

Original Paper

Allicin Overcomes Hypoxia Mediated Cisplatin Resistance in Lung Cancer Cells through ROS Mediated Cell Death Pathway and by Suppressing Hypoxia Inducible Factors

Namita Pandey^a Gunjan Tyagi^b Prabhjot Kaur^a Shrikant Pradhan^a
M. V. Rajam^a Tapasya Srivastava^a

^aDepartment of Genetics, University of Delhi South Campus, New Delhi, India, ^bDepartment of Chemical Engineering, Imperial College London, London, United Kingdom

Key Words

NSCLC • Hypoxia • Allicin • Reactive Oxygen Species (ROS) • Cisplatin

Abstract

Background/Aims: The hypoxic microenvironment in NSCLC has been widely accepted as a contributor to both therapeutic resistance and tumor progression. In this study, we have explored Allicin, a key organosulfur compound present in garlic for its previously unreported effectiveness in the heterogeneous hypoxic tumor microenvironment of NSCLC. **Methods:** The effect of Allicin on the viability of NSCLC cells was determined by MTT assay. To determine the migration rate of treated cells compared to the control, scratch and transwell migration assays were performed. Flowcytometry was done to explore cell cycle distribution, apoptosis and ROS production in cells. Fluorescence microscopy was used to examine autophagy and DNA damage in cells. Dot blot was done to check genome wide methylation. RNA expression was detected by RT-PCR and protein expression by western blotting. **Results:** Allicin significantly decreases cell viability, proliferation and migration of NSCLC cells in both normoxia and hypoxia. It elicits both apoptosis and autophagy pathway in A549 cells by ROS accumulation and facilitating S/G2-M phase arrest in both normoxia as well as hypoxia. We suggest that ROS/MAPK and ROS/JNK signaling pathway together govern the cytotoxic effect of allicin in NSCLC cells. Notably, allicin suppresses the expression of HIF-1 α and HIF-2 α in hypoxic cells, pointing towards a mechanism of its effectiveness in hypoxia. A long term passive demethylation was observed, with decreased mC and no change in TET expression, thereby ruling out active demethylation by allicin. Furthermore, allicin synergistically enhances growth inhibitory

activity of low dose cisplatin to effectively overcome hypoxia induced cisplatin resistance in A549 cells. **Conclusion:** Altogether, our results elucidate a potential use of allicin in sensitizing hypoxic and chemoresistant NSCLC to cisplatin-based chemotherapy and provide new, affordable therapeutic strategy with reduced side effects.

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Introduction

Non small cell lung cancer (NSCLC) constituting ~80% of all lung cancer cases is a leading cause of cancer related death worldwide [1]. With ~40% of newly diagnosed cases at late stage of disease, its treatment has become a formidable clinical challenge [2]. Despite recent advances in potential therapy, NSCLC continue to be treated with cytotoxic combination chemotherapy with limited advancement in their potential to address recurrence and metastasis [2, 3]. Hypoxia, a common feature of solid tumors has been reported to be a major obstacle in treating lung cancer cells exhibiting increased metastasis and resistance to standard treatment [4]. Understanding of molecular pathway leading to tumor progression and metastasis has led to identification of specific inhibitors and modulators that when used in combination with chemotherapy or radiotherapy resulted in better clinical outcome. For example, hypoxia specific cytotoxin Tirapazamine in combination with radiotherapy and cisplatin showed promising median survival in several phase II trials [5]. Similarly, VEGF inhibitor, Bevacizumab when used in combination with paclitaxel and carboplatin improves survival in patients with metastatic NSCLC [6]. Recently, immune checkpoint modulators Nivolumab has shown promise in many solid tumors including NSCLC [7]. In our previous study conducted on NSCLC cell lines we observed that a pan-Histone Deacetylase inhibitor Scriptaid when used in combination with the low dose of cisplatin, synergistically increases the efficacy of cisplatin and reduces migration even in hypoxic condition [8]. Various natural compounds having cytotoxic activities with reduced side effects are now emerging as novel therapeutic candidates for treatment of cancer [9]. With the FDA approval to plant derived alkaloids, like vincristine, vinblastine, paclitaxel and many more, studies investigating the molecular mode of action of various natural compounds and developing them as a potential anticancer agent is underway [9].

Allicin (2-propene-1-sulfinothioic acid S-2-propenyl ester) is a key biological active molecule derived from garlic which was first known for its anti-bacterial properties [10]. In the Indian traditional treatment method, garlic extract itself is used widely for treating respiratory diseases however, lacks experimental validation. Further research conducted on this active compound revealed its potential to confer anti-fungal, anti-hypertensive, cardioprotective, anti-inflammatory and anti-cancer activities [11-14]. Allicin has been shown to be effective in killing cancer cells derived from kidney [15], liver [16, 17], ovary [18], pancreas [19], stomach [20, 21], brain [22], colon [23], bone [24] and lung [25]. Allicin can induce cell death through either autophagy or apoptosis and its mechanism of action may vary in a tissue specific manner involving antioxidant enzyme systems, p53 gene modulation, and activation of MAPK/ERK signaling pathways and/or PI3K/AKT/mTOR pathways [15-27]. Overall, the underlying mechanism of allicin induced cell death; especially in the heterogeneous tumor microenvironment lung cancer has not been addressed.

Keeping in mind the need to develop therapies that are effective in late stage tumors, we have conducted our analysis in both normoxic and hypoxic conditions. This work, hence, comprises assessment of cytotoxicity of allicin in heterogeneous lung tumor microenvironment, elucidating the molecular mechanism of allicin action and studying the effectiveness of allicin in combination with other standard anti-cancer therapies.

Materials and Methods

Cell culture

The human NSCLC cell lines A549 (adenocarcinoma) and NCI-H460 (large cell carcinoma) were purchased from National Centre for Cell Science (NCCS, Pune, India) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific, USA) and 5µg/ml ciprofloxacin (Sigma, USA) at 37°C in a humidified incubator with 5% CO₂. The *in vitro* hypoxic conditions were created using Anoxomat chambers (Mart® Microbiology, and the Anoxomat™ system, Netherland), as described before [8]. Culture conditions with 1% O₂ is referred to as 1% hypoxia and with 18% oxygen concentration as normoxia.

Cell viability and proliferation assay

The NSCLC cell lines' viability was assessed using water soluble compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as reported earlier [8]. The relative cell viability (%) was assessed separately for normoxic and hypoxic condition by comparing the absorbance of treated cells in normoxia/hypoxia with that of untreated control cells in normoxia/hypoxia respectively as (OD of treated cells/OD of untreated cells) × 100. For clonogenic assay, 1000 cells/well were grown in 6-well plates, treated with allicin and incubated in normoxia and 1% hypoxia for 2 hrs at 37°C. Subsequently, medium from each well was aspirated and cells were grown in regular growth medium for about 2 weeks until cells in control plates have formed colonies with considerably good size (50 cells per colony). The colonies were fixed with methanol: acetic acid (3:1) and stained with 0.5% crystal violet for visualization.

Wound healing and migration assay

For wound heal (scratch) assay, cells were seeded in 24-well plate at density of 1×10⁵ cells per well, the monolayer of cells was gently scratched using a pipette tip and wells were treated with required dose of allicin. The images were captured at 0hr and 24hr to identify the migration rate of treated cells compared to the control. The transwell migration assay was performed as described in our earlier study [8].

Cell cycle analysis

To determine cell cycle distribution, 48 hour treated NSCLC cells were processed as described before [8]. Propidium iodide (25µg/ml, Sigma, USA) stained cells were then analyzed using flow cytometer (BD FAC Calibur). The percentage of cells at different stages of cell cycle was calculated using BD CellQuest™ software.

DAPI staining for assessment of nuclear morphology

Cells cultured in four well chamber slides, after giving allicin treatment for 24hrs were fixed in 4% paraformaldehyde for 10min and stained with 1µg/ml DAPI (Sigma, USA) for 5min. Morphological changes were analyzed by fluorescence microscopy.

