Novel Radiosensitizers

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Ferroptosis Induction by Combining Clobetasol Propionate with Radiation Results in Radio-sensitization of Keap-1 Mutant Human Lung Cancer Cells

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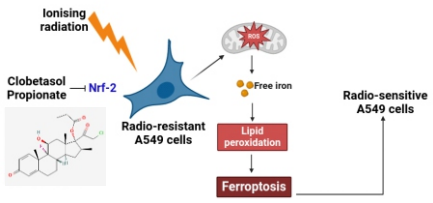

Radio-sensitization of A549 cells

ABSTRACT

Combining radiotherapy with an Nrf-2 inhibitor could be effective against Keap-1 mutant radioresistant lung cancer cells. The present study explored the use of Nrf-2 inhibitor, clobetasol propionate (CP), to enhance sensitivity of A549 lung cancer cells to radiation induced killing. CP treatment followed by radiation induced ferroptosis, a form of cell death triggered by iron induced oxidative stress and lipid peroxidation. The study identified the role of mitochondrial ROS in inducing ferroptosis. Nrf-2 overexpression in A549 cells resisted CP+4 Gy induced radio-sensitization. Thus, repurposing of US FDA approved anti-psoriatic drug, CP, presents a promising strategy for combating radioresistant lung cancer cells.

KEYWORDS: *Iron homeostasis, Glucocorticoid, Lipid peroxidation, Clobetasol propionate, Radio-sensitization, Non-small cell lung carcinoma*

Introduction

Lung cancer, responsible for roughly 18% of cancer-related fatalities worldwide, poses a significant challenge due to its resistance to radiotherapy [1]. Despite considerable efforts, the development of effective radiosensitizers that can be used in clinic remains limited, necessitating urgent exploration for safer and more efficient alternatives.

Clobetasol propionate (CP), originally approved by the US- Food and Drug Administration (FDA) for treating skin conditions like eczema and psoriasis due to its anti-inflammatory properties, has emerged as a promising candidate for repurposing in lung cancers characterized by mutations in Keap-1, a negative regulator of Nrf-2 [2]. Upregulation of Nrf-2, linked to poor prognosis in lung cancer patients, affects approximately one-third of non-small cell lung cancers (NSCLC). Further, exposure to radiation also activates Nrf-2 leading to radioresistance [3,4]. Small molecule inhibitors targeting Nrf-2 have shown promise in sensitizing cancer cells to chemotherapy, suggesting their potential as adjuvants to radiotherapy [5]. Hence, CP was combined with radiation to assess their potential to sensitize Keap-1 mutant human lung cancer cells in the current investigation. Inhibiting Nrf-2 with CP and exposure to radiation facilitated ferroptosis induction, thereby enhanced radiosensitivity in NSCLC cells [6]. Ferroptosis, an iron-dependent form of non-apoptotic cell death triggered by intracellular lipid peroxide accumulation, is hindered by Nrf-2 [7,8]. This approach underscores the potential of CP in overcoming radioresistance in lung cancer cells.

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Materials and Methods

A549 human lung adenocarcinoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS at 37^{0}C in a 5% CO_2 incubator. Cells were treated with indicated concentrations of CP and doses of γ-radiation. Cell viability was assessed using a cell titre Adenosine triphosphate (ATP) based luminescent assay. Live cell imaging was carried out in treated groups using Incucyte system. Sub-G1 population analysis was conducted using flow cytometry. Clonogenic assay was performed and colonies were counted after crystal violet staining. Spheroid formation and quantification was carried by propidium iodide (PI) staining microscopically.

Nrf-2 expression was studied by immunofluorescence microscopy and Western blotting. Gene expression studies were conducted by RNA Seq and real time PCR. Mitochondrial reactive oxygen species (ROS) levels were studied by Mitosox Red (MSR) staining, mitochondrial membrane potential (MMP) was estimated by JC-1 assay and Transmission Electron Microscope (TEM) was used for ultrastructure study.

