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Radiosensitizing potential of Plumbagin in B16F1 melanoma tumor cells through mitochondrial mediated programmed cell death

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Abbreviations:
DCFH-DA, 2′,7′-dicholorofluorescein diacetate
Ψm, mitochondrial membrane potential
PARP, poly (ADP-ribose) polymerase
PI, propidium iodide
PLB, Plumbagin
RIPA, radioimmunoprecipitation assay
ROS, reactive oxygen species
RT, radiation
SER, sensitization enhancement ratio
SF, surviving fraction

ABSTRACT

The radiosensitizing potential of Plumbagin (PLB) against chemo- and radioresistant B16F1 melanoma cells growing *in vitro* was investigated. Clonogenic assay revealed a sensitization enhancement ratio (SER) of 1.5 for PLB treatment in combination with radiation. PLB pretreatment for 1 h prior to radiation resulted in elevated intracellular ROS levels compared to the group treated with radiation alone. Alkaline comet assay analysis revealed PLB's potential to enhance the radiation induced DNA damage. Cell cycle studies have shown enhanced G_2/M arrest for combination treatment of PLB with radiation. Cell death exerted by PLB combination was mainly through programmed cell death, involving the depletion of mitochondrial membrane potential, increase in the expression of p53, Bax, Cytochrome c, PARP and Caspase 3 cleavage. In conclusion, this study demonstrates the radiosensitizing potential of PLB to inhibit the growth of melanoma cells *in vitro*, which may be attributed to the oxidative stress and DNA damage leading to enhanced mitochondria-mediated programmed cell death. Also, this study demonstrate the ability of PLB to augment ionizing radiation induced tumor cell kill which further warrant the avenue for the development of a clinically useful radiosensitizer.

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Introduction

Radiation therapy, till date, remains the main non-surgical armamentarium against cancer either alone or in combination with chemotherapy. As individual entities, the failure of chemo- or radiation therapy in treating cancer is due to the resistance of tumor cells, heterogeneity in the tumor, and severe normal tissue toxicity caused by the curative doses of radiation and/or chemotherapeutic drugs. Major avenues of progress in overcoming these limitations have integrated the combined/multi modality approaches in a highly significant strategy as a curative and palliative intention wherein the design is to damage cancerous cells as much as possible, while limiting the harm to the nearby healthy tissues. Combined modality treatment employing a variety of chemotherapeutic agents and ionizing radiation is considered to be one of the strategies to enhance therapeutic efficacy, as compared to individual treatment alone (Bernier et al., 2004; Horsman et al., 2006). Research in this direction continues in the search of novel natural or synthetically derived molecules that can be combined with radiation to enhance the therapeutic outcome.

PLB (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinine (Fig. 1A) derived from *Plumbagineae* and *Droseraceae* families, has been widely demonstrated to possess anticancer potential. Besides its anticancer potential, it exhibits other biological activities, which include anti-inflammatory, antibacterial, and antifungal activities (Padhye et al., 2012). The anticancer activity of PLB has been well established in numerous cancer types (Srinivas et al., 2004; Hsu et al., 2006; Wang et al., 2008; Xu and Lu, 2010). It has been reported that quinine containing compounds exert their toxicity by interacting with cellular macromolecules such as glutathione and sulfhydryl-containing proteins (Babich and Stern, 1993). In addition, DNA remains to be the main target for all these quinoid antitumor agents and most of them belong to the group of DNA intercalating agents (Yamashita et al., 1991; Prasad et al., 1996; Xu and Lu, 2010; Padhye et al., 2012). PLB has been reported to induce mammalian topoisomerase II mediated DNA cleavage *in vitro* (Fujii et al., 1992). Suppression of NF-κB and its regulated gene products by modulation of p65 and IκBα kinase activation by PLB was also demonstrated (Sandur et al., 2006) and inhibition of microtubule polymerization has been reported recently (Acharya et al., 2008). A recent report published by Pan and colleagues (2015) also advocate the anti-cancer property of PLB

