**ABSTRACT**

The major drawback of anticancer therapy is the development of resistance against drugs and radiation at the later phase of treatment which may lead to recurrences of the disease. Therefore, strategy was taken to enhance radiation sensitivity of lung (A549) and liver (HepG2) carcinoma cells by treatment with ferulic acid (FA) prior to irradiation. FA pre-treatment initially decreased reactive oxygen species (ROS) level in carcinoma cells which induced reductive stress and cytostasis. To overcome this stress, cellular mechanism increased the Keap1 level to down-regulate nuclear localisation of Nrf2 and its dependent antioxidant system. The antioxidant system reached the lowest level after 3 and 6 h of FA treatment in A549 and HepG2 cells respectively. As endogenous ROS were still being generated at same rate, ROS level was clearly higher than control which changed the reductive stress to oxidative stress. Exposure to gamma-radiation in this condition further increased ROS level and caused radio-sensitisation of carcinoma cells. Combination of irradiation (IR) and FA activated mitochondrial apoptotic pathway and concomitantly inhibited the cell cycle progression and survival pathway over the IR group. Moreover, the combination treatment showed significant tumour regression, caspase 3 activation and nuclear fragmentation in tumour tissue compared to radiation alone. In contrast, FA pre-treatment protected peripheral blood mononuclear cells (PBMC) and normal lung fibroblast WI38 cells from radiation damage. Together, combination treatment offers effective strategy of killing cancer cells and demonstrates its potential for increasing the efficacy of radio-therapy.

**INTRODUCTION**

In recent years, the morbidity and mortality of cancer have been increasing at an alarming rate, thereby posing a major public health threat worldwide. Despite the progress being made in the field of targeted therapy and immunotherapy, the chemotherapy and radiotherapy still remain as a promising treatment strategy for most of the cancer types. Radiotherapy is localised, penetrative, and an effective treatment modality, yet a large number of tumour cells eventually develop resistance to the radiation doses [1]. Additionally, radiotherapy generates severe side effects by affecting adjacent and nonadjacent normal cells, as well as immune networks [2]. Therefore, it is obvious that an agent which selectively increases the effect of radiation on carcinoma cells will have strong biological significance because a reduced dose of radiation will sustain an efficient killing of tumour cells in presence of that adjuvant. There are many chemical radio-sensitizers available. However, the high systemic toxicity has restricted their therapeutic application [3]. Thus, the search for a new compound from natural origin that can increase the sensitivity of cancer cells towards radiation while showing comparatively low toxicity to normal cells is one of the major focus of novel cancer drug development.
A large number of studies have been focussed on plant-derived compounds because of their antineoplastic potential with negligible systemic toxicity [4]. FA (ferulic acid, chemically named as 4-Hydroxy-3-methoxycinnamic acid) which is structurally related to curcumin, has been reported for its antioxidant and anti-inflammatory activities [5]. Reports are available on the protective effect of FA against gamma radiation induced DNA damage in the normal cells [6, 7]. Several in vitro and in vivo studies on radio-protective effect of FA reiterate its lower systemic toxicity with a significant protective ability [7–12]. In addition to that, the anti-proliferative, anti-metastatic and anticarcinogenic activities of FA have also been reported for thyroid cancer cells [13]. Interestingly, till now, only a few sporadic reports exist that elucidated the radio-sensitising ability of FA. In an earlier attempt, FA has been shown to augment the radiation effect on cervical cancer cells [14]. FA and 2 deoxy glucose in combination with radiation induce the death of non-small cell lung carcinoma (NSCLC) cells [15]. However, neither of these studies elucidated the detailed molecular mechanism. With this perspective in the current study, we wanted to delineate the radiation sensitising effect of FA in three different types of cancer cell lines (HepG2, A549, and CT26) and murine tumour model with underlying mechanistic aspects. A549 cells and mouse colon carcinoma cells (CT26) express PECAM1 protein, whereas HepG2 cells do not. The expression of PECAM1 is responsible for showing aggressive metastatic behaviour of A549 and CT26 cells [16]. Thus, to check whether this radiation sensitisation effect of FA is only specific to a particular cancer cell type or it is generalised, we choose these three different cancer cell lines. The CT26 cells were used to develop solid tumour in BALB/c mice.

This study uniquely identified the radiation sensitisation effect of FA on HepG2, A549, and CT26 cells. It was observed that combination treatment efficiently decreased cell survival markers’ expression with the augmentation of G2/M phase arrest, and activation of intrinsic apoptotic pathway compared to either radiation or FA treatment alone. Moreover, the in vivo experimental data showed prominent shrinkage in tumour tissue with massive damage and apoptotic body formation in neoplastic region. In contrast, FA did not show any toxicity to peripheral blood mononuclear cell (PBMC) and to normal lung fibroblast WI-38 cells, rather combination of IR and FA treatment protected them from the radiation hazards.

**Materials and methods**

**Chemicals**

HepG2, CT26, A549, and WI-38 cell lines were from ATCC. DMEM, FBS, and 0.05% trypsin-EDTA and antibiotic cocktail were purchased from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). FA was purchased from Sigma-Aldrigh (St. Louis, MO, USA). Anti-mouse/rabbit monoclonal antibodies against NF-κB, Nrf2, phos-pho-Akt, phos-pho-p38 MAPK, Cox-2, active caspase 3,9,7, cleaved PARP, Akt, and MAPK, were purchased from Cell Signalling Technology (Danvers, MA, USA). Matrix metalloproteinase 9 (MMP9), vascular endothelial growth factor (VEGF), p53, p21, Bax, Bcl-2, cyclin B1, Cdc25C, FITC, and Texas Red (TR) tagged secondary antibody was obtained from Santa Cruz (Dallas, TX, USA). Annexin-APC and JC1 were obtained from BD Bioscience (San Jose, CA, USA). The dead-end colorimetric TUNEL (terminal deoxynucleotidyl transferase UTP nick end labelling) assay kit was purchased from Promega (Madison, WI, USA). Chemical inhibitors, such as SB203580, LY294002, and Cdc25C phosphatase Inhibitor II, NSC 663284 were obtained from Sigma and CST. All other chemicals used were of highest purity grade.

**Maintenance of cell lines**

The HepG2, A549, CT26 and WI-38 cells were cultured with DMEM complete medium and PBMC was cultured with RPMI-1640 complete medium in T25 Flask and 60 mm plates. All complete media contained 10% FBS and 1 x antibiotic cocktail. The cells were kept in a CO2 incubator at a temperature of 37 °C with 5% CO2 pressure.

**FA and radiation doses**

FA solution was prepared in 0.1-M potassium phosphate buffer, and then passed through a syringe filter for sterilisation. 10⁴ cells were seeded in each well of 96 well plates. After seeding of cells, increasing concentration of FA (10–400 μM) was added and incubated for 72 h to determine the IC₅₀ concentration of FA. To find out IC₅₀ dose of radiation, the cells were exposed to different doses of gamma radiation 5, 7.5, 10 and 15 Gy (⁶⁰Co) at the dose rate of 1 Gy/min and were incubated for 72 h. For the combination treatment, the A549 cells were exposed to 8 Gy dose of irradiation after 3 h of 90 μM of FA treatment. The HepG2, PBMC and WI-38 cells were exposed to 7.5 Gy dose of irradiation after 6 h of 100 μM of FA treatment. The CT26 cells were exposed to 4 Gy dose of irradiation after 4 h of 300 μM of FA treatment. Soon after irradiation, the cells were kept in 5% CO₂ in a 37 °C humidified incubator for 72 h followed by MTT assay was performed.

