



## ANTIPROLIFERATIVE EFFECT OF HESPERETIN IN SMALL AIRWAY EPITHELIAL CELLS

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### ABSTRACT

*IL-1 $\beta$  is a pluripotent cytokine involved in various inflammatory disorders including lung cancer and implicated in the cancer initiation by upregulating the cell growth. Hesperetin, a citrus isoflavone, is known for its anti-inflammatory and anti-cancer property. However, its role in IL-1 $\beta$  induced upregulation of airway epithelial cell proliferation is not known. We have therefore investigated the effect of hesperetin on IL-1 $\beta$  induced increased cell proliferation in human small airway epithelial cells (SAECs). The SAECs were starved for ~18 h with/without hesperetin 100  $\mu$ M for 24 h and stimulated with IL-1 $\beta$  for different time points. Cell proliferation was assessed by MTT assay; protein expression was analyzed by immunoblotting. Our findings demonstrate the no significant cytotoxic effects of hesperetin at 50, 100 and 150  $\mu$ M dose in SAECs. Further, out of different doses of IL-1 $\beta$  (0, 5, 10, 20 and 50 ng/mL) used to stimulate the SAECs, 10 ng/mL showed enhanced cell proliferation at 24 h, while 50 ng/mL showed decreased cell growth, however, at 48 h cell growth showed decreasing trend. Pretreatment with hesperetin (100  $\mu$ M) reversed IL-1 $\beta$  (10 ng/mL) induced in SAECs growth. Furthermore, IL-1 $\beta$  stimulation enhanced the CDK4 and 6 expressions at 24 h, and hesperetin pretreatment inhibited IL-1 $\beta$  induced increase in the levels of phospho-CDK6 and NF- $\kappa$ B-p65. Lastly, IL-1 $\beta$  induced decrease in the p53 expression was enhanced by hesperetin at 24 h. Collectively, our results demonstrated that hesperetin restricts IL-1 $\beta$  induced cell proliferation in SAECs which may be mediated through the inactivation of p53 and inhibition of NF- $\kappa$ B-p65 indicating that hesperetin may emerges as a potential chemopreventive agent against IL-1 $\beta$  induced tumor initiation in lungs.*

### KEY WORDS

*lung cancer; chemoprevention; hesperetin; human small airway epithelial cells; interleukin-1 $\beta$ .*

### 1. INTRODUCTION

Various exogenous stimuli such as air pollutant, tobacco cigarette smoke and lipopolysaccharides as well as endogenous stimuli such as pro-inflammatory cytokines for example tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and tumor growth factors induce inflammation in lungs. Unresolved acute inflammation leads to chronic inflammation and endogenous factors start accumulating in inflammatory microenvironment. Accumulated inflammatory cells and pro-inflammatory

cytokines contributed to exaggerated chronic responses which could facilitate the tumor initiation.

Lung cancer is one of the prominent causes for global mortality and morbidity [1, 2]. Approximately 25% of inflammatory etiologies are related to the cancer development including lung cancer [3]. Chronic inflammation is one of the critical risk factor for lung cancer and also participate in all inflammatory disorders in the lungs such as acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disorder (COPD), pulmonary emphysema, ventilation caused pneumonia

and chronic bronchitis [4]. IL-1 $\beta$ , an inducible cytokine in response to varied stimuli, is critical component of chronic inflammatory microenvironment and plays decisive role in neoplastic transformation. IL-1 $\beta$  is not expressed in healthy or normal tissues [5] however, it has been shown that it plays potent role in the tumor promotion and progression when present persistently [4, 6, 7].

Cell growth is an important step in tumor initiation. The pro-inflammatory cytokine IL-1 $\beta$  regulate the cell proliferation, migration, and metastasis, the hallmarks of carcinogenesis. Uncontrolled cell division indicate the role of cyclin dependent kinases (CDKs) which could also be regulated through activation of NF- $\kappa$ B pathway by IL-1 $\beta$ . NF- $\kappa$ B-p65 is key transcription factor which functions in a positive feedback loop and associated with maintenance of the critical concentration of IL-1 $\beta$  in tumor microenvironment resulting into increased cell proliferation and tumor progression [8]. Thus, inhibiting the CDKs and NF- $\kappa$ B pathway may be an important strategy in the inflammation-induced cancer chemoprevention