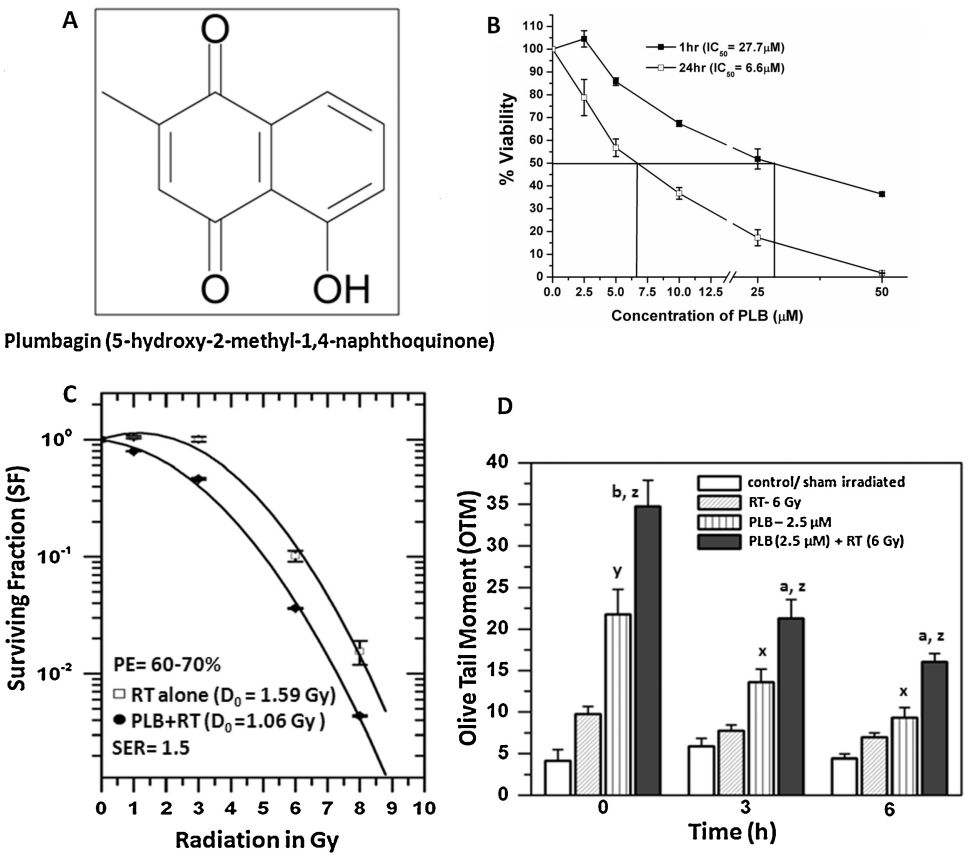

Fig. 1 – (A) Chemical structure of Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), (B) graph indicating the effect of PLB (2.5–50 μM) on cell viability as assessed at 1 h and 24 h time interval by MTT assay, (C) radiosensitizing potential of PLB *in vitro* against B16F1 melanoma cells assessed using the clonogenic assay (SER = 1.5), (D) radiosensitization potential of PLB against melanoma cells *in vitro* assessed using the alkaline comet assay. [x,y,z]Statistically significant when compared to respective control/sham irradiated group; [a,b]Statistically significant when compared to respective radiation treated group; RT – radiation.

against human tongue squamous cell carcinoma cells through the activation of ROS/GSK3β/p38 MAPK and PI3K/Akt/mTOR-mediated pathways. However, the radiosensitizing potential of PLB has not been explored much (Adhikari et al., 2005; Bischoff et al., 2009), except a previous study conducted by Nair and colleagues (2008) which showed the radiation-sensitization potential of PLB in cervical cancer cells. Hence, in light of the above-published reports, the aim of the present study was to assess the radiosensitizing potential of PLB against chemo- and radiation resistant B16F1 melanoma cells growing *in vitro*. We made an attempt to harness the pro-oxidant and genotoxic property of PLB in combination with ionizing radiation which further aided in enhanced cell killing by augmented expression of proteins responsible for programmed cell death.

Materials and methods

Chemicals and reagents

Plumbagin, Eagle's minimum essential medium (MEM), 2′,7′-dicholorofluorescein diacetate (DCFH-DA), L-glutamine, gentamycin sulfate, fetal bovine serum, RNase A, propidium iodide (PI), ethidium bromide (EtBr), ethylene diamine tetra-acetic acid (EDTA), triton-X 100, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St Louis, MO). Other chemicals were procured from Qualigens Fine Chemicals (Mumbai, India).

Cell line and culture

Mouse melanoma tumor cells (B16F1) obtained from the National Center for Cell Sciences (Pune, India) were routinely grown in 25 cm^2 T-flasks containing MEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 80 μg/mL gentamycin sulfate at 5% CO_2 in humidified air at 37 °C (NuAire, Plymouth, MN, USA).

Irradiation

Irradiation was done using ^{60}Co Gamma teletherapy facility (Theratron Atomic Energy Agency, Canada). For *in vitro* experiments, exponentially growing cells, with and without drug treatment, were irradiated at a dose rate of 1 Gray (Gy)/minute (min). For clonogenic cell survival assay, cells were exposed to different doses of radiation as mentioned in the methodology section. For assessment of cell cycle analysis, cells were irradiated with 2 Gy. To study the mechanistic aspects of cell death a dose of 6 Gy was selected.

Preparation of PLB solution and treatment of cells

PLB was dissolved in 0.02% DMSO to prepare a stock concentration of 1 mM and then diluted in MEM to make the working solutions for the experiments. For assessing the radiosensitizing potential of PLB, B16F1 cells were exposed to PLB for 1 h. Next, PLB containing media was aspirated aseptically and the cells were exposed to radiation in sterile PBS. After irradiating the cells with respective doses of γ-rays, the cultures were incubated in growth media and harvested accordingly for different assays.

Cell viability measurement by MTT assay

The effect of PLB on cell viability was assessed by the ability of the cells to reduce MTT to formazan crystals. Briefly, cells were seeded on 96-wells plates (10^4 cells/well) and incubated at 37 °C for overnight attachment. Next, cells were incubated with different concentrations of PLB (2.5–50 μM) for 1 or 24 h. Next, PLB-containing media was discarded and the cells were treated with MTT reagent (100 μL; 1 mg/mL) and further incubated at 37 °C for 4 h following which, 100 μL of DMSO was added to each well for dissolving the formazan crystals formed. Absorbance of dissolved formazan crystals was collected at 540 nm using multi-well plate reader (Tecan M200, Austria). Cell viability was calculated as mentioned earlier (Kumar et al., 2009) using the formula:

$$\%\,\text{Viability} = [(\text{Test}_{OD} - \text{Blank}_{OD}) - (\text{Control}_{OD} - \text{Blank}_{OD})] \times 100$$

Radiosensitizing potential by clonogenic assay

Exponentially growing melanoma cells (10^6) were seeded into individual T-25 flasks and allowed to grow further for 24 h to promote attachment and were then treated as follows: (a) control/sham irradiated, (b) radiation (1–8 Gy), (c) 5 μM PLB for 1 h followed by radiation (1–8 Gy). Post treatment, appropriate number of viable cells were seeded on to 6 cm^2 culture plate containing 5 mL of growth media and incubated in CO_2 incubator for two weeks for colony formation. Colonies (consisting of more than 50 cells) were counted after staining with 0.1% crystal violet. Plating efficiency and surviving fraction were calculated as described earlier (Aithal et al., 2012), and sensitization enhancement ratio (SER) was determined as reported earlier (You et al., 2010).