After determining the dose and time point of irradiation, the HepG2, A549, CT26, WI-38, and PBMC were
divided into four experimental groups. In control group
the cells were treated with 0.1-M potassium phosphate
buffer, the vehicle. In the IR group, vehicle + 7.5-Gy
radiation were used for HepG2, PBMC and WI-38 cells;
vehicle + 4-Gy radiation for CT26 and vehicle + 8-Gy
radiation were used for A549 cells. In the FA group,
100 μM of FA was used for HepG2, WI38, and PBMC;
90 μM of FA was used for A549 and 300 μM of FA was
used for CT26 cells. In IR + FA group, HepG2, WI-38 and
PBMC were treated with 100 μM of FA + 7.5-Gy radia-
tion; A549 cells were treated with 90 μM of FA + 8-Gy
radiation and CT26 cells were treated with 300 μM of
FA + 4-Gy radiation.

The cells were harvested at different time intervals,
for analysis of cellular parameters. ROS generation was
measured within 1 h of irradiation as it was the immedi-
ate early response of radiation exposure. The external-
isation of phosphatidyl serine, and mitochondrial
membrane potentials were measured after 3 h of irradia-
tion, and the level of p-Akt was measured after 6 h of
irradiation. The GSH (reduced glutathione) content,
TBARS level, p-NF-κB, p-STAT3, cell cycle arrest, expres-
sion of MMP-9, VEGF, PDGFRα/β, PECAM1, Cdc25C, cyc-
clin B1, p21, and nuclear localisation of p53 protein were
measured after 24 h of irradiation as it was the immedi-
ate early response of radiation exposure. The external-
isation of phosphatidyl serine, and mitochondrial
membrane potentials were measured after 3 h of irradia-
tion, and the level of p-Akt was measured after 6 h of
irradiation. The GSH (reduced glutathione) content,
TBARS level, p-NF-κB, p-STAT3, cell cycle arrest, expres-
sion of MMP-9, VEGF, PDGFRα/β, PECAM1, Cdc25C, cyc-
clin B1, p21, and nuclear localisation of p53 protein were
measured after 24 h of irradiation. The level of active
caspase 9,7,3, cleaved PARP, expression of COX-2, and
 Measurement of mitochondrial membrane
potential and externalisation of phosphatidyl
serine (PS)
The 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazo-
lylcarbocyanine iodide (JC-1) was used to measure the
mitochondrial membrane potential (Ψm) of tumour
cells, WI-38 and PBMC. The externalisation of phospha-
tidyl serine in the outer leaflet of the membrane was
determined by annexinV-APC, and propidium iodide
(PI) staining. The cells accepting the colour of APC rep-
resented the early apoptotic population, while cells
accepting both colours of APC and PI represented the
late apoptotic population. Both assays were performed
following the earlier protocol [18]. The cells (1 × 10^6)
were seeded for each experimental group. After 3 h of
incubation post irradiation, the cells were treated with
either JC-1 (2.5 μg/ml) or annexinV-APC/PI for 20 min at
room temperature. The fluorescence of 10^4 cells was
measured by using a flow cytometry (Becton Dickinson,
Franklin Lakes, NJ, USA). The data were analysed using
the software FlowJo 8.0.

ROS generation assay
The intracellular production of ROS was assessed using
the H2DCF-DA (2′, 7′-dichlorodihydrofluorescein diace-
tate) [18]. The intracellular ROS, particularly H2O2 oxi-
dises H2DCF to 2′, 7′-dichlorofluorescein (DCF) whose
fluorescence (excitation, 488 nm; emission, 520 nm) was
measured by flow cytometry after 1 h of gamma radia-
tion exposure following the earlier protocol [18]. The
cells were trypsinized and washed with medium con-
taining serum followed by a wash with 1 × PBS. The
H2DCF-DA was added to each experimental group at
required concentration. The cells were incubated for
5 min and then analysed by flow cytometry.

Cell homogenate preparation
Cells were homogenised following the protocol of pre-
vious study [18], although with some modifications. In
brief, HepG2, A549, PBMC and WI-38 cells (2 × 10^6)
were suspended in 70 μl of cytosolic extraction buffer

Treatment with signalling inhibitors
According to the protocol of Cheng et al. 2006 [17], the
cells were treated with chemical inhibitors. The inhibitor
of p38-MAPK (SB203580), p-Akt (LY294002), and Cdc25C
phosphatase (Cdc25C phosphatase Inhibitor II, NSC
663284) were applied an hour prior to irradiation. The
concentrations of SB203580, LY294002, and Cdc25C
phosphatase Inhibitor II, NSC 663284 used for the cell
lines were 5, 50 μg/ml, and 10 μM respectively. The cells
were harvested after 24 h of incubation post irradiation.

MTT assay
In this experiment, 3-(4, 5-dimethylthiazolyl-2)-2, 5-
diphenyltetrazolium bromide (MTT) assay was consid-
ered as cell proliferation assay. As per the previous
protocol [18], the assay was performed for six inde-
pendent times, and every time, triplicates of a condition
were taken. The cells were treated with either FA/radi-
ation or their combination. After incubation for 72 h,
MTT assay was performed. For combination treatment,
cell were initially treated with FA either for 6 h (HepG2,
PBMC and WI-38) or for 3 h (A549) followed by expo-
sure of 7.5 Gy (HepG2, PBMC and WI-38) or 8 Gy (A549)
dose of irradiation. In CT26 cells were initially treated
with FA for 4 h followed by exposure of 4 Gy dose of
radiation. The cells were incubated for 72 h after irradia-
tion and MTT assay was performed to observe cell pro-
liferation and viability.
(10 mM HEPES, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl and 1 mM dithiothreitol, pH 7.9), containing a protease inhibitor cocktail, and sonication (20 kHz ultrasonic pulse) was performed. Thereafter, the sample was centrifuged at 700 x g for 10 min at 4 °C, and the supernatant was collected as a cytosolic fraction. The resultant nuclear pellet was washed for removing cytosolic contaminants, and was resuspended in 40 μl of nuclear extraction buffer (20 mM HEPES, 1.5 mM MgCl\textsubscript{2}, 0.42 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol and 25% (v/v) glycerol, pH 7.9), containing the protease inhibitor cocktail, followed by sonication. Finally, the nuclear suspension was centrifuged at 16 000 x g for 5 min at 4 °C to collect the supernatant as a nuclear fraction. The tubes were placed in ice during sonication (20 kHz) process and short pulses of 5 seconds were given intermittently for maximum 10 minutes time period. All the processes were done either at 4 °C or in ice. Protein concentration was determined following the standard protocol with slight modification [19].

**Reduced glutathione (GSH) activity and lipid peroxidation assay**

Following the protocol of the previous study with minor modification [20], the GSH level from cell lysate was measured. The GSH formed a yellow complex with DTNB, whose absorbance was measured at 412 nm against a substrate blank. Thiobarbituric acid reactive substance (TBARS) in the cell lysate was estimated by following a standard protocol [18]. The absorbance of the supernatant was measured at 535 nm against the substrate blank.

**Immunoblot assay**

Fifty micrograms of protein was loaded on 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) for electrophoresis, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin (BSA), whose absorbance was measured at 412 nm against a substrate blank. Thiobarbituric acid reactive substance (TBARS) in the cell lysate was estimated by following a standard protocol [18]. The absorbance of the supernatant was measured at 535 nm against the substrate blank.

