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**Original Research Article**

# Dihydropyrano[2,3-c]pyrazole-induced apoptosis in lung cancer cells is associated with ROS generation and activation of p38/JNK pathway

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## Abstract

***Purpose:*** *To investigate the effect of 2,4-dihydropyrano[2,3-c]pyrazole (DHPP) on lung cancer cells, and the associated mechanism.*
***Methods:*** *The effect of DHPP on cell proliferation was measured using sulphorhodamine B (SRB) assay. Apoptosis of cells was determined using Olympus IX71 inverted microscope connected to FITC and rhodamine filters.*
***Results:*** *DHPP significantly suppressed the proliferation of A549 and H1299 cells at doses of 0.5-8.0 μM, but did not affect normal cells (MRC5 and BEAS-2B). In DHPP-treated A549 and H1299 cells, caspase-3 activity was markedly enhanced. At 24 h of treatment with 8.0 μM DUPP, apoptosis in A549 and H1299 cells was increased to 67.89 and 61.35 %, respectively. Phosphorylation levels of JNK-1/2 and p38 in DHPP-treated A549 and H1299 cells were markedly enhanced. The p-ERK-1/2 expressions in DHPP-treated A549 and H1299 cells were suppressed significantly at 24 h. In DHPP-treated A549 and H1299 cells, DCF-fluorescence was increased 10 folds and 8.5 folds, respectively. Pretreatment with FeTMPyP, an antioxidant, effectively alleviated DHPP-induced increase in expressions of p-p38 and p-JNK, and suppression of expression of p-ERK-1/2. In FeTMPyP-pre-treated cells, the DHPP-induced increase in caspase-3 activity was markedly reduced.*
***Conclusion:*** *DHPP selectively inhibits lung cancer cell growth via oxidative stress which subsequently causes cell apoptosis. Moreover, it activates caspase-3 protein and p38/JNK signaling, with simultaneous inactivation of ERK-1/2. Therefore, DHPP has a potential to be developed for the treatment of lung cancer. However; more studies are required to confirm these findings.*

***Keywords:*** *Lung cancer, Anti-oxidant, Apoptosis, Caspase-3, Chemotherapy*



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## INTRODUCTION

Lung cancer is the most common cause of mortality associated with cancers, and it has very high metastatic potential [1]. Causative molecular mechanistic studies have very effectively helped in development of treatment strategies for lung cancer, as well as improvements in patient prognosis [2]. However, despite improved treatment

strategies, the 5-year survival of lung cancer patients is only 17 % [3]. Therefore, further mechanistic investigations and search for effective therapeutic compounds are required for pulmonary cancer treatment.

Reactive oxygen species (ROS), the bye products generated during various metabolic processes, have either beneficial or toxic effects [4]. Abnormally increased ROS levels initiate oxidative stress leading to activation of secondary events like apoptotic, autophagic and necrotic cell death [5]. The ROS-induced stress activates mitogen-activated protein kinase (MAPK) comprising extracellular signal-related kinases (ERKs), c-Jun $NH_2$-terminal kinases (JNKs) and p38 MAPKs at cellular level [6]. Oxidative stress-mediated cellular apoptosis is linked to phosphorylation of p38 and JNK proteins [7]. The vital roles of MAPKs have been clearly demonstrated in multiple cellular processes such as proliferation and apoptosis [8]. Many anticancer drugs rely on apoptosis activation to eliminate cancerous cells [9].

Heterocyclic molecules bearing pyrazole rings connected to pyrans possess diverse pharmacological properties. For example, compounds such as 1,4- and 2,4-dihydropyrano[2,3c]pyrazoles exhibit significant anticancer [10] and anti-inflammatory effects [11]. Studies have shown that the substituted 2Hfuro[2,3-c]pyrazoles possess anti-platelet and anti-allergic properties [12, 13]. The present study investigated the anti-proliferative effect of 2,4-dihydropyrano[2,3-c]pyrazole (Figure 1) on lung cancer cells, and the underlying mechanism.