Quantification of apoptosis by Annexin V/PI staining

The percentage of early apoptotic, late apoptotic and necrotic cells was quantitated using an Annexin V-FITC/PI apoptosis detection kit (BD Pharmingen™, USA). Briefly, cells were seeded in 6-well culture plate and given indicated treatment for 48hrs. The cells were then harvested, washed with PBS and dual stained with FITC-AnnexinV and PI using manufacturer's protocol. The stained cells were analyzed by flow cytometry within 1hr and obtained data were accessed using BD CellQuest™ software.

Detection of Reactive oxygen species (ROS)

Intracellular ROS was detected using fluorescent dye 2',7'-dichlorofluorescein-diacetate (DCFHDA). Cells seeded in 6-well culture plate were treated with allicin in the absence or presence of 5mM N-Acetyl Cysteine (NAC) and incubated in their respective conditions (normoxia and 1% O₂) for 12hrs at 37°C. After incubation cells stained with 40µM DCFHDA dye for 1hr were harvested from the monolayer by trypsinization, resuspended in 500µl PBS and assessed immediately for ROS by flow cytometry (BD FACS Calibur) using the 488 nm lasers for excitation and 535 nm for detection.

Detection of Autophagy using LysoTracker dye

Cells seeded in cell culture dishes were given allicin treatment for 24hrs. The cells were then gently washed with PBS, flooded with fresh medium containing 100nM LysoTracker Red DND-99 (Invitrogen, USA) and incubated for 1hr in the dark at 37°C. Thereafter, the cells were inspected for LysoTracker accumulation by confocal microscopy (Leica TCS SP5) and flow cytometry (BD FACS Calibur).

γH2AX immunostaining for DNA damage

DNA damage was detected by microscopic analysis of γH2AX foci after immunostaining cells with γH2AX antibody and Alexa Fluor® 488 (Invitrogen, USA) (1:100) for 1hr at room temperature. Actin fibers were stained with phalloidin (Cytoskeleton, USA) and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, USA). The slides were mounted with Fluoroshield (Sigma, USA), sealed and observed under confocal microscope (Leica TCS SP5).

RNA isolation and Real-time PCR

RNA was extracted from allicin treated and untreated A549 cells cultured in normoxia and 1% hypoxia for 24hr using Trizol (Invitrogen, USA) according to the manufacturer's instructions. About 2μg of total RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen, USA) following manufacturer's instructions. The detailed primer sequences are given in Supplementary Methods (for all supplementary material see www.cellphysiolbiochem.com).

Western Blotting analysis for protein expression

Treated cells were washed with PBS and homogenized using lysis buffer (50mM Tris-Cl pH-8, 150mM NaCl, 0.02% sodium azide, 0.01% SDS, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, Protease inhibitor cocktail and Phosphatase inhibitor cocktail) as preparation for protein analysis. Protein estimation, polyacrylamide gels, electrotransfer were performed as before [8]. Immunoblots were detected with the chemiluminescence reagent Luminol (Thermo Scientific, USA), and developed by ImageQuant LAS4000 (Fujifilm, Japan). β-Actin was used as loading control for the total protein content. List of antibodies used in this study are given in Supplementary Methods.

Dot blot analysis of 5mC and 5hmC

Cells seeded in cell culture dishes were given allicin treatment (2μg/ml) and cultured in normoxia for 7 and 14 days. On attaining 75-80% confluence cells were passaged at a split ratio of 1:3. The time of passaging that includes trypsinization and replating was kept low (5-10 min) and the cells were immediately given allicin treatment. For analysis, the cells were collected from the monolayer by trypsinization and genomic DNA was extracted using QiaAmp® DNA blood Mini Kit (Qiagen, Germany) by following the manufacturer's instructions. The isolated DNA was then processed for dot blot analysis as described in our earlier study [28].

Statistical analysis

All experiments were performed at least in three biological replicates for reproducibility of data. Bars in the graph represent +standard deviation of at least three independent experiments. Two-tailed Student-t test was used to analyze the difference between two samples for which raw data was evaluated and $p < 0.05$ was considered as statistically significant.

Results

Allicin suppresses cell growth and inhibits proliferation and migration of human NSCLC

The anticancer activity of allicin was evaluated by examining the cellular morphological changes of two NSCLC cell lines, A549 and NCI-H460 after allicin exposure for 48hrs. As shown in Supplementary Fig. 1, allicin treatment led to cell death with features of fragmentation indicating apoptosis in A549 and NCI-H460 cells in both normoxia and hypoxia. The detached cells were confirmed to be unviable by Trypan blue exclusion assay (figure not shown). Further, MTT assay was performed to evaluate percentage viability in both the

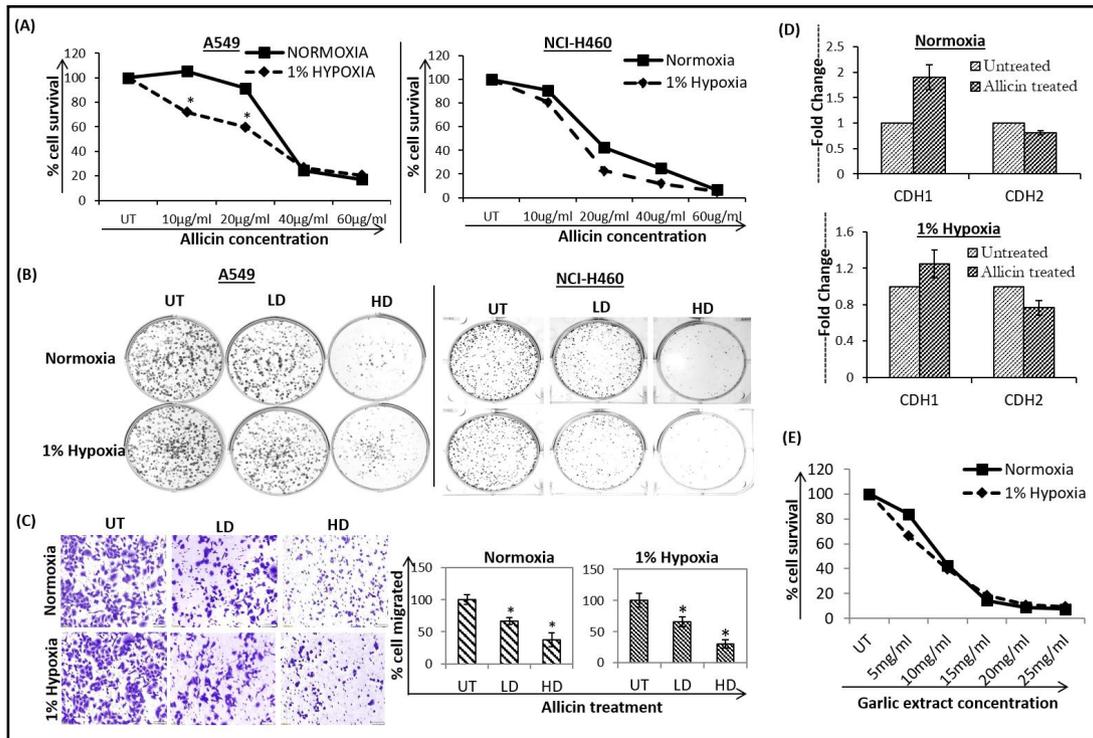


Fig. 1. Cytotoxic effect of alicin on NSCLC cells cultured in normoxia and 1% hypoxia. (A) A549 and NCI-H460 cells were treated with different concentrations of alicin (10–60 µg/ml) for 72hrs, and cell viability was assessed using the MTT assay. Data represent the mean of six technical replicate of three independent experiments. * represents $p < 0.05$ relative to its normoxic counterpart. (B) Clonogenic assay showing a significant inhibition of colony formation in low dose (LD) of 10µg/ml as well as high dose (HD) of 40µg/ml alicin treated samples compared to untreated (UT) sample in normoxia and 1% hypoxia. (C) Photomicrograph and plot representing percentage of A549 cell migrated in untreated and alicin treated conditions. The relative cell migrated (%) was assessed separately for normoxic and hypoxic condition by comparing the migration of alicin treated cells in normoxia/hypoxia with that of untreated control cells in normoxia/hypoxia respectively. The error bars indicate standard deviations. * represents $p < 0.05$ relative to its UT counterpart. (D) Real-time PCR of EMT markers in response to alicin (40µg/ml) treatment shows down-regulation of mesenchymal markers and up-regulation of epithelial marker in normoxic and hypoxic conditions. Results indicate fold change relative to untreated cells after normalization to the 18s expression. The bars represent the mean \pm standard deviation calculated from three independent experimental values. CDH1= cadherin1/E-cadherin; CDH2= cadherin1/N-cadherin. (E) MTT Assay representing cytotoxic effect of garlic extract on A549 cells cultured in normoxia and 1% hypoxia for 72hr.