**Immunofluorescence**

The cells were fixed with 4% paraformaldehyde at 4 °C for immunofluorescence study and then permeabilized with 0.2% Triton X-100, followed by blocking (2% BSA, and 0.1% Triton X-100 in PBS, and without Triton-X for GLUT1/4) for 1 h. The slides were incubated with GLUT 1, GLUT 4, p-NF-κB, p-STAT3, and isotype IgG primary antibody at a ratio of 1:250 each in the blocking solution. After washing, incubation with FITC-labelled anti-mouse, and Texas Red (TR) labelled anti-rabbit antibodies at a ratio of 1:250 each in the blocking solution was performed. The slides were washed, covered with mounting solution, and visualised using the EVOS microscope (Shinjuku, Tokyo, Japan) [18].

**Cell cycle analysis**

The cell cycle analysis was performed following the protocol of the previous study [18]. In brief, the cells were fixed in 70% ethanol at 4 °C for overnight. After treatments with 2 mg/ml RNase A, cells were stained with 50 mg/ml propidium iodide (PI), containing 0.1% Triton X-100, and EDTA (0.02 mg/ml). Using BD FACS Calibur, the cells (2 x 10\textsuperscript{6}) were quantified to determine the cell distribution in different phases. The results were analysed using the FlowJo software [18].

**Analysis of protein expression by flow cytometry**

The cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature and permeabilized in 0.1% Triton X-100 in PBS with 0.1% FBS for 5 min (not for GLUT1 and GLUT4). After washing twice in PBS with 3% FBS, the permeabilized cells were incubated with primary antibody (active caspase 9,3,7, active PARP, p-STAT3, p-p38 MAPK, p-Akt, GLUT1/4) on ice for 2 h and washed twice in PBS. The cells were then incubated with FITC-conjugated goat anti-rabbit/anti-mouse IgG as a secondary antibody for 30 min on ice and washed twice in PBS. The stained cells were acquired and analysed against isotype control using BD FACS Calibur, equipped with FlowJo software [18].

**TUNEL assay**

The TUNEL assay was performed according to the kit protocol of Promega. In brief, the DAB system was used to detect TUNEL positive nuclei. The slides were counterstained with haematoxylin to differentiate TUNEL negative nuclei from TUNEL positive nuclei.
Animal experiment

Mice were maintained according to the guidelines set by the Institutional Animal Ethics Committee (IAEC), maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%), and 12 hours of light-dark cycle. Animals were given standard mice feed (CNCI, Kolkata), and water ad libitum. The protocols were as per the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India), and the IAEC, University of Calcutta (“Ferulic acid as a modulator of radiation sensitive pathway” Permit Number: IAEC IV/ proposal/SD-03/2014, dated 05/09/2014). All efforts were made to minimise the suffering of the animals during treatment.

Following the growth and expansion of the cell line (CT26) in vitro, cells were harvested by trypsinization, washed, counted by trypan blue dye exclusion method and cell density was adjusted to 10 × 10⁶/mL in PBS. To build the heterotopic syngeneic tumour model, a suspension of 2 × 10⁵ viable tumour cells in 0.2 ml PBS was inoculated subcutaneously into the left and right rear flanks of the male BALB/c mice. On 15th day, mice with appropriate tumour size (150–200 mm³ volume) were divided into four groups. In tumour control group, after tumour development mice were given i.p. of 0.1-M potassium phosphate buffer from 16th day to 24th day at every alternative day. In IR group, after tumour development mice were given i.p. of 0.1-M potassium phosphate buffer from 16th day to 24th day at every alternative day. In IR + FA group, after tumour development mice were given i.p. doses of FA (50 mg/kg body weight dissolved in 0.1-M potassium phosphate buffer) from 16th to 24th day at every alternative day.

Tumour volume and mice body weight were measured every 3 days. Tumour volume was calculated as mm³ = 0.5 × length (mm) × width² (mm²).

Determination of FA dose for the treatment of syngeneic mice model

The 300 μM of FA (IC⁵₀ 400 μM) and 4-Gy radiation showed prominent radio-sensitisation effect on C726 cells under in vitro condition. Therefore, 300 μM dose is equivalent to 300 μmoles/l i.e. 0.3 μmoles/ml. The MW of FA is 198.18 g/moles i.e. 198.18 μg/μmoles. Therefore, 0.3 μmoles/ml × 198.18 μg/μmoles = 59.45 μg/ml. According to the guidelines of Organisation for Economic Co-operation and Development (OECD) for the testing of chemicals, the standard phytochemical (curcumin) suppressed 50% cellular proliferation (IC⁵₀) with 50 μg/ml dose which was in vitro dose equivalent to 50 mg/kg body weight in an experimental tumour model [21]. Here the in vitro dose of FA is almost similar to curcumin IC⁵₀ dose. Moreover, in previous literature it was observed 50 mg/kg body weight dose of FA showed significant tumour regression in C57BL/6 mice without any toxicity to the normal cells [22]. In our laboratory we observed that 50 mg/kg body weight dose of FA for 5 days did not alter the liver function and conferred protection to mice exposed to 10 Gy dose of irradiation [7–10]. Therefore, the dose 50 mg/kg body weight of FA was used here for in vivo study.

Histology

To perform the histopathological studies, a small tumour tissue mass was taken and fixed in formalin solution. After dehydration followed by embedding, the paraffin block of tumours was prepared. Four micrometres thick sections were cut using microtome and the sections were then fixed on a slide by Mayer’s albumin solution. After deparaffinization, the tumour sections were stained with haematoxylin and eosin (H&E) staining solution. The mounted sections were then observed under a light microscope (Olympus 207 444, Tokyo, Japan) at 400× magnification and the images were captured using the Canon PowerShot S70 digital camera [8] for the histological evaluations of the tumour tissue.

Immunofluorescence in the tumour section

Fluorescence staining was carried out on formalin-fixed-paraffin-embedded sections with cleaved caspase 3 antibody and DAPI was used to stain nuclei. Briefly,
depurated sections were used for unmasking of antigens by heating the sections at 90 °C for 10 minutes in 10-mM citrate buffer, pH 6 and cooled at room temperature for 30 min. To quench endogenous peroxidase activity in samples, the sections were treated with 3% hydrogen peroxide for 10 minutes. After blocking in 5% BSA, each section was treated with primary antibody of cleaved caspase 3 (1:200) overnight at 4 °C. After washing the sections were incubated with secondary antibody (1:250) that was tagged with fluorescein isothiocyanate (FITC) for 2 h. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). Fluorescent signals were viewed under microscope at 600x magnification (EVOS FL cell imaging system of Invitrogen) [10]. Quantification was done by evaluating the colour intensity using ImageJ software (1.42q version).

Statistical analysis

The numerical values of results were given as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with Tukey’s post hoc test was done for statistical evaluation of the data and for the determination of level of significance in various groups using the software Origin Lab 8.0. In all cases, a value of $p < .05$ was considered significant. In all experiment the significance of differences were calculated between the *** control vs. irradiation group and # irradiation vs. combination (IR + FA) group until and unless it was mentioned in the figure legend.

Results

FA in combination with radiation inhibits the proliferation of HepG2 and A549 cells

As obtained from the MTT assay, the IC$_{50}$ concentration of FA for HepG2 was 200 μM, and for A549 cells was 180 μM (Figure 1(B,C)). Henceforth, we chose 100 and 90 μM doses of FA for HepG2 and A549 cells, respectively. The radiation dose profile revealed 69.67 ± 1.07% and 73.77 ± 1.5% viability in HepG2 (Figure 1(D)) and A549 cells (Figure 1(E)) post 7.5 and 8 Gy doses of irradiation, respectively. The combination of 100 μM of FA and 7.5 Gy dose of radiation to HepG2 cells showed significantly ($p < .05$) lower cell viability (37.45 ± 1.28%) after 72 h (Figure 1(F)). A similar effect was also obtained in case of A549 cells (28.77 ± 1.6% viable cells) upon combination treatment (Figure 1(G)). The percentage of cell viability in the combination groups was approximately equivalent to effect of 15-Gy irradiation dose for both cell lines. The combination treatment was more effective in killing A549 cells than HepG2. The bright field microscopy revealed pronounced cellular damage and morphological alterations in combination groups, compared to either radiation or the FA treatment alone (Figure 1(H,I)).