**Figure 1:** Chemical structure of 2,4-dihydropyrano[2,3-c]pyrazole

## EXPERIMENTAL

### Cell culture

The A549 and H1299 cancer cells and normal cells (BEAS-2B and MRC-5 cell lines) were obtained from ATCC, USA. The cell lines were grown in DMEM containing 10 % FBS and the antibiotics penicillin and streptomycin, each at a concentration of 100 U/mL. The cells were grown overnight under humidified atmosphere containing 5 % $CO_2$ in an incubator at 37 ºC.

### Cytotoxicity assay

Changes in cell proliferation due to treatment with DHPP at doses of 0.5, 1.0, 2.0, 4.0 and 8.0 µM DHPP were measured using SRB assay [17]. Cells in DMEM in 24-well plates at 90 % confluence were treated with DHPP for 24 h. Thereafter, the cells were fixed for 1 h with 30 % trichloroacetic acid at 4 ºC and rinsed with de-ionized water, followed by air-drying at room temperature. Then, the cells were incubated at room temperature with SRB (0.04 %) for 40 min, after which they were washed thrice in 1 % acetic acid, air-dried and put in Tris-base (10 mM). The absorbance of the cells was read at 568 nm using EnSpire multimode plate reader.

### Assay of caspase-3 activity

Cells pretreated with MAP kinase inhibitor for 1 h were exposed to 8.0 µM DHPP for 24 h, and caspase-3 activation was determined using AFC-conjugated tetrapeptide substrates. Termination of incubation was followed by PBS washing and lysis with ice-cold buffer (50 mM HEPES, pH 7.4, containing 5 mM CHAPS and 5 mM DTT). The lysate was centrifuged at 4 ºC for 15 min at 12,000 g and the supernatant was mixed with assay buffer [HEPES (40 mM), pH 7.4, containing CHAPS (0.2 %), EDTA (4 mM) and DTT (10 mM)] and caspase-3 substrate (40 nM Ac-DEVD-7AFC). The mixture was incubated at 37 ºC, and fluorescence was measured after 5 min using multimode plate reader (Perkin Elmer).

### Annexin-V/FITC staining

The cells were cultured on coverslips of 6-well plates and exposed for 24 h to 8.0 µM DHPP. After washing the cover-slips in PBS, the cells were treated with 1X-binding buffer and subsequently stained using Annexin-V/FITC antibodies for 40 min in the dark. Thereafter, the cells were treated with 1X binding buffer as per protocol of the manufacturer (Clontech Inc, USA). The cells were fixed in 2 % formaldehyde prior to monitoring of fluorescence using OlympusIX71inverted microscope connected to FITC and rhodamine filters.

### Measurement of ROS production

Abnormally high production of ROS was measured in cells using carboxy-H2-DCFDA (molecular Probes [14]. Termination of cellular incubation in 12-well plates was followed by

aspiration of the medium and subsequent cell washing with PBS. Serum-free DMEM was added to 10 mM carboxy-H2-DCFDA and incubation was carried out for 25 min at 37 ºC. Then, the cells were washed with PBS and fluorescence measurement was done using OlympusIX71 inverted microscope connected to FITC filters.

### Western blot assay

Cells treated with 8.0 µM DHPP were rinsed with PBS and subjected to centrifugation for 5 min at 450 g. The cells were re-suspended in RIPA (100 mL) containing protease inhibitors and 10 mM sodium ortho-vanadate (Sigma). The lysates were centrifuged at 13000 g for 15 min at 4 ºC, and their protein levels were estimation using Bradford's method. Thereafter, 25 mg protein samples were subjected to 10% SDS-PAGE and subsequently transferred to PVDF membranes. Protein probing was made by incubation overnight at 4 ºC with primary antibodies against p-ERK-1/2, p-p38, p-JNK, α-tubulin, ERK-1/2, p38 and JNK (Cell Signaling Technology). This was followed by washing and 1 h incubation with anti-rabbit Ig-G-conjugated secondary antibody (Sigma-Aldrich) at room temperature. Band development was made using ECL Prime blotting reagent (GE, Life Sciences). The intensities of the bands were calculated with GeneTools software.

