Original Paper

Emodin Inhibits ATP-Induced Proliferation and Migration by Suppressing P2Y Receptors in Human Lung Adenocarcinoma Cells

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Key Words

Emodin • ATP • A549 cells • Suramin • NF-κB

Abstract

Background/Aims: Extracellular ATP performs multiple important functions via activation of P2 receptors on the cell surface. P2Y receptors play critical roles in ATP evoked response in human lung adenocarcinoma cells (A549 cells). Emodin is an anthraquinone derivative originally isolated from Chinese rhubarb, possesses anticancer properties. In this study, we examined the inhibiting effects of emodin on proliferation, migration and epithelial-mesenchymal transition (EMT) by suppressing P2Y receptors-dependent Ca^{2+} increase and nuclear factor-κB (NF-κB) signaling in A549 cells. **Methods:** A549 cells were pretreated with emodin before stimulation with ATP for the indicated time. Then, intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured by Fluo-8/AM staining. Cell proliferation and cell cycle progression were tested by CCK8 assay and flow cytometry. In addition, wound healing and western blot were performed to determine cell migration and related protein levels (Bcl-2, Bax, claudin-1, NF-κB). **Results:** Emodin blunted ATP/UTP-induced increase of $[\text{Ca}^{2+}]_i$ and cell proliferation concentration-dependently. Meanwhile, it decreased ATP-induced cells accumulation in the S phase. Furthermore, emodin altered protein abundance of Bcl-2, Bax and claudin-1 and attenuated EMT caused by ATP. Such ATP-induced cellular reactions were also inhibited by a nonselective P2Y receptors antagonist, suramin, in a similar way to emodin. Besides, emodin could inhibit activation of NF-κB, thus suppressed ATP-induced proliferation, migration and EMT. **Conclusion:** Our results demonstrated that emodin inhibits ATP-induced proliferation, migration, EMT by suppressing P2Y receptors-mediated $[\text{Ca}^{2+}]_i$ increase and NF-κB signaling in A549 cells.

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Introduction

Over one million people die of lung cancer every year worldwide. Primary malignant lung cancers can be classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma). NSCLC accounts for 80%~85% of total lung cancers with 5-year survival rate of 10-15% [1]. It has been found ATP level in the tumor micro-environment is several hundred even thousand times higher than those in normal tissues [2]. ATP regulates biological functions mainly through purinergic P2 receptors [3], which can be classified into ligand-gated ion channel P2X receptors and G-protein coupled P2Y receptors [4]. In human lung adenocarcinoma cells (A549 cells), ATP stimulates cell proliferation by activating P2Y receptors [5]. Activation of P2Y receptors triggers $[Ca^{2+}]_i$ increase, which is one of the key regulators of not only cell survival but also cell death [6]. In A549 tumor cells, P2Y receptors-induced proliferation also depends on activation of NF- κ B, which can translocate to nucleus [5]. It promotes transcription of downstream target genes such as Bax [7]. Inhibition of NF- κ B signaling pathway suppresses cell proliferation and induces cell cycle arrest in G2/M phase accompanied by regulation of Bcl-2 family proteins (anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax) in prostate cancer cells [8]. The Bcl-2/Bax ratio is increased in lung cancer cells after stimulation with ATP. Moreover, elevation of Bcl-2/Bax ratio in lung cancer cells may enable tumor cells to survive [9].

Epithelial-mesenchymal transition (EMT) is characterized by the loss of cell-cell adhesion molecules, such as E-cadherin, and up-regulation of mesenchymal markers, such as fibronectin and vimentin [10]. EMT inhibition by down-regulating metastasis associated lung adenocarcinoma transcript 1 (MALAT1) represses lung cancer cell invasion [11]. The intercellular cell-adhesion molecule-1 (ICAM-1), which participates in EMT and tumor invasion is also involved in survival of NSCLC patients and tumor differentiation [12]. Extracellular ATP can decrease expression of the claudin-1, a kind of tight junction protein [13].

Traditional Chinese medicine has many biologic functions such as anti-apoptotic, anti-lipid aggregation [14]. Emodin, a natural anthraquinone isolated from rhubarb, possesses properties of anti-bacterial [15], anti-inflammatory [16], vasorelaxant [17], and triggering erythrocyte cell membrane scrambling [18]. It can ameliorate diesel exhaust particles-induced impairment of vascular and cardiac homeostasis in mice [19]. Recent studies have shown emodin can significantly inhibit tumor growth such as melanoma [20], tongue cancer as well as NSCLC [21]. It is reported that emodin suppresses cell growth and triggers cell apoptosis of NSCLC [22, 23]. Extracellular ATP promotes tumorigenesis [24]. In lung cancer cells, ATP promotes cell survival via regulation of $[Ca^{2+}]_i$ and Bcl-2/Bax ratio [9]. However, it remains unknown if emodin can improve lung adenocarcinoma tumors by inhibiting ATP-induced $[Ca^{2+}]_i$ increase and its downstream signaling pathway.

