(-)- β -hydrastine suppresses the proliferation and invasion of human lung adenocarcinoma cells by inhibiting PAK4 kinase activity

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Abstract. (-)- β -hydrastine is one of the main active components of the medicinal plant, *Hydrastis canadensis*, which is used in many dietary supplements intended to enhance the immune system. However, whether (-)- β -hydrastine affects the tumor signaling pathway remains unexplored. In the present study, we found that (-)- β -hydrastine inhibited the kinase activity of p21-activated kinase 4 (PAK4), which is involved in the regulation of cytoskeletal reorganization, cell proliferation, gene transcription, oncogenic transformation and cell invasion. In the present study, (-)- β -hydrastine suppressed lung adenocarcinoma cell proliferation by inhibiting expression of cyclin D1/D3 and CDK2/4/6, leading to cell cycle arrest at the G₁ phase, in a PAK4 kinase-dependent manner. Moreover, inhibition of PAK4 kinase activity by (-)- β -hydrastine also promoted the early apoptosis of lung adenocarcinoma cells through the mitochondrial apoptosis pathway. In addition,

(-)- β -hydrastine significantly suppressed the migration and invasion of human lung adenocarcinoma cells in conjunction with concomitant blockage of the PAK4/LIMK1/cofilin, PAK4/SCG10 and PAK4/MMP2 pathways. All of these data indicate that (-)- β -hydrastine, as a novel PAK4 inhibitor, suppresses the proliferation and invasion of lung adenocarcinoma cells. Taken together, these results provide novel insight into the development of a PAK4 kinase inhibitor and a potential therapeutic strategy for lung cancer.

Introduction

Hydrastine derivatives are composed of a phthalide and an isoquinoline alkaloid and exist in two configurations: (1R,9S)- β -hydrastine [(-)- β -hydrastine] and (1S,9R)- β -hydrastine [(+)- β -hydrastine] (1). (-)- β -hydrastine is one of the main active components of the medicinal plant, *Hydrastis canadensis*, which is used in many dietary supplements intended to enhance the immune system (2). *Hydrastis canadensis* has been reported to contain major bioactive compounds such as isoquinoline alkaloids berberine, hydrastine, palmatine and canadine (3). Cancer chemoprevention involves the use of natural or synthetic chemicals to prevent the tumorigenesis of cancer (4,5). (-)- β -hydrastine has been used for the treatment of a wide variety of ailments including gastrointestinal disturbances, urinary tract disorders and inflammation. It was reported that (-)- β -hydrastine has a mild cytotoxic effect and at higher concentration ranges aggravates L-DOPA-induced cytotoxicity in PC12 cells (1). The chemotherapeutic potential of *Hydrastis canadensis* extract on HeLa cells was also noted *in vitro*, indicating its drug-DNA interaction and apoptosis induction ability (6). Moreover, *Hydrastis canadensis* extract inhibited the proliferation of A375 cells through G2/M arrest (7). Noscaphine, which has structural similarities with hydrastine, has been reported to exhibit antitumor activity (8,9). All of these findings indicate that (-)- β -hydrastine may have antitumor activity via certain signaling pathways.

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The p21-activated kinases (PAKs) are a family of serine/threonine protein kinases which act as effectors for Rac and Cdc42 (10). PAKs play important roles in cytoskeletal reorganization, cell survival, hormone signaling, gene transcription and tumorigenesis (10,11). There are six mammalian members of PAK which can be classified into group I PAKs (PAK1-3) and group II PAKs (PAK4-6) (12). PAK4 is the most extensively and profoundly studied member among the group II PAKs. Overexpression of PAK4 has been found in a variety of cancer cell lines, including lung, prostate, gall bladder and stomach (13-15), and also in several primary tumors (16). Subsequent studies have demonstrated that PAK4 promotes cell proliferation and survival (13,17), inhibits cell adhesion and promotes anchorage-independent growth (18), and it also enhances cell migration (15), invasion (19) and metastasis (20). Many of these functions rely on PAK4 kinase activity. Therefore, PAKs which belong to the protein kinases are important therapeutic targets of tumors and are considered highly able to be used as agents owing to their conserved ATP-binding pocket (21). At present, many small-molecule inhibitors target PAKs (22,23).