Effect of FA on redox status

The higher concentration of FA exhibits prooxidant effect on carcinoma cells. However, we found that 90 and 100 μM of FA treatment initially scavenged ROS. As shown in Figure 2(A,B), FA effectively reduced intracellular ROS in a time-dependent manner. However, 6 h (HepG2) and 3 h (A549) of FA treatment showed an increased level ($p < .05$) of ROS than the control group. This indicated that FA has dual effect on the redox status, resulting in reductive stress and eventually the oxidative stress, depending on the treatment duration and concentration. To find out the reason, we evaluated GSH level in carcinoma cells. As shown in Figure 2(C), at initial time points, the GSH level was noticeably higher in FA treated groups compared to control. This GSH level was significantly ($p < .05$) declined in HepG2 and A549 cells after 6 and 3 h of FA treatment, respectively (Figure 2(C)). We also evaluated the nuclear localisation of Nrf2 at different time points after the FA treatment. After 6 h (HepG2) and 3 h (A549), Nrf2 nuclear localisation reached the lowest level compared to the control group (Figure 3(A–D)). Thus, the compromised antioxidant level was induced by FA treatment. Furthermore, as presented in Figure 3, it was also observed that after treating the cancer cells with FA, the expression of Keap1 was greatly increased at the same time points when Nrf2 nuclear level was decreased (6 h in case of HepG2 cells; 3 h in case of A549). The probable explanation is that the FA treatment decreased ROS initially and induced reductive stress or cytostasis in carcinoma cells. To adapt in this reductive stress state, cellular machinery increased the Keap1 level to inhibit nuclear translocation of Nrf2 and activation of antioxidant system (GSH) at that time points. This impaired the ROS metabolism and its neutralisation. As the endogenous ROS were still produced, the newly formed ROS were accumulated at a faster rate and the level was significantly ($p < .05$) increased in HepG2 cells after 6 h of the FA treatment. A similar modulation was observed for A549 cells after 3 h of the FA treatment (Figure 2(A,B)). Consequently, the oxidative stress induced by FA resulted in a redox imbalance between the excess production of ROS and a defect in the antioxidant defence. The exposure to ionising radiation on top of this situation led to massive oxidative burst and caused radio-sensitisation of the carcinoma cells.
Combination treatment enhances oxidative stress in cancer cells

To investigate whether the effect of combination treatment is able to enhance oxidative stress in cancer cells, the ROS generation was measured after 1 h of irradiation by H$_2$DCFDA using flow cytometry. The histograms in Figure 4(A,B) represented the ROS level in HepG2 and A549 cells, respectively. The shift of histogram towards the right side indicated higher ROS generation. The ROS level in IR + FA treated groups were 1.43 fold (HepG2), and 1.86 fold (A549) higher compared to radiation alone (Figures 4(A, B) and S3(A,B)). Compared to IR and FA alone, the decreased level of reduced glutathione content (Figure S3(C,D)) along with higher rate of TBARS formation in combination groups (Figure S3(E,F)) further confirmed the prevalence of oxidative burst in both the cancer cells.
FA and IR combined treatment increases externalisation of PS and decreases p-Akt/p-p38MAPK level

The externalisation of PS in outer leaflet of cell membrane is the immediate early event associated with apoptosis. Although irradiation and FA treatment alone increased the early apoptotic population in both cancer cells, the combination treatment significantly increased both early (annexinV-APC+PI-) and late apoptotic (annexinV-APC+PI+) populations (Figure 4(C,D)). The HepG2 cells showed 47.55 ± 3.27% early apoptotic and 12.18 ± 0.22% late apoptotic population in the combination treatment whereas, A549 showed 50.74 ± 2.03% early apoptotic population, and 16.98 ± 0.54% late apoptotic population (Figure 4(C,D)). This finding indicated that the combination treatment was more effective in inducing the apoptotic pathway in cancer cells.

To comprehend the role of diverse intracellular signalling network in promoting cell survival, the involvement of p38-MAPK and p-Akt were elucidated. The combination treatment showed 5.62-fold, and 3.37-fold decrease in p-p38 MAPK level in HepG2 (Figure 4(E)) and A549 cells (Figure 4(F)), respectively when compared to IR alone. This was reciprocally increased after 6 h of radiation in both carcinoma cells (Figure 4(E,F)). To elucidate the factors driving higher cell viability in radiation group compared to combination group, the activation of PI3K-Akt survival pathway was studied by flow cytometry. Irradiation increased the level of phosphorylated Akt by 2.47 fold in HepG2 cells compared to IR+FA treated cells after 6 h of irradiation (Figure 4(G)). Similar pattern was observed for A549 cells (Figure 4(H)), indicating that the combination treatment could prevent the activation of Akt/MAPK pathway in both cancer cells.

Figure 2. Effect of FA on cellular redox status: (A) The histogram plots indicate the ROS level in HepG2 and A549 cells at different time points after FA treatment. The shift of histogram towards right indicates the higher ROS generation. (B) The bar diagram indicates the DCF fluorescence intensity in different experimental groups. (C) The bar diagram shows the reduced glutathione content at different time points and was expressed as nmol/mg of protein. Error bars represent mean ± SEM for n = 3. p<.05 was considered significant. Statistical comparison was done between control vs. FA group designated by “*” for HepG2 cells, and control vs. FA group designated by “#” for A549 cells.
Suppression of NF-κB, Nrf2 and STAT3 activation and expression of downstream Cox-2 by combination treatment

We hypothesised that the activation of Akt pathway emulates nuclear translocation of NF-κB and confers resistance against radiotherapy or chemotherapy. In order to test it, we examined nuclear translocation of NF-κB and Nrf2 by immunoblot. Both HepG2 and A549 cells showed higher nuclear localisation of Nrf2 under control, and irradiated conditions (Figures 5(A,B) and S4(A,B)). Combination treatment significantly ($p < .05$) decreased Nrf2 activation followed by nuclear localisation by 3.01-fold in HepG2 and by 7.5-fold in A549 cells, compared to irradiated group (Figure S4(A,B)). In comparison to control, irradiation significantly increased the nuclear localisation of NF-κB/p65 by 1.27-fold and 1.43-fold in HepG2 cells, and A549 cells, respectively (Figures 5(A,B) and S4(C,D)). However, when compared to combination groups in HepG2 and A549 cells, the combination treatment prevented this translocation process by 1.65-fold and 1.81-fold, respectively (Figures 5(A,B) and S4(C,D)) than IR groups. Similar result was obtained using fluorescence microscopy in which the combination treatment strikingly decreased the translocation of NF-κB (p65) (green fluorescence) into the nuclei (DAPI region) of both carcinoma cells – HepG2 (Figure 5(C)) and A549 (Figure 5(D)). The irradiation groups showed the maximum nuclear localisation of NF-κB among all other groups.