Hesperetin is a flavanone known for its anti-inflammatory [9-11], anti-oxidant [12] and anti-cancer properties against prostate [13], breast [14, 15], colon [16], gastric [17] and thyroid [18] cancer. Based on the available reports and affinity of hesperetin for better membrane interaction as compare to other flavanone group derivatives [19]; we hypothesized that hesperetin may inhibit the IL-1 $\beta$ -induced cell proliferation in human small airway epithelial cells and therefore, may inhibit the neoplastic transformation and hence tumor initiation in lung. Therefore, in this study we have investigated the mechanism of action of hesperetin in the prevention of IL-1 $\beta$ -induced cell proliferation in human small airway epithelial cells.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and reagents

Hesperetin, methanol, dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trizma base, sodium dodecyl sulphate (SDS) and  $\beta$ -mercaptoethanol (BME) were procured from Sigma Aldrich (St. Louis, USA). Phospho-CDK6 antibody was purchased from Abcam (Cambridge, UK). NF- $\kappa$ B-p65, p53, GAPDH and  $\beta$ -actin antibodies were purchased from Cell Signaling Technology, USA.

Human small airway epithelial cells (SAEC) will be procured from Lonza (Walkersville, MD, USA).

### 2.2 Cell culture and treatment condition

SAEC cultured in Small Airway Epithelial Cell Growth Medium (SAGM) supplemented with Bullet-kit. SAECs were maintained, passaged and used for experiment, incubated in a humidified atmosphere under 5% CO<sub>2</sub> and 37°C temperature in CO<sub>2</sub> incubator (Thermo Fisher Scientific, USA).

Hesperetin was dissolved in DMSO to make 200 mM stock solution and stored at 4°C until used. Cells were seeded as per the requirement of experiments, allowed to adhere for 24 to 48 h and then starved for ~18 h in starvation medium containing 0.2% serum with or without HN 100  $\mu$ M. After starvation, cells were treated with IL-1 $\beta$  (10 ng/mL) in presence or absence of HN and incubated as per the plan of experiment.

### 2.2 MTT assay

Approximately  $8 \times 10^3$  cells were plated in a 96-well plate under standard conditions. After the completion of incubation time with MTT dye (5 mg/mL) for 24 and 48 h, absorbance were measured at 570 nm [20].

### 2.3 Immunoblotting

SAECs were starved and treated with or without HN as described in earlier sections. The whole cell lysates were prepared, and proteins separated by SDS-PAGE followed by immunoblotting as described [20, 21].

### 2.4 Statistical analysis

The statistical analysis was performed using unpaired Student's t-test by using GraphPad Prism software version 6 (GraphPad, LaJolla, CA, USA). The data are represented as Mean  $\pm$  standard error mean (SEM),  $p$  values of  $<0.05$  was considered statistically significant.

## 3. RESULTS

### 3.1 Effect of hesperetin on SAECs viability

First of all, we assessed the cytotoxic effect of hesperetin on SAECs by MTT assay at 24 and 48 h. After the completion of incubations with hesperetin treated cells with 50, 100 and 150  $\mu$ M doses, MTT assay was performed, which showed that there was no significant change in the cell viability in the treated groups as compared to control. These results showed that hesperetin exhibited almost no cytotoxic effects on SAECs at 24 and 48 h. From this experiment we chose 100  $\mu$ M dose of hesperetin for further experiments.

### 3.2 Effect of IL-1 $\beta$ stimulus on SAECs proliferation

Next we performed the dose-dependent effect of IL-1 $\beta$  on SAECs by MTT assay. The serum-starved SAECs were treated with different concentration of IL-1 $\beta$  (5, 10, 20 and 50 ng/mL) for 24 and 48 h. The viable cells converted the tetrazolium salt of MTT into formazan crystals and absorbance was measured at 570 nm. The absorbance was directly proportional to the cell proliferation. The results showed that upto 20 ng/mL concentration there was dose-dependent enhanced cell proliferation at 24 h in SAECs, whereas there was decrease in cell growth at 50 ng/mL concentration. At 48 h however, only 5 and 10 ng/mL concentration showed enhanced cell proliferation in SAECs. The decreased cell proliferation was observed at 20 and 50 ng/mL doses at 48 h. From this experiment we decided to use 10 ng/mL dose of IL-1 $\beta$  in further experiments.