### Statistical analysis

Data are expressed as mean ± S.D. Differences were determined statistically using one-way analysis of variance (ANOVA). The data was analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). Differences were taken as significant at $p < 0.05$.

## RESULTS

### DHPP decreased viability of lung cancer cells

The anti-proliferative potential of DHPP against cancer cells (A549 and H1299) and normal cells (MRC5 and BEAS-2B) are shown in Figure 2. The cells were exposed to increasing doses of DHPP (0.5, 1.0, 2.0, 4.0 and 8.0 µM) for 24 h. The toxicity of DHPP was more prominent against A549 cells than H1299 cells. Treatment with 8.0 µM DHPP decreased the viabilities of A549 and H1299 cell lines to 23 and 29 %, respectively. However, DHPP did not induce significant toxicity in MRC5 and BEAS-2B cell cultures at the tested concentration range (0.5 - 8.0 µM).

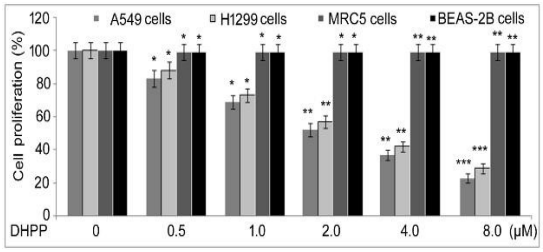


**Figure 2:** Effect of DHPP on the viabilities of normal and lung cancer cells. Normal cell lines (MRC5 and BEAS-2B) and cancer cell lines (A549 and H1299) were incubated with DHPP for 24 h and analyzed using SRB assay. *$p < 0.0487$, **$p < 0.0198$, ***$p < 0.0099$, vs. control cells

### Induction of cell apoptosis in lung cancer cells by DHPP

The effect of 24 h treatment with DHPP on caspase-3 activities in A549 and H1299 cells is shown in Figure 3 A. The DHPP treatment promoted caspase-3 activities in A549 and H1299 cells in a dose-dependent manner. Moreover, DHPP enhanced apoptosis of A549 and H1299 cells (Figure 3 B). At 24 h of treatment with DHPP (8.0 µM), apoptosis in A549 and H1299 cells increased to 67.89 and 61.35 %, respectively.

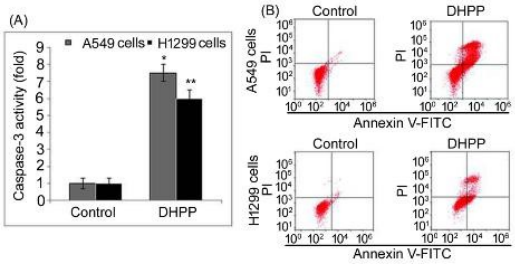


**Figure 3:** Effect of DHPP on cellular apoptosis. (A) Caspase-3 expressions in A549 and H1299 cells after 24 h of incubation with DHPP, as assayed with western blotting. (B) Apoptosis in control and 8.0 µM DHPP-treated cells, as analyzed using Annexin-V/FITC and PI staining, and fluorescence. *$p < 0.0187$, **$p < 0.0099$, vs. control cells

### DHPP promoted p38/JNK pathway activation

Figure 4 shows the effect of DHPP on ERK, p38 and JNK activation in A549 and H1299 cells after 24 h. There were marked increases in p38-phosphorylation in DHPP-treated A549 and H1299 cells, relative to control. The 24 h DHPP treatment also significantly elevated p-JNK-1/2 expressions in A549 and H1299 cells, but suppressed p-ERK-1/2 expressions in these cells. Pre-treatment of A549 and H1299 cells with PD169316 (p38 inhibitor) or SP600125 (JNK inhibitor) significantly reversed DHPP-induced promotion of caspase-3 activity. However, U0126

(MEK inhibitor) pretreatment did not suppress the DHPP-induced promotion of caspase-3 activities in A549 and H1299 cells.