To further elucidate the intracellular signalling network in promoting cell survival, the involvement of STAT3 was elucidated, in addition to Akt-NF-κB pathway. The fluorescence microscopy images clearly showed the inhibition of STAT3 phosphorylation and its nuclear localisation due to the combination treatment in both cancer cells (Figure 5(E,F)). The lower infiltration of red fluorescence into the DAPI region indicated lower STAT3 phosphorylation and activation. The flow cytometry data showed significantly increased level of p-STAT3 after 24 h of incubation post irradiation (Figure 5(G,H)). The combination treatment resulted in a decreased level of p-STAT3 (3.86 in A549 cells, and 5.03-fold in HepG2 cells) compared to irradiation alone. To evaluate the induction of the downstream pathway of NF-κB, the expression of Cox-2 was studied (Figure 5(A,B)). Irradiation prominently increased the expression of Cox-2 in HepG2 cells by 1.33-folds (Figures 5(A) and S4(E)). Analogous finding was also observed in case of A549 cells (Figures 5(B) and S4(F)). The combination treatment significantly prevented the Cox-2 protein expression in both HepG2 (3.44-fold), and A549 cells (5.09-fold) compared to irradiation treatment (Figure S4(E,F)).
Figure 4. Enhancement of oxidative stress, early apoptosis and alteration in Akt/MAPK signalling pathway by combination treatment: (A) The histogram plot of ROS generation in HepG2 cells and (B) in A549 cells. DCF intensity is taken along the X-axis, FL1-H channel and count is taken along the Y-axis. (C) The externalisation of phosphatidyl serine (PS) and propidium iodide (PI) incorporation in HepG2 cells and (D) in A549 cells. The Q1 quadrant represents the viable cell populations. Q2 quadrant represents the early apoptotic cell populations (only annexinV-APC positive cells). The Q3 quadrant represents the late apoptotic cell population (Both annexinV-APC & PI positive cells). (E) The histogram plots show p-p38 MAPK level in HepG2 cells, and (F) in A549 cells. (G) The histogram plots represent the level of p-Akt in different experimental groups of HepG2 cells, and (H) of A549 cells. The fluorescence intensity in FL1-Channel is taken along the X-axis and count is taken along the Y-axis. SEM for \( n = 3 \) (three independent set). \( p < .05 \) was considered significant. Statistical comparison was done between control vs. IR and IR vs. FA + IR group.
Figure 5. Effect of combination of radiation and FA on STAT3/NF-κB activation and related downstream pathway in both A549 and HepG2 cells: (A) The expression of different signalling proteins with respect to loading control in HepG2 cells and (B) in A549 cells. (C) Immunocytochemistry data show subcellular localisation of NF-κB/p65 in HepG2 cells and (D) in A549 cells. (E) Immunocytochemistry data represent the subcellular localisation of STAT3 in HepG2 and (F) in A549 cells. The Nuclei were stained by DAPI (nuclear stain); NF-κB and STAT3 were stained by FITC and Texas red (TR) tagged secondary antibody respectively. The merged and 3D plot images show the infiltration of green or red fluorescence into DAPI region which indicates nuclear localisation of NF-κB/p65 and STAT3 in both HepG2 and A549 cells. Four different sections were studied for each individual group (technical replicates). \( n = 2 \) (two independent sets or biological replicate) for immunocytochemistry. (G) The histogram plots show level of p-STAT3 in HepG2 cells and (H) in A549 cells. Three independent experiments were performed \( (n = 3) \). \( p < .05 \) was considered significant. Statistical comparison was done between control vs. IR, and IR vs. FA + IR.
Combination of IR and FA treatment alters the expression of angiogenesis markers

The expression of PDGFRβ, PDGFRα, PECAM1 along with MMP-9 and VEGF were studied in the cell lines. We found decreased expression of MMP-9 (2.87-fold for HepG2, and 3.35-fold for A549) and VEGF (3.11-fold for HepG2, and 4-fold for A549) upon combination treatment as compared to radiation alone (Figure 6 (A–D)). We did not find any alteration in PDGFRα level. The irradiated HepG2 cells showed 1.53-fold higher expression of the PDGFRβ receptor compared to control cells (Figure 6(E)). The irradiated A549 cells showed 1.75-fold higher expression of same receptor compared to control (Figure 6(F)). However, a remarkable decrease in PDGFRβ was noticed in both carcinoma cells in response to combination treatment compared to IR alone. We also assessed the expression of important metastatic factor, PECAM1 by flow cytometry. The data revealed higher PECAM1 expression (1.87-fold) in A549 cells after 24 h of incubation post irradiation compared to the control cells (Figure 6(G)). The combination treatment significantly prevented PECAM1 expression compared to radiation treatment. It was further noted that only the FA treatment could inhibit the PECAM1 expression, which indicated that FA alone is capable of inhibiting the migration of A549 cells. However, there was no PECAM1 expression found in HepG2 cell.

FA and IR combination treatment promotes mitotic arrest in carcinoma cells

The combination prevented the survival pathway activation in carcinoma cells due to irradiation. We further studied the cell cycle progression of carcinoma cells via flow cytometry. The result revealed blockade at G2/M phase in both HepG2 and A549 cells due to combination treatment (Figure 7(A,B)). The irradiated cells showed an insignificant increase in G2/M phase population with respect to control. However, combination
treatment arrested 38.53 ± 2.52% of HepG2 and 40.39 ± 2.67% of A549 cells at G2/M phase (Figure 7(A,B)). To find out the underlying molecular basis of G2/M phase arrest, expressions of p53 protein, p21waf1/cip1, Cdc25C, and cyclin B1 were examined in both cell types (Figure 7(C,D)). The combination therapy showed 1.79 fold and 2.08 fold higher expressions of p53 protein in HepG2 and A549 compared to IR alone (Figure 7(C,D)). Almost a similar pattern was obtained for p21waf1/cip1. The combination of IR and FA decreased the Cdc25C expression by 1.78 and 1.9 fold in both HepG2 and A549 cells respectively compared to the irradiated groups (Figure 7(C,D)). An elevated level of cyclin B1 was found due to the reduced level of Cdc25C phosphatase which caused the mitotic arrest of A549 and HepG2 cells. The exposure of HepG2, and A549 cells to NSC 663284 (phosphatase inhibitor II) resulted in distinct G2/M phase arrest which corroborated with the result obtained by combination of IR and FA treatment (Figure 7(A,B)). This finding confirmed that the
compromised expression of Cdc25C due to combination therapy was responsible for mitotic arrest of carcinoma cells (Figure 7(A,B)).

**Activation of mitochondrial apoptosis pathway by combination of radiation and FA**

Oxidative damage to mitochondria irrevocably altered its membrane potential and caused the leakage of cytochrome C into the cytosol. A significant increase in population at lower quadrant with a loss of potential was observed in the IR plus FA treatment compared to IR treatment alone. In HepG2 cells, 46.3 ± 2.2% population was shifted to lower quadrant following combination treatment (Figure 8(A)). Radiation plus FA showed maximum Bax expression in HepG2 cells with significant ($p < .05$) downregulation in Bcl-2 expression (1.89-fold) compared to IR alone (Figures 8(B) and S5 (A,B)). The alteration of membrane potential and higher Bax/Bcl-2 ratio obtained by combination treatment illuminated the leakage of cytochrome C. To understand the downstream effect, the levels of active caspase 9, 3 and 7 and cleaved PARP were evaluated using flow cytometry after 48 h of incubation post-irradiation. The combination treatment significantly ($p < .05$)
increased the level of active caspase 9 by 1.78 fold in HepG2 cells (Figure 8(C)) compared to the radiation only. The higher level of active caspase 3 in the combination group compared to irradiation asserted the activation of intrinsic pathway (Figure 8(D)). As compared to the irradiation group, combination treatment showed 1.64 fold higher level of active caspase 7 in HepG2 cells (Figure 8(E)). Consequently, the level of cleaved PARP was also elevated in the combination group in comparison to any other individual treatments (Figure 8(F)). Similar findings were observed for A549 cells. Combination treatment to A549 cells showed 55.4 ± 2.9% cells with loss of membrane potential (Figure 8(G)) with higher Bax expression and 5.22 fold decrease in Bcl-2 expression (Figures 8(H) and S5(C,D)). This activated the intrinsic caspase cascade (Figure 8(I–L)). The activation of PARP was relatively higher in the A549 cells compared to the HepG2 cells. These results suggested that the involvement of PARP in inducing cellular apoptosis was more pronounced in the A549 cells than the HepG2 cells.