Increasing data implicate PAK4 in tumor proliferation and metastasis (24). In the present study, we report the identification and characterization of (-)- β -hydrastine as a novel inhibitor targeting PAK4 in human lung adenocarcinoma cells with the expected cellular functions of a PAK4 inhibitor. Collectively, these studies expand the scope of exploitation and application of PAK4 inhibitors, and provide a new therapeutic strategy for the targeting of lung adenocarcinoma cells by inhibiting PAK4 kinase activity and its signaling pathways.

Materials and methods

Cell lines and culture condition. Human lung adenocarcinoma cell lines A549 and LTEP-A-2, large cell lung cancer cell line NCI-H460, and small cell lung cancer cell lines NCI-H446, NCI-H292 and HEK-293 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (both from Invitrogen) at 37°C in an incubator with a humidified atmosphere of 5% CO₂ and 95% air.

Reagents. Hydrastine, with chemical name [S-(R*,S*)]-6,7-dimethoxy-3-(5,6,7,8-tetrahydro-6-methyl-1,3-dioxolo(4,5-g)isoquinolin-5-yl)-1(3H)-isobenzofuranone was purchased from ChromaDex, Inc (Irvine, CA).

MTT assays. Human cancer cells (1x10⁴/well) were plated in 0.1 ml of medium containing 10% FCS in 96-well Corning plates; 24 h later, the medium was removed and replaced with 0.1 ml medium containing the indicated concentrations of (-)- β -hydrastine for 12, 24, 36 or 48 h respectively. Next, the capability for cellular proliferation was assessed by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this, 0.01 ml of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well. After a 4-h incubation at 37°C, the medium was replaced with 0.1 ml dimethylsulfoxide (DMSO). After a 15-min incubation at 37°C, the optical density at 490 nm was measured using a microplate reader (Bio-Rad).

Cell cycle analysis. A549 cells were incubated with the indicated concentrations of (-)- β -hydrastine for 24 h. The cells were then collected, rinsed with PBS, and suspended in staining buffer (10 μ g/ml propidium iodide, 0.5% Tween-20, 0.1% RNase in PBS). The cells were analyzed using a FACS Vantage flow cytometer with Cell Quest acquisition and analysis software program (Becton-Dickinson and Co., San Jose, CA, USA). Gating was set to exclude cell debris, doublets and clumps.

Cell migration and invasion assays. Migration and invasion assays were performed using modified Boyden chambers with a polycarbonate nucleopore membrane. Pre-coated filters (6.5 mm in diameter, 8- μ m pore size and Matrigel 100 μ g/cm²) for the invasion assay were rehydrated with 100 μ l medium. Then, 1x10⁵ cells in 100 μ l serum-free DMEM supplemented with 0.1% bovine serum were placed in the upper part of each chamber, whereas the lower compartments were filled with 600 μ l DMEM containing 10% serum. After incubating for 12 h at 37°C, the non-invaded or non-migrated cells were removed from the upper surface of the filter with a cotton swab, and the invaded/migrated cells on the lower surface of the filter were fixed, stained, photographed and counted under high-power magnification.

Cell apoptosis. Analysis of cell apoptosis was carried out using the V-FITC apoptosis detection kit according to the manufacturer's instructions as follows. A549 cells were incubated with the indicated concentrations of (-)- β -hydrastine for 24 h. After incubation, the cells were rinsed twice with cold PBS, and then 5 μ l of Annexin V-FITC and 10 μ l of PI were added. The cells were incubated in the dark at room temperature for 15 min, and 400 μ l binding buffer was added to each tube. Finally, the apoptosis rate was measured by flow cytometry within 1 h.