NSCLC cell lines after alicin treatment. Alicin significantly decrease the viability of both the A549 and NCI-H460 cell lines in a dose dependent manner (Fig. 1A) in both conditions. The IC50 value for alicin in normoxia and hypoxia was $\sim 30\mu\text{g/ml}$ and $\sim 25\mu\text{g/ml}$ respectively in A549 and $\sim 20\mu\text{g/ml}$ and $15\mu\text{g/ml}$ respectively in NCI-H460, indicating effectiveness in both hypoxia and normoxia. Fewer and smaller colonies in alicin treated A549 and NCI-H460 cells compared to untreated cells in the clonogenic assay indicate inhibition of proliferation of the two NSCLC cell lines in both normoxia and hypoxia (Fig. 1B). Alicin repressed wound healing of A549 cells (Supplementary Fig. 2) and significantly inhibited migration capacity in both normoxia ($p < 0.05$) and hypoxia ($p < 0.05$) (Fig. 1C). Overexpression of epithelial marker, Cadherin1 (CDH1) together with down regulation of mesenchymal marker, Cadherin2 (CDH2) (Fig. 1D), further confirmed that alicin inhibits A549 cell migration in both normoxia and hypoxia. We have also shown that freshly crushed garlic, which has alicin as a key component, exhibits anti-cancer activity (Fig. 1E).

Allicin induces both apoptosis and autophagy in A549 adenocarcinoma cells

To further investigate the allicin induced cell death mechanism in NSCLC cells; the allicin treated cells were stained with DAPI and the nuclear changes were observed under microscope. Cells showed characteristic signs of apoptosis with condensed nuclei, blebbing and budding of apoptotic bodies at both the low dose and high dose of allicin in both normoxia and hypoxia (Fig. 2A). Annexin V-FITC/PI staining and flow cytometry analysis showed that in both normoxia and hypoxia, allicin effectively increased the early apoptotic (Annexin V⁺⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) cells in dose dependent manner (Fig. 2B). The cleavage of poly-ADP ribose polymerase (PARP) by caspases marks one of the important features of cell undergoing apoptosis [29]. We observed cleaved/active form of the executioners caspase-8 and PARP in A549 cells after allicin treatment in both normoxia and hypoxia suggesting that allicin mediated cytotoxicity is apoptotic in nature (Fig. 2E).

It is known that more than one pathways of cell death may be active at one time and it has been shown by others that allicin induces autophagy mediated cell death pathway in hepatic cancer cells [16, 17]. In order to study allicin mediated autophagy induction in our model of NSCLC cells, the A549 cells were stained with the fluorescent acidotropic probe, LysoTracker Red DND-99 that labels acidic organelles like lysosomes and analyzed by both the confocal microscopy and flow cytometry techniques. Allicin treatment markedly increased

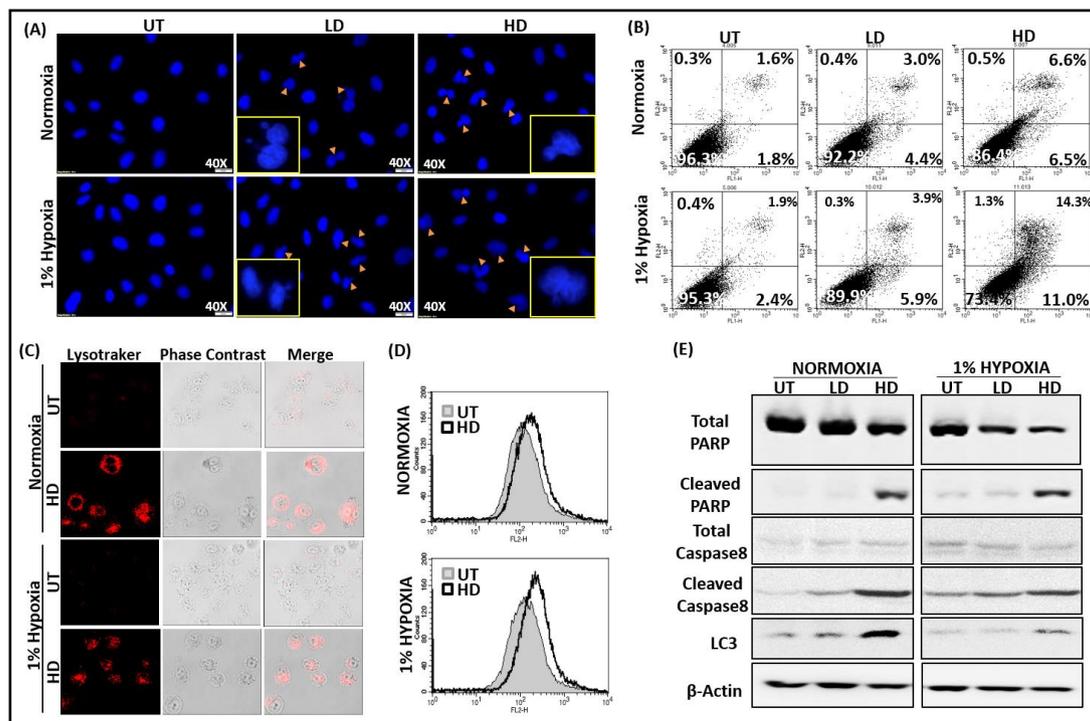


Fig. 2. Induction of apoptosis and autophagy by allicin in A549 cells in normoxia and 1% hypoxia. (A) Representative microphotograph of DAPI stained A549 cells after treatment with 10µg/ml (LD) and 40µg/ml (HD) allicin for 24hr in normoxic and hypoxic condition at 40X magnification. Yellow triangle indicates cells undergoing apoptosis. Inset represents enlarged image of apoptosing cells with condensed nuclei, blebbing of the membrane and budding of apoptotic bodies. (B) Flow cytometry analysis of Annexin V-FITC/PI stained A549 cells showing an increase in the percentage of the early apoptotic (Annexin V⁺⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) cells after allicin treatment in dose dependent manner in both normoxia and 1% hypoxia (C, D) Representative microphotograph at 60X magnification and Histogram overlay after LysoTracker Red DND-99 staining in A549 cells in response to 40µg/ml (HD) allicin treatment for 24hr in normoxic and hypoxic condition. (E) The protein levels of cleaved PARP, cleaved Caspase 3 and LC3 confirmed the induction of apoptosis and autophagy by allicin in A549 cells in normoxic as well as hypoxic conditions. UT= untreated.

the LysoTracker Red fluorescence in A549 cells in both normoxia and hypoxia (Fig. 2C). This was quantified using flow cytometry which showed a noticeable shift and increase in fluorescence (Fig. 2D) suggesting that allicin treatment caused induction of autophagy in A549 cells in both normoxia and hypoxia. This was further confirmed by noting an increased expression of LC3 protein in both normoxia and hypoxia (Fig. 2E).

Together, these results revealed that allicin can induce both apoptosis and autophagy in A549 cell lines.