DNA damage by alkaline comet assay

Exponentially growing cells (10^6), were treated as follows: (a) control/sham irradiated, (b) 6 Gy, (c) 2.5 μM PLB for 1 h and (d) 2.5 μM of PLB for 1 h prior to 6 Gy. At 0, 3 and 6 h of post-treatment, cells were harvested and processed for the alkaline comet assay as described earlier (Kumar et al., 2009). Briefly, treated cells were suspended in low melting agarose (0.75%) and layered on to slides previously coated with normal agarose (1.5%). Embedded cells were lysed using ice-cold buffer (10 mM Trizma base, 100 mM EDTA, 2.5 M NaCl, 1% Triton-X 100, 10% DMSO), followed by 20 min of unwinding in chilled alkaline electrophoresis buffer (200 mM EDTA, 10 M NaOH; pH 13). The electrophoresis was performed for 20 min at 20 V and 300 mA. Next, the slides were rinsed with neutralization buffer (400 mM Tris–Cl, pH 7.5) and stained with EtBr (20 μg/mL). Slides were scanned using Olympus BX51 (Olympus Microscopes, Tokyo, Japan) and images of comets were captured with CCD camera (Cool SNAP-Pro cf Digital Color Camera Kit Version 4.1, Media Cybergenetics, Silver Spring, Maryland). The comets were analyzed using Komet software (Version 5.5, Kinetic Imaging Ltd., Bromborough, UK). The mean Olive Tail

Moment (OTM = (Head Mean) × Tail % DNA/100) was used as a parameter to assess the level of DNA damage.

Cell cycle analysis by flow cytometry

The effect of PLB, alone or in combination with 2 Gy radiation, on cell cycle at different time points was studied by using flow cytometry after propidium iodide staining (Devi et al., 2000). Exponentially growing cells (10^6) were subjected to various treatments as follows: (a) control/sham irradiated, (b) 2 Gy of radiation, (c) 5 μM PLB for 1 h, and (d) 5 μM of PLB for 1 h followed by 2 Gy of radiation. After treatment, cells were trypsinized, washed in PBS, fixed in 70% ice-cold alcohol and treated with RNase A (1 mg/mL) and incubated at 37 °C for 30 min. The cells were stained with propidium iodide (50 μg/mL in PBS) for 15 min at 4 °C in dark and analyzed in a FACS Calibur using Cell Quest software (Becton Dickinson, USA). The percentage of cells in G_0/G_1, S and G_2/M phases were analyzed using Win MDI 2.8 program.

Detection of cytosolic ROS and mitochondrial membrane potential (Ψm)

Exponentially growing cells (5×10^4) were seeded in 24-well plate and incubated for 24 h following which they were treated as follows: (a) control/sham irradiated, (b) 6 Gy of radiation, (c) 5 μM PLB for 1 h and (d) 5 μM PLB for 1 h followed by 6 Gy of radiation. After treatment, the ROS generation was assessed by incubating the cells with 5 μM of DCFH-DA and the fluorescence was measured using Infinite®M200 (TECAN, Switzerland) with excitation and emission wavelengths of 488 nm and 525 nm respectively (Kumar et al., 2009). For determination of changes in the Ψm the above treated groups were further incubated in fresh media for 24 h and changes in the Ψm was determined by staining the cells with 5 μM of Rhodamine 123 and the fluorescence was measured using Infinite®M200 (TECAN, Switzerland) with excitation and emission wavelengths of 488 nm and 525 nm respectively (Kanno et al., 2013).

Analysis of cell death by flow cytometry

Exponentially growing cells (10^6) were subjected to various treatments as follows: (a) control/sham irradiated, (b) 6 Gy of radiation, (c) 5 μM PLB for 1 h and (d) 5 μM of PLB for 1 h followed by 6 Gy of radiation. After treatment, the cells were further incubated for 24 h and then analyzed for cell death using Annexin V-FITC/PI staining method. The detailed methodology is described in an earlier report (Kumar et al., 2009).

Western blotting analysis

Exponentially growing cells (10^6), were subjected to various treatments: (a) control/sham irradiated, (b) 6 Gy of radiation, (c) 5 μM PLB for 1 h and (d) 5 μM of PLB for 1 h followed by 6 Gy of radiation. After treatment, cells were further incubated 24 h and protein lysates were prepared using standard method. Briefly, cells were lysed with ice cold RIPA lysis buffer containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris (pH 8.0), 1 mM sodium fluoride, 1 mM sodium orthovandate, 1:100 protease inhibitor cocktail (Sigma-Aldrich Co., St Louis, MO). Protein concentration was determined by standard Bradford assay and 20 μg of proteins were resolved in 12–15% SDS-polyacrylamide gels and electroblotted to nitrocellulose membrane. Blotted membranes were probed with a panel of primary antibodies namely PARP (1:5000), p53 (1:5000), Bax (1:5000), Bcl-2 (1:5000), Caspase 3 (1:5000), Cytochrome c (1:5000) and β-actin/β-tubulin (1:8000) (Cell Signaling Technology, Inc., USA) followed by detection of expression levels using enhanced chemiluminescence detection reagents (Super-Signal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Statistical analysis

Each experiment was performed in triplicates. Student's *t*-test and One way ANOVA, followed by Bonferroni's *post hoc* test were used to compare the significance between different treatment groups using the GraphPad Prism version-4 (California, USA) and statistical significance was evaluated at the significance level $2\alpha = 0.05$.