Hoechst 33258 staining. A549 cells were incubated with the indicated concentrations of (-)- β -hydrastine for 24 h. After incubation, the cells were fixed with 4% polyoxymethylene, and washed twice with PBS, incubated with 10 μ g/ml Hoechst 33258 for 5 min at room temperature, and then washed with PBS for 3 times. The cells were observed using a fluorescence microscope.

Mitochondrial membrane potential. The cells (1x10⁵) were cultured in 6-well plates for the assay. The cells were then collected, centrifuged and resuspended in 0.5 ml DMEM. The cells were washed twice in staining buffer and then incubated in 0.5 ml JC-1 staining buffer at room temperature in the dark. Flow cytometry was used to determine the fluorescence intensity of the red/green ratio semi-quantitatively.

Transfection of shRNA. To stably silence PAK4, the cells were transfected with pRS-shPAK4 (Shanghai GeneChem Co.), and then selected with puromycin (1.5 μ g/ml). The pRS vector was used as the control.

Western blot analysis. To determine the expression of protein, whole cell extracts were prepared from 1x10⁶ cells in lysis buffer (20 mmol/l Tris pH 7.4, 250 mmol/l sodium chloride, 0.1% Triton X-100, 2 mmol/l EDTA, 10 μ g/ml leupeptin,

10 $\mu\text{g/ml}$ aprotinin, 0.5 mmol/l phenylmethylsulfonyl fluoride, 4 mmol/l sodium orthovanadate and 1 mmol/l DTT), and 60 μg of total protein was resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electro-transferred to a PVDF membrane (Amersham). The membrane was blocked with 5% (or 2.5% for phospho-antibodies) non-fat dry milk in TBS-T (20 mmol/l Tris, pH 7.6, 137 mmol/l NaCl, 0.1% Tween-20) for 1 h at room temperature, and then probed with specific antibodies against cyclin D1, cyclin D3, CDK2, CDK4, CDK6, LIMK1, phospho-LIMK1 (Thr508), cofilin, phospho-cofilin (Ser3), PAK4, phospho-PAK4 (Ser474) (Cell Signaling Technology), MMP2 (Bioworld), SCG10, phospho-SCG10 Ser50, caspase 3, caspase 8, PARP, Bax and Bcl-2 (Neomarkers, Fremont, CA, USA). To assure equal loading, the membranes were probed with antibodies against GAPDH (Kangchen Biotech Inc., Shanghai, China). All PVDF membranes were detected by chemiluminescence (ECL; Pierce Technology).

Kinase Glo luminescent assay. The indicated concentrations of (-)- β -hydrastine and 1 μl of PAK4 kinase were mixed and filled with kinase buffer (50 mmol/l HEPES, pH 7.5, 10 mmol/l MgCl_2 , 2 mmol/l MnCl_2 and 0.2 mmol/l DTT) to 20 μl . The mixture was incubated at 37°C for 3 h and then mixed with 20 μl kinase Glo Luminescen (Promega, Madison, WI, USA) reaction solution, and 384 fluorescence values were measured after standing for 10 min.

Kinase assay. PAK4 kinase assays were performed using the exogenous MBP as a substrate. PAK4 kinase (Invitrogen) was pre-incubated with the indicated concentrations of (-)- β -hydrastine. Kinase activity was measured in 30 μl of kinase buffer (50 mmol/l HEPES, pH 7.5, 10 mmol/l MgCl_2 , 2 mmol/l MnCl_2 and 0.2 mmol/l DTT) containing 10 μCi of [γ - ^{32}P]-ATP (5,000 Ci/mmol) for 20 min at 30°C. Reactions were stopped by adding 6X SDS sample buffer and resolved on a 10% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, and ^{32}P -labeled proteins were visualized by autoradiography with Molecular Imager RX (Bio-Rad). To assure equal loading, MBP was detected by Ponceau staining.

Statistical analysis. All statistical analyses were carried out using SPSS 16.0 software, and the results were considered to be statistically significant at a P-value <0.05.