Allicin causes ROS mediated apoptosis and autophagy in A549 cells accompanied with DNA damage

Oxidative stress regulations are known to play key role in anticancer therapy and an excessive ROS level is associated with severe cellular damage leading to cell death [30]. The role of ROS in allicin induced cell death pathway was studied using 2',7'-dichlorofluorescein diacetate probe that turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. Allicin treated cells showed notable enhancement of high intensity fraction and geometric mean fluorescence intensity calculation suggested significant ($p < 0.05$) increase in the ROS level in both normoxia and hypoxia (Fig. 3A, 3B). The antioxidant NAC could quench the increase in ROS in allicin treated cells thereby leading to a reversal in the observed shift of fluorescent intensity (Fig. 3C). Pretreatment with NAC significantly overcomes allicin induced cell death in both normoxia ($p < 0.05$) and hypoxia ($p < 0.05$) suggesting that allicin induces a ROS dependent mechanism of cytotoxicity (Fig. 3D).

To further elucidate the mechanism of reversal of cell death in NAC treated samples, we investigated the apoptotic fraction with flow cytometry analysis of Annexin V/PI stained A549 cells treated with allicin in presence of NAC. NAC treatment markedly reduces allicin induced early apoptotic (Annexin V⁺/PI⁻), (lower right quadrant) and late apoptotic (Annexin V⁺/PI⁺) cells (upper right quadrant) in both normoxia and hypoxia (Fig. 3E).

Pretreatment with NAC inhibited allicin induced cleavage of caspase 8 and PARP and decreased the expression of LC3 confirming that allicin induced apoptosis and autophagy in A549 cells is largely mediated by increased ROS accumulation (Fig. 3F). The antiproliferative activity of allicin was also found to be reversed by NAC pretreatment in NSCLC cell lines (Fig. 3G).

In our previous study we have reported binding of allicin to minor groove of DNA. The nucleic acid binding properties of allicin can cause DNA damage leading to cell cycle arrest or cell death [8]. DNA damage induced by allicin was evident by an increase in the formation of γ -H2AX foci, observed by immunofluorescence analysis and increased protein expression of allicin treated cells in normoxia and hypoxia (Fig. 4). Again, the ROS scavenger, NAC significantly abrogated the expression of γ -H2AX suggesting that allicin induced γ -H2AX phosphorylation is ROS dependent. Taken together these observations indicate that allicin mediated induction of ROS leads to DNA damage.

Allicin activates ROS dependent and p53-mediated cell cycle arrest via induction of p21

An understanding of the mechanism governing anti-proliferative effect of allicin is essential to determine its potential in therapy, hence the cell cycle profile of A549 cells treated with allicin and on quenching the ROS was studied in both normoxia and hypoxia. As shown in figure (Fig. 5A and Supplementary Fig. 3), allicin treatment led to accumulation of cells in the S and G₂-M phase in both normoxia and hypoxia. High dose of allicin caused ~30% cells to accumulate in G₂-M phase as compared to only 11% untreated cells in normoxia and 5% in hypoxia. The S-phase population was also slightly increased from 9% (control) to 15% in cells exposed to high dose allicin in normoxia and from 3% to 9% in allicin treated cells in hypoxia. NAC pretreatment markedly overcomes allicin induced S and G₂-M phase arrest and decreased sub-G₁ population suggesting that ROS accumulation by allicin induced G₂/M phase arrest in A549 cells.

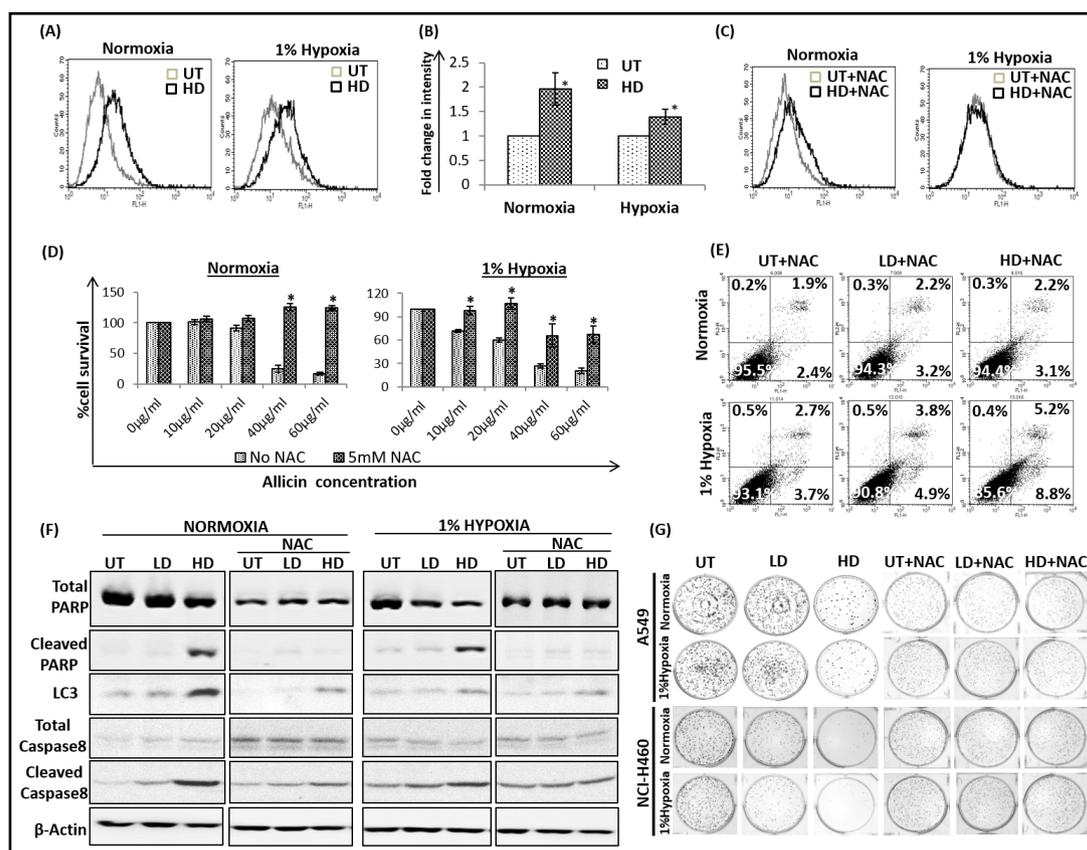
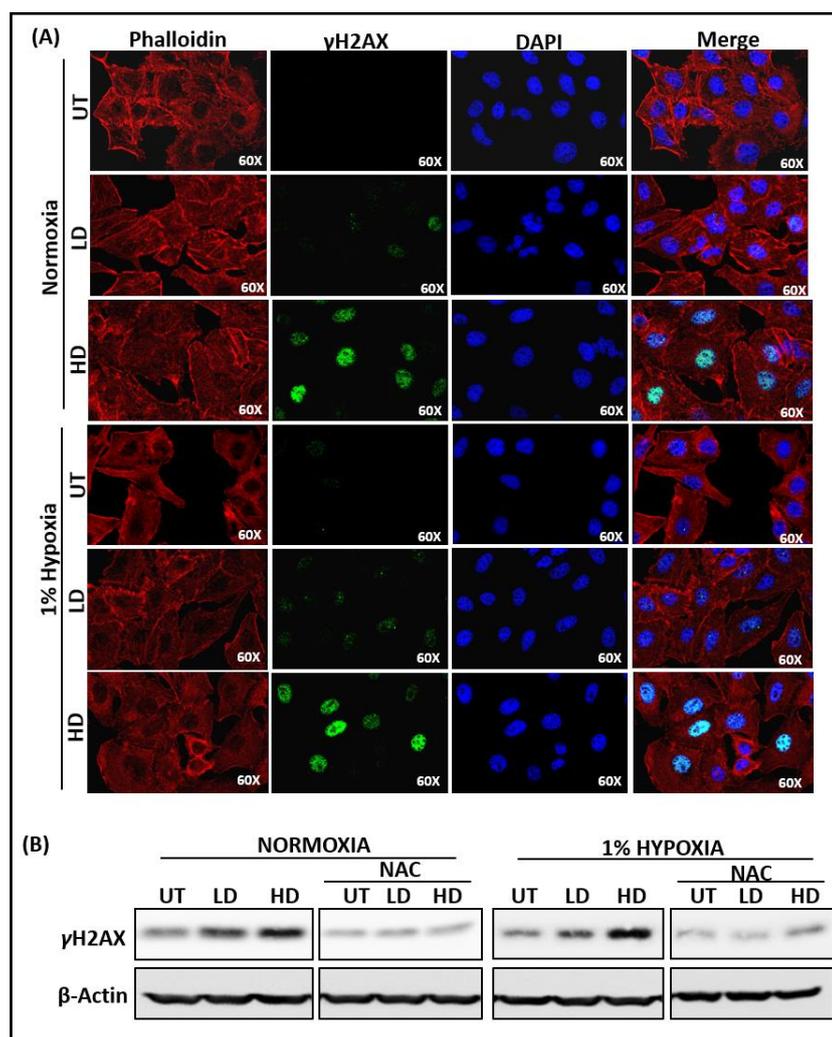