### 3.3 Effect of hesperetin on IL-1 $\beta$ -induced SAECs proliferation

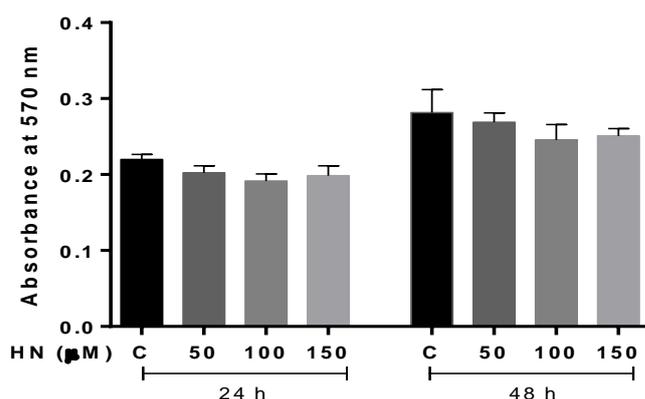
We next performed MTT assay to investigate the effect of hesperetin (100  $\mu$ M) on IL-1 $\beta$  (10 ng/mL)-induced cell proliferation at 24 and 48 h. Our results showed a significant ( $p < 0.05$ ) increase in IL-1 $\beta$ -induced SAECs proliferation at 48 h which was significantly ( $p < 0.05$ ) prevented by hesperetin (100  $\mu$ M) in. At 24 h, there was slight increased cell proliferation which was prevented by hesperetin.

### 3.4 Effect of IL-1 $\beta$ on the expression of CDKs in SAECs

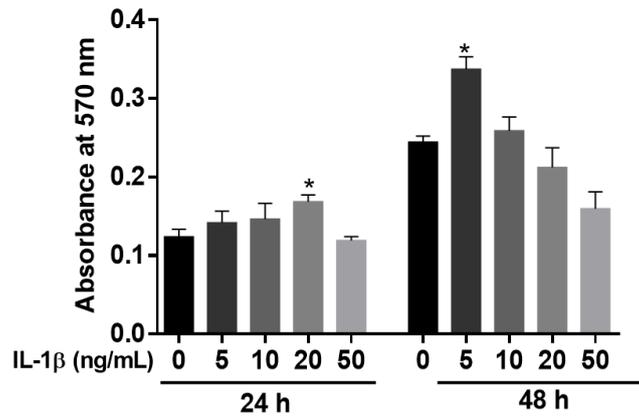
The uncontrolled cell proliferation is important hallmark of tumor initiation and CDKs are important regulators of cell cycle events [22-24]. We therefore assessed the expression of CDKs in response to IL-1 $\beta$  stimulus. Our immunoblotting results showed a dose-dependent enhancement in the expression of CDK4 and CDK6 at 24 h. Thus, IL-1 $\beta$ -induced enhanced cell proliferation may be due to its effect on CDKs expression in SAECs.

### 3.5 Effect of hesperetin on CDK phosphorylation and expression of p53 and NF- $\kappa$ B-p65 proteins

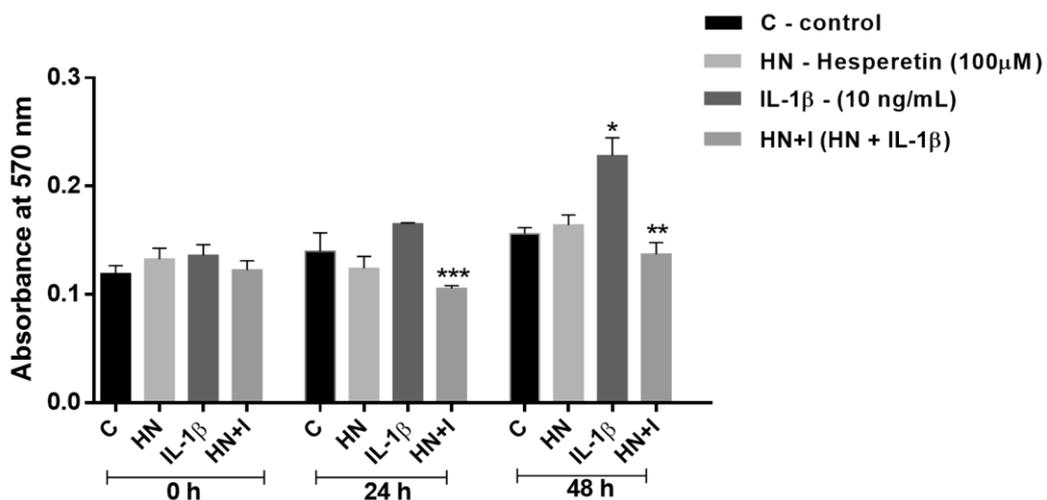
The cell cycle events determine the fate of cells towards survival and growth which is an important factor for tumor promotion and progression [23, 24]. Such important events in inflammatory tumor microenvironment are regulated through the inactivation of tumor suppressor protein p53 and activation of NF- $\kappa$ B-p65 pathway [25-27]. Our results showed that IL-1 $\beta$  stimulus increased the level of phospho-CDK6 to 1.5 fold, and decreased the expression of p53 to 0.2 fold, while expression of NF- $\kappa$ B-p65 increased by 8.6 fold as compared to respective controls at 24 h. Hesperetin very effectively restored these changes and reduced the CDK6 phosphorylation to near control; increased the level of p53 to nearly 1.4 fold and decreased the expression of NF- $\kappa$ B-p65 to 3.8 fold in SAECs. The reversal of changes is important from the point of view of chemopreventive role of hesperetin.



