Hesperetin, a Citrus bioflavonoid, prevents IL-1β-induced inflammation and cell proliferation in lung epithelial A549 cells

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Hesperetin, a *Citrus* bioflavonoid, exhibits anticancer, anti-inflammatory and antioxidant properties. However, its action and mechanism in inflammation-induced lung cancer is unknown. We have investigated anticancer effects of hesperetin in IL-1β-stimulated lung adenocarcinoma cell proliferation and COX-2 -mediated inflammation. The human lung adenocarcinoma A549 cells were serum-starved with or without HN (100 μM) for overnight and stimulated with IL-1β for varying durations. Cell viability and proliferation were assessed by MTT and wound healing assays. Cell cycle progression was measured by flow cytometry, and RT-PCR and immunoblotting methods were used to examine the expression COX-2 mRNA and protein, respectively. Protein stability assessed by cycloheximide chase assay. IL-1β caused a time- and dose-dependent increase in cell viability and proliferation, expression of COX-2 at transcription as well as translation levels, increased the stability of COX-2 protein, and PGE2 production while HN significantly decreased these changes. Further, IL-1β stimulated increased phosphorylation of ERK-1/2 and p65 subunit of NF-κB, which were reversed by HN in A549 cells. These results show that HN could inhibit IL-1β-stimulated cell proliferation, COX-2 expression and its regulation at translation level and PGE2 synthesis in A549 lung epithelial cells, indicating its anti-inflammatory and anticancer potential in lung cancer cells.

Keywords: Anticancer, Anti-inflammatory, Antioxidant, COX-2, Lung cancer

Lung cancer has become one of the prominent cancer-related mortality worldwide including the United States and India[1]. Rudolf Virchow, for the first time, established the link between inflammation and cancer development[2]. Approximately, 15% of all cancers are attributed to inflammatory etiologies providing evidence for the association between inflammation and cancer[3,4]. The unresolved chronic inflammation and prolonged presence of pro-inflammatory cytokines modulate the inflammatory microenvironment that helps in the initiation of tumour formation.

A pro-inflammatory cytokine, interleukin (IL)-1β, is known to play prominent role in lung carcinoma and implicated in increased cell proliferation, angiogenesis, adhesion, invasion, promotion and metastasis through the expression of concomitant biomarker proteins[5,6]. In addition to promoting cell proliferation through upregulation of mitogen-promoting kinases, IL-1β is also involved in the induction of inflammatory markers such as cyclooxygenase 2 (COX-2)[6,7]. Several studies have

shown the role of COX-2 against inflammatory symptoms such as pain, redness or fever which strongly suggest its role in inflammation[8,9]. It is an inducible isoform transcribed by nuclear factor-kappa B (NF-κB-p65) upon IL-1β stimulus and also regulates the inflammatory response in tumor microenvironment[9,10]. The upregulated COX-2 expression is associated with its increased catalytic activity and enhanced PGE2 synthesis. Both these properties have been observed in the pre-malignant as well as malignant tissues[11,12]. In eukaryotic cells, the expression of COX-2 is regulated at both transcription and translation levels through NF-κB-p65 pathway. Accordingly, upstream regulator kinases, such as ERK1/2, p38[13], JNK/SAPK and PI3K/Akt pathways are implicated in the regulation of COX-2/PGE2 inflammatory cascade[7,14]. Thus, targeting this pathway could be an important approach for treatment and prevention of lung cancer.

Hesperetin (HN), a *Citrus* bioflavonoid, is a potent anti-inflammatory and antioxidant compound. Its anticancer property has been reported in several cancers[15-17]. However, its anticancer effect in lung cancer is unknown, especially in the settings of inflammatory microenvironment. We have

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hypothesized that HN could prevent IL-1β-mediated increased proliferation and inflammation in lung cancer cells through blocking the relevant pathways. In this study, we evaluated the effect of HN on IL-1β stimulated cell proliferation, COX-2 induction and regulation at transcription and translation levels, activation of related signaling intermediates in A549 lung epithelial cells.

Materials and Methods

Chemicals and Reagents

The procurement of hesperetin (HN), dimethysulfoxide (DMSO) and MTT (dimethylthiazol-2-yl diphenyl tetrazolium bromide) was done from Sigma-Aldrich (St. Louis, MO, USA). The COX-2, phospho-ERK1/2, ERK1/2 and p65 subunit of NF-κB antibodies were purchased from Cell Signaling Technologies (Beverley, MA, USA). Phospho-NF-κB-Ser536 were purchased from Abcam (Cambridge, UK). RPMI 1640 medium was from HiMedia (Pennsylvania, USA), Fetal bovine serum (FBS) (Gibco, certified US origin), IL-1β were procured from Thermo-Fisher Scientific (Waltham, CA, USA). PVDF membrane and Luminata Crescendo Western HRP substrate as detection reagents were obtained from EMD Merc Millipore (Billerica, MA, USA). The kit for assessment of Prostaglandin E2 (PGE2) was procured from Cayman (Ann Arbor, MI, USA).