Fig. 3. Allicin induced ROS regulate autophagy and apoptosis in A549 cells. (A, B) Histogram overlay of 2',7'-dichlorofluorescein fluorescing untreated (UT) and 40 μg/ml (HD) allicin treated A549 cells cultured in normoxia and hypoxia as determined by flow cytometry and plot representing fold change in geometric mean fluorescence intensity of allicin treated cells wherein geometric mean fluorescence intensity of untreated cells was arbitrary set as 1.0. The bars represent the mean ± standard deviation calculated from three independent experimental values. * represents $p < 0.05$ compared to untreated cells. (C) The pretreatment of cells with antioxidant N-acetylcysteine (NAC) quenches allicin induced increase in ROS leading to a reversal in the observed shift of fluorescent intensity in allicin treated cells. (D) MTT Assay representing cytotoxic effect of allicin in absence and presence of 5mM NAC on A549 cells cultured in normoxia and 1% hypoxia for 72hrs. The bars represent the mean ± standard deviation calculated from three independent experimental values. * represents $p < 0.05$ relative to its NAC untreated counterpart. (E) Flow cytometry analysis of Annexin V-FITC/PI stained A549 cells showing NAC treatment markedly reduced allicin induced percentage of the early apoptotic (Annexin V⁺⁺/PI⁻) and late apoptotic (Annexin V⁺/PI⁺) cells in both normoxia and 1% hypoxia (F) Western blot analysis of total PARP, cleaved PARP, total caspase8 and cleaved caspase8 and LC3 proteins in NAC pretreated A549 cells confirming allicin induced apoptosis and autophagy in A549 cells as largely mediated by increased ROS accumulation. The western blot images of NAC untreated samples are same as in Fig. 2E, the picture is reused for comparison only. (G) Clonogenic assay showing NAC treatment markedly overcome allicin induced inhibition of colony formation in low dose (LD) of 10 μg/ml as well as high dose (HD) of 40 μg/ml allicin treated samples compared to untreated (UT) sample in normoxia and 1% hypoxia.

DNA damage signaling pathway can lead to activation of tumor suppressor gene p53 which further activates downstream effector genes causing either cell cycle arrest or apoptosis [31]. Allicin led to a marked increase in the transcript and protein expression of p53 and p21 protein in both normoxia and hypoxia, along with decreased expression of cyclin B1. These modulations were reverted in presence of ROS scavenger NAC (Fig. 5B).

Fig. 4. Accumulation of DNA damage in Allicin treated A549 cells. (A) Confocal images of A549 cells expressing γ -H2AX after treatment with 10 μ g/ml (LD) and 40 μ g/ml (HD) allicin for 24hr in normoxic and hypoxic condition at 60X magnification. Phalloidin was used to stain actin fibres and DAPI was used to stain nuclei (B) Western blot analysis of γ -H2AX in A549 cells treated with 10 μ g/ml (LD) and 40 μ g/ml (HD) allicin in absence and presence of antioxidant NAC and cultured in normoxia and 1% hypoxia for 48hr. UT= untreated.



p53 is a transcription factor that can regulate transcription of pro- and anti-apoptotic members of the Bcl-2 family thereby favoring apoptosis [32]. We observed that allicin significantly increased the expression of pro-apoptotic gene Bax and decreased the expression of anti-apoptotic gene Bcl2 (Fig. 5C) suggesting that allicin mediated apoptosis may involve the p53 dependent regulation of Bcl-2 family genes.

These results reveal that allicin mediated G2-M arrest and apoptosis in A549 cells is through ROS dependent alterations of p53, p21 and other downstream effectors.

ROS/MAPK and ROS/JNK signaling pathway, together regulate cell survival and death in allicin treated cells

Mitogen-activated protein kinase (MAPK) pathways are known to regulate various cellular processes like growth, proliferation, differentiation, migration and apoptosis [33]. We investigated the MAPK and JNK signaling cascade for our study. The expression of both total p38MAPK and phosphorylated p38MAPK decreased in a dose dependent manner in both normoxia and hypoxia. NAC treatment could revert this allicin mediated change indicating that the MAPK signaling network was regulated atleast in part, by allicin induced ROS (Fig. 5D). In contrast, phosphorylation level of JNK was markedly elevated even when expression of total JNK was decreased after allicin treatment and this was reversed by NAC pre-treatment suggesting that ROS/JNK pathway may be mediating allicin induced apoptosis of A549 cells in both normoxia and hypoxia. Hypoxia induces phosphorylation of p38MAPK, as

