate that PL overcomes both hypoxic and intrinsic radioresistance of colorectal cancer cells and, subsequently, delays tumor growth and improves the survival rate of tumor-bearing mice. The mechanisms underlying the radiosensitizing effect is linked to DNA damage, cell cycle arrest, and inhibition of cellular respiration, which is induced by upregulated ROS production due to the decrease of GSH and inhibition of TrxR.

Enhancement of intrinsic radiosensitivity by PL has been reported in breast cancer cells, ascribed to the increase of intracellular ROS levels and, consequently, augmentation of radiation-induced apoptosis [35]. Likewise, in anoxia-tolerant lung cancer cells, PL overcomes intrinsic radioresistance and slightly delays tumor growth [45]. However, in

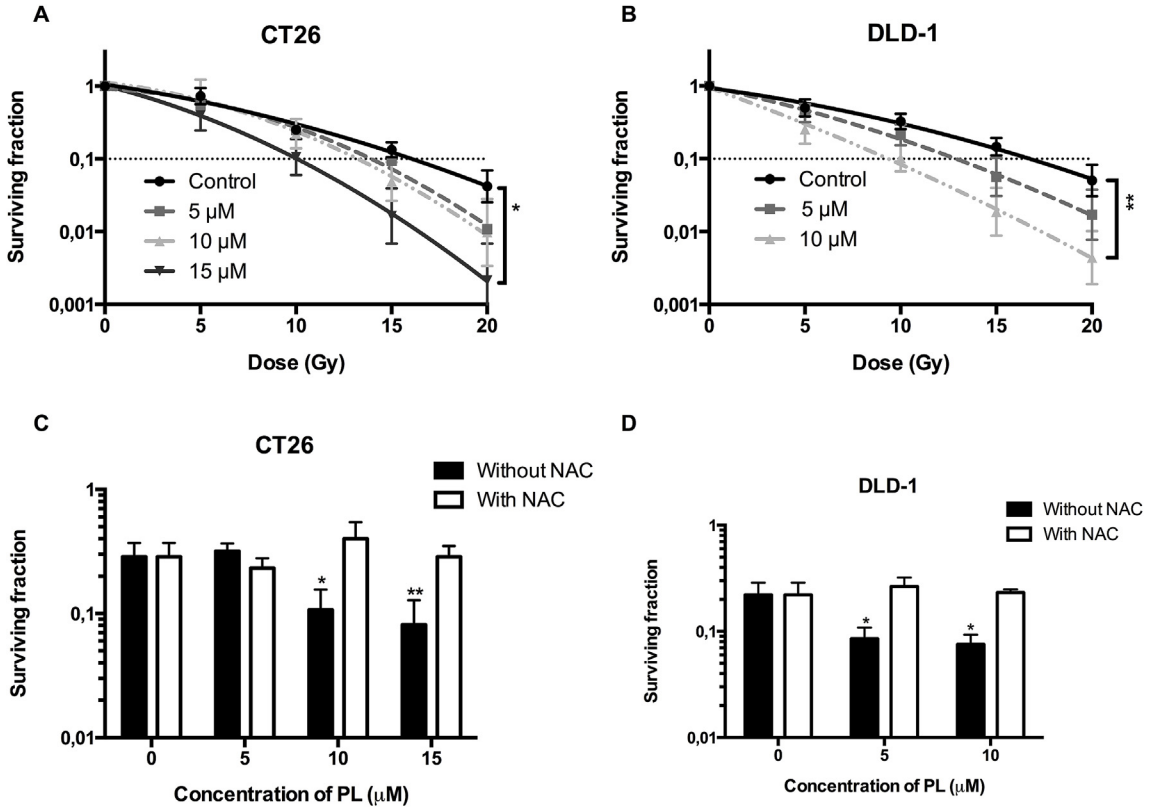

Fig. 5. PL sensitized hypoxic tumor cells to radiation. CT26 and DLD-1 cells were treated with PL for 3 h at indicated concentrations, while NAC (10 mM) was added 1 h prior and during treatment. Hypoxia was induced by micropellet model as described in methods and material. (A–B) Dose-response curves of tumor cells upon exposure to different concentrations of PL. (C–D) Pretreatment of NAC reversed radiosensitizing effect of different concentrations of PL at 10 Gy in hypoxic condition. Data are shown as mean ± SD (n ≥ 3). Two-way ANOVA with multiple comparison test was used to calculate statistics: $^{*}p < 0.05$, $^{**}p < 0.01$.

these two studies, the cellular target of PL, ROS mediated biological changes, and whether the *in vivo* effect is associated with ROS production are not well defined. Hereby, in our study, we first found that PL improved the response of colorectal cancer cells to radiation with ROS as the primary effector molecules. Next, we revealed that ROS production was triggered by depletion of cellular antioxidant GSH and decrease of TrxR activity. It is well known that ROS as signalling transducers and super active chemicals can interact with various cellular molecules, for instance DNA, cell cycle checkpoints, mTOR, and P53, which are reported to be targeted by PL as well but not yet linked to radioresponse [15,19,21,44]. In colorectal cancer cells, we found that ROS production induced by PL led to augmented ds-DNA damage and cell cycle arrest in G2/M phase, acting as downstream effects of ROS and attributing to radiosensitization. Yet we cannot exclude that other signalling pathways activated by ROS might implicate in PL-induced intrinsic radiosensitization, which deserves further investigation [15,16,18–21].