Results

NCI-H460, NCI-H446, NCI-H292, HEK-293, A549 and LTEP-A-2 cells were used to detect the inhibitory effects of (-)- β -hydrastine on cell growth. As determined by the MTT assay, (-)- β -hydrastine treatment inhibited the proliferation of A549 and LTEP-A-2 cells in a concentration-dependent manner, but had little effect on the NCI-H460, NCI-H446, NCI-H292 and HEK-293 cells (Fig. 1A). To further investigate the mechanisms by which (-)- β -hydrastine inhibited the growth of human lung cancer cells, A549 cells were exposed to various concentrations of (-)- β -hydrastine for 24 h, and then cell cycle analysis was performed. (-)- β -hydrastine prominently induced a dose-dependent increase in the percentage of cells in the G_1 phase and decreased the percentage of cells in the S phase compared with the control (Fig. 1B), indicating that

(-)- β -hydrastine arrested the A549 cells at the G_1 phase of the cell cycle. Since cyclin D1/3 and CDK2/4/6 are key regulators in the G_1 phase of the cell cycle, we examined the expression level of the indicated regulators in (-)- β -hydrastine-treated lung cancer cells. Western blot analysis showed that exposure of the A549 cells to 10 $\mu\text{mol/l}$ (-)- β -hydrastine for 24 h markedly decreased the protein expression of cyclin D1 and CDK2/6 and gently decreased the protein expression of cyclin D3 and CDK4 (Fig. 1C), indicating that (-)- β -hydrastine arrested the cells at the G_1 phase and then suppressed lung adenocarcinoma cell growth via cyclin D1 and CDK2/6.

PAK4 is a type of serine/threonine kinase and promotes cell survival, inhibits cell adhesion and promotes anchorage-independent growth (13,18). Therefore, the levels of PAK4 and phospho-PAK4 (Ser474) (active form of PAK4) were assessed in the lung cancer cell lines. Notably, the level of activated PAK4 was higher in the lung adenocarcinoma A549 and LTEP-A-2 cells when compared with that in the other lung cancer and HEK-293 cells (Fig. 2A), indicating that PAK4 may be targeted by (-)- β -hydrastine and may be involved in (-)- β -hydrastine-induced growth inhibition of lung adenocarcinoma cancer cells. Then, the inhibitory effect of (-)- β -hydrastine on PAK4 kinase was examined by *in vitro* kinase assay. (-)- β -hydrastine markedly inhibited PAK4 kinase activity in a dose-dependent manner (Fig. 2B). The inhibitory effect of (-)- β -hydrastine on other PAK family members such as PAK1, PAK5 and PAK6 was detected. The results showed that (-)- β -hydrastine weakly inhibited PAK1 and PAK5 kinase activity, but did not inhibit PAK6 kinase activity (Fig. 2C). (-)- β -hydrastine inhibited PAK4 activity with an IC_{50} value of 28.05 $\mu\text{mol/l}$ (Fig. 2D). Furthermore, we examined whether (-)- β -hydrastine inhibited the PAK4 kinase activity in lung adenocarcinoma cells. (-)- β -hydrastine at 10 $\mu\text{mol/l}$ markedly inhibited the level of phospho-PAK4 (Ser474) (Fig. 2E). These results indicate that (-)- β -hydrastine inhibits PAK4 kinase activity selectively in lung adenocarcinoma cells.