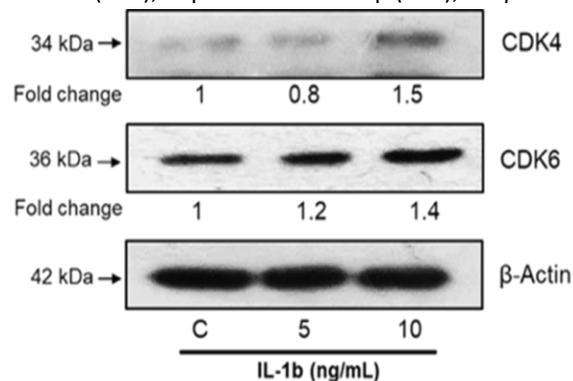
**Figure 1.** The cytotoxic effects of hesperetin on small airway epithelial cells. MTT assay was performed with serum-starved SAECs treated with different doses, 50, 100 and 150  $\mu$ M of hesperetin in for 24 h and 48 h. The bars represent Mean  $\pm$  SEM, (n=3). C, control, HN, hesperetin.



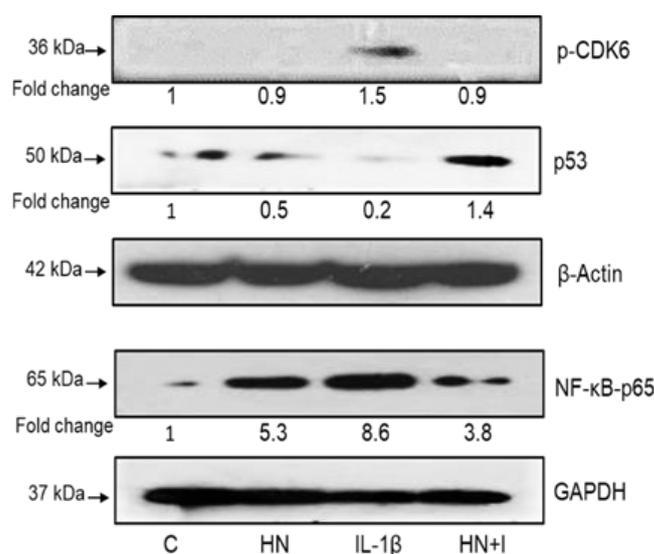
**Figure 2: Effect of IL-1 $\beta$  stimulus on the cell proliferation of SAECs.** MTT assay was performed after serum-starved SAECs were treated with 5, 10, 20 and 50 ng/mL doses of IL-1 $\beta$  for 24 h and 48 h. The bars represent Mean $\pm$ SEM, (n=3); \* $p$ <0.05 vs. respective controls.



**Figure 3: HN decreases the IL-1 $\beta$ -induced cell proliferation in SAECs.** MTT assay was performed with the serum-starved SAECs with/without HN 100  $\mu$ M treated with 10 ng/mL dose of IL-1 $\beta$  for 24 h and 48 h. The bars represent Mean $\pm$ SEM, (n=3); \* $p$ <0.05 vs. control (48h); \*\* $p$ <0.0001 vs. IL-1 $\beta$  (48h); \*\*\* $p$ <0.0001 vs. IL-1 $\beta$  (24h).



**Figure 4: Dose-dependent effect of IL-1 $\beta$  on expression of CDK4 and CDK6 in SAECs.** Immunoblotting was done to analyze dose-dependent (5 and 10 ng/mL) expression of IL-1 $\beta$ -induced CDKs for 24 h in SAECs. Numbers below the bands are fold change calculated against the loading control  $\beta$ -actin bands for each sample. Data shown are representative of two experiments.