Cell culture and treatments

Human lung adenocarcinoma A549 cells were procured from the National Centre for Cell Science (NCCS, Pune), grown in complete RPMI 1640 media containing FBS (10%) and antibiotics mixture (containing 1% P/S/A) in a CO_2 incubator (Thermo Scientific, Waltham, CA, USA) aptly humidified at 37°C

The stock solution (200 mM) of HN was prepared in DMSO. The cells were treated with HN in such way that final DMSO concentration was ≤0.1% in culture medium, which was not toxic. The stock solution of IL-1β (0.1 mg/mL) was prepared in autoclaved deionized water. At 70% confluency, the A549 cells were starved for overnight (~18h) with low-serum (containing 0.2% FBS) medium containing 100 μM of HN. Subsequently, the cells were stimulated with different doses of IL-1β (as required and mentioned in the figures) without or with HN in the medium and incubated for different time intervals as required for different experiments.

MTT assay

Approximately 8×10^3 cells were plated in a 96-well plate under standard conditions. After starvation with 0.2% FBS media for overnight with or without HN, the cells were stimulated with 0, 5, 10, 20 and 50 ng/mL concentrations of IL-1 β with or without HN and incubated for 0, 12, 24 and 48 h. After the completion of incubations, MTT dye (5 mg/mL) was added and cells were further incubated for 4 h. Subsequently the media was removed and formazan crystals formed in cells were dissolved in DMSO and absorbance was measured at 570 nm[18].

Wound healing assay

Approximately 1×10^5 A549 cells were seeded in a 6-well plate. After starvation with 0.2% FBS media for overnight with or without HN, a scratch was made using pipette (20-200 μL) tips and followed by treatment with IL-1β (10 ng/mL) with or without HN (100 μM) and incubated for 24 h. Images of the scratch in each sample were acquired at zero hour and 24 h after incubation with IL-1β. The relative width of wound closure was averaged from 5 random fields of each sample.

Cell cycle analysis

Approximately 1×10^5 A549 cells were plated in 6-well plate. After starvation with 0.2% FBS media for overnight with or without HN, the cells were stimulated with 10 ng/mL concentrations of IL-1 β and incubated for 48 h. After the completion of treatment, the cells were processed for propidium iodide staining as described[18]. The cell cycle analysis was performed using FACS Aria III flow cytometry systems and data analysed using FlowJo software.

RT-PCR

Total RNA was isolated using Trizol reagent and cDNA was synthesised from RNA using reverse transcriptase PCR kit (Clonetech, TaKaRa, CA, USA). The 25 cycles of regular PCR reactions were carried as described[17], Step 1: First denaturation step at 95℃ for 180 s, Step 2: Denaturation at 94°C for 30 s; annealing at 55℃ for 30 s; elongation/extension at 72℃ for 90 s, Step 3: Final elongation at 72℃ for 5 min. The specific sequences of primers include the followings: COX-2: Forward primer 5' TTCAAATGAGATTGTGGAAAAATTGCT 3'; Reverse primer 5' AGATCATCTCTGCCTGAGT ATCTT 3'; Actin: Forward primer 5' AGCCATG TACGTAGCCATCC 3' Reverse primer 5' TCTCAG CTGTGGTGGTGAAG 3'.

Western blot analysis

Approximately, 2.5×10^5 A549 cells were seeded in 60 mm culture dishes, starved with 0.2% FBS containing media for overnight with or without HN and the cells were stimulated with 10 ng/mL concentrations of IL-1β and incubated for 48 h or as required for the experiment. Subsequent to the completion of incubations, the cells were washed using chilled 1X PBS and lysed using RIPA lysis buffer containing phosphatase (1X) and proteases (1X) inhibitor cocktail (Roche Applied Sciences, Penzberg, Germany). The whole cell lysates were prepared, and protein was estimated by Bradford assay. Equal amount of proteins from each sample was subjected to SDS-PAGE followed by immunoblotting as described[17]. Briefly, the separated proteins were electro-transferred onto PVDF membrane. The membranes were blocked using 3% defatted milk powder (HiMedia, Pennsylvania, USA) for 1 h at room temperature and probed with respective primary antibodies by incubating for overnight at 4℃. The membranes were washed and probed with HRP-conjugated secondary antibody at room temperature for two hours. The corresponding protein bands were detected using ECL. Anti-β-actin and GAPDH antibodies were used as loading control after stripping and reprobing the membranes.

Cycloheximide chase (CHX) assay

Approximately 1×10^5 A549 cells in 6-well plates were starved as described earlier and incubated with IL-1β for 24 h followed by treatment with 10 μg/mL of CHX (HIMEDIA Laboratories, Mumbai, India) in fresh starvation media for additional 2, 4 and 8 h. Western blotting was performed as described earlier[17].

PGE2 assay

Approximately 1×10^5 A549 cells in 6-well plates were starved and incubated with IL-1β for 24 h. The medium from each well was collected and processed as per the instructions given in manufacturer's protocol and absorbance were measured at 420 nm by multi-plate reader. The cells were harvested and subjected to immunoblotting using anti-COX-2 antibodies as described earlier[17].