Lipid peroxidation and iron levels were quantified using respective fluorescent probes (Liperfluo and BODIPY iron probe, respectively). Intracellular GPX4 levels were studied by fixation, permeabilization, staining with anti-GPX4 mAb

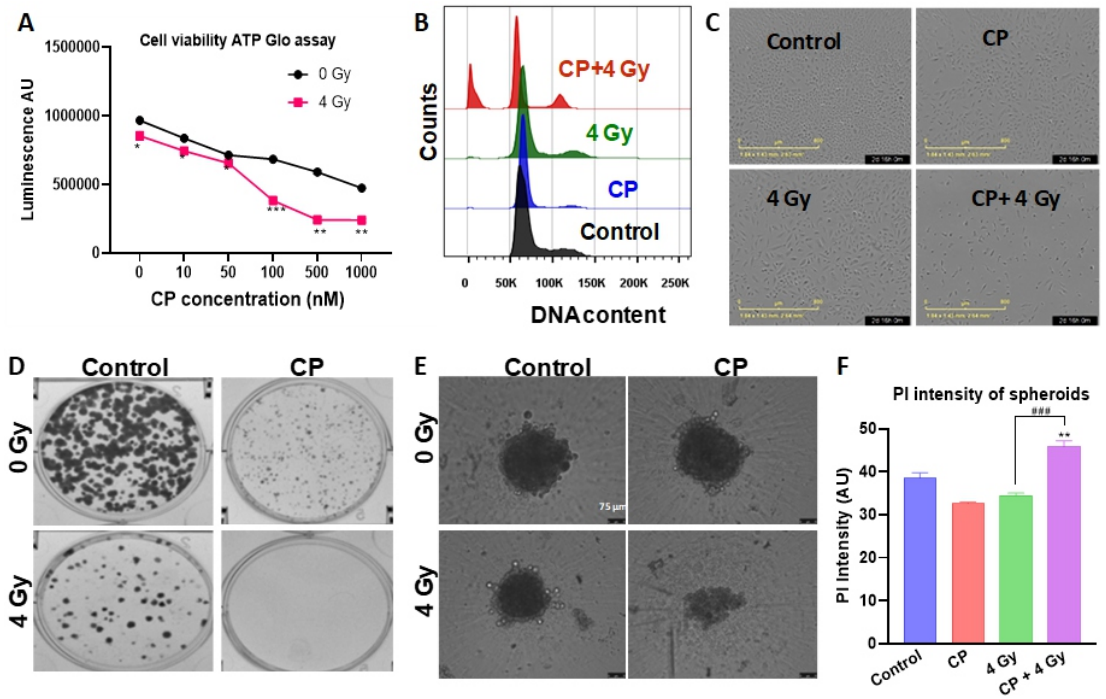


Fig.1: A) Cell viability was studied using Cell Titre ATP Glo assay in A549 cells with indicated treatments. B) Overlaid flow cytometric histograms of propidium iodide stained cells. C) Incucyte images of cells showing confluence post 64 hours of indicated treatments. D) Representative images of macroscopic colonies formed after 14 days of indicated treatments. E) Images of lung cancer spheroids 72 hours post treatment. F) Quantitation of spheroids after staining with PI is shown as bar graph. (Adapted from Rai et al., Acta Pharmacol Sin., 2024).