To determine the anticancer effect of emodin and its underlying mechanism, we examined the effect of emodin on ATP signaling and its induced proliferation, migration and EMT. We found that emodin could inhibit the ATP-induced proliferation, migration and EMT of A549 cells via inhibiting P2Y receptors-induced $[Ca^{2+}]_i$ increase and NF- κ B activation. The new finding may offer a new target for future anticancer therapy.

Materials and Methods

Chemicals and reagents

Emodin was purchased from Shanghai future industry Limited by Share Ltd (Shanghai, China). Adenosine 5-triphosphate disodium salt hydrate (ATP) and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Suramin hexasodium salt was purchased from TOCRIS (Bristol, UK). Rabbit anti-Bcl-2, anti-Bax were purchased from Santa Cruz Biotechnology (CA, USA), anti-claudin-1, anti-NF- κ B p65, anti-p-NF- κ B p65, anti-E-cadherin, anti-Snail and anti-GAPDH were purchased

from Cell Signaling Technology (Beverly, MA, USA), anti-fibronectin was purchased from ABCam (ABcam, MA, USA), RIPA lysis buffer was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture

The human A549 cell was purchased from Fudan IBS Cell Center (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, 4.5 g/L glucose) (Life Technologies, USA) supplemented with 10% fetal bovine serum and antibiotics (100 KU/L penicillin and 100 mg/L streptomycin) in an incubator at 37 °C with 5% CO₂.

Quantitative real-time PCR

Total RNA was extracted from A549 cells with Trizol Reagent (Invitrogen Corporation, CA, USA). First-strand cDNA was amplified from 0.2 µg of total RNA with the ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). Then the mRNA levels of P2Y1, P2Y2, P2Y4, P2Y6 were analyzed by quantitative real-time PCR (iCycler iQReal-time PCR Detection System, Bio-Rad Laboratories Inc., USA) using SYBR Green Real-time PCR MasterMix (Toyobo, Japan) in a total volume of 20 µl. Expression levels were normalized to GAPDH. The target genes and their primer sequences were shown in Table 1.

Immunofluorescence staining

A549 cells were seeded on sterile glass slides. After fixation with 4% paraformaldehyde for 10 min, the slides were washed with PBS three times. Then cells were blocked with 2% BSA at room temperature for 1 h and incubated with primary antibodies (NF-κB, 1:100) at 4 °C overnight. After incubation, samples were washed three times with PBS and incubated with secondary antibodies at room temperature for 2 h. The nuclei were stained with DAPI. After washing with PBS, coverslips mounted in 90% glycerol in PBS, and fluorescence was detected using a Zeiss LSM 710 confocal laser system.

CCK8 assay

The viability of the A549 cells was determined by the CCK8 assay. Cells were seeded in 96-well plates at $\sim 5 \times 10^3$ and treated with medium alone (control) or with emodin and suramin in the presence of ATP (100 µM). Then 10 µl of CCK8 was added to each well and incubated for 2 h in an incubator at 37 °C with 5 % CO₂. The absorbance was recorded at a wavelength of 450 nm.

Cell cycle analysis

For cell cycle analysis, A549 cells were cultured in a 60 mm dish and pretreated with suramin or emodin 15 min before ATP stimulation. Thereafter, ATP (100 µM) was applied to A549 cells for 4 h. Then the cells were washed, fixed, stained with propidium iodide and analyzed for DNA content on a BD FACSCalibur flow cytometer (BD Biosciences).

Western Blot

Protein was extracted from cultured A549 cell lysates with RIPA lysis buffer with phosphatase inhibitor (Roche). After that protein was loaded into each lane onto SDS-polyacrylamide gels and transferred to a PVDF membrane, the membrane was incubated with primary anti-Bcl-2 antibody (1:1000), anti-Bax antibody (1:500), anti-claudin-1 antibody (1:1000), mouse anti-GAPDH polyclonal antibody (1:2000) at 4°C overnight, mouse anti-GAPDH polyclonal antibody (1:2000 dilution). HRP-labeled Goat Anti-Rabbit IgG (H+L) (1:1000 dilution) and HRP-labeled Goat Anti-Mouse IgG (H+L) (1:2000 dilution) antibodies were used as the secondary antibodies. The optical density was normalized to that of GAPDH (1:1000, Beyotime), which represented as relative optical density.