Statistical analysis

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

Results and Discussion

Hesperetin decreased IL-1β-stimulated cell viability and proliferation

First, we examined the role of pro-inflammatory cytokine IL-1β on A549 cell proliferation. The A549 lung epithelial cells were starved stimulated with diverse doses (5, 10, 20 and 50 ng/mL) of IL-1β for different durations, such as 12, 24 and 48 h. As shown in Fig. 1A, IL-1β-induced a dose- and time-dependent increase in cell proliferation, specifically at 48 h, with a significant increase of approximately 49%
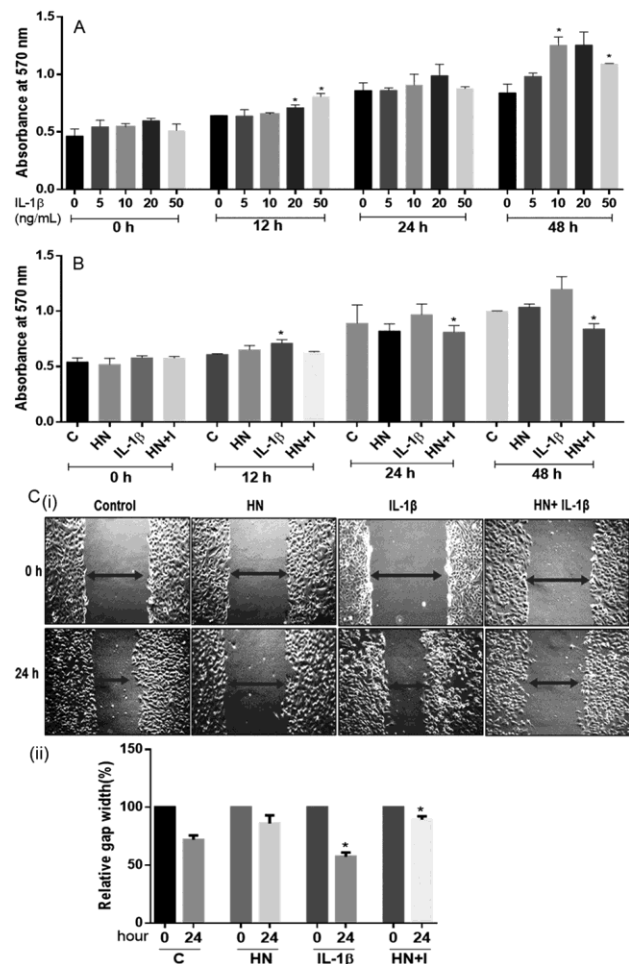

Fig. 1 — HN regulates IL-1β-stimulated cell viability and proliferation in A549 cells. For MTT assay, (A) serum-starved A549 cells were stimulated with 0, 5, 10, 20 and 50 ng/mL doses of IL-1β for 0, 12, 24 and 48 h in 0.2% serum media; and (B) serum-starved A549 cells with or without HN were treated with IL-1β (10 ng/mL) for 0, 12, 24 and 48 h. *P <0.05 vs. Control (n=3); and (Ci) IL-1β-stimulated cell proliferation was determined by scratch healing assay and representative microscopic images of five random microscopic fields for 0 and 48 h (n = 3) are shown; and (Cii)Quantification of relative scratch width (%) at 0 and 24 h. *P <0.05 vs. 24 h control (n=3). [C, control; HN, hesperetin 100 μM; IL-1β, interleukin-1beta 10 ng/mL; HN+I, hesperetin + IL-1β]

with 10 ng/mL (P <0.03) and 20 ng/mL (P <0.02) doses of IL-1β. However, with increased dose of IL-1β at 50 ng/mL, decreased cell proliferation at both 24 and 48 h was observed (Fig. 1A). On the basis of these results, we selected 10 ng/mL dose of IL-1β for further experiments.

Next, we examined the cytotoxicity of HN on lung adenocarcinoma cells using 0, 50, 100 and 150 μM doses, and observed that up to 100 μM doses cells were not affected significantly, while at 150 μM dose cell viability decreased in A549 cells at 48 and 72 h durations (data not shown). From this experiment we selected 100 μM dose of HN for further experiments. Further, we examined whether IL-1β-induced A549 cell proliferation could be regulated by HN (100 μM). As shown in Fig. 1B, there was a time-dependent increase in cell viability by IL-1β which was significantly decreased (by 35.8%; P <0.02) by HN at 48 h (Fig. 1B).

The wound healing assay demonstrated that IL-1β-stimulated cell proliferation was increased in A549 cells which led to increased closure of scratched wound gap. The gap width of 42% *(P* <0.003*)* was observed with IL-1β-stimulus in A549 cells indicating increased cell growth, while a significantly more gap width of 89.47% (P <0.01) wound was observed in HN-pretreated IL-1β-stimulated A549 cells indicating cell growth inhibition by HN (Fig. 1C).