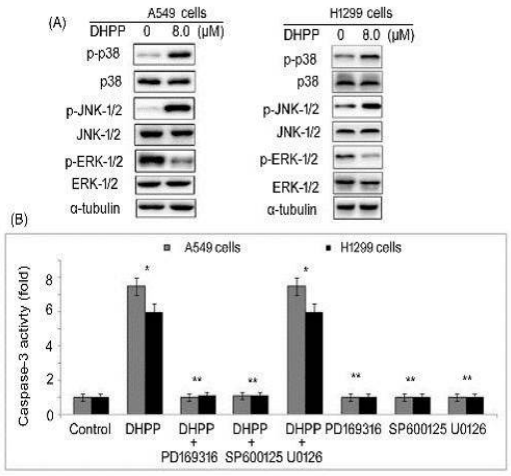


**Figure 4:** Effect of DHPP on p38/JNK activation. (A) The 8.0 μM DHPP treated or control cells were analyzed for changes in ERK, p38 and JNK activation at 24 h using western blotting. (B) Pre-treatment with p38/JNK/MEK inhibitors, each at a dose of 5 mM for 1 h, and subsequent incubation with 8.0 μM DHPP was followed by caspase-3 activation assessment in A549 and H1299 cells

### DHPP promoted ROS production

Treatment of A549 and H1299 cells with DHPP for 24 h resulted in increases in ROS levels, relative to control cells. The DHPP treatment elevated DCF-fluorescence in A549 and H1299 cells by 7.6 and 6.1 folds, respectively. However, the DHPP-induced increases in DCF-fluorescence were effectively suppressed in A549 and H1299 cells pretreated with 10 mM FeTMPyP, an antioxidant.

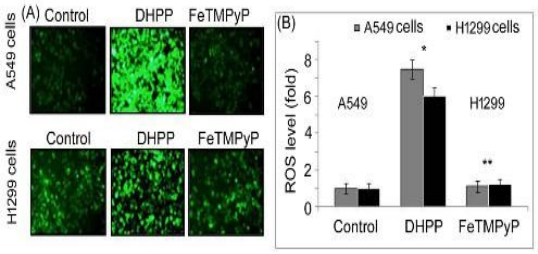


**Figure 5:** Effect of DHPP on ROS levels in A549 and H1299 cells. (A) ROS levels in DHPP-exposed cells with or without pretreatment with FeTMPyP, as determined using DCF-fluorescence. Fluorescence in cells was captured using Olympus–IX71 microscope. (B) Mean cellular fluorescence intensities (as obtained using ImageJ software from three different fields). *$p$ < 0.0487, vs. control cells

### FeTMPyP reversed DHPP-induced p38/JNK/ERK-1/2 activation

Pretreatment with FeTMPyP effectively reversed DHPP-induced increases in p-p38 and p-JNK expressions in A549 and H1299 cells (Figure 6 A). The DHPP-mediated suppression of p-ERK1/2 expression was also prevented in A549 and H1299 cells by FeTMPyP pre-treatment. In FeTMPyP-pre-treated A549 and H1299 cells, the DHPP-induced increases in caspase-3 activity were markedly suppressed (Figure 6 B).