To confirm the involvement of PAK4 in (-)- β -hydrastine-induced A549 cell growth arrest, wild-type PAK4 (PAK4WT) and kinase dead type PAK4 (PAK4KM) plasmids were transfected into A549 cells. After treatment with 10 $\mu\text{mol/l}$ (-)- β -hydrastine for 24 h, cell proliferation was analyzed by MTT assay. Compared to the vector control, PAK4WT, but not PAK4KM, promoted A549 cell proliferation which was markedly inhibited by 10 μM (-)- β -hydrastine (Fig. 3A). Identical results were obtained using LTEP-A-2 and NCI-H460 cells (Fig. 3B and C). Furthermore, (-)- β -hydrastine showed almost no inhibitory effect of cell growth in PAK4-silenced A549 cells (Fig. 3D). It was reported that cyclin D1 expression is involved in PAK4-regulated cell proliferation (25). PAK4 was also found to be involved in the PI3K/AKT pathway in gastric cancer cells (26), AKT regulates GSK3 β and affects CDK2 expression (27,28), indicating that PAK4 may regulate CDK2 expression via the AKT/GSK3 β pathway. Thus, cell cycle regulators involved in G_1 -S phase such as cyclin D1/D3 and CDK2/4/6 were detected by western blot analysis. PAK4WT overexpression in the A549 cells promoted the expression of phospho-PAK4 (Ser474), cyclin D1/3, CDK2/6, while (-)- β -hydrastine treatment markedly decreased the PAK4-mediated effect (Fig. 3E). PAK4 knockdown also suppressed the expression of cyclin D1/3 and CDK2/4/6,

Figure 1. (-)- β -hydrastine suppresses the proliferation of lung adenocarcinoma cells. (A) HEK-293, NCI-H446, NCI-H292, NCI-H460, A549 and LTP-A-2 cells were cultured with the indicated concentrations of (-)- β -hydrastine for the indicated hours in 96-well plates, and the MTT assay was performed. The results represent the mean $\pm$ SD of three experiments carried out in triplicate. (B) A549 cells were pre-incubated with (-)- β -hydrastine for 24 h, and then the cells were analyzed using a FACS Vantage flow cytometer with the Cell Quest acquisition and analysis software program. The experiment was repeated 3 times. (C) A549 cells were treated with DMSO alone or the indicated concentrations of (-)- β -hydrastine for 24 h, and proteins were extracted and subjected to western blot analysis. The membrane was sequentially probed with the indicated antibodies.

while (-)- β -hydrastine treatment showed a weaker inhibitory effect (Fig. 3F). To confirm whether (-)- β -hydrastine inhibits CDK2 expression via the PAK4/AKT/GSK3 β pathway, phospho-AKT (Ser308) and GSK3 β expression was examined in the A549 cells following (-)- β -hydrastine treatment. As expected, the expression levels of phospho-AKT (Ser308) and GSK3 β were decreased in the (-)- β -hydrastine-treated A549 cells (Fig. 3G). Further silencing of PAK4 suppressed the expression of phospho-PAK4 (Ser474), phospho-AKT (Ser308) and GSK3 β while (-)- β -hydrastine treatment showed a weaker inhibitory effect (Fig. 3H). All these data demonstrated that (-)- β -hydrastine suppressed lung adenocarcinoma cell growth by inhibiting expression of cyclin D1/D3 and CDK2/4/6, leading to cell cycle arrest at the G₁ phase in a PAK4 kinase-dependent manner.

Apoptosis also affects cell growth, therefore, we aimed to ascertain whether (-)- β -hydrastine induces the apoptosis of A549 cells. As determined by Annexin V-FITC staining,

apoptosis was increased in the A549 cells following treatment with (-)- β -hydrastine compared with that of the control cells (Fig. 4A). Hoechst 33258 staining was performed to observe the (-)- β -hydrastine-induced apoptotic nuclei of the A549 cells. Condensed chromatin was observed in the (-)- β -hydrastine-treated A549 cells (Fig. 4B). Furthermore, the expression levels of apoptosis regulators were examined. The expression of Bcl-2 was obviously decreased and the levels of caspase 3 and 8, PARP, and Bax were increased in the (-)- β -hydrastine-treated A549 cells (Fig. 4C). As known, there are two pathways noted in apoptotic cells: the receptor-mediated pathway and the non-receptor-mediated pathway (29). An obvious decrease in Bcl-2 in the (-)- β -hydrastine-treated A549 cells indicated that the non-receptor apoptotic pathway may be the key pathway in the (-)- β -hydrastine-induced apoptotic adenocarcinoma cells. It has been reported that PAK4 leads to an increase in the phosphorylation of the pro-apoptotic protein Bad and