[Ca²⁺]_i Measurement

A549 cells were grown at 50%–60% confluence on 25-mm diameter circular glass coverslips. Then, cells were pretreated with emodin or suramin at indicated concentrations for 10 min. Next, cells were loaded

Table 1. The target genes and their primer sequences

Gene	The Sequence of Gene-Specific Primer
P2Y1	F-GCCGCCGTCTCCTCGTCGTT- R-CCATTCTGCTTGAACCTCAG-
P2Y2	F-CGAGGACTTCAAGTACGTGC- R-GTTCTCGCTACCAGCCGGCG-
P2Y4	F-GCTGCCTGTGAGCTATGCAG - R-GAGCTGACGGAGCTGACGTCG -
P2Y6	F-GTTCAGGCTGAGGAGATG- R-GGAAGCTGATGCAGGTGA-
GAPDH	F-TGACTTCAACAGCGACACCCA R-CACCCTGTTGCTGTAGCCAAA-

with 10 μ M Fluo-8/AM and 0.02% pluronic F-127 for 30 min. After washing with normal physiological solution (NPSS) containing 1 mmol CaCl_2 , 140 mmol NaCl, 5 mmol KCl, 1 mmol MgCl_2 , 10 mmol glucose, 5 mmol Hepes, coverslip with cells was mounted into the recording chamber. The fluorescence signals were recorded by Zeiss 710 confocal laser system and $[\text{Ca}^{2+}]_i$ changes were showed as a ratio of fluorescence ($F1/F0$).

Wound healing assay

A549 cells were seeded in a six-well plate and pretreated with emodin and suramin then stimulated with ATP for the indicated time. Three parallel lines were drawn on the underside of each well to demarcate the wound areas for analysis.

A scratch was made in the center of the well using a sterile 1000 μ l micropipette tip before the cells were fully confluent. The wounds were observed using bright field microscopy (Olympus, Tokyo, Japan) after the scratch at 0 h, 6 h, 12 h, 24 h. Images were analyzed by Image J software. The rate of migration = (scratch widths before migration-scratch widths after migration)/scratch widths before migration $\times 100\%$.

Statistical analysis

Data are expressed as mean $\pm$ SD. The significance of differences among groups was evaluated by one-way ANOVA test. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of emodin on ATP/UTP-induced $[\text{Ca}^{2+}]_i$ increase in A549 cells.

We detected the expression of P2Y receptors in A549 cells using RT-PCR (Fig. 1A). As expected, mRNA level of P2Y2 and P2Y6 was more than P2Y1 and P2Y4. Then the effect of emodin on ATP-induced $[\text{Ca}^{2+}]_i$ increase was examined. Application of ATP (100 μ M) evoked a

Fig. 1. Emodin inhibited ATP/UTP-induced Ca^{2+} increase in a concentration-dependent manner: (A) mRNA level of P2Y1, P2Y2, P2Y4, P2Y6 were measured by real-time qualitative PCR. The expression of P2Y2 and P2Y6 receptor were much higher than that of P2Y1 and P2Y4 in A549 cells. Values are mean $\pm$ SD, $n = 6$, $**p < 0.01$. (B) Representative tracings of $[\text{Ca}^{2+}]_i$ for cells treated with ATP (100 μ M) alone and cells pretreated with suramin (100 μ M) or emodin (1 and 5 μ M). Suramin and emodin was added 15 min before the addition of ATP. Arrows indicated applications of ATP. (C) Representative $[\text{Ca}^{2+}]_i$ traces for stimulating A549 cells with UTP (100 μ M) after pretreatment with suramin or emodin for 15 min. Arrows indicated the application of UTP. (D-E): Summarized data showing ATP and UTP-induced $[\text{Ca}^{2+}]_i$ rise after application of ATP or UTP alone and in the presence of emodin or suramin. Values are mean $\pm$ SD, $n = 6$, $*P < 0.05$, $**p < 0.01$.