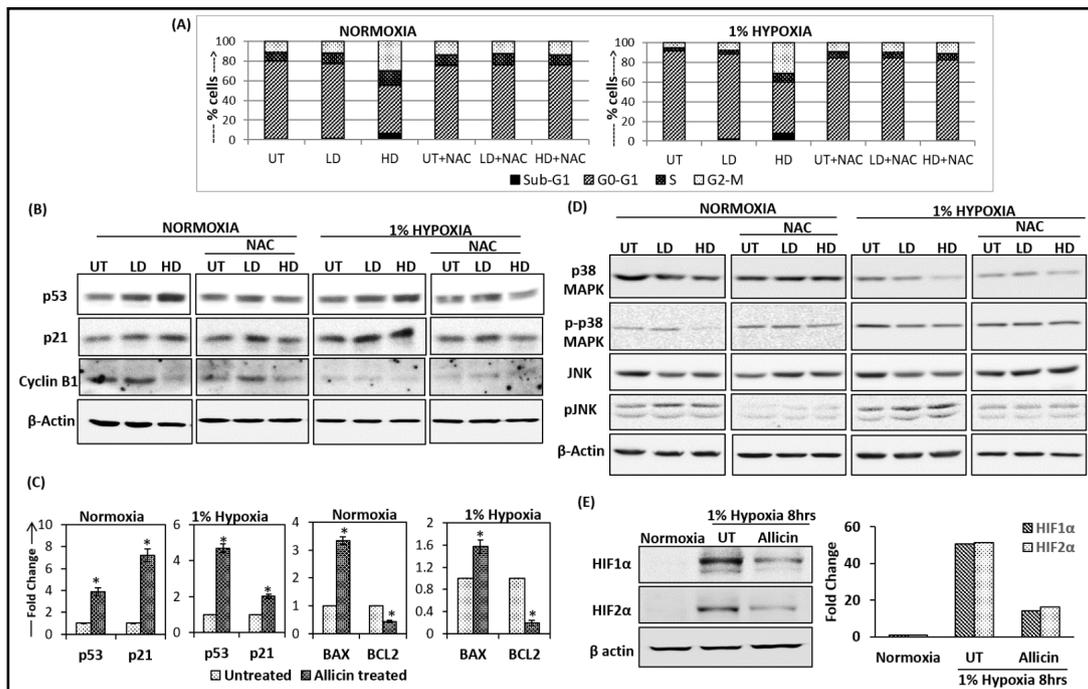


Fig. 5. Involvement of p53, p21 and MAPK signaling cascades in allicin mediated cell cycle arrest and cell death process in A549 cells. (A) Graph representing percentage of A549 cells present in different stages of cell cycle after treatment with allicin in absence and presence of antioxidant NAC for 48hr in normoxic and hypoxic condition as determined by flow cytometry. (B) Immunoblot analysis of p53, p21 and cyclin-B1 in A549 cells treated with allicin in absence and presence of antioxidant NAC and cultured in normoxia and 1% hypoxia for 48hr. (C) mRNA quantification of p53, p21, BAX and BCL2 by real-time PCR in untreated and 40µg/ml allicin treated A549 cells cultured in normoxia and 1% hypoxia. Results indicate fold change relative to untreated cells after normalization to the 18s expression. The bars represent the mean ± standard deviation calculated from three independent experimental values. * represents p<0.05 compared to untreated cells. (D) Western blot analysis of p38 MAPK, phosphorylated p38 MAPK (p-p38 MAPK), JNK and phosphorylated JNK (pJNK) in A549 cells treated with allicin in absence and presence of antioxidant NAC and cultured in normoxia and 1% hypoxia for 48hr. (E) Representative western blot analysis and corresponding graph depicting fold change in IDV of HIF-1α and HIF-2α in A549 cells treated with 40µg/ml allicin and cultured in 1% hypoxia for 8hr. UT= untreated, LD=10µg/ml allicin and HD=40µg/ml allicin.

evident from the increased expression of phosphorylated p38MAPK in untreated hypoxia sample when compared to its normoxic counterpart (Fig. 5D). Studies suggest that inhibition of p38MAPK activity can suppress HIF-1α expression and the consequent phosphorylation suggesting that activation of p38 MAPK is necessary for HIF-1α stabilization and nuclear accumulation [34, 35]. Thus, we examined the effect of allicin on HIF protein expression in hypoxia. As shown in Fig. 5E, expression of both HIF-1α and HIF-2α decreased with allicin treatment suggesting that allicin regulates HIF expression possibly via ROS/MAPK pathway in A549 cells in hypoxia.

Long-term allicin treatment leads to passive genome hypomethylation

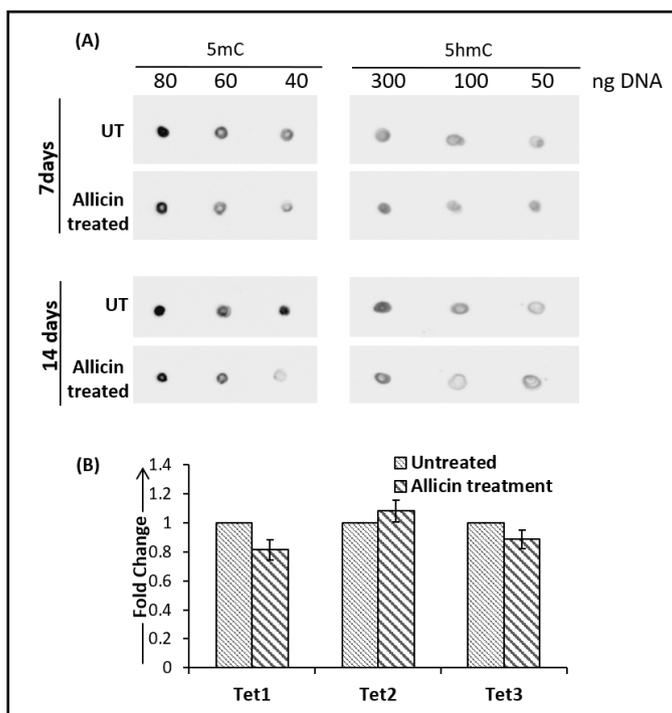
DNA methylation is one of the major epigenetic mechanisms involved in regulation of gene expression and global genomic hypomethylation together with regional hypermethylation of tumor suppressor genes is known to play important role in tumorigenesis [36, 37]. To understand the role of allicin in the epigenome, we investigated long-term effect of allicin on DNA methylation using dot- blot analysis of methylated cytosine residues (5'mC) and the de-methylated isoform 5-hydroxymethyl cytosine (5'hmc) which is formed due to active de-methylation activity of DNA demethylase enzyme, Ten-Eleven Translocation (TET).

Our observations from dot blot analysis revealed relatively unchanged level of 5'hmC levels from Day0 to Day14 of treated cells suggesting that allicin does not cause active DNA demethylation. However, a reduced 5'mC indicates that the existing methylation levels are not maintained in subsequent replication cycles (Fig. 6A). The relatively unchanged transcript levels of Tet1, Tet2, Tet3 genes as observed by real time analysis (Fig. 6B) further confirmed non-involvement of an active DNA demethylation pathway. Overall, these observations indicate that DNA is hypomethylated as a result of passive demethylation in long-term treatment of allicin to A549 cells.

Allicin synergizes the growth inhibitory activity of cisplatin on A549 cells and induces an enhancement of apoptosis in ROS dependent manner

Various studies suggest that enhanced cellular ROS accumulation sensitizes cancer cells to cisplatin induced cytotoxicity [38, 39]. Therefore, we investigated the potential of allicin in combination with low dose standard-of-care drug for lung cancer, cisplatin. The combination of 10µg/ml allicin followed by 2µg/ml cisplatin decreased A549 cell viability by 65% and was found to synergistically (Supplementary Methods) improve the efficacy of low dose cisplatin in normoxic conditions ($p < 0.05$) (Supplementary Fig. 4 and Fig. 7A). In hypoxia, the combination proved equally effective where it decreased cell viability to ~20%, thus having the potential to overcome hypoxia induced cisplatin resistance (Supplementary Fig. 4 and Fig. 7A). NAC, which effectively inhibited allicin-induced accumulation of ROS, significantly attenuated the combination induced cytotoxicity in both normoxia and hypoxia (Fig. 7A). These results suggest that allicin induced increase in ROS accumulation enhances cisplatin sensitivity even at low doses in A549 cells. The increased expression of cleaved PARP and relatively unchanged expression of LC3 in combination treatment as shown by western blot analysis further suggested that in A549 cells in both normoxia and hypoxia the combination therapy induced toxicity shifts the mechanism of cell death towards more apoptosis, unlike the contribution of autophagy with only allicin (Fig. 7B).

Fig. 6. Long-term effect of allicin on DNA methylation. (A) Dot blot analysis of 5-methyl cytosine (5'mC) and 5-hydroxymethyl cytosine (5'hmC) in A549 cells treated with 2µg/ml allicin and cultured in normoxia for 7days and 14days (B) mRNA quantification of Tet1, Tet2, Tet3 genes by real-time PCR in untreated and the 2µg/ml allicin treated A549 cells. Results indicate fold change relative to untreated cells after normalization to the 18s expression. The bars represent the mean ± standard deviation calculated from three independent experimental values. UT= untreated.