Results

MTT assay

Cellular viability was assayed by treating B16F1 melanoma cells with various concentrations of PLB. Cells exposed to PLB showed a dose and time dependant decrease in cell viability with the IC_{50} being 27.7 and 6.6 for 1 h and 24 h exposure time intervals (Fig. 1B).

Clonogenic assay

The effect of PLB on the radiation survival curves was studied by performing colony-forming assay in melanoma cells. Cells were pre-treated with 5 μM of PLB for 1 h before treating with different doses of gamma radiation (1–8 Gy). As shown in Fig. 1C, treatment of melanoma cells with PLB prior to treatment with different doses of radiation resulted in a clear, dose-dependent decrease in cell survival ($SF_2 = 0.31$, $D_o = 1.06$ Gy for PLB plus radiation) compared to the radiation alone group ($SF_2 = 0.54$, $D_o = 1.59$ Gy) suggesting the potential of PLB to increase the radiosensitivity of melanoma cells. The SER, calculated based on the D_o was 1.50 for PLB plus radiation treated group.

PLB enhances radiation-induced DNA damage

Alkaline comet assay was performed to study the extent of DNA damage induced upon treatment of melanoma cells with PLB alone or in combination with radiation. The genotoxicity concentration of 2.5 μM (approximately 1/10th dose of IC_{50}) was selected on the basis of MTT results obtained from 1 h exposure of PLB. This concentration was selected to ensure only the induction of DNA damage without direct cell death.

Treated cells were harvested at 0, 3 and 6 h post incubation time points and further processed for comet assay. Fig. 1D demonstrates that the melanoma cells treated with PLB alone showed a significant increase in OTM values at 0 h interval when compared to control/sham irradiated whereas at 3 h and 6 h the OTM values decreased compared to 0 h interval, which indicates the DNA damage repair at the latter time points. Further, the results showed that DNA damage caused by radiation alone to melanoma cells was almost repaired within 6 h of incubation. But the cells subjected to a combination treatment of PLB plus radiation showed an increase in DNA damage levels with OTM value of 16.03 as against the 4.42 for control/sham irradiated group at 6 h incubation period.

Cell cycle analysis

Fig. 2 and Table 1 summarize the percentage of cells in different phases of the cell cycle (G_0/G_1, S and G_2/M) after treatment with PLB in combination with radiation. The rationale for using a lower dose of 2 Gy was to observe whether PLB was effective in inducing cell cycle arrest in combination with radiation without inducing cell death. It can be seen that the percentage of cells in G_2/M phase of the radiation alone treatment group was found to be $39.41 \pm 4.47\%$ at 6 h time interval after which a maximal G_2/M block with $41.74 \pm 4.22\%$ cells was observed after 12 h incubation period. However, the percentage of G_2/M phase cells decreased to