Fig. 2. Emodin suppressed ATP-induced cell proliferation. (A) A549 cells were stimulated with ATP for 4 h, 8 h, 12 h, 16 h, 20 h and 24 h. The cell viability was measured by CCK8 assay. Viability of cells increased 4 h after ATP stimulation and then decreased and reached the baseline at 12 h. (B) The cell viability of A549 cells was measured after treated with emodin of 0.1, 0.5, 1, 5, 10 μ M for 4 h. Cells treated with DMSO served as control. Low doses of emodin had no effect on cell viability, while high dose of emodin (10 μ M) inhibited cell viability slightly. (C)

Cells were pre-cultured with suramin (100 μ M) or emodin at different concentrations (0.5, 1, 5 μ M) before ATP (100 μ M) stimulation. Either suramin or emodin attenuated the proliferating effect of ATP. Additionally, the inhibitory effect of emodin on ATP-induced proliferation was dose-dependent. (D-E) Cell cycle profiles were further evaluated by flow cytometry. The G0/G1, S, G2/M cell populations were assessed after propidium iodide staining. Stimulation with ATP caused more cell populations in the S and G2/M phases. While pretreatment with either emodin or suramin eventually led to an accumulation of cells in the G0/G1 phase and decreased cell population of S and G2/M phases. Values are mean $\pm$ SD, $n = 6$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: S, DNA duplication phase; G0/G1, gap between end of M-phase and start of S-phase; G2, gap between end of S-phase and start of M-phase.

rapid increase in $[Ca^{2+}]_i$ in A549 cells (Fig. 1B). ATP-evoked $[Ca^{2+}]_i$ increase was significantly reduced by pretreatment with emodin at 1, 5 μ M. UTP, a selective agonist for P2Y receptor, also induced $[Ca^{2+}]_i$ response in A549 cells [25]. Thus, UTP was used as a positive control to determine the potency and specificity of emodin. As shown in Fig. 1C, UTP-triggered $[Ca^{2+}]_i$ increase was also remarkably inhibited by emodin dose dependently. Therefore it was well established that P2Y receptors are highly expressed in A549 cells. These results indicated that emodin could suppress ATP/UTP-induced $[Ca^{2+}]_i$ increase in A549 cells.

Emodin inhibited ATP-induced proliferation.

Since ATP-induced proliferation time dependently, cells were treated with ATP for 4, 8, 12, 16, 20 and 24 h. Cell proliferation degree peaked at 4 h after ATP stimulation and then reduced (Fig. 2A). Thus, 4 h was selected as the best stimulating time. We then tested the effect of emodin on cell viability of A549 cells. Cells were treated for 4 h with increasing doses of emodin from 0.1 μ M to 10 μ M. Emodin at a concentration of ≤ 5 μ M had no discernible effect, however, 10 μ M caused a very mild but significant reduction in cell viability (Fig. 2B). To investigate the inhibitory effect of emodin on extracellular ATP-induced proliferation, cells were pretreated with emodin (0.5, 1, 5 μ M) or suramin (100 μ M) and then stimulated with ATP (100 μ M). As shown in Fig. 2C, stimulation with ATP evidently

Fig. 3. Emodin inhibited ATP induced cell migration. (A) Wound healing assay was used to assess the effect of emodin and suramin on A549 cells migration. The wound closure was photographed at post-scratching 0 h, 6 h, 12 h, 24 h. Original magnification, $\times 50$. ATP promoted migration of cells. Emodin and suramin attenuated the effect of ATP. (B-D) Representing the migration rate was calculated by Image J software. The group data reflected the wound closure rate at 6 h, 12 h, 24 h. Values are mean $\pm$ SD, $n = 6$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (E-I) Representative images and summarized data showing the expression levels of Bcl-2, Bax, claudin-1. The expression level of Bcl-2, Bax, claudin-1 were normalized to GAPDH level in the same sample. ATP increased the expression of Bcl-2 and decreased the expression of Bax and claudin-1. Similar to suramin, emodin inhibited the effect of ATP. Values are mean $\pm$ SD, $n = 6$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

The expression level of Bcl-2, Bax, claudin-1 were normalized to GAPDH level in the same sample. ATP increased the expression of Bcl-2 and decreased the expression of Bax and claudin-1. Similar to suramin, emodin inhibited the effect of ATP. Values are mean $\pm$ SD, $n = 6$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

enhanced A549 cells proliferation. However, emodin inhibited ATP-induced proliferation in a concentration-dependent manner with the most significant inhibition at concentration of 5 μ M. Besides, ATP-induced cell proliferation was suppressed by suramin in a similar fashion. To further confirm the effect of emodin on the A549 cells proliferation, we analyzed cell cycle by propidium iodide staining. The data showed that stimulation with ATP caused more cell