**Figure 5: HN modulates IL-1 $\beta$ -induced expression of p53 and NF- $\kappa$ B-p65 for 24 h in SAECs.** Serum-starved cells were treated with the IL-1 $\beta$  with/without HN for 24 h in SAECs. Immunoblotting was done to analyze the IL-1 $\beta$ -induced expression of p53 and NF- $\kappa$ B-p65 in SAECs. Numbers below the bands are fold change calculated against the loading control  $\beta$ -actin bands for each sample. Data shown are representative of two experiments.

#### 4. DISCUSSION

Chemoprevention based on the bioflavonoids is a potential strategy in cancer therapeutics. Hesperetin, a citrus bioflavonoid exhibits the better membrane interactions which results in the improved bioavailability compared to other bioflavonoids [19, 28-30]. Although, this compound has been reported to possess anti-cancer property in *in-vitro* models, the cytotoxic effects of the compound on normal cells or tissues has not been investigated earlier in terms of assessing its chemoprevention properties and plausible mechanism of action. Our findings suggested two prong novel information, one, that hesperetin exhibited no significant cytotoxic effects on human small airway epithelial cells which implicated its safe use in chemoprevention strategy. Secondly, hesperetin showed inhibitory effect on IL-1 $\beta$ -mediated cell proliferation in SAECs through enhancing the level of p53, a tumor suppresser protein, and decreased the expression of CDK 4, 6 and that of transcription factor NF- $\kappa$ B. These findings clearly suggest the anti-proliferative and chemopreventive property of hesperetin. Therefore, hesperetin may restrict the inflammation-induced activation of survival cascade which leads to neoplastic transformations.

During the chronic inflammatory responses mononuclear phagocytes are primary source of IL-1 $\beta$  in lung. This leads to activation of alveolar macrophages

along with other inflammatory cells which further activate the lung epithelial cells and manifest the secretion of IL-1 $\beta$  and establishment of inflammatory microenvironment [31]. IL-1 $\beta$  stimulus plays central role in recruitment of inflammatory cells to the site of infection or injury followed by activation of granulocytes macrophage colony stimulating factor. This activation is associated with the acquired resistance to apoptosis [32, 33]. Pro-inflammatory cytokines, including IL-1 $\beta$ , participate in the coordinated network to initiate the signaling cascade for sustained neoplastic cell proliferation as well as invasion [34]. This implicate the role of IL-1 $\beta$  in the several inflammatory events in lung that links it to carcinogenesis related events such as adhesion, migration, activation of collagenases and matrix metalloproteinases [35].

Uncontrolled tumor cell proliferation is regulated through the activation of cyclin-cdk complexes and inactivation of the tumor suppressor proteins during tumor initiation process. The inflammatory mediators exaggerated the chronic responses that create and accelerate the cancer promoting microenvironment. This microenvironment induces the oncogenic activation or mutations in the cells followed by genetic and epigenetic alterations causing neoplasia [36]. The inactivation of tumor suppressor proteins also promote the cancer related inflammation which implicated in tumor promoting effects [6, 35-37]. Our finding

suggested that pro-inflammatory cytokine IL-1 $\beta$  was responsible for the phosphorylation/activation of CDK6, increased expression of CDKs and decreased expression of tumor suppressor proteins p53 which may contribute towards increased cell proliferation, indicating the likely uncontrolled cell growth in the inflammatory microenvironment leading to tumor initiation. Further, the presence of hesperetin reverses these changes to near control group which suggest its role in modifying the inflammatory microenvironment and its chemoprevention potential.

Among the important hallmarks of cancer, unregulated cell proliferation is a prominent characteristic; and tumor microenvironment is critically regulated by the chronic inflammation [24]. Our findings in the present study demonstrated that IL-1 $\beta$  may alter the transcription factors involved in survival signaling which could enhance the proliferation of human small airway epithelial cells. Hesperetin inhibited IL-1 $\beta$ -induced increased expression of NF- $\kappa$ B-p65 in SAECS, which indicated its anti-inflammatory and anti-proliferative function. These finding suggest that hesperetin could be an effective chemo-preventive agent. Taken together, the finding of this study entails that hesperetin may be used as a chemopreventive agent as it exhibited no significant toxicity in the primary small airway epithelial cells and also it is potent inhibitor of CDKs and NF- $\kappa$ B and inducer of p53, a tumor suppressor protein.

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