**FA and radiation-induces apoptosis culminates into DNA double-strand break**

TUNEL assay was performed to confirm the caspase-mediated cellular apoptosis. The control group showed a healthy morphology in both A549 and HepG2 cells. The irradiation and FA treatment alone caused an unusual swelling of nuclear bodies in A549 cells and to some extent in HepG2 cells. However, most of the cells showed a distinct hematoxylin counterstain and were apparently not apoptotic in nature. In contrast, the combination treatment dramatically increased the number of cells with TUNEL positive nuclei. These cells showed a massive alteration in the cellular morphology akin to apoptotic cells in both cancer cell types (Figure 9(A,B) HepG2 and Figure 9(C,D) A549 cells). The TUNEL index (calculated by dividing the number of TUNEL positive cells with total number of cells) plot of HepG2 cells and A549 cells showed a higher index value for A549 cells compared to HepG2 cells under the combination treatment (Figure 9(A–D)). These findings indicated that the combination treatment exerted more robust effect on A549 cells over HepG2 cells.

![Figure 9](image_url)
PI3K-Akt and p38MAPK mediated NF-κB activation underpins radiation-induced MMP-9 expression

The specific inhibitors against PI3K (LY294002), p38MAPK (SB203580) and Cdc25C phosphatase (NSC 663284 phosphatase inhibitor II) were used to confirm the involvement of these potential driver cascades. The immunoblot data revealed that LY294002/SB203580 inhibited radiation-induced nuclear translocation of NF-κB and expression of MMP-9 (Figures 10(A,B) and S6(B-E,G-J)) in both cell lines. Similar results ascertained that combination treatment is capable of inhibiting PI3K/Akt and p38 MAPK followed by NF-κB activation and MMP-9 expression. Therefore, FA along with radiation inactivated PI3K/Akt-NF-κB-MMP-9 and p38 MAPK-NF-κB-MMP-9 axis which together encompasses multiple events culminating in cell proliferation and angiogenesis of carcinoma cells (Figure 10(A–D)). However, the inhibition of nuclear translocation of NF-κB and MMP-9 expression was higher in cells treated with LY294002 compared to the cells treated with SB203580 indicating the critical driver role of Akt over p38 MAPK. The microscopic images showed that cell death was more in both carcinoma cells when treated with Akt inhibitor LY294002 compared to p38MAPK inhibitor SB203580 (Figure 10(E,F)).

FA protects the normal lung fibroblast WI-38 cells from radiation damage

The effect of this combination treatment was studied on normal lung fibroblast cells WI-38 (Figure 11) along with PBMC (Figures S7 and S8). FA alone did not show any toxic effect on either PBMCs (Figure S7) or WI-38 (Figure S9) rather protected them from radiation damage (Figures 11 and S7). WI-38 cells were treated with 10–500 μM of FA and incubated for 6 h, prior exposure to different doses of ionising radiation. WI-38 cells were treated with four different doses of gamma radiation 3, 5, 7.5 and 10 Gy. Among them 7.5 Gy dose of radiation showed 66.67 ± 5.75% viability and therefore, chosen to observe the radio-protective effect of FA. With the gradual increase of radiation dose there was a prominent decrease in cell viability was observed (Figure 11(A–D)). FA treatment prior to radiation successfully prevented (p < .05) the radiation induced cell death.

In order to observe the effect of FA on the cellular redox status, the cells were treated with 100 μM of FA and harvested after 1, 3, 6, 12 and 18 h of treatment. The ROS generation and nuclear translocation of Nrf2 were studied at different time points. The Nrf2 nuclear localisation was increased with FA treatment compared to the control group. The nuclear localisation was significantly (p < .05) high at 3 and 6 h time points. After that, there was a steady state level of Nrf2 observed in the nucleus (Figure 11(E)). It was also observed that the ROS level was continuously decreased with the increasing time points after FA treatment (Figure 11(F and G)). Although at 1 and 3 h time points the differences were not significant (p < .05), ROS level was low at the later time points (6, 12, 18 h). Therefore, treatment of normal cells with FA increased Nrf2 activation and dependent endogenous antioxidant system which reduced the ROS level and did not show any reductive stress response. This situation was altogether reversed compared to carcinoma cells (HepG2, A549, and CT26). As the carcinoma cells require ROS for their normal metabolism and signal transduction pathway, the elimination of ROS by FA treatment induced reductive stress. The cellular mechanism then inhibited nuclear translocation of Nrf2 level to increase the availability of ROS. The level of Nrf2 inhibitor, Keap1 was also significantly (p < .05) low in WI-38 cells under FA treated condition.

To find out whether FA pre-treatment was able to prevent ROS generation in WI-38 cells, we measured the ROS level under different experimental conditions. Exposure to 7.5 Gy dose of radiation increased the ROS level by 3.4 fold compared to control group. The FA treatment prior to radiation significantly decreased the ROS level compared to radiation treatment (Figure 11(H)). The externalisation of PS and PI incorporation were estimated by annexin-APC/PI staining. Irradiation increased the level of both early and late apoptotic populations after 24 h. The irradiated cells showed 12.99 ± 1.40% early apoptotic population and 7.75 ± 0.09% late apoptotic population whereas, the combination group showed 2.99 ± 0.023% early apoptotic population and a negligible percentage of late apoptotic population (Figure 11(I)). This result indicated a prominent protection to WI-38 cells by FA treatment prior to irradiation. Similar observation was obtained in case of mitochondrial membrane potential. The JC1 staining showed significantly high percentage of cells (20.91 ± 1.02%) with loss of membrane potential due to irradiation and this condition was reversed by combination of IR and FA treatment (8.89 ± 0.98%) (Figure 11(J)). The expression of cell survival protein like p-Akt, and apoptotic proteins like p53 protein, cleaved caspase 9 and 3 was observed by flow cytometry. The overlapped histogram showed a significant decrease in the p-Akt level under irradiated condition compared to the control. The treatment with FA before radiation exposure significantly (p < .05) increased the p-Akt level compared to radiation alone. This indicated that FA and...
IR combination treatment promoted the cell viability and increased the activation of Akt survival pathway (Figure 11(K)). Irradiation alone increased the level of p53 protein, p-p65, cleaved caspase 9 and 3 (Figure 11(L–O)). The higher level of p53 protein, cleaved caspase 9 & cleaved caspase 3 indicated the activation of...
Figure 11. Effect of combination treatment on normal lung fibroblast cells WI-38: (A) The figure shows the percentage of WI-38 cells viability after 3 Gy dose of irradiation in presence or absence of increasing concentration of FA. (B) The figure shows the percentage of WI-38 cells viability after 5 Gy dose of irradiation in presence or absence of increasing concentration of FA. (C) The figure shows the percentage of WI-38 cells viability after 7.5 Gy dose of irradiation in presence or absence of increasing concentration of FA. (D) The diagram shows the percentage of WI-38 cells viability after 10 Gy dose of irradiation in presence or absence of increasing concentration of FA. (E) Expression of Keap1, nuclear and cytosolic localisation of Nrf2 at different time points after FA treatment. (F) and (G) Level of ROS at different time points after FA treatment. (H) ROS generation under different experimental conditions. (I) Externalisation of phosphatidyl serine and PI incorporation under different experimental conditions. (J) Detection of mitochondrial membrane potential by JC1 staining. (K) The overlapped histogram plot represents the p-Akt level. (L) The overlapped histogram plot represents the p-p65 level in different groups. (M) The overlapped histogram plot represents the p53 protein level in different groups. (N) The overlapped histogram plot represents the cleaved caspase 9 and (O) cleaved caspase 3 level in different groups. Data were obtained from 3 independent experiments. SEM for n = 3, p < .05 was considered significant. Statistical comparison was done between “$” control vs FA, “***” control vs. IR, and “#” IR vs. FA + IR.
cellular apoptotic pathway in WI-38 cells due to radiation exposure. The higher level of p-p65 indicated the activation of inflammatory pathway in radiation group. The combination treatment successfully prevented the activation of inflammatory as well as apoptotic pathway in normal lung fibroblast WI-38 cells (Figure 11(L–O)).