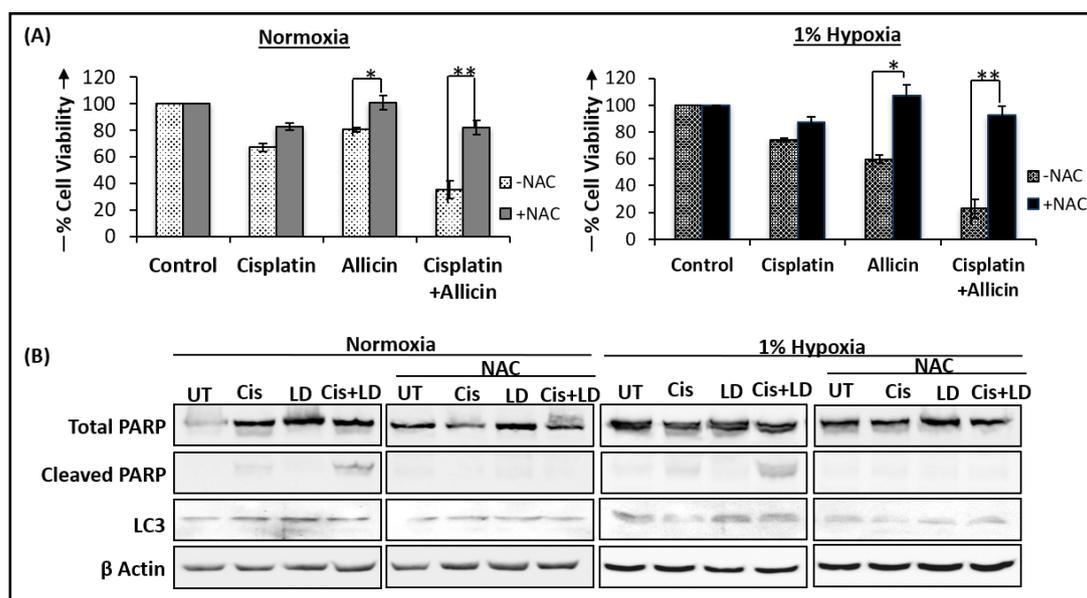


Fig. 7. Allicin treatment resulted in synergistic cytotoxicity with cisplatin. (A) MTT Assay- Cytotoxic effect of 2µg/ml cisplatin, 10µg/ml allicin and combination of 2µg/ml cisplatin and 10µg/ml (LD) allicin in absence and presence of 5mM NAC on A549 cells cultured in normoxia and 1% hypoxia for 72hrs. The bars represent the mean ± standard deviation calculated from three independent experimental values. * represents $p < 0.05$ and ** represents $p < 0.005$. (B) Western blot analysis of total PARP, cleaved PARP and LC3 in A549 cells treated with 2µg/ml cisplatin (Cis), 10µg/ml allicin (LD) and combination of 2µg/ml cisplatin and 10µg/ml allicin (Cis + LD) in absence and presence of antioxidant NAC (5mM) and cultured in normoxia and 1% hypoxia for 48hr. β-actin is used as loading control. UT= untreated.

Discussion

Efforts for improving the 5-year survival rate of patients with lung cancer continue to occupy cancer researchers. Debilitating side effect of radio and chemotherapy significantly diminish the quality of life of cancer patients [40], therefore, there is a renewed interest in a wide spectrum of pharmacologically safe molecules derived from the natural products and their derivatives as potential therapy [41]. Our focus has been in identifying molecules that overcome chemoresistance in the solid tumor microenvironment such as hypoxia and regulatory signaling networks that promote more malignant phenotype [42].

Allicin is a key organosulfur compound present in garlic and responsible for its anti-cancer activities. It is relatively unstable, and instantly decomposes to form other bioactive oil-soluble organosulphur compounds such as diallyl sulfide, diallyl disulfide, diallyltrisulfide, dithiins, and ajoene and some water-soluble organosulphur compounds such as S-allyl cysteine (SAC) and S-allylmercaptocysteine (SAMC) [43]. Despite its transient nature, allicin has been identified as the most active anticancer compound in aqueous garlic extract [44]. The ability of allicin to readily permeate the cell membrane may contribute to its pharmacological activity [45].

This study is the first to demonstrate the cytotoxic activity of allicin in hypoxic tumor environment of NSCLC cells and understand its mechanisms of cell death in both normoxia and hypoxia. We show that allicin is effective in killing NSCLC cells equally, if not more effectively, in hypoxia compared to normoxia and reducing cell viability of the two NSCLC cell lines (A549 and NCI-H460). Allicin also suppresses proliferation and significantly inhibits the migratory potential of cells in normoxic and hypoxic lung cancer cells.

Some of the earliest work has been reported in ancient Sanskrit vedic texts of 200CE, Charak Samhita, which describe garlic derivatives as being 'tumor-killing'. Garlic finds its

place in both Charaka as well as Sushruta Samhita, the earliest known texts of medicine and surgery respectively, as being the best chemical formulation identified by the knowledgeable practitioners of the ancient India [46]. More recently, its various derivatives have been shown to have anti-cancer activity in several cancers of different tissue origin. Several studies have looked at the potential of allicin as an anticancer agent and a few towards understanding the underlying molecular mechanisms.

Our work demonstrates new insights into the pathway leading to allicin mediated cytotoxicity in NSCLC cells which will help in utilizing allicin in combination with other drugs. Our study shows that allicin induced cell death in NSCLC engages both apoptosis and autophagy. Oxidative homeostasis plays a critical role in maintaining normal cellular processes and its deregulation is frequently observed in cancer cells [47]. It has also been established that ROS trigger cell death when its generation reached toxic threshold level by overcoming the antioxidant capacity of the cell and inducing irreversible oxidative modifications of lipid, protein or DNA [30, 48]. Various antineoplastic drugs are known to promote accumulation of ROS [30, 49]. For example, chemotherapeutic drugs such as paclitaxel and doxorubicin mediate anticancer effect by generating high level of ROS thereby leading to cell death [50, 51]. We observed significant ROS accumulation in allicin treated cells in both normoxia and hypoxia, which was inhibited by pretreatment of cells with antioxidant N-acetylcysteine (NAC). NAC also reversed allicin induced apoptosis and autophagy. Increased ROS level is a feature of hypoxic tumors that makes them more sensitive to oxidative stress [52] and our results demonstrate that in hypoxic conditions even low dose allicin causes elevation of ROS above the toxic threshold level disturbing redox balance, leading to loss of cellular integrity and cell death. Thus, allicin provides a therapeutic strategy to selectively kill hypoxic cancer cells using ROS-mediated mechanisms.

Autophagy induction by ROS is known to behave either as a cell survival mechanism against stress induced killing or a cell death mechanism against increased cytotoxicity [53]. From our experimental observations, we interpret that low dose allicin causes moderate accumulation of ROS that induces autophagy, which might be cytoprotective to the cell. It appears that increased production of ROS at high dose allicin causes lysosomes disruption in NSCLC cells thereby leading to autophagic cell death. Some anticancer agents that cause autophagic cell death via ROS generation are known to prevent lysosomal fusion with autophagosomes eventually leading to lysosomal disruption and release of hydrolytic enzyme causing cell death [54, 55]. Various studies describe complex cross-talk between autophagy and apoptosis, involving molecular machineries that respond to a specific condition by either co-operating or combating with each other, to activate either cell survival or cell death pathway [56, 57]. While our study comprehensively establishes a role for both these events, further work is required to elucidate their individual contribution in determining cell fate after allicin treatment.

We demonstrate for the first time an allicin mediated disruption of cell cycle progression and S/G₂-M phase arrest coupled with increased sub-G₁ cell population in NSCLC cells. In hypoxic condition, cells are known to divide slowly and get arrested at G₀-G₁ phase thereby, mitigating the cytotoxic effect of various chemotherapeutic agents that act through cell cycle regulation [58]. However, our results suggest that allicin induced S/G₂-M arrest in NSCLC cells overcomes the hypoxia mediated G₁ arrest. NAC treatment prior to allicin completely reverses the allicin induced S/G₂-M arrest confirming that that the observed stalling of the cell cycle is largely due to oxidative stress.