Hesperetin inhibited IL-1β-induced expression of cell cycle regulatory proteins

Inhibition of cell growth through cell cycle arrest is considered one of the mechanisms exhibited by anti-cancer agents[19]. HN is known to inhibit cancer cell proliferation through cell cycle arrest in colon, cervical and breast cancers[15,17,20,21]. Therefore, we studied whether HN could impact IL-1β-induced cell cycle progression. As shown in Fig. 2A, IL-1β induced a significant G1 phase arrest (74.7%) at 48 h (P <0.001), which was reversed by HN (62.1%) near to control. Further, we assessed the various cell cycle regulatory proteins including CDKs and CDK inhibitors (CDKi). Although, no significant changes in the expression of CDK-1, -2 and -6 was observed at 48 h, there was significant decrease in the expression of CDKi, especially p21 protein (Fig. 2B). HN treatment significantly upregulated the IL-1β-stimulated decreased expression of p21 and p27 at 48 h in A549 cells. These results indicate the preventive role of HN against cell cycle progression in lung cancer cells through the modulation of CDKIs.

Hesperetin decreased IL-1β-induced COX-2 expression and release of PGE2 in A549 cells

COX-2 has been implicated in the establishment and maintenance of inflammatory microenvironment and cancer progression[7,11,19]. Thus, we determined whether HN could regulate IL-1β-induced COX-2 expression. As can be seen in Fig. 3A, IL-1β treatment time-dependently increased the expression of COX-2 at both mRNA (Fig. 3Ai) and protein (Fig. 3Aii) level till 24 h. We also examined dose-dependent effect of IL-1β-stimulated COX-2 expression and observed that IL-1β increased COX-2 expression dose-dependently at both 24 and 48 h time points in A549 cells (Fig. 3B).

COX-2 is known to catalyze the production of PGE2 which has impacts on cancer cell proliferation and survival. The role of pro-inflammatory cytokine IL-1β-stimulated PGE2 has been reported in human colorectal and lung cancers[22]. Therefore, we quantified IL-1β-stimulated PGE2 secretion in A549 cells. As shown in Fig 3Ci, a significant (P <0.05) enhancement in PGE2 level from 157.7 pg/mL in control to 1559.9 pg/mL in IL-1β-stimulated A549

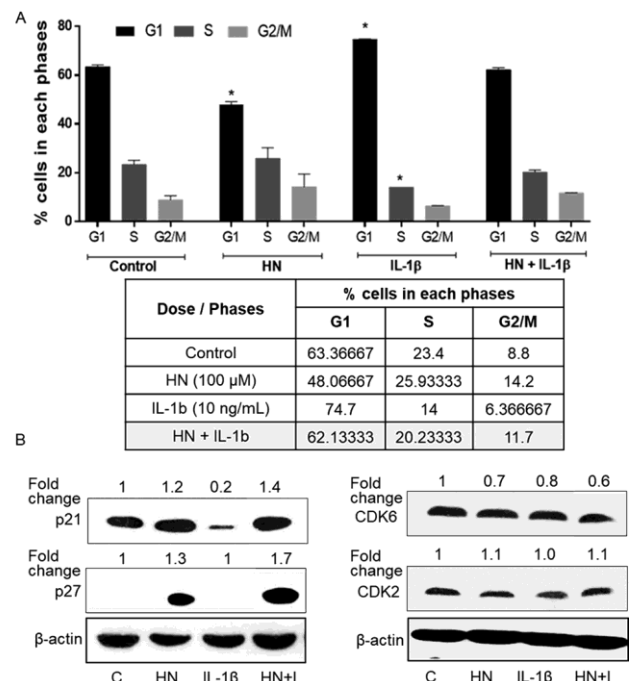| Dose / Phases	% cells in each phases		
	G1	S	G2/M
Control	63.36667	23.4	8.8
HN (100 μM)	48.06667	25.93333	14.2
IL-1b (10 ng/mL)	74.7	14	6.366667
HN + IL-1b	62.13333	20.23333	11.7