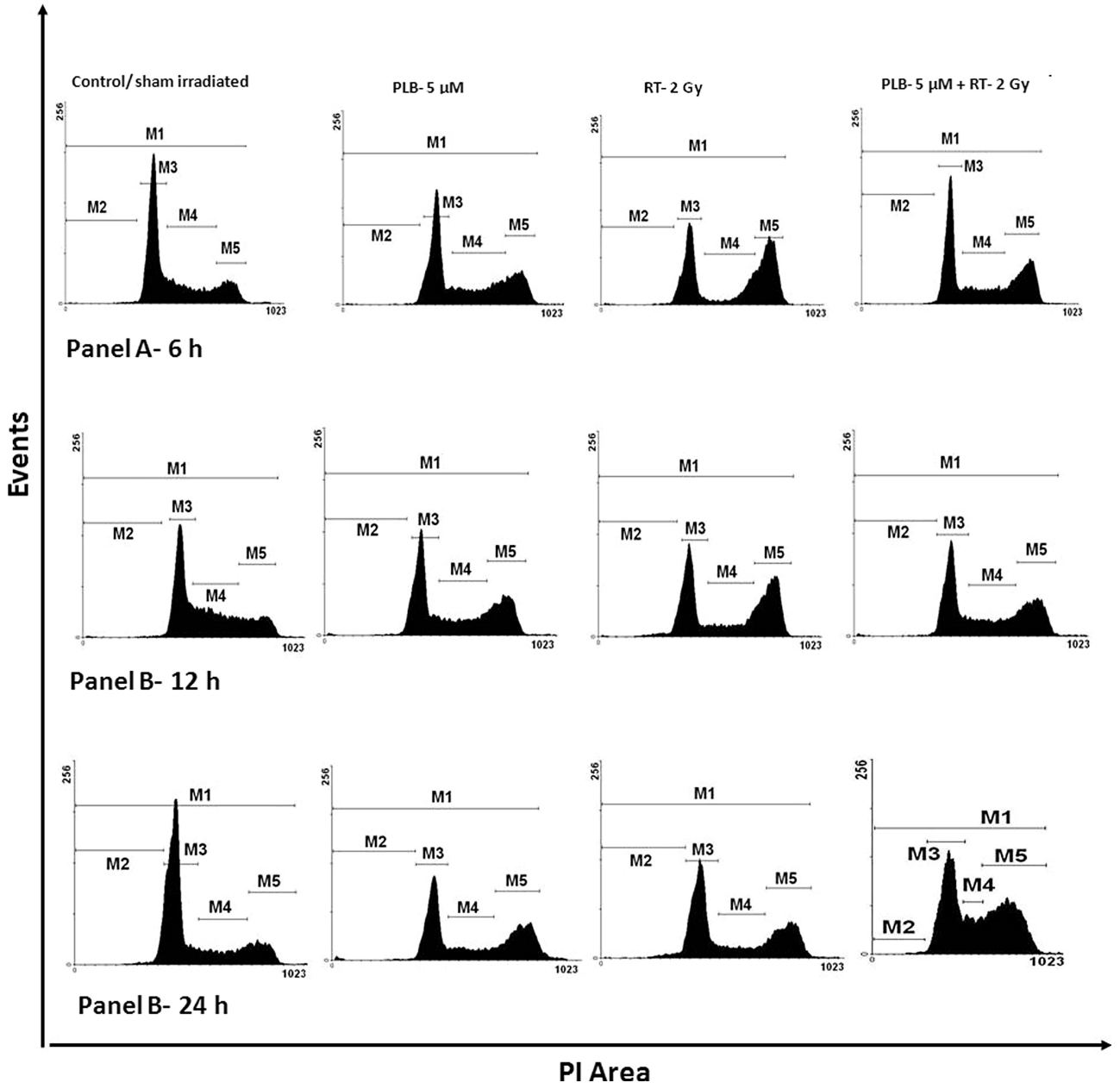

Fig. 2 – DNA histograms of B16F1 melanoma cells analyzed by flow cytometry after treatment with PLB in combination with or without radiation (2 Gy). Gates M_1 = total cells; M_2 = % sub G_0/G_1 phase; M_3 = % G_0/G_1 phase; M_4 = % S phase; M_5 = % G_2/M phase. The cell cycle phase distribution is provided in Table 1; RT – radiation.

Table 1 – Cell cycle changes after treatment with PLB with or without radiation (RT) (mean ± SD).

Post treatment time	Treatment group	M2 (% sub G_0/G_1)	M3 (% G_0/G_1)	M4 (% S)	M5 (% G_2/M)
6 h	Control/sham irradiated	1.46 ± 0.15	56.65 ± 2.17	23.91 ± 4.75	17.25 ± 1.41
	PLB-5 μM	2.44 ± 0.69	43.51 ± 2.33^{z}	28.66 ± 1.44	22.27 ± 1.71
	RT-2 Gy	2.98 ± 0.88	34.56 ± 2.23^{z}	20.98 ± 3.97	39.41 ± 4.47^{y}
	PLB-5 μM + RT-2 Gy	2.45 ± 0.51	43.05 ± 1.68^{a,y}	22.78 ± 2.52	31.26 ± 4.05^{z}
12 h	Control/sham irradiated	1.91 ± 0.24	47.14 ± 6.4	32.11 ± 4.12	18.57 ± 2.10
	PLB-5 μM	3.32 ± 1.31	41.78 ± 3.45	23.48 ± 1.33	28.92 ± 3.38^{x}
	RT-2 Gy	2.71 ± 0.34	37.08 ± 5.05	14.50 ± 5.31	41.74 ± 4.22^{y}
	PLB-5 μM + RT-2 Gy	3.54 ± 1.07	42.13 ± 3.45	24.78 ± 1.07	28.62 ± 2.78^{x,a}
24 h	Control/sham irradiated	2.65 ± 0.36	57.03 ± 2.29^{y}	22.49 ± 1.49	19.20 ± 2.01
	PLB-5 μM	3.31 ± 0.92	41.92 ± 0.31^{y}	24.79 ± 1.14^{y}	28.62 ± 3.47^{x}
	RT-2 Gy	2.91 ± 0.78	48.36 ± 3.86^{y}	19.15 ± 1.48^{x}	27.50 ± 2.31^{x}
	PLB-5 μM + RT-2 Gy	3.38 ± 0.31	36.97 ± 0.84^{a,y}	19.57 ± 0.47^{x}	40.20 ± 2.59^{a,y}

^{x,y,z}Statistically significant when compared to control.
^{a} Statistically significant when compared to RT.

27.50 ± 2.31% at 24 h. In the PLB alone treatment group there was a persistent block of G_2/M phase cells at 6 h, 12 h and 24 h time intervals when compared to control/sham irradiated. But in case of combination treatment (PLB with radiation), there was an increase in the G_2/M phase cells from 31.26 ± 4.05% at 6 h time interval to 40.20 ± 2.59% at 24 h. From the results, it was observed that there was 12.7% increase in the percentage of G_2/M phase cells for the combination