Fig. 4. Emodin inhibited ATP-induced EMT in A549 cells. (A) The protein levels of E-cadherin, fibronectin and Snail were measured by western blot. ATP decreased the expression of E-cadherin and increased the expression of fibronectin and Snail. Emodin as well as suramin inhibited the effect of ATP. (B-D) The group data from A. The expression level of E-cadherin, fibronectin and Snail were normalized to GAPDH level in the same sample. Values are mean $\pm$ SD, n = 6, *P<0.05, **P<0.01.

populations in the S phase compared with control. While pretreatment with either emodin or suramin eventually led to an accumulation of cells in the G0/G1 phase and decreased cell populations in S phase (Fig. 2D). These data suggested that emodin inhibited ATP-induced cell proliferation through blocking P2Y-mediated Ca^{2+} increase and inducing cell cycle arrest in A549 cells.

Emodin inhibited ATP-induced migration in A549 cells.

Since ATP promotes cell invasion and metastasis through P2Y receptors in prostate cancer cells [13], we tested whether emodin acts as a P2Y antagonist could inhibit ATP-induced migration. Our results showed that emodin as well as suramin inhibited ATP-induced migration (Fig. 3A-D), indicating that emodin may inhibit ATP-induced migration by blocking P2Y receptors. As it is well documented that mitochondrial signaling pathway crucially participates in cell apoptosis [26], we examined the expression level in apoptosis-related proteins within the mitochondrial pathway to elucidate the intracellular signaling pathway. Immunoblotting data suggested that ATP (100 μM) treatment increased the expression of

Fig. 5. Emodin inactivated Ca^{2+} dependent NF- κB signaling pathways. (A) Immunofluorescence staining of NF- κB was performed. ATP significantly promoted translocation of NF- κB /P65 from cytoplasm to nuclear. Treatment of emodin inhibited ATP induced translocation of NF- κB /P65, which was consistent with the effect of suramin. Original magnification, $\times 400$. (B-C) Representative images and summarized data showing the expression levels of p-NF- κB and NF- κB . A549 cells were treated with and without ATP (100 μM) and/or suramin, emodin. The expression level of p-NF- κB was normalized to GAPDH level. ATP stimulation increased the expression of p-NF- κB /P65. However, treatment with suramin and emodin caused a significant decreased expression of p-NF- κB /P65. Values are mean $\pm$ SD, $n = 6$, ** $P < 0.01$, *** $P < 0.001$

Bcl-2 but decreased the expression of Bax and migration-related protein claudin-1. However, treatment with either suramin or emodin (5 μM) caused a significant decrease in Bcl-2 while increase in Bax and claudin-1. These data suggested that emodin reversed ATP-induced protein level changes of Bcl-2, Bax, claudin-1 (Fig. 3E-I).

Emodin inhibited ATP-induced EMT in A549 cells.

EMT is a frequent behavior which can enhance cancer cell migration and invasion [27, 28]. To determine the effect of ATP and emodin on EMT of A549 cells, we examined expression of mesenchymal markers fibronectin, Snail and epithelia marker E-cadherin. ATP (100 μM) treatment for 6 h, 12 h, 24 h increased expression of mesenchymal markers, fibronectin and Snail, but decreased the expression of E-cadherin, which is defined as cell-cell adhesion protein (Fig. 4). Emodin suppressed ATP-induced EMT after stimulation of 6 h, 12 h, and 24 h, which was consistent with suramin (Fig. 4). Thus, we suggested that emodin inhibited ATP-induced EMT and invasion of A549 cells via antagonizing P2Y receptors.

Emodin suppressed ATP-activated Ca^{2+} dependent NF- κB signaling.

In A549 cells, a Ca^{2+} -dependent activation of NF- κB may be one of the main signaling pathways [5]. Therefore, we investigated whether emodin inhibited ATP-induced activation of Ca^{2+} dependent NF- κB signaling in A549 cells. As the results showed, ATP significantly promoted translocation of NF- κB /P65 from cytoplasm to nuclear when compared with control cells. However, NF- κB /P65 was detected largely in the cytoplasm after emodin treatment, consistent with the effect of suramin (Fig 5A). Besides, ATP stimulation increased the expression of p-NF- κB /P65. However, treatment with suramin and emodin caused a significant decreased p-NF- κB /P65 expression (Fig 5B-C). These data suggested that emodin inhibits ATP-induced activation of Ca^{2+} dependent NF- κB signaling pathway in A549 cells.