Fig. 2 — HN regulates IL-1β-induced cell cycle arrest and cell cycle regulatory proteins in A549 cells. (A) Fluorescence-activated cell sorting analysis was done to analyze the percent cells in different phases. *P <0.005 *vs.* respective phases of control; and (B) Immunoblotting was done to analyze the expression of cell cycle regulatory proteins CDK 2 and 6, and CDK inhibitors p21 and p27 and the representative blots are shown from at least three independent experiments (n=3).
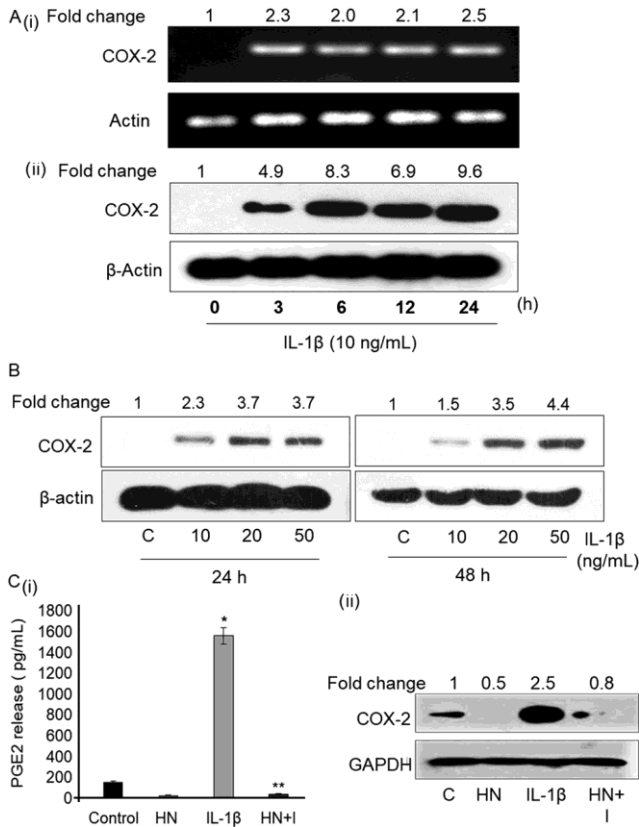
Fig. 3 — IL-1β-stimulated COX-2 expression at mRNA and protein levels in A549 cells. IL-1β-induced time-dependent expression of COX-2 at (Ai) mRNA and (Aii) protein levels were determined by RT-PCR and immunoblotting, respectively; (B) A dose-dependent effect of IL-1β induction on COX-2 protein expression was determined by immunoblotting and the representative blots are shown from at least three experiments (n=3); (Ci) IL-1β-stimulated PGE2 release (pg/mL) in A549 cells were measured in the media supernatant using colorimetric assay. *P <0.05 *vs*. Control and **P <0.02 *vs*. IL-1β (n=3); and The COX-2 protein expression was assessed by immunoblotting from same experiments.
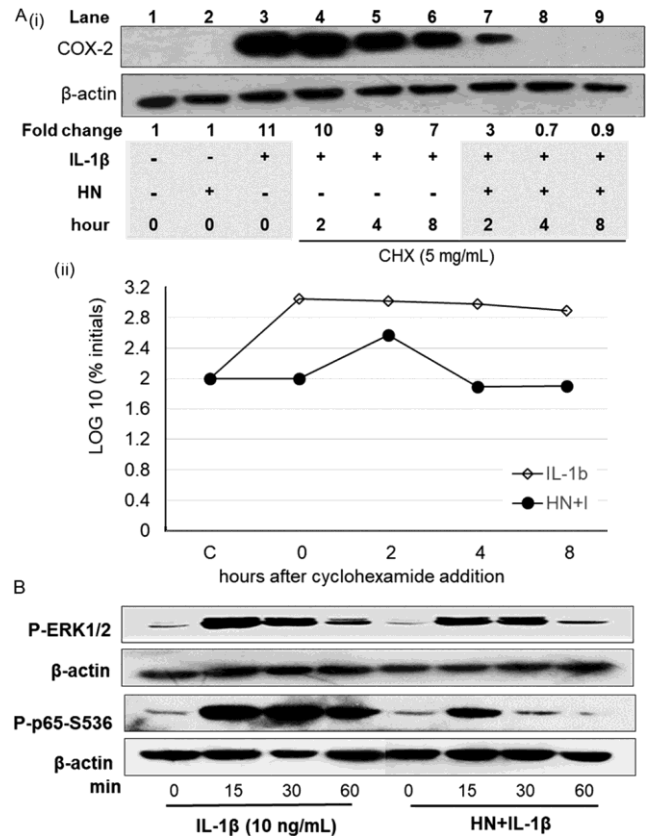
Fig. 4 — HN regulates IL-1β-stimulated PGE2 production and COX-2 level in A549 cells. (Ai) Serum-starved A549 cells treated with translation inhibitor, cycloheximide (CHX) for 2, 4 and 8 h followed by immunoblotting; (Aii) Quantified values (fold-change) for turnover of COX-2 protein at respective chase times are shown; and (B) Effect of HN on IL-1β-stimulated increased phosphorylation of signaling intermediates ERK1/2 (Thr202/Tyr204) and NF-κB-p65 (Ser536) for different time intervals 15, 30 and 60 min was assessed by immunoblotting and representative blots along with loading control are shown (n=3).

cells at 24 h was observed. HN treatment significantly (P <0.05) decreased PGE2 level to 39.4 pg/mL which was near the control value. In the same experiment, the cytosolic extract was used to measure the expression of IL-1β-stimulated COX-2 protein, which increased significantly by approximately 2.5 folds as compared to control, and in HN pre-treated A549 cells it decreased significantly near the control level (Fig. 3Cii). These results suggest that HN efficiently modulated COX-2 activity and prevented the production of inflammatory mediator PGE2.