Hypoxia, as a result from an imbalance between oxygen supply and consumption, is a common feature of the tumor microenvironment and a well-defined factor for clinical radioresistance [46]. Intriguingly, in tumors a partnership exists between hypoxia and ROS. Hypoxia enhances ROS generation via prolongation of the lifetime of the semiquinone radicals; reciprocally, ROS assist tumor cells to adapt to hypoxia via stabilization of HIF1-α [47]. However, extracellular insults could break down this partnership via triggering excessive ROS production, which impairs mitochondrial respiration and thus decreases hypoxia fraction in tumor [38,43]. In this context, arsenic trioxide inhibits the oxygen consumption of tumor cells through increase of intracellular ROS, leading to enhanced radioresponse [43]. Hypoxic radioresistance, mechanically, is due to repair of radiation-induced DNA lesions by compounds containing sulfhydryl groups such as GSH and Trx [48]; thus, diminution of them contributes to hypoxic radiosensitization as well. Indeed, dimethylfumarate, buthionine sulphoximine (BSO), and auranofin radiosensitize hypoxic tumors cell via depletion of thiols [38,39,49]. Consistent with these findings, in our study, PL depleted GSH and inhibited oxygen consumption rate, resulting in improved hypoxic radioresponse of colorectal and breast cancer cells.

Pharmacological agents, targeting diverse components of GSH and Trx systems, have been explored as radiosensitizers since decades and succeeded to demonstrate radiosensitizing effects in various *in vitro* models [50–53]. However, the majority of them displayed disappointing results in tumor bearing mice. In this respect, BSO as a classical drug to inhibit the production of GSH exhibits substantial radiosensitizing effect in lung, breast, head and neck, and renal cancer cells in both oxygenated and hypoxic conditions [54–56], but with a marginal effect *in vivo*. Likewise, auranofin, a well-characterized irreversible TrxR inhibitor, demonstrates considerably radiosensitizing effect *in vitro*, while fails to significantly enhance radioresponse *in vivo* [38]. A possible interpretation of this discrepancy is due to the interaction between GSH and Trx systems. These two systems back each other up to maintain cellular redox homeostasis, and inhibiting one of them activates another to compensate the loss of antioxidant [37,57]. Therefore, it is presumable that simultaneously inhibiting both systems, excessive ROS and the radiosensitizing effect might be more attainable with lower and tolerable concentrations of the agents. Indeed, dual targeting of GSH and Trx systems by BSO and auranofin substantially enhances radioresponse and increases survival rate of tumor-bearing mice without noticeable toxicity [38]. Consistently, in our syngeneic colorectal tumor model, PL as an inhibitor of both GSH and Trx systems improved radioresponse via augmentation of ROS, verified by decreased GSH and increased lipid peroxidation. We cannot exclude that