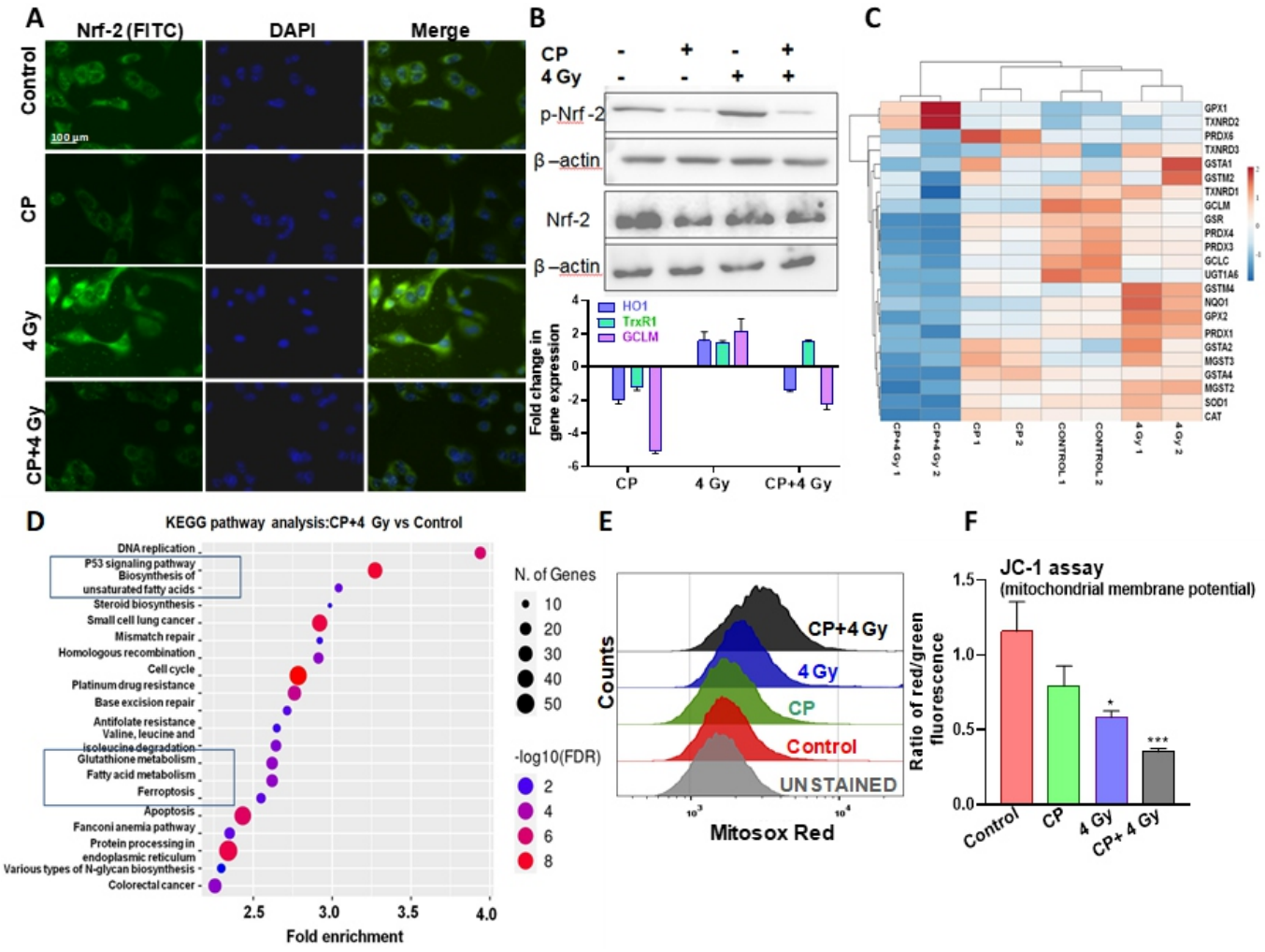


Fig.2: A) Immuno-fluorescence images showing nuclear translocation of Nrf-2 at 4 hours. B) Western blot images for Nrf-2 and phospho-Nrf-2 in cells treated with indicated groups. C) Heatmap of Nrf-2 dependent antioxidant genes studied by RNA Sequencing. D) KEGG pathway analysis of cells treated with CP+4 Gy was compared with control. E) Overlaid flow cytometric histograms showing mitochondrial ROS levels in indicated groups. F) Bar graph showing changes in MMP levels. (Adapted from Rai et al., Acta Pharmacol Sin., 2024).