Hesperetin decreased IL-1β-induced increase in COX-2 protein stability

The stability of intracellular COX-2 protein is important characteristic of tumor microenvironment to assist the progression of tumor cell proliferation which aids in tumor growth and metastasis[6]. Therefore, we investigated whether HN could alter IL-1β-stimulated COX-2 stability in A549 cells using CHX assay. As shown in Fig. 4Ai, stimulation with IL-1β increased and maintained the stability of COX-2 protein till 8 h, while HN pretreated cells showed loss of COX-2 protein at 4 and 8 h. The turnover of COX-2 protein was shown in percent value and calculated from the Log 10 values (% of initial value) of fold-change of treatment groups IL-1β and HN+IL-1β at respective chase times (Fig. 4Aii). The expression of β-actin protein was unaffected at various time points and treatment groups. These results suggest that HN could be a potential inhibitor of IL-1β-stimulated COX-2 expression and the

regulation of COX-2 may be controlled by affecting its stability at post-translationally.

Hesperetin inhibited IL-1β-induced phosphorylation of signaling intermediates

Both cytokine-induced cancer cell growth and enhanced inflammation are regulated by phosphorylation of ERK1/2 MAPK [7] and NF-κB transcription factor[7,23]. Activation of these signaling molecules reveals their role in IL-1β-mediated survival of tumor cells and maintenance of tumor microenvironment as well. Therefore, we next examined the effect of HN on IL-1β-stimulated phosphorylation of these signaling molecules. Serum-starved A549 cells pretreated with or without HN were stimulated with IL-1β for 0, 15, 30 and 60 min followed by Western blotting. As demonstrated in Fig. 4B, IL-1β induction increased phosphorylation of ERK1/2 as well that of NF-κB-p65 in A549 cells and HN decreased these changes. These findings suggest that IL-1β-stimulus increases the phosphorylation of kinases such as ERK1/2 and transcription factor NF-κB-p65 and pretreatment of HN decreased their activation which could be responsible for its anticancer and anti-inflammatory properties in A549 cells.

The novel findings of present study are HN could regulate the inflammatory microenvironment and cancer cell growth by modulating the expression and activities of key inflammatory protein COX-2 and cell cycle regulators i.e., CDKi in A549 cells. IL-1β is a known for its role in tumor promotion and progression in several cancers including lung cancer[4,6,24]. It is also known to modulate the inflammatory tumor microenvironment and stimulates cancer cell proliferation which lead to malignancies[2]. Thus, inhibition of IL-1β-mediated enhanced cell proliferation and inflammation could be effective strategy to restrict the lung cancer.

The use of synthetic and nonsteroidal anti-inflammatory drugs (NSAID) against lung cancer has been practiced in clinics for many years despite their side effects[9]. To minimize their side effects, phytochemical-based chemotherapy could be a better approach. In the present study, the anti-inflammatory and anticancer properties of HN[21,25,26] were assessed in IL-1β-induced inflammation and cell growth in A549 cells. Numerous studies have revealed anticancer property of HN against colorectal[16,20], breast[17,21], cervical[15] and prostate[27] cancer. However, its role against lung cancer and potential mechanism involved is still unknown.

Antiproliferative effect of HN was assessed by evaluating its effect on IL-1β-stimulated cell growth and viability in A549 cells, which showed that HN significantly inhibited cancer cell growth induced by IL-1β at 24 and 48 h. These results clearly indicate that inhibition of cancer cells proliferation could be one of the pathways to restrict lung carcinoma *in vitro*. Since cell proliferation has direct relationship with cell cycle events, we examined the effect of HN on IL-1β-stimulated cell cycle progression. The IL-1β showed cell cycle arrest in G1-phase in A549 cells (increased by 11.33%) which was reversed to near control value by HN and cells were slightly pushed to G2/M phase. HN alone could also cause a G2/M phase arrest in A549 cells which indicates its anticancer property.

The cell cycle events are regulated through cyclin dependent kinases (CDKs) and inhibitors of CDKs (CDKIs) in different cell cycle phases[28]. We observed that in A549 cells the expression of CDKs was largely unaffected by IL-1β stimulation and also there was no significant effect of HN on expression of CDKs. However, CDKIs (especially p21 and p27) expression decreased significantly after IL-1β stimulation, whereas HN treatment restored the expression of these CDKIs. Downregulation of CDKIs by IL-1β suggests the role of IL-1β in promoting cell proliferation through removing the breaks on CDKs that promotes uncontrolled cell cycle progression. On the other hand, HN upregulates that CDKIs expression which could restrict cell proliferation through putting the breaks in place, indicating a possible route through which HN could control tumor growth.