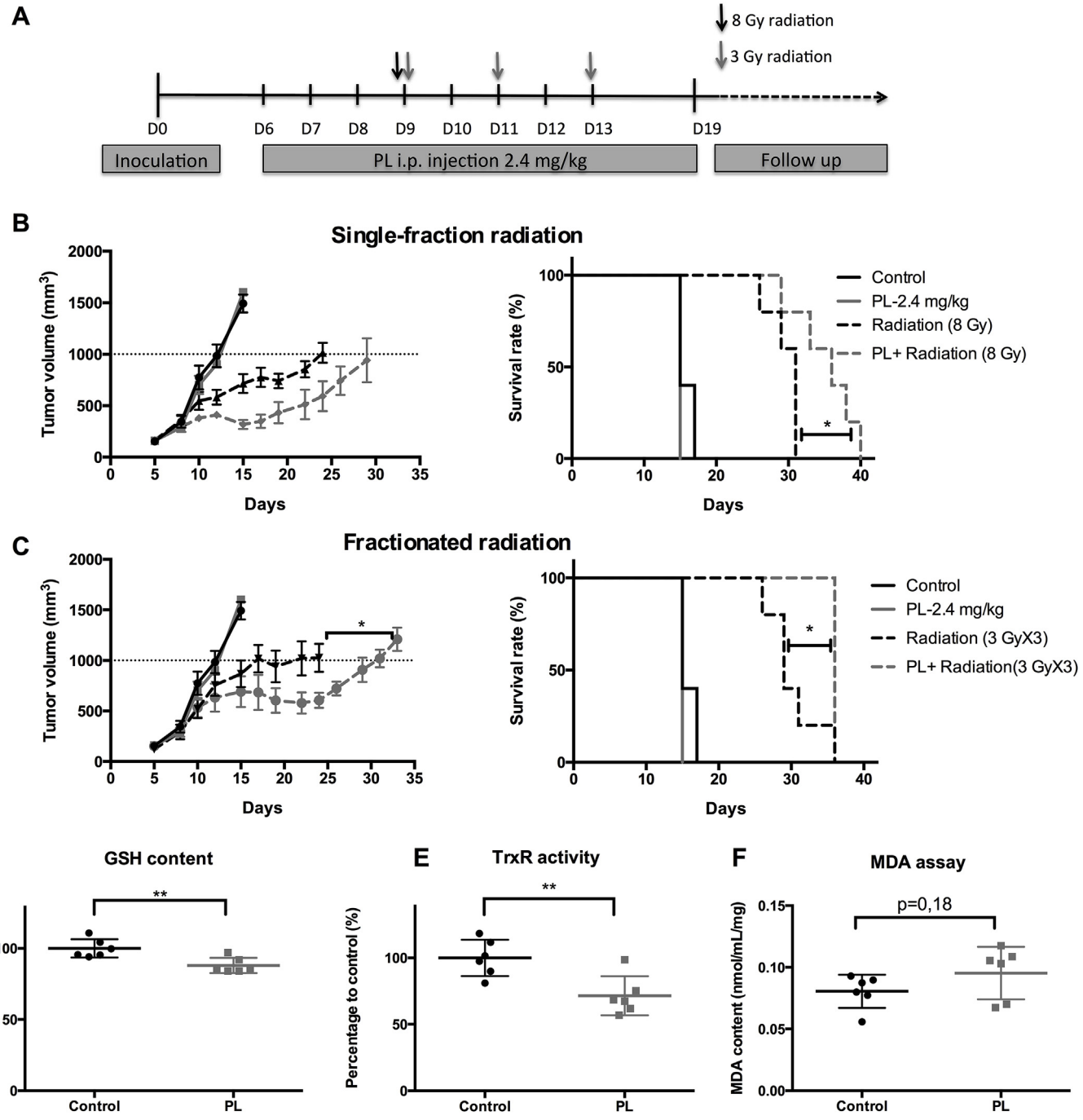

Fig. 6. PL enhanced the radioresponse of CT26 tumor via increase of oxidative stress. After inoculation of CT26 tumor cells into mice (taken as day 0), PL (2.4 mg/kg) was administered intraperitoneally for consecutive 13 days starting at day 6. Tumors were radiated either with a single fraction of 8 Gy or with 3 times 3 Gy every other day starting after 3 times administration of PL. Tumor was measured by electronic calliper every other day. The toxicity was assessed by body weight loss, and mice were euthanized at diameter of 15 mm. (A) Experimental scheme. (B) Tumor growth and survival curves after treatment with PBS, PL, 8 Gy, and PL plus 8 Gy. (C) Tumor growth and survival curves after treatment with PBS, PL, 3 × 3 Gy, and PL plus 3 × 3 Gy. Regarding oxidative stress, tumors were resected after administration of either PBS or PL (2.4 mg/kg) for 3 consecutive days, and analysed by commercial kits according to manufacturer's instructions. (D) GSH content. (E) TrxR enzymatic activity. (F) Lipid peroxidation by MDA assay. Data are shown as mean ± SD (n = 6). One-way ANOVA with Bonferonni's multiple comparison test was used to calculate statistics: $^*p < 0.05$. Log-rank test was used to test overall survival of mice receiving radiation treated with or without PL: $^*p < 0.05$.

the significant radiosensitizing effect and the weak systemic toxicity of PL is also due to the high bioavailability of PL *in vivo* and the high demanding of GST and TrxR in detoxifying ROS in tumor cells as compared with normal cells, which have been reported previously [9]. Furthermore, we have to admit that the simultaneous inhibition of GST and TrxR by PL may cause experimental issues, since silencing of both enzymes may result in severe cytotoxicity towards tumor cells, making the evaluation of radioresponse impossible [57–60]. Nevertheless, as a consequence of the increased oxidative stress in tumor, the radiosensitizing effect of PL in cell cultures translated into a significantly enhanced radioresponse in tumor-bearing mice, regardless in combination with single or fractionated radiation.

In colorectal cancer patients, the major obstacle to improve the overall survival lies at the distance metastases [61]. Growing evidence

suggests that radiotherapy can elicit a pronounced anti-tumor immune effect and convert the radiated tumor into an in situ vaccine [62–64]. In rare circumstances, radiotherapy can augment the innate and adaptive immune response to induce tumor regression at non-irradiated sites, known as abscopal effect, though this phenomenon to radiotherapy alone is rare [65,66]. So combining radiotherapy with immunotherapy stands great chance to tip the balance of the host immune response to suppress and even completely eradicate the metastases outside the radiation filed. So as the next step, it could be of great clinical relevance, to investigate the combinatorial effect of radiation, PL, and immune modulator with the hope to eliminate both local and distance tumor.

Taken together, our results demonstrate that PL radiosensitizes both aerobic and hypoxic colorectal cancer cells, mediated by ROS-induced DNA damage, cell cycle arrest, and inhibition of cellular respiration. Moreover, PL significantly improved radioresponse of colorectal tumor by inhibition of both GSH and Trx systems. Our study provides a rationale for the development of PL as a radiosensitizer for colorectal cancer, warranted further investigation.

Conflicts of interest

The authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.034.

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