Fig. 6. NF- κ B inhibitor PDTC blocked ATP-induced proliferation and migration in A549 cells. (A) Proliferation of A549 cells was measured by CCK-8, after treatment with PDTC alone, stimulation with ATP at presence and absence of PDTC. Treatment with PDTC alone had no discernible effect on A549 cell viability while PDTC significantly decreased ATP-induced cell viability. Values are means $\pm$ SD, $n = 6$, $*P < 0.05$. (B) Wound healing assay was used to assess the effect of PDTC on A549 cells migration. The wound closure was photographed at post-scratching 0 h, 6 h, 12 h, 24 h. Original magnification, $\times 50$. (C-E) The group data from B. Values are mean $\pm$ SD, $n = 6$, $*P < 0.05$, $***P < 0.001$.

Inhibition of NF- κ B suppressed ATP-induced biological effects in A549 cells

To investigate whether NF- κ B signaling is involved in ATP-induced proliferation and migration in A549 cells, Pyrrolidine dithiocarbamate (PDTC, an NF- κ B inhibitor), was applied to address the effect of NF- κ B. A549 cells were treated with PDTC (20 μ M) and then stimulated with ATP (100 μ M) for 6 h, 12 h, 24 h. Treatment with PDTC alone had no discernible effect on A549 cells proliferation or viability while it significantly decreased ATP-induced proliferation and viability (Fig. 6). This result indicated inhibition of NF- κ B suppressed ATP-induced proliferation and migration, therefore NF- κ B signaling is involved in ATP-induced reactions in A549 cells.

Since it has been demonstrated that inhibition of NF- κ B suppressed ATP-induced proliferation and migration, we next examined effect of NF- κ B on Bcl-2, Bax and claudin-1. ATP increased the expression of Bcl-2 but decreased the expression of Bax. However, treatment with PDTC caused a significant decrease in Bcl-2 while increase in Bax. Additionally, pretreatment with PDTC before stimulation with ATP significantly up-regulated protein level of claudin-1 (Fig. 7). These data suggested that inhibition of NF- κ B repressed ATP-induced protein level change of Bcl-2, Bax and claudin-1.

Fig. 7. NF- κ B inhibitor suppressed ATP-induced expression of Bcl-2, Bax, claudin-1. (A) A549 cells were treated with and without ATP (100 μ M) and/or PDTC. Expression of Bcl-2, Bax, claudin-1 were analyzed by western blot. (B-E) summarized data showing the quantitative densitometry of different samples. Expression of Bcl-2, Bax, claudin-1 were normalized to GAPDH level in the same sample. ATP increased the expression of Bcl-2 but decreased the expression of Bax. However, treatment with PDTC caused a significant decrease in Bcl-2 while increase in Bax. Additionally, pretreatment with PDTC before stimulation with ATP significantly up-regulated protein level of claudin-1. Values are shown as the mean $\pm$ SD. n = 6. *P<0.05, **P<0.01.

To identify whether NF- κ B is involved in EMT induced by ATP in A549 cells, PDTC was applied before stimulation with ATP. Then we measured the expression of E-cadherin, fibronectin, Snail by western blot (Fig. 8). PDTC suppressed ATP-induced EMT significantly at 6 h, 12 h, and 24 h. As verified above, NF- κ B signaling is involved in ATP-induced proliferation and migration, thus we demonstrated that emodin also prevented ATP-induced EMT of A549 cells via inhibition of NF- κ B signaling.

Discussion

It is well known that intratumoral ATP concentration is much higher than that of normal tissues [29]. The elevated concentration of ATP is found to act as a stimulus in the cancer progression [30]. Additionally, extracellular ATP enhances migration of cancer cells, which is a crucial determinant of cancer invasion and metastasis [31]. Although emodin has been shown to suppress lung cancer metastasis, the inhibitory effect of emodin on ATP-induced promotion of cell survival via regulation of $[Ca^{2+}]_i$ and NF- κ B signaling pathways in lung cancer cells has not been reported. In the present work, we aim to investigate the effects of emodin on cell proliferation and migration by detecting the influences of emodin on P2Y receptors-mediated $[Ca^{2+}]_i$ increase and NF- κ B signaling pathway.