The prolonged presence of IL-1β is responsible for virulent tumor phenotype, particularly through the presence of mediators like COX-2 and PGE2[23]. We investigated if HN reversed IL-1β-mediated increased expression of COX-2 at transcriptional and translational level, translational stability and its activity in terms of PGE2 release. Transcriptional induction of COX-2 by IL-1β was observed at 3 h and its expression was consistent through 24 h. Similarly, increased translation of COX-2 protein was also observed. HN decreased the expression of COX-2 both at mRNA and protein levels. These results suggest that not only IL-1β induces inflammatory mediators expression early, it also tend to maintain its levels for prolonged duration, suggesting that IL-1β could be a prominent factor regulating COX-2 at
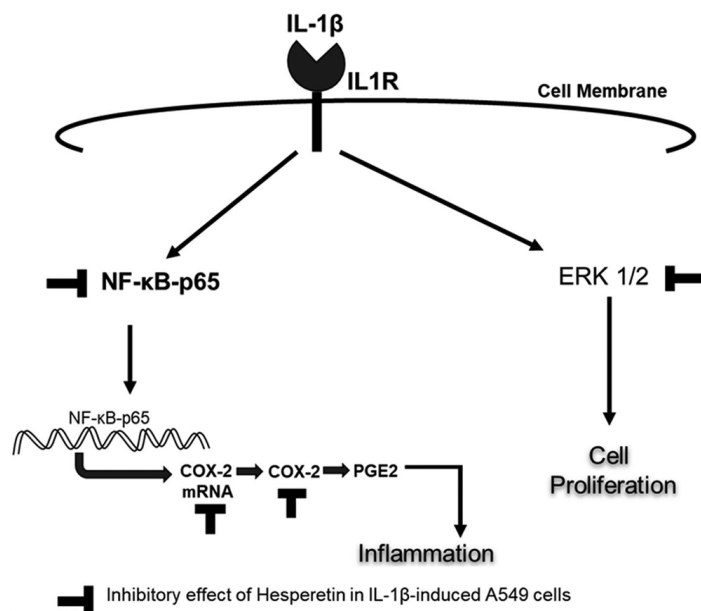
Fig. 5 — Schematic representation of HN's role in the prevention of A549 cell proliferation and inflammation

transcriptional and translational levels in A549 cells, and HN could prevent these changes indicating towards its potential as anti-inflammatory properties.

COX-2 can contribute to several events of carcinogenesis only when the intracellular level of protein is stable and present for prolonged time. CHX assay showed that IL-1β increased the stability of COX-2 protein and HN effectively reversed this effect and caused the early loss of COX-2 protein, although its mechanism is not known at this time. Nonetheless, these findings suggest that HN could be an effective pharmacological agent against persistent inflammation by affecting the turnover of COX-2 protein in A549 cells. Also, increased enzymatic activity of COX-2 is associated with increased PGE2 synthesis and release. Therefore, we examined effect of HN on COX-2 enzyme activity by measuring the production of PGE2. IL-1β-stimulus increased PGE2 secretion, which was reversed with HN treatment. These results give further insight into anti-inflammatory role of HN through inhibition of transcriptional and translational expression, stability and enzyme activity of COX-2.

Presence of IL-1β leads to enhanced phosphorylation of MAPK, Akt and NF-κB-p65 signaling in several cancers[29]. In order to determine the mechanism of IL-1β-stimulated A549 cell growth and inflammation and its inhibition by HN, we examined changes in MAPKs/NF-κB signaling and showed that IL-1β activated ERK1/2 and transcription factor NF-κB-p65, which are known for their role in survival and inflammation in lung cancer A549 cells, were inhibited by HN. Thus, this study reveals the preventive role of HN against IL-1β-stimulated phosphorylation of ERK1/2 as well as p65 in A549 lung epithelial cells, which could be responsible for decreased inflammation and A549 cell growth.

Taken together, our results provide evidence that inhibition of IL-1β-stimulated survival of lung adenocarcinoma cells by HN could be through the inhibition of proliferation by regulating cell cycle progression and modulation of mitogenic kinases such as ERK1/2 and NF-κB-p65 (Fig. 5). Further, inhibition of COX-2 expression as well as its enzyme activity by HN, could be responsible for its anti-inflammatory properties.

Acknowledgment

References

1   Siegel R, Naishadham D & Jemal A, Cancer statistics, 2013. CA *Cancer J Clin*, 63 (2013) 11.
2   Coussens LM & Werb Z, Inflammation and cancer. *Nature*, 420 (2002) 860.
3   Mantovani A, The inflammation - cancer connection. *FEBS J*, 285 (2018) 638.
4   Lewis AM, Varghese S, Xu H & Alexander H R, Interleukin-1 and cancer progression: the emerging role of interleukin-1

receptor antagonist as a novel therapeutic agent in cancer treatment. *J Transl Med*, 4 (2006) 48.

5   Apte RN & Voronov E, Immunotherapeutic approaches of IL-1 neutralization in the tumor microenvironment. *J Leukoc Biol*, 102 (2017) 293.

6   Yano S, Nokihara H, Yamamoto A, Goto H, Ogawa H, Kanematsu T, Miki T, Uehara H, Saijo Y, Nukiwa T & Sone S, Multifunctional interleukin-1beta promotes metastasis of human lung cancer cells in SCID mice via enhanced expression of adhesion-, invasion- and angiogenesis-related molecules. *Cancer Sci*, 94 (2003) 244.

7   Chen KH, Weng MS & Lin JK, Tangeretin suppresses IL-1beta-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells. *Biochem Pharmacol*, 73 (2007) 215.