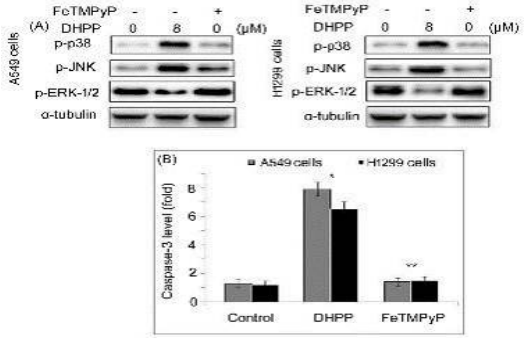


**Figure 6:** Effect of FeTMPyP on DHPP-induced alterations in p38/JNK/ERK-1/2. (A) p38/JNK/ERK-1/2 expressions in FeTMPyP-pre-treated or untreated A549 and H1299 cells after incubation with DHPP. (B) Caspase-3 levels after 24 h in FeTMPyP-pre-treated and untreated A549 and H1299 cells after incubation with DHPP. *$p$ < 0.0487, vs. control cells

## DISCUSSION

The present study demonstrated that DHPP specifically exhibits anti-proliferative effect on A549 and H1299 lung cancer cells without affecting the growth of MRC5 and BEAS-2B (normal cell lines). Oxidative damage and apoptosis were induced in A549 and H1299 cells by DHPP through the MAPK pathway. Various cellular events which regulate diverse processes such as viability, differentiation, death and survival are controlled by MAPK proteins [15]. Activated ERK-1/2 is important in the proliferation of cancer cells, and studies have shown that direct or indirect targeting of ERK-1/2 expression has immense significance for arresting tumor growth [15,16]. Activation of stress activated kinase, JNK is linked to different kinds of stress stimuli such as DNA damage, altered cytokine levels, heat shock and exposure to UV radiation [15]. The JNK phosphorylation is most commonly accompanied by simultaneous activation of p38 protein [15]. It is well established that ERK-1/2 activation leads to anti-apoptotic changes and phosphorylation of p38/JNK has pro-apoptotic effect [17].

The present study demonstrated that DHPP treatment promoted p38-phosphorylation in A549 and H1299 cells. In DHPP-treated A549 and H1299 cells, p-JNK-1/2 expression also showed significant increases. The ERK-1/2 activations in A549 and H1299 cells were markedly down-regulated on treatment with DHPP. These findings indicate that DHPP effectively induces pro-apoptotic changes and deactivates anti-apoptotic cellular processes. In order to confirm these results, A549 and H1299 cells were pre-treated with PD169316 (p38 inhibitor) or SP600125 (JNK inhibitor) prior to incubation with DHPP. Data showed that DHPP-mediated caspase-3 activation was alleviated in PD169316- or SP600125-pretreated A549 and H1299 cells. Reactive oxygen species are ubiquitous molecules, examples of which are hydrogen peroxide, nitric oxide, hydroxyl radical and singlet oxygen. These ROS influence multiple biological processes. Some crucial steps involved in signal transduction and several other cellular processes are regulated by ROS. Reports have demonstrated that oxidative stress regulates the activation of proteins involved in the MAPK pathway [18]. Abnormal increases in ROS levels are responsible for mitochondrial damage and ultimately results in activation of pro-apoptotic signaling pathway [1921]. In the present study, DHPP treatment of A549 and H1299 cells significantly increased DCF-fluorescence, relative to control cells. However, the DHPP-induced increases in DCF-fluorescence were effectively suppressed in A549 and H1299 cells on pretreatment with 10 mM FeTMPyP, an antioxidant. Thus, DHPP activated p38/JNK signaling *via* oxidative stress which subsequently caused apoptosis of A549 and H1299 cells.

## CONCLUSION

The results obtained in this study indicate that DHPP selectively targets lung cancer cell growth through oxidative stress which subsequently results in cell apoptosis. Moreover, in lung cancer cells, DHPP treatment activates caspase-3 protein and p38/JNK signaling, with simultaneous inactivation of ERK-1/2. Thus, DHPP has inhibitory effect on lung cancer cells, and so possesses some potentials for use in the treatment of lung cancer.

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhaohong Chen - conceived and designed the study; Miao He, Chao Li, Yingying He, Guangquan Yang, Youcai Zhang - collected and analyzed the data; Miao He, Chao Li, Yingying He, Guangquan Yang -wrote the manuscript. All authors read and approved the manuscript for publication.

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