Several subtypes of P2Y receptors such as P2Y1, P2Y2, P2Y4, P2Y6 and P2Y13 were found to be existed in human lungs [4]. In the present study, we examined the expression of different subtypes of P2Y receptors in A549 cells. Our results indicated that P2Y2 and P2Y6 are abundant in this cell line (Fig. 1A). In airway epithelial cells, extracellular ATP or UTP regulates intracellular response via P2Y2 and P2Y1 receptors [32]. Our data showed that both ATP and UTP, agonists of P2Y receptors, evoked a strong elevation of $[Ca^{2+}]_i$. The results demonstrate that ATP-induced $[Ca^{2+}]_i$ increase mainly through activation of P2Y receptors in A549 cells. It is reported that intracellular Ca^{2+} could promote radioresistance of NSCLC cells [33]. Elevation of $[Ca^{2+}]_i$ stimulates lung cancer cells proliferation by promoting cell

Fig. 8. NF- κ B inhibitor suppressed ATP-induced EMT. (A) The protein expression was measured by stimulating with and without ATP (100 μ M) and/or PDTC. Representative images of EMT markers proteins including E-cadherin, fibronectin and Snail. PDTC suppressed ATP-induced EMT. (B-D) The expression levels of E-cadherin, fibronectin, Snail were normalized to GAPDH level in the same sample. Values are shown as the mean $\pm$ SD. n = 6. *P<0.05, **P<0.01.

cycle progression [34]. That implicates that Ca^{2+} plays critical role in progression of lung cancer cells. Then the effect of emodin on ATP/UTP-induced $[\text{Ca}^{2+}]_i$ increase (Fig. 1B-E) was observed. Moreover, the efficient inhibition of emodin on P2Y-mediated calcium response was similar to that of the nonselective P2Y receptors inhibitor, suramin (Fig. 1B-D). These results suggest that emodin may inhibit ATP-induced $[\text{Ca}^{2+}]_i$ increase via blocking P2Y receptors.

P2Y receptors are involved in mediating cell growth of melanoma, lung cancer, breast cancer [5, 35-37]. Extracellular ATP leads to increase of intracellular ATP concentration, thus promotes cell growth and survival of A549 cells [29]. Recent study shows that emodin inhibits cell proliferation by modulating cell cycle markers expression during oral carcinogenesis [38]. Subsequently, we investigated the effect of emodin on ATP-induced proliferation in A549 cells. We found that ATP promoted A549 cells proliferation time dependently. Consistently, ATP accelerated DNA synthesis of A549 cells, thus caused an obvious increase of cell population in the S phase (Fig. 2D). Emodin and suramin inhibited ATP-induced proliferation in a concentration-dependent manner through decreasing cell population of S phase, thus

led to accumulation of cells in G0/G1 (Fig. 2C, D). Our data on this profile was in agreement with a previous study that in lung squamous cell carcinomas, emodin has been reported in cancer prevention [39]. Besides, emodin triggers apoptosis of human lung cancer cells through a ROS-dependent signaling pathway [22]. These data suggests that the inhibitory effect of emodin on cell cycle may lead to suppression of ATP-induced proliferation.

In order to investigate the molecular mechanism of emodin on ATP-induced proliferation and migration, we determined related protein level of ATP downstream. The expression of claudin-1, which has significant roles in migration and metastasis, antiapoptotic protein Bcl-2, proapoptotic protein Bax is examined. We found that stimulation with ATP dramatically up-regulated Bcl-2 expression and down-regulated the expression level of Bax and claudin-1 as shown in Fig. 3E. It has been reported that ATP promotes A549 cell survival via regulation of $[Ca^{2+}]_i$ and increase of Bcl-2/Bax ratio [9]. That implies elevation of Bcl-2/Bax ratio by high extracellular ATP may be an important latent reason for promoting cancer cells progression. Besides, we found that emodin and suramin treatment before ATP stimulation up-regulates the protein level of claudin-1 and Bax, down-regulates the protein level of Bcl-2. In prostate cancer cells, decreased expression of claudin-1 by ATP leads to invasiveness and EMT [13]. Based on data above, we conclude that emodin inhibits ATP-induced up-regulation of Bcl-2/Bax ratio and down-regulation of claudin-1 by antagonizing P2Y receptors.

Calcium signal plays critical role in the cancer progression, and is also required for the occurrence of EMT [6]. It is well accepted that EMT participates in tumour progression and metastasis formation [40]. Inhibition of EMT can repress lung cancer cells invasion [11]. Moreover, in the peripheral leading edge of NSCLC, the expression of EMT biomarkers (vimentin and N-cadherin) is markedly high [41]. In addition emodin ameliorates high glucose induced-podocyte EMT *in vitro* and *in vivo* [42]. Thus, we wonder whether EMT is induced by ATP stimulation and inhibited by emodin treatment. Our data showed that ATP caused gradually lessened expression of epithelial marker, E-cadherin and increased expression of mesenchymal markers, fibronectin, Snail significantly. However, such ATP-induced protein level change was suppressed by emodin, suggesting that emodin represses ATP-induced lung cancer cells progression via inhibition of EMT (Fig. 4).