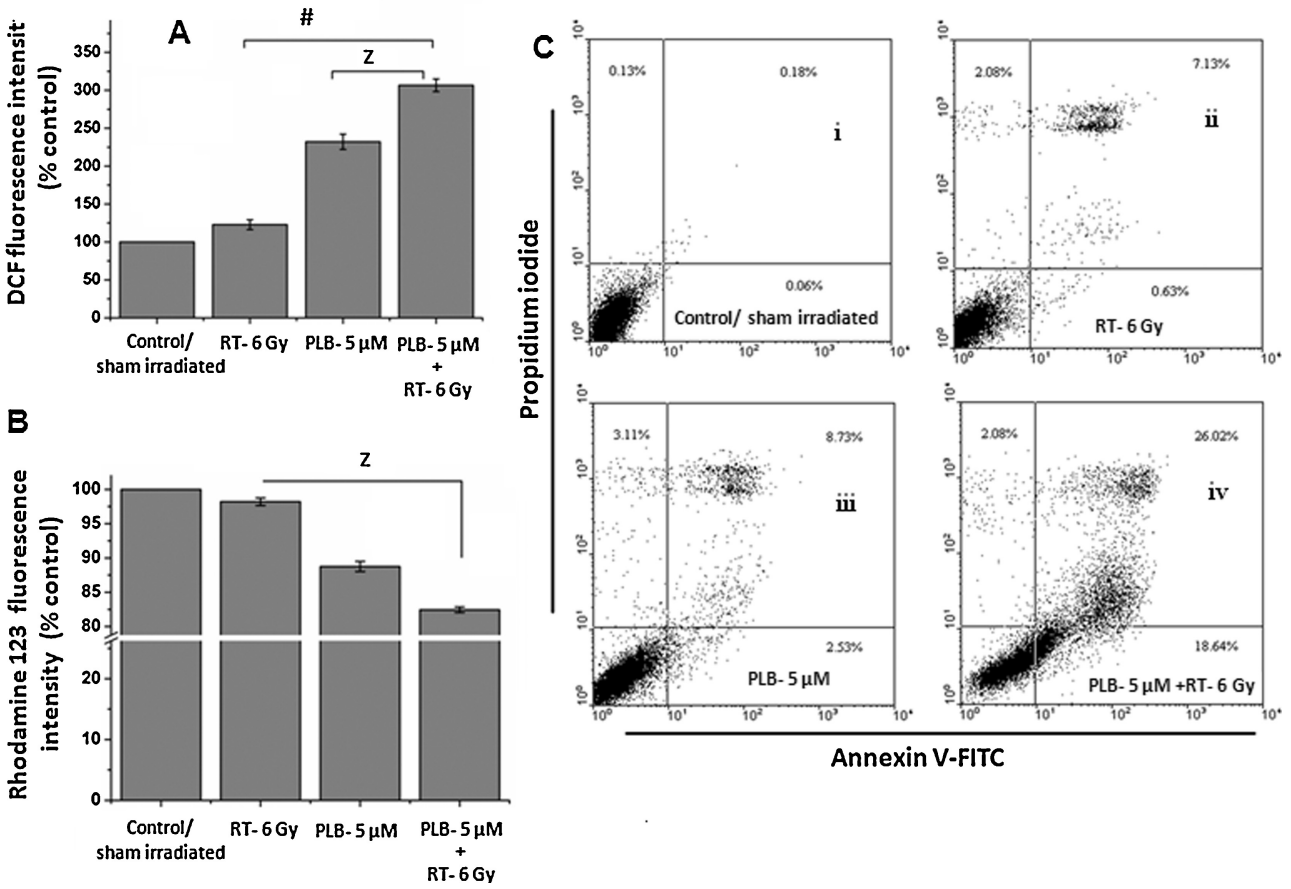

Fig. 3 – (A) Effect of PLB with or without radiation treatment (6 Gy) on the intracellular ROS levels in melanoma cells assessed using DCFH-DA assay. (B) PLB induced increase in the reduction of Ψm in combination with radiation. (C) Flowcytometric analysis of apoptosis in melanoma cells by Annexin V-FITC/PI staining. Cells pretreated with PLB followed by radiation exposure showed a marked increase in apoptotic population (early apoptotic shown in lower right quadrant & late apoptotic shown in upper right quadrant); (i) control/sham irradiated; (ii) RT alone; (iii) PLB alone; (iv) PLB+RT. $^{\#,z}$Statistically significant when compared to control/sham irradiated; RT – radiation.

treatment group at 24 h time point in comparison to the radiation alone treated group. The percent of G_0/G_1 phase cells at 24 h decreased to 36.97 ± 0.84% at 24 h from 43.05 ± 1.68% at 6 h.

Intracellular ROS and Ψm measurement

ROS generated during radiosensitizing effect of PLB was measured by increase in the fluorescence of DCFH-DA. The results of ROS generation after the treatment with PLB with or without radiation (6 Gy) have been shown in Fig. 3A Pretreatment of cells with 5 μM PLB for 1 h prior to radiation exposure helped in the significant elevation of ROS levels when compared to PLB or radiation alone group, showing the additive effect of PLB.

Along with increased ROS levels a concomitant depletion in Ψm was observed by decrease in Rhodamine 123 intensity in case of cells pretreated with 5 μM PLB prior to radiation exposure when compared to PLB or radiation alone group showing the radiation sensitizing potential of PLB (Fig. 3B).

Cell death by apoptosis

The mode of cell death induced in B16F1 cells by PLB radiosensitization was assessed by flowcytometry using Annexin V-FITC/PI staining method. The Annexin V-FITC-positive population of the cells (apoptotic cells; lower right quadrant) in case of PLB alone and radiation alone treatment groups was found to be 8.73% and 7.13% respectively. Treatment of cells with PLB prior to radiation exposure increased the apoptotic population to 26.02% showing its radiosensitizing potential (Fig. 3C).

Western blot analysis of apoptotic proteins

As demonstrated in Fig. 4 cells pre-conditioned with PLB prior to radiation exposure showed a marked increase in PARP cleavage along with higher expression of p53, Bax, Cytochrome c, cleaved Caspase 3 and a concomitant decrease in the Bcl-2 expression levels indicating the radiosensitizing potential of PLB that induces the process of intrinsic pathway for programmed cell death.