8   Todoric J, Antonucci L & Karin M, Targeting Inflammation in Cancer Prevention and Therapy. *Cancer Prev Res* (Phila), 9 (2016) 895.

9   Williams GW, An update on nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors. *Curr Pain Headache Rep*, 9 (2005) 377.

10 Capece D, Verzella D, Tessitore A, Alesse E, Capalbo C & Zazzeroni F, Cancer secretome and inflammation: The bright and the dark sides of NF-kappaB. *Semin Cell Dev Biol*, 78 (2018) 51.

11 Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C & Kaidi A, The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis*, 30 (2009) 377.

12 Sobolewski C, Cerella C, Dicato M, Ghibelli L & Diederich M, The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies. *Int J Cell Biol*, 2010 (2010) 215158.

13 Jang BC, Sanchez T, Schaefers HJ, Trifan OC, Liu CH, Creminon C, Huang CK & Hla T, Serum withdrawal-induced post-transcriptional stabilization of cyclooxygenase-2 mRNA in MDA-MB-231 mammary carcinoma cells requires the activity of the p38 stress-activated protein kinase. *J Biol Chem*, 275 (2000) 39507.

14 Lee JS, Lee EJ, Na HK & Surh YJ, From Inflammation to Cancer: Opportunities for Chemoprevention via Dietary Intervention. In: *Immunity and Inflammation in Health and Diseases*, 1st Edn. (Eds. Chatterjee S, Jungraithmayr W & Bagchi D; Academic Press, Elsevier, Massachusetts, United States), 2017, 203.

15 Alshatwi AA, Ramesh E, Periasamy VS & Subash-Babu P, The apoptotic effect of hesperetin on human cervical cancer cells is mediated through cell cycle arrest, death receptor, and mitochondrial pathways. *Fundam Clin Pharmacol*, 27 (2013) 581.

16 Sivagami G, Vinothkumar R, Preethy CP, Riyasdeen A, Akbarsha M A, Menon VP & Nalini N, Role of hesperetin (a natural flavonoid) and its analogue on apoptosis in HT-29 human colon adenocarcinoma cell line--a comparative study. *Food Chem Toxicol*, 50 (2012) 660.

17 Yang Y, Wolfram J, Boom K, Fang X, Shen H & Ferrari M, Hesperetin impairs glucose uptake and inhibits proliferation of breast cancer cells. *Cell Biochem Funct*, 31 (2013) 374.

18 Singh N, Nambiar D, Kale RK & Singh RP, Usnic acid inhibits growth and induces cell cycle arrest and apoptosis in human lung carcinoma A549 cells. *Nutr Cancer*, 65 Suppl 1 (2013) 36.

19 Arif E, Vibhuti A, Deepak D, Singh B, Siddiqui MS & Pasha MA, COX2 and p53 risk-alleles coexist in COPD. *Clin Chim Acta*, 397 (2008) 48.

20 Aranganathan S & Nalini N, Antiproliferative efficacy of hesperetin (citrus flavanoid) in 1,2-dimethylhydrazine-induced colon cancer. *Phytother Res*, 27 (2013) 999.

21 Choi E J, Hesperetin induced G1-phase cell cycle arrest in human breast cancer MCF-7 cells: involvement of CDK4 and p21. *Nutr Cancer*, 59 (2007) 115.

22 Seyedrezazadeh E, Kolahian S, Shahbazfar AA, Ansarin K, Pour Moghaddam M, Sakhinia M, Sakhinia E & Vafa M, Effects of the flavanone combination hesperetin-naringenin, and orange and grapefruit juices, on airway inflammation and remodeling in a murine asthma model. *Phytother Res*, 29 (2015) 591.

23 Chun KS & Surh YJ, Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol*, 68 (2004) 1089.

24 Mantovani A, Barajon I & Garlanda C, IL-1 and IL-1 regulatory pathways in cancer progression and therapy. *Immunol Rev*, 281 (2018) 57.

25 Zhang Y, Kong W & Jiang J, Prevention and treatment of cancer targeting chronic inflammation: research progress, potential agents, clinical studies and mechanisms. *Sci China Life Sci*, 60 (2017) 601.

26 Yang Y, Wolfram J, Shen H, Fang X & Ferrari M, Hesperetin: an inhibitor of the transforming growth factor-beta (TGF-beta) signaling pathway. *Eur J Med Chem*, 58 (2012) 390.

27 Shirzad M, Heidarian E, Beshkar P & Gholami-Arjenaki M, Biological Effects of Hesperetin on Interleukin-6/Phosphorylated Signal Transducer and Activator of Transcription 3 Pathway Signaling in Prostate Cancer PC3 Cells. *Pharmacognosy Res*, 9 (2017) 188.

28 Lapenna S & Giordano A, Cell cycle kinases as therapeutic targets for cancer. *Nat Rev Drug Discov*, 8 (2009) 547.

29 Tanabe T & Tohnai N, Cyclooxygenase isozymes and their gene structures and expression. *Prostaglandins Other Lipid Mediat*, 68-69 (2002) 95.