Recent studies have demonstrated UTP-induced activation of nuclear factor (NF- κ B) in activated T cells [43]. Emodin protects against concanavalin A-induced hepatitis through inhibiting activation of the p38 MAPK-NF- κ B signaling pathway in mice [44]. In prostate cancer cells, inhibition of NF- κ B signaling pathway suppresses cell proliferation and induces cell cycle arrest in G2/M phase associated with regulation of Bcl-2 family proteins (Bcl-2, Bax) [8]. What's more, P2Y receptors-induced proliferation of A549 cells depends on activation of NF- κ B [5], which promotes transcription of downstream target genes such as Bax [7]. Therefore we detected the effect of emodin on ATP-induced activation of NF- κ B and found that ATP significantly promoted translocation of NF- κ B from cytoplasm to nuclear, while, emodin and suramin treatment inhibited this effect (Fig. 5). That is in agreement with a previous study that in human skin squamous cell carcinoma, emodin was detected to inhibit TPA-induced nuclear translocation of p65 and NF- κ B DNA-binding activity [45]. The results demonstrate that emodin suppresses ATP-induced activation of Ca^{2+} -dependent NF- κ B signaling pathways. Thus it indicates that emodin acts as a P2Y blocker and contributes to inactivation of NF- κ B.

In order to explicate whether NF- κ B participate in ATP-induced proliferation and migration, we applied an NF- κ B inhibitor, PDTC. Our data revealed that PDTC treatment inhibited ATP-induced proliferation and migration remarkably (Fig. 6). The role of claudin-1, Bcl-2, Bax in NF- κ B signaling pathway in A549 cells has not been reported, previously. Recent study demonstrated that inhibition of NF- κ B can ameliorate gastric destruction by increasing protein level of claudin-1 [46]. In this case, we examined the protein level change of Bcl-2, Bax and claudin-1 in the presence and absence of PDTC before ATP stimulation. Our results showed that inhibition of NF- κ B increased the expression of claudin-1 and Bax and decreased the expression of Bcl-2 (Fig. 7) thus inhibited ATP-induced cell proliferation and migration (Fig. 6). It is documented that suppression of NF- κ B inhibits prostate cancer growth and

EMT in prostate cancer cells [47]. Inhibition of NF- κ B leads to deregulation of EMT and neural invasion in pancreatic cancer [48]. In our study, we found the expression of epithelial marker, E-cadherin was increased and the mesenchymal markers, fibronectin and Snail were decreased after inhibition of NF- κ B. Therefore, inactivation of NF- κ B also led to inhibition of EMT provoked by ATP (Fig. 8).

Thus, we conclude that emodin may act as a latent therapeutic drug for the treatment of lung cancer, whose advantage lies in the following aspects: the safety of emodin has been long demonstrated. In addition, it is economical and practical with regard to patients, because it can be obtained from plants with high content of emodin.

Nevertheless, we have to point out the limitation in our investigation is that we did not examine the effects of emodin on lung cancer *in vivo*, whether it plays protective role in treating lung cancer remains unknown. Therefore it is necessary to further explore its biological function and action mechanism in anti-cancer *in vivo*.

In summary, our results indicates that high concentration of extracellular ATP promotes proliferation, migration, EMT of A549 cells by activating P2Y receptors, inducing $[Ca^{2+}]_i$ increase and activating NF- κ B signaling. Additionally, our data confirms that emodin inhibits activation of P2Y-receptors/NF- κ B pathway as well as ATP-induced carcinogenic effect (Fig. 9). Taken together, emodin may be a potent P2Y antagonist and suppresses ATP-induced proliferation and migration by inhibiting P2Y-mediated $[Ca^{2+}]_i$ increase and NF- κ B activation.

Fig. 9. A model for summarizing emodin inhibits ATP-induced proliferation and migration pathway in A549 cells. Extracellular ATP promotes proliferation, migration, EMT of A549 cells by activating P2Y receptors, the activation of which stimulates $[Ca^{2+}]_i$ increase and activates NF- κ B signaling. Emodin inhibits activation of P2Y-receptors/ Ca^{2+} /NF- κ B pathway as well as ATP-induced carcinogenic effect.

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Disclosure Statement

The authors declare no Disclosure Statement.

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