Discussion

There is an immediate need for new radiosensitizing agents against solid tumor to improve the success of radiotherapy (Adhikari et al., 2005). In this regard, various chemotherapeutic agents such as cisplatin, gemcitabine, paclitaxel, docetaxel, and irinotecan have been used in combination with radiation, however with varying degrees of success (Horsman et al., 2006) and have been associated with normal tissue toxicity (Bischoff et al., 2009). The present experiments demonstrated the radiosensitizing potential of PLB against radioresistant B16F1 melanoma tumor. Exposure of melanoma cells to PLB prior to treatment with different doses of γ-radiation resulted in a significant reduction in tumor cell surviving fraction (SF) in comparison to the radiation alone group with a sensitizer enhancement ratio (SER) of 1.5 for PLB. Earlier, Chung and

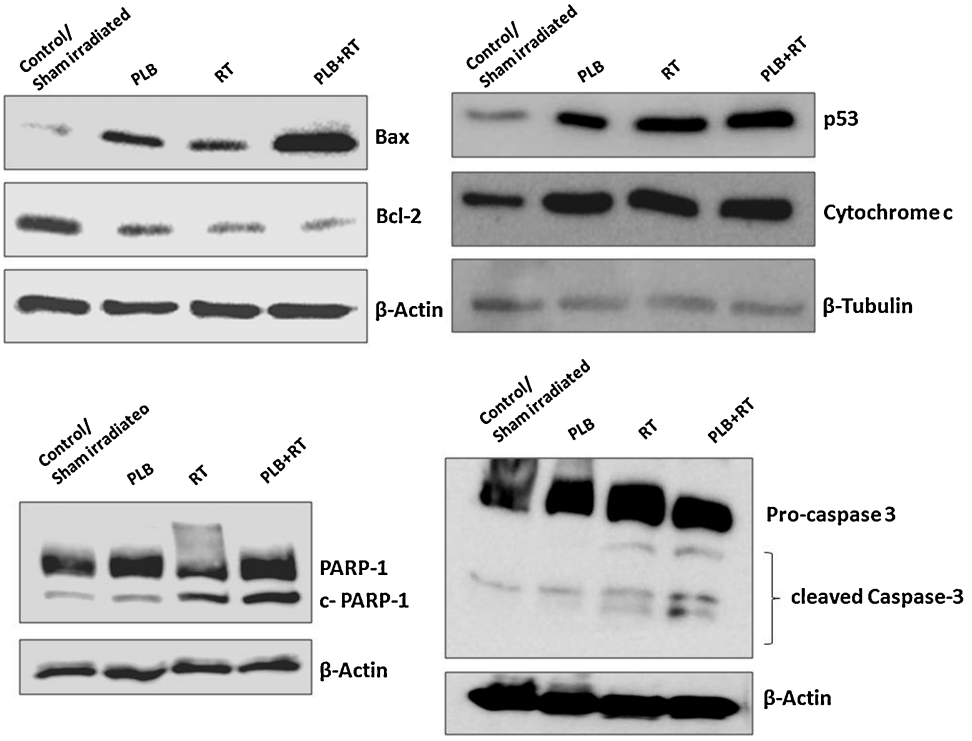

Fig. 4 – Effect of PLB and radiation combination treatment on the expression of: Bax, Bcl-2, p53, Cytochrome c, PARP and cleaved PARP (c-PARP), Pro-caspase 3 and cleaved Caspase 3 protein levels. Cell lysates were analyzed by western blotting using the indicated antibodies. The levels of β-actin/β-tubulin served as a loading control; RT – radiation.

colleagues (2009) have reported that agents which induce a SER ratio of greater than 1 is considered as potential radiation sensitizers. Therefore, these results showed that PLB could augment the sensitivity of B16F1 cells to irradiation, indicating its radiosensitizing potential.

Radiation induced ROS aiding in altering the lipid bilayer fluidity and permeability properties (Shankar et al., 2003), damage DNA and proteins, eventually leading to cellular dysfunction or cell death (Girdhani et al., 2005). Radiation-induced oxidative stress plays a key role in causing DNA damage. It was clear that the combination treatment of PLB with radiation resulted in a significant enhancement of intracellular ROS levels *versus* independent treatments. This elevation in the oxidative stress by the combination treatment acts as a causative agent, enhancing DNA damage (higher values of OTM). Similar results were reported, wherein the potential of juglone, a quinone containing moiety enhanced the radiation-induced DNA damage and delay in repair capacity *via* ROS mediated oxidative stress mechanism in B16F1 melanoma cells *in vitro* (Aithal et al., 2012).

It has been suggested that DNA damage by ROS leads to the formation of single and double stranded breaks resulting in cell cycle arrest and affect the recruitment of DNA repair enzymes to rescue cells from the damage (Maity et al., 1994). Beta lapachone, a plant based quinone with its established anticancer properties has been shown to enhance the radiosensitivity of cancer cells by the inhibition of DNA repair mechanisms (Horsman et al., 2006), considered to be a key determinant in radiosensitization. Earlier studies reported that PLB pretreatment before radiation significantly enhanced the tumor growth inhibitory effect when compared to radiation or PLB alone treatment (Prasad et al., 1996). Sandur and co-authors (2006) have reported PLB as a potent inhibitor of NF-kB activation pathway leading to suppression of NF-kB regulated gene products, which explains its cell growth modulatory, anticarcinogenic and radiosensitizing effects. In general, quinones act *via* two distinct mechanisms, (a) by the process of redox cycling resulting in production of semiquinone radicals and ROS, and (b) depleting intracellular reservoirs of GSH. PLB has been shown to react stoichiometrically with GSH (Inbaraj and Chignell, 2004). In addition, the hydroxyl group present in the 5th position of the aromatic ring of PLB helps in exerting its toxic effect through uninterrupted redox signaling, as reported by Padhye and colleagues (2012). Checker and colleagues (2010) reported a similar finding of an irreversible conjugation of GSH with PLB. As GSH forms an integral component of a cell's antioxidant/detoxification repertoire, inhibiting it would result in the outbreak of numerous pathways leading to cellular dysfunction/death.