**Regression of murine tumour by combination of IR and FA treatment**

We determined the effects of this combination on the tumour growth in vivo. The schematic diagram in Figure 12(A) showed the design of the experiment. As shown in Figure 12(B,C), tumour volume in the radiation group decreased 1.22-fold on 30th Day (after exposure to two fractionated doses of radiation). The five consecutive treatments with FA (50 mg/kg body weight) at every alternative day significantly inhibited the tumour growth by 2.5 fold on the 30th day. IR alone inhibited the tumour growth moderately. Treatment with FA (50 mg/kg body weight) at every alternative days for five times and followed by two fractionated doses of IR (2 Gy each) significantly (p < .05) inhibited tumour growth by 3.5 fold on the 30th day. Figure 12(D) showed the animal with tumour under different experimental conditions and in Figure 12(E), the tumour region (marked by white circle) also showed the prominent tumour regression in FA as well as in combination treatment condition.

Further profiling of this efficacy revealed that the DAPI staining and the histological section confirmed nuclear damage in different groups. The control tumour (treated with 0.1-M potassium phosphate buffer) showed normal nuclear morphology throughout the section. In IR group along with normal tumour nuclei, there were few numbers of fragmented nuclei were observed which indicated radiation-induced disintegration of nuclear structure of the tumor tissue. The FA treated condition significantly increased the number of fragmented nuclei compared to IR group. This observation was clearly in the line with of tumour size regression data which prominently proved the anticarcinogenic effect of FA for CT26 colon carcinoma cells. The nuclear damage was severely high in combination group compared to all other treatment conditions. Almost all the nuclei showed damaged and fragmented morphology that led to severe impairment in tumour tissue (indicated by arrow in Figure 12(F)). Moreover, the immunohistochemistry data showed higher activation of cleaved caspase 3 due to combination treatment compared to IR and FA treatment alone. The high level of cleaved caspase 3 confirmed the apoptosis of the tumour cells by combination treatment (Figure 12(G)).

The mode of tumour growth inhibition and histological tumour shrinkage was further supported by H&E data. The major effect of the combination treatment was the damage in tumour cell parenchyma and massive shrinkage in tissues which was characterised by fragmented appearance. The apoptosis of tumour cells was supported by nuclear fragmentation and apoptotic body formation at the neoplastic region. On the contrary, there was an abundance of mitotic features in the control tumour tissue indicating rampant tumour growth (Figure 12(H)).

**Discussions**

The radiotherapy increases ROS level beyond the threshold limit to promote cellular death [23]. The proliferative cancer cells maintain a high redox level where elevated ROS level and activated antioxidant defence system seemingly meet the equilibrium to promote neoplastic growth [24–26]. Reactive oxygen species at low and moderate level promote the cancer cell proliferation and survival through post-translational modification of multiple cellular proteins and kinases [27–29]. Therefore, in the present study a strategy was designed to down-regulate their antioxidant defence system initially, followed by the induction of oxidative stress upon exposure of ionising radiation. This was the basis of interrogating our model cell lines and murine tumour with the combination treatment.