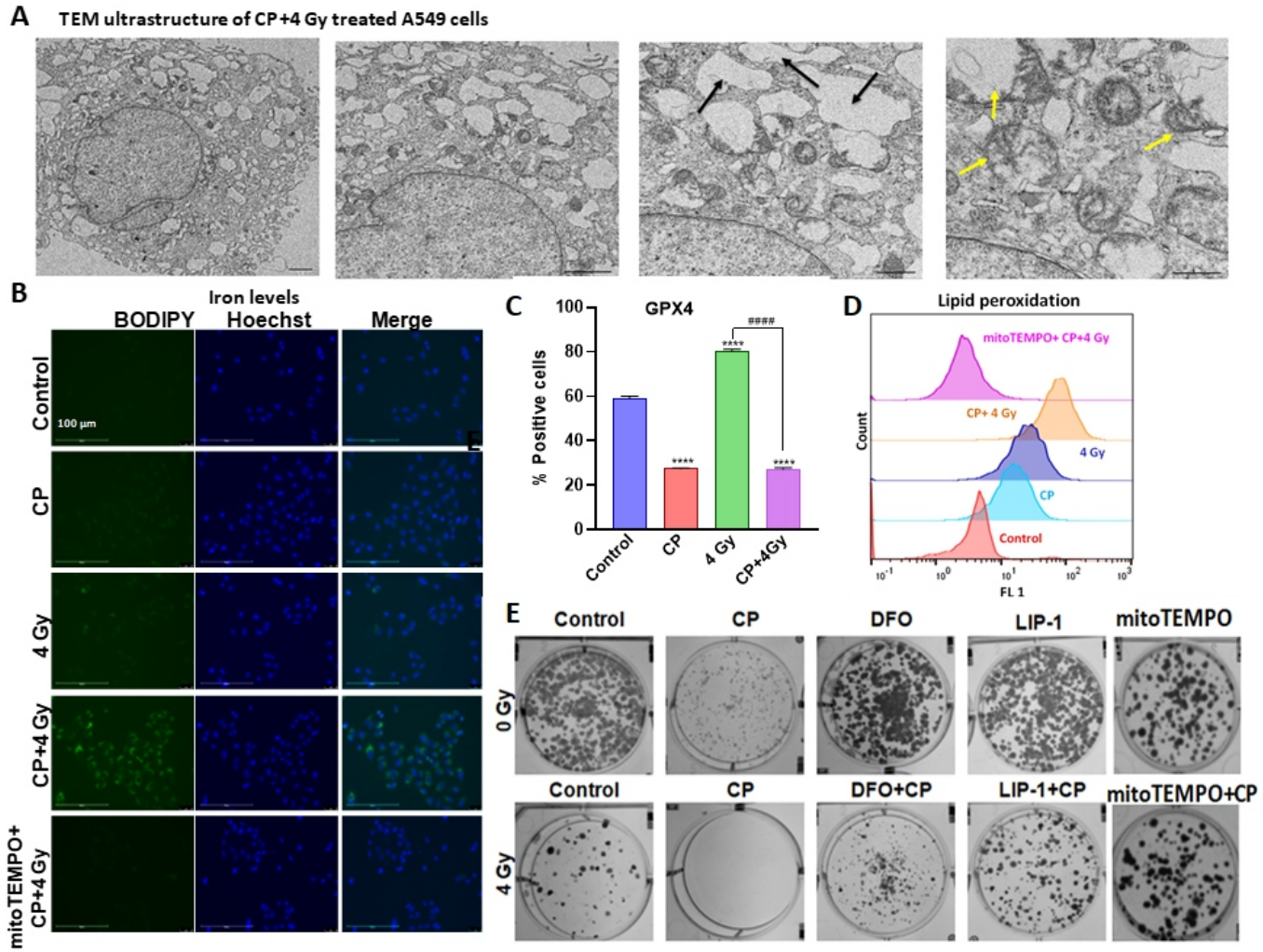


Fig.3: A) TEM images of cells treated with CP+4 Gy for 24 hours indicating increased vacuolisation (black arrows) and loss of cristae (yellow arrows). B) Images representing intracellular free iron levels in indicated groups. C) Bar graph showing GPX4 levels as studied by flow cytometry in cells treated with indicated groups. D) Overlaid flow cytometric histogram representing lipid peroxidation. E) Representative images of colonies of lung cancer cells treated with indicated groups. (Adapted from Rai et al., Acta Pharmacol Sin., 2024).

followed by PE-conjugated secondary antibody and acquired using a flow cytometer. Nrf-2 overexpression was achieved via lipofectamine based transfection. Animal experiments were performed using NOD/SCID BALB/c mice. Statistical analysis was conducted using Graphpad Prism 9.0 software.

Results and Discussion

CP sensitized radiation induced killing of lung cancer cells: Combination of CP and radiation resulted in significant loss of viability of A549 cells along with increase in cell death, as compared to individual CP or 4 Gy alone treated group (Fig.1 A, B). CP+4 Gy treatment resulted in loss of confluence due to cell death and severely diminished the clonogenic potential of A549 cells as compared CP or 4 Gy alone (Fig. 1 C, D). A549 tumor spheroid size and integrity were reduced along with higher PI uptake in the presence of CP+4 Gy treatment (Fig.1 E, F), as compared to CP or 4 Gy group. These results strongly suggested that CP enhanced radiation induced killing of Keap-1 mutant A549 cells.

CP inhibited Nrf-2 and upregulated genes involved in ferroptosis: Treatment of A549 lung cancer cells with CP downregulated the constitutive as well as radiation induced expression and nuclear translocation of Nrf-2 (Fig.2A, B). Due to inhibition of Nrf-2, several Nrf-2 anti-oxidant and cytoprotective genes were also downregulated by CP as studied by RT-PCR (Fig.2B). Transcriptomics data also suggested upregulation of genes involved in ferroptosis in CP+4 Gy treated cells (Fig.2C, D). To validate the role of ferroptosis, mitochondrial parameters were studied. Mitochondrial ROS was elevated and the mitochondrial membrane potential was compromised in CP+4 Gy treated cells as compared to CP or 4 Gy group (Fig. 2E, F).