Cell cycle analysis was performed to observe the effect of combination treatment on cell cycle changes in asynchronously growing B16F1 melanoma cell. PLB treatment alone caused G_2/M cell cycle arrest, and persisted up to 24 h compared to the respective control/sham irradiated group. Cells exposed to radiation showed a G_2/M block up to 12 h post incubation and later the G_2/M phase cells decreased at 24 h. In contrast, there was a significant increase in G_2/M phase cells at 24 h post incubation period in combination treatment (PLB plus radiation) compared to radiation alone group indicating its radiosensitization potential. Cell cycle arrest is associated with altered activity of cyclins/CDKs responsible for guiding a cell through different stages of the cell cycle. Although, in the present investigation we have not assessed the alterations in the expression of cyclins/CDKs which may aid in PLB mediated cell cycle arrest. However, there are several earlier reports that have indicated the ability of PLB mediated S-G_2/M cell cycle arrest. Further, it has been reported that PLB exhibited cell proliferation inhibition by inducing cells to undergo G_2/M arrest in MCF-7 and MDA-MD-231 human breast cancer cells (Kuo et al., 2006) and A549 human non-small cell lung cancer cells (Hsu et al., 2006). For instance, Jaiswal and colleagues (2002) showed that PLB mediated cell cycle arrest through the induction of p21. Findings of Hsu and co-workers (2006) advocated the ability of PLB to induce cell cycle arrest by p53/p21 mediated cascades in A549 cells. Recently published reports (Pan et al., 2015; Wang et al., 2015) also support the ability of PLB to induce cell cycle arrest *via* cyclin B1/p53/p27 Kip1/p21 Waf1/Cip1 pathways.

Combination of PLB with radiation caused cell death predominantly by programmed cell death as shown by increase in the Annexin V-FITC/PI positive cells. This observation may be attributed to the fact that PLB pre-exposure augmented ROS formation and increased DNA damage. Additionally, the present data also showed a marked decrease in the Ψm during PLB radiosensitization process. Although, ROS serve as ''redox messengers'' in a variety of signaling cascades, their overproduction is detrimental to the fate of a cell leading to oxidation of important macromolecules such as lipids, proteins and DNA. In addition, the mitochondria can be the direct targets for ROS, which causes membrane damage, depletion of Ψm and modifying mitochondrial permeability transition pore complexes allowing the release of pro-apoptotic factors in the cytosol inducing cell death (Marchi et al., 2012). Western blot analysis of proteins revealed that PLB helped in the radiosensitizing process by enhancing the expression of pro-apoptotic proteins namely, p53, Bax, cleaved PARP and Caspase 3, Cytochrome c and down-regulation of Bcl-2 indicating that PLB pre-exposure aided in the mitochondrial mode of cell death. Also, the upregulation of p53 may be attributed to the occurrence of cell cycle arrest in case of cells pretreated with PLB followed by radiation exposure. Expression of apoptotic regulatory molecules such as Bcl-2, Bax and Survivin was also modulated by PLB in combination with 2 Gy of radiation as reported in an earlier literature (Nair et al., 2008). Previous reports have also demonstrated the same phenomenon wherein PLB have been shown to be efficacious against a variety of models causing cell death *via* mitochondrial pathway (Srinivas et al., 2004; Hsu et al., 2006; Wang et al., 2008). Also our present findings indiacated that PLB pretreatement led to increased PARP cleavage which is in line with previous reports (Hsu et al., 2006; Sandur et al., 2006) that also advocate the ability of PLB to induced PARP cleavage leading to cell death. More recent reports published by Pan and co-workers (2015) and Wang et al. (2015) strongly supported the ability of PLB to decrease Bcl-2 levels leading to cellular demise under *in vitro* conditions using tongue squamous cell carcinoma cell lines. It has also been reported that PLB may induce cell death by the activation of programmed cell death and autophagy by triggering ROS along with p38 MAPK- and PI3K/Akt/mTOR signaling cascades

(Wang et al., 2015). Therefore, in the light of above supporting evidences it may be ascertained that PLB may act as a potent Bcl-2 inhibitor enhancing tumor cell death and this phenomenon may also be extended to its radiosensitization potential.

Conclusion

In conclusion, the present study demonstrates the potential of PLB to inhibit the growth of melanoma cells *in vitro*. Although an earlier report suggested the growth inhibitory effects of PLB against mouse melanoma cells (Prasad et al., 1996), this study demonstrates the mechanism behind the radiosensitization of PLB in chemo- and radioresistant melanoma cells. The observed effect of PLB was basically by the virtue of its ability to induce redox signaling facilitating mitochondrial mode of programmed cell death. It is clear that enhancement of radiation-induced oxidative stress by PLB resulted in the formation of increased DNA damage, depleted Ψm resulting in cell death. Also, this study demonstrate the ability of PLB to augment ionizing radiation induced cell killing, further providing scope for the development of PLB as radiosensitizer. Further understanding of the *in vivo* mechanisms of PLB in modulating tumor microenvironment along with tumor regression would be of utmost importance for its clinical impact.

Conflict of interest

The authors declare that there are no conflicts of interest.

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