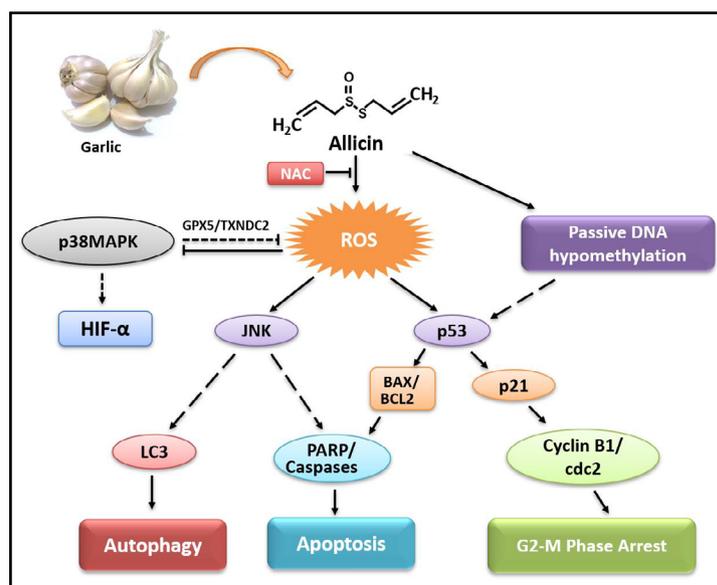
Allicin induced ROS is known to cause DNA damage in liver cancer cells [17]. Previously we reported for the first time a direct binding of allicin to DNA [25]. Consistent with these studies, we observed that allicin treatment elicits DNA damage in A549 cells grown in both normoxia and hypoxia as evident from increased expression of DNA double strand break marker, γ -H2AX. DNA damage can block cells at G₂-M phase by either p53 dependent or p53 independent pathway [59]. We have shown that allicin treatment to A549 cells in both normoxia and hypoxia causes ROS accumulation, which facilitated DNA damage and p53 activa-

tion. It is likely that activated p53 increases the expression of p21 and downregulates cyclin B1 level thus possibly preventing cdc2-cyclin B1 complex formation and activation of cdc25 thereby arresting cell at G₂-M phase.

Garlic is consumed extensively in cooked food, as off-the-shelf tablets, powder and juice; and as traditional medicine in India. Hence, we decided to investigate allicin's long-term epigenetic effect on tumor cells. We know that DNA methylation inhibitors like azacytidine and decitabine cause growth arrest and apoptosis in cancer cells by inducing re-expression of silenced tumor suppressor genes [60], while no such information is available for allicin. Long-term effect of allicin on DNA methylation suggests that while allicin reduces the expression of 5mC, the expression of 5hmC and DNA demethylase enzyme isoforms Tet1, Tet2 and Tet3 are unchanged. Our results suggest that allicin does not induce active demethylation appears to induce passive genome wide hypomethylation in A549 cells.

Finally, we shed more light on the molecular mechanism governing the anti-cancer effect of allicin by investigating two oft-altered pathways in the cell proliferation and survival network. The mitogen-activated protein kinases (MAPKs) constituting the extracellular signal-regulated kinases (ERKs), the C-Jun N-terminal kinases (JNKs) and the p38 MAPKs has been known to play role in regulation of cell cycle transition and cell growth stimulation, apoptosis and even tumorigenesis [61, 33]. Several reports indicate that both the p38 MAPK and JNK are involved in oxidative stress induced cell death cascade [62, 63]. It is known that p38 MAPK signaling negatively regulates the JNK pathway and p38 MAPK inhibition results in ROS upregulation, in turn activating the JNK pathway via inactivation of phosphatases [64]. Our results show that in both normoxia and hypoxia, allicin treatment decreases both the total p38MAPK and the phosphorylated p38MAPK expression level leading to marked increase in the phosphorylation status of JNK even with a decrease in total JNK. This phenomenon is reverted with NAC pretreatment. A concomitant decrease in the expression of HIF-1 α and HIF-2 α was also observed in allicin treated A549 cells grown in hypoxia suggesting a new mechanism of allicin mediated cell death in NSCLC cells. Allicin shows, with sufficient effectiveness in difficult-to-treat hypoxic conditions, an ability to suppress HIF expression as the possible mechanism of its efficacy. Based on the work done by other researchers and our own results, we propose that allicin induced ROS accumulation leads to phospho-p38 inhibition which then increases ROS accumulation and exacerbates cellular toxicity. It has been reported that p38MAPK can positively regulate antioxidant gene like GPX5 and TXNDC2 and thus can negatively regulate ROS accumulation [64]. The increased ROS may mediate the observed JNK activation and cell death in NSCLC cells in both normoxia and hypoxia. Stabiliza-

Fig. 8. A proposed model representing the pathways involved in allicin mediated cell death in A549 cells in normoxia and hypoxia. The solid arrows represent pathways that we have analyzed with our results while the dotted arrows represent those needing further validation.



tion of HIF protein in solid tumors can induce resistance to various chemotherapeutic drugs [65, 66]. We have observed a repression of HIF with allicin treatment and it is likely that this assists in increasing cytotoxic potential of allicin in hypoxic condition. A hypothetical model representing the pathways involved in allicin mediated cell death in lung cancer cells is shown in Fig 8, although further studies using inhibitors of the two concerned pathways are required to validate the proposed hypothesis.

Standard chemotherapeutic regimes are still the gold standard in post-operative management as well as a first line of treatment. We envisage a potential for allicin to be used as a chemotherapeutic drug in the future. As an initial step towards this goal, the efficacy of low dose allicin was tested with low dose of cisplatin. Cisplatin (cis-diamminedichloroplatinum) is the platinum based chemotherapeutic agent commonly used for treatment of a wide variety of solid tumors including lung cancer [67]. Cisplatin interacts directly with DNA to form inter and intra strand DNA adduct, cause DNA damage, interrupt DNA replication and activate signaling pathway that involves ATR, p53 and MAPK thereby inducing apoptosis in cancer cells [67, 68]. The elevated incidence of chemoresistance due to several reasons including tumor hypoxia and diverse undesirable side effects such as neuro- and/or renal-toxicity is the main limitation of cisplatin use in clinical practice [69, 70]. To overcome drug-resistance and reduced toxicity, combination therapies of cisplatin with other drugs have been considered [68]. Various studies suggest that the sensitivity of tumor cells to cisplatin can be enhanced by selectively increasing cellular ROS accumulation [71, 72]. Our study suggests that low dose of allicin synergises the growth inhibitory activity of cisplatin and can effectively overcome hypoxia induced cisplatin resistance in A549 cells. We have observed that the combination of allicin and cisplatin enhances apoptosis in a ROS dependent manner in both normoxia and hypoxia.

Therapeutic efficacy of phytochemicals like allicin depend heavily on its effective delivery to the tumor site. Unlike oral administration where the metabolic pathways of gastrointestinal tract epithelial cells might mitigate its efficacy, the intraperitoneal injection of raw garlic extract could successfully cure the sarcoma S180-bearing mice [73]. Similarly, the aged garlic extract was found to be more effective in the treatment of a bladder tumor mice model through intralesional administration [74]. Use of nanoparticle in delivery of phytochemicals has also shown promise [75]. We hope that our work boosts the on-going efforts for making allicin a deliverable drug. Our study proposes new insights and suggests that allicin could be effectively used to sensitize lung cancer cells for the cisplatin-based chemotherapy and provide new therapeutic strategy for lung cancer with reduced side effects.

Acknowledgements

NP was a recipient of UGC-BSR fellowship, PK is a recipient of ICMR-SRF fellowship. We thank Dr. Neeru Saini from the Institute of Genomics and Integrative Biology (IGIB), Delhi, for generously giving us p83MAPK antibody for our experiments.

Author Contributions

NP: Concept and design, execution of experiments, data interpretation and analysis, manuscript writing; GT: Standardization, execution of preliminary experiments; PK: executing experiments; SP: Standardization, execution of preliminary experiments; MVR: Material, resource, research facility and manuscript reading; TS: Concept and design, data interpretation and analysis, financial support, manuscript writing, final approval of manuscript.

Funding

The work did not have a direct funding. We are grateful to Science and Engineering Research Board, SERB, India for grant support to TS. Support from University of Delhi R&D

fund to TS was helpful. We thank UGC for SAP (BRS III), DST for FIST (level II) program, DU-DST PURSE (phase II) and the Central Instrumentation Facility at University of Delhi South Campus for supporting our work.

Statement of Ethics

This study did not require any ethics approval.

Disclosure Statement

The authors declare no competing financial and non-financial interests exist.

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