The phenylpropanoids, mostly the naturally occurring phytochemicals found in plants, have been identified as potential radio-therapeutic agents due to their anticancer activity and relatively safe levels of cytotoxicity to the normal cells. These compounds could not only sensitize cancer cells to radiation resulting in inhibition of growth and cell death but also protect normal cells against radiation-induced damage [30]. However, the effect of these compounds on perturbing the redox status of carcinoma cells has remained unexplored. In this study, the pre-treatment of cancer cells with FA eliminated the intracellular ROS initially. ROS has crucial role in modification of kinases and activation of several transcription factors for cell proliferation. To ensure the availability of ROS and to maintain cellular redox homeostasis, the FA treated cancer cells by themselves impeded the nuclear localisation of Nrf2 by over expressing Nrf2 inhibitor Keap1. The neutralisation of ROS was therefore hampered due to the down-regulation of Nrf2 and antioxidant pathway dependency. As the endogenous ROS were still being generated at the
Figure 12. Effect of combination treatment on murine tumour: (A) Schematic line diagram shows the experimental design. (B) The images of tumours in different experiment groups. (C) The bar diagram represents the change in tumour volume under different experimental conditions. (D) The images of tumour bearing mice after completion of treatment. (E) The images of tumour bearing mice with marked tumour region which shows a prominent decrease in tumour volume at the end of the treatment. (F) DAPI staining of tumour tissue section to observe nuclear damage. The arrows indicate the nuclear fragmentation. (G) The level of cleaved caspase 3. DAPI was used to stain the nuclei and cleaved caspase 3 was stained by FITC tagged secondary antibody. The scale bar in image indicates 600X magnification. (H) The histopathological images of tumour tissue under different experimental conditions. The shrinkage in tumour tissue with severe damage and nuclear fragmentation are clear in combination group. The scale bar in image indicates 400X magnification. SEM for \( n = 3 \). \( p < .05 \) was considered significant. Statistical comparison is done between "**" control vs. IR, and "#" IR vs. FA + IR.
same rate, the newly formed ROS were accumulated at a faster rate at specific time points (documented at 3 and 6 h) after FA treatment in A549 and HepG2 cells. This converted the reductive stress to oxidative stress. It was also observed that FA treatment decreased the surface level and expression of GLUT1 and 4 in both the carcinoma cells (Figures S1(B,D) and S2(C,F)) and therefore, hindered the uptake of glucose [31–33]. An exposure to ionising radiation (7.5 Gy for HepG2 and 8 Gy for A549) on top of this situation showed a detrimental effect on carcinoma cells. The entire strategy escalated the oxidative stress burden, cellular damage and activated the apoptotic cascades in the cancer cells. Another benefit of this treatment was that a reduced dose of radiation (7.5 and 8 Gy) in the presence of FA was capable of showing the equivalent effect imparted by 15 Gy dose alone. Although in the cell culture model, we relied on non-fractionated dose of radiation, the tumour bearing mice were treated with fractionated dose of irradiation (two irradiations with 2 Gy dose) in presence or absence of FA. FA in presence of fractionated doses of radiation successfully regressed the tumour size in mouse tumour model. The histopathology and tissue immunofluorescence experiments also suggested the pronounced tumour cell death compared to radiation alone. The 7.5 and 8 Gy are the sub-lethal doses of radiation for HepG2 and A549 cells, respectively (Figure 1(C,F)). The earlier reports showed that the exposure to sub-lethal dose of radiation enhanced the invasiveness of HCC cells [17]. Therefore, sub-lethal dose of radiation was chosen for the treatment of cancer cells. Here in this study, we also found that sub-lethal dose of radiation promulgated the expression of different angiogenesis and metastatic proteins. FA and IR combined treatment prominently decreased the expression of angiogenesis and metastatic proteins. Indeed, radiation induced the activation of PI3K-Akt pathway in HepG2, A549 and CT26 (Figure S10) cancer cells along with the GLUT (not studied for CT26). Earlier reports showed that Akt stimulates glycolysis by phosphorylating key glycolytic enzymes and also increasing the expression as well as membrane translocation of GLUTs [34] which gives the clue for high expression and surface level of GLUT1 and 4 in carcinoma cells after irradiation. Thus, it can be suggested that radiation treatment activated ROS-Akt-GLUT-Glycolysis axis in carcinoma cells. The combination treatment induced heighten ROS generation which inhibited Akt activation followed by reduced the GLUT1 and GLUT4 expression. This mechanistically explains that combination treatment effectively disrupted the ROS-Akt-GLUT-Glycolysis network. The high level of lipid peroxidation and compromised level of reduced glutathione content in the combination group compared to IR highlight the enhanced oxidative stress condition in the carcinoma cells. We observed a coordinated hyper activation of Akt, NF-κB and STAT3 in response to radiation which increased the survivability of cancer cells. Several studies have explored the linkage between hyper activation of Akt-NF-κB-STAT3 and radioresistant nature of carcinoma cells [35–39]. The combination treatment downregulated Akt, NF-κB and STAT3 activation, inhibited RelA (STAT3-NF-κB) survival complex formation and henceforth, repressed the cell survival pathway [37]. We further noticed that irradiation increased the expression of invasion markers such as MMP-9, VEGF, PDGFRβ and PECAM1 expression in cancer cells whereas; the combination treatment significantly suppressed them. Moreover, combination treatment decreased the Cdc25C level thereby inhibiting cyclin B1 degradation. The high expression of p53 protein, p21 and accumulation of cyclin B1 induced the mitotic arrest in HepG2 and A549 cells. The wild-type p53 protein downregulates the death signalling suppressor Bcl-2 and upregulates the death pathway promoter Bax, thereby orchestrating the Bax/Bcl-2 dynamics towards programmed cell death [40,41]. In this study, the higher level of p53 protein in combination group also increased the Bax/Bcl-2 ratio which sustained the pore formation in mitochondrial membrane and facilitated cytochrome C leakage. The leakage of cytochrome C led to the activation of caspase 9,3,7 and PARP. Finally, the novel combination treatment made more DSB and significantly increased the number of TUNEL positive nuclei compared to the IR and FA treatment alone. Thus, combination treatment activated ROS-p53-Bax-Cytochrome C-intrinsic caspases-DNA damage axis in both carcinoma cells thereby programming them for apoptosis. The data also proved that radiation activated PI3K/Akt, and p38 MAPK pathway and ultimately increased MMP9 expression via NF-κB activation. The treatment of cancer cells with specific inhibitors against PI3K/Akt and p38 MAPK showed notable down regulation in NF-κB activation and MMP-9 expression. A similar kind of observation with the combination of FA and IR treatment strongly depicted that the FA treatment prior to irradiation, selectively inhibited the ROS-PI3K/Akt-p38MAPK-NF-κB-MMP-9 network in carcinoma cells. We also noticed that the involvement of PI3K/Akt pathway in activation of NF-κB-MMP-9 axis was prominent more in both the A549 and HepG2 cells compared to p38 MAPK pathway.

In addition, FA did not show any toxicity to PBMC (Figure S7) and WI-38 cells (Figure S10) even at higher
dose (400 μM) of treatment. Moreover, the FA treatment increased the level of p-Akt and facilitated nuclear translocation of Nrf2 which strengthened the antioxidant defence system. We evaluated the time-dependent nuclear localization of Nrf2 and expression of keap1 in WI-38 cells under FA alone treated condition. It was observed that FA treatment increased the nuclear localization of Nrf2 in all time points compared to control and also reduced the ROS level. As the normal cells maintain low redox level and the requirement of ROS is comparatively lower than carcinoma cells, this elevated antioxidant level did not induce reductive stress. Our previous studies using murine model also established that FA activated Nrf2-ARE antioxidant defence pathway in liver and intestinal epithelial cells [7,10]. This observation further strengthened the biphasic action of FA in response to differential redox status of normal and carcinoma cells. Combination treatment decreased in the level of p53 protein, cleaved caspase 3, and increased p-Akt which indicated the obvious protection of PBMC and WI-38 from radiation damage.

The antitumor effect of this FA plus IR combination treatment was also established in the syngeneic model. Before developing the murine tumour model, the radiation sensitisation effect of FA on CT26 cells was studied under in vitro condition (Figure S10). The combination treatment (4-Gy radiation + 300 μM of FA) showed increased level of ROS generation, PS externalisation and caspase 3 activation with compromised p-Akt level in CT26 cells compared to radiation alone (Figure S10). The tumour regression was due to the additive effect of this combination therapy. The tumour cell death occurred due to the activation of intrinsic apoptotic pathway which was similar to pathway observed under in vitro condition. This finding strengthened the radiation sensitising effect of FA. Earlier study showed that caffeic acid which is structurally similar to FA also increased radiation sensitivity of CT26 colorectal adenocarcinoma without affecting bone marrow cells [42]. Our previous study showed that 50-mg/kg body-weight dose of FA for 5 consecutive days protected Swiss albino mice from radiation injury [7–10]. The survivability experiment showed no toxic effect even with 100 mg/kg body weight dose of FA. Thus, FA in one hand has a potential to reduce the normal tissue damage caused by irradiation, on the other hand it can selectively increase the radiation sensitivity of tumour cells serving itself as potential radio-therapeutic agent.

Earlier investigation suggested that antioxidants when used before radiation treatment, selectively inhibit radiation damage repair of cancer cells, but protect normal cells [43,44]. Clinical study also showed that antioxidants enhanced the killing of cancer cells while decreasing the side effect of radiation in normal tissues.

**Figure 13.** Schematic representation of the overall events: The diagram represents the main signalling pathways on which the focus was given in the present study. The left box shows the effect of combination treatment in normal cells. The right box shows the effect of the same combination treatment on cancer cells. The down arrows indicate the down-regulation of that particular marker and the up arrows indicate just the opposite scenario. Combination treatment induces apoptosis in cancer cells while showing protection to the normal cells.
In this report, we established that FA instigates a differential modulatory effect on normal and carcinoma cells depending on the period of treatment and cellular redox status. An exposure to gamma-radiation after 3 and 6 h of FA treatment showed radiosensitisation effect on cancer cells which might provide a temporally critical therapeutic window. Similar observation in earlier study further justified our findings. Thus, taken together, the current findings offer new insights to radiation based therapy where mechanism driven rational approach would enhance the efficacy of existing and emerging treatment paradigms while keeping the systemic toxicity at reasonably low level (Figure 13). To our knowledge, this is the first report showing the linkage between metabolic and redox signalling events and their modulation by combination treatment. Indeed, further validation of these observations in more complex and heterogeneous preclinical platforms are warranted before considering its translational application.

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Disclosure statement

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