Validation of ferroptosis by CP+4 Gy treatment: TEM studies indicated severe damage to mitochondrial ultrastructure like loss of cristae and increased vacuolisation strongly associated with ferroptosis in CP+4 Gy treated cells (Fig.3A). The combination treatment resulted in increase in intracellular free iron levels which was abrogated by using mitochondrial ROS scavenger mitoTEMPO (Fig.3B). CP and CP+4 Gy treated cells had downregulation of GPX4 which is reported to inhibit ferroptosis (Fig.3C). Lipid peroxidation caused by increase in iron levels was high in CP+4 Gy treated cells as compared to CP or 4 Gy group. This effect was abrogated by mitoTEMPO (Fig.3D). Scavenging mitochondrial ROS (mito-TEMPO) or pre-treatment with iron chelator (DFO) or ferroptosis inhibitor (Liproxstatin) resulted in abrogation of ferroptosis induced by CP+4 Gy treatment (Fig.3E).

Role of Nrf-2 in CP+4 Gy mediated radio-sensitization: Overexpression of Nrf-2 resulted in abrogation of lipid peroxidation and eventually inhibited CP mediated radiosensitization (Fig.4A,B). This substantiated the role of Nrf-2 in ferroptosis induced by combination treatment of CP

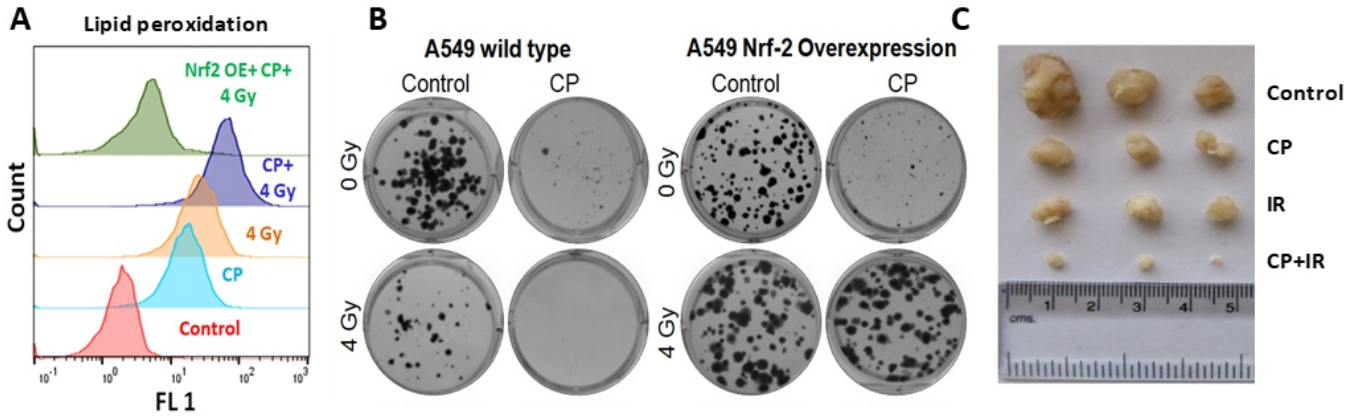
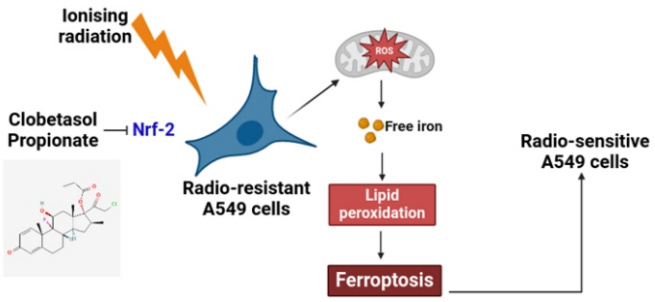


Fig.4: A) Overlaid flow cytometric histograms showing lipid peroxidation in indicated groups. B) Representative images of colonies formed by cells treated with indicated groups. C) Representative images of tumors excised from SCID mice transplanted with A549 cells from indicated groups. (Adapted from Rai et al., Acta Pharmacol Sin., 2024).



Scheme 1: Nrf-2 inhibition by CP prior to radiation exposure increases mitochondrial ROS mediated ferroptosis which results in radio-sensitization of A549 cells.

and 4 Gy. The in vivo studies clearly showed reduction in tumor burden in A549 xenograft tumor bearing SCID mice administered with CP and 3 x 2 Gy radiation (Fig.4C). The results strongly suggested that Nrf-2 inhibition by CP can exhibit radiosensitization in Keap-1 mutant Nrf-2 overexpressing lung cancer cells.

Conclusion

Multiple studies have suggested the potential of ferroptosis in overcoming therapy resistance and achieving better clinical outcome [9,10]. This underscores the promise of novel radiosensitizers that induce ferroptosis. The current study has identified clobetasol propionate, an FDA-approved agent, as a novel inducer of ferroptosis through Nrf-2 inhibition when combined with radiation (Scheme 1).

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