Figure 2. (-)-β-hydrastine inhibits PAK4 kinase activity. (A) Total proteins of the lung cancer cell lines or the HEK-293 cell line were extracted to detect PAK4 and activated PAK4 (phospho-Ser474) levels. (B) PAK4 kinase was pre-incubated with the indicated concentrations of (-)-β-hydrastine for 1 h, and then an *in vitro* kinase assay was performed. (C) PAK1/4/5/6 kinases were pre-incubated with the indicated concentrations of (-)-β-hydrastine for 1 h respectively, then kinase Glo assay was performed. (D) PAK4 kinases were pre-incubated with the indicated concentrations of (-)-β-hydrastine for 1 h respectively, and the kinase Glo assay was performed and the inhibition rate was calculated. Linear fit curve was drawn with an equation of $y = 4.32439x - 71.31834$. (E) A549 cells were treated with DMSO alone or the indicated concentrations of (-)-β-hydrastine for 24 h, and proteins were extracted and subjected to western blot analysis. The membrane was probed sequentially with the PAK4 antibody and the phospho-PAK4Ser474 antibody.

inhibition of caspase activation (30). To confirm whether the PAK4 pathway was involved in the (-)-β-hydrastine-induced A549 cell apoptosis, PAK4 was overexpressed or silenced in the A549 cells. Subsequently, flow cytometric assay was performed after (-)-β-hydrastine treatment. PAK4WT, but not PAK4KM, decreased the percentage of apoptotic A549 cells, whereas (-)-β-hydrastine treatment increased the percentage of apoptotic cells transfected with PAK4WT or PAK4KM (Fig. 4D). PAK4 knockdown led to increased apoptosis, while (-)-β-hydrastine treatment showed no further effect (Fig. 4E), indicating that (-)-β-hydrastine promotes the apoptosis of lung cancer cells mainly via its inhibitory effect on PAK4 kinase activity. Furthermore, the expression of apoptotic proteins in the PAK4-overexpressing or PAK4-knockdown A549 cells was examined after treatment with (-)-β-hydrastine. The results showed that PAK4 overexpression increased Bcl-2 and decreased caspase 3 levels, while (-)-β-hydrastine abrogated these levels (Fig. 4F). While PAK4 knockdown decreased the Bcl-2 level and increased the caspase 3 level when compared to the control cells,

(-)-β-hydrastine treatment had no further effect. Notably, the caspase 8 level was not altered to a significant degree in both the PAK4-overexpressing and PAK4-silenced cells, but increased along with (-)-β-hydrastine treatment, suggesting that (-)-β-hydrastine may induce the receptor-mediated apoptosis pathway independent of PAK4. The loss of mitochondrial membrane potential ($\Delta\Psi_m$) has been regarded as one of the early events in the apoptotic pathway, and can trigger the release of cytochrome *c* and other apoptotic molecules after induction by various stimuli. To detect the change in mitochondrial membrane potential, flow cytometric assay following JC-1 staining was performed. The results showed that the number of cells with lost $\Delta\Psi_m$ increased after treatment with (-)-β-hydrastine (Fig. 5A). PAK4WT, but not PAK4KM, decreased the percentage of cells with lost $\Delta\Psi_m$ and then (-)-β-hydrastine treatment increased the percentage of cells with lost $\Delta\Psi_m$ more than that of the vector or PAK4KM-overexpressing cells (Fig. 5B). PAK4 knockdown markedly increased the percentage of cells with lost $\Delta\Psi_m$ and (-)-β-hydrastine treatment showed no further effect (Fig. 5C).

Figure 3. PAK4 is involved in the inhibitory effect of (-)- β -hydrastine on lung adenocarcinoma cell growth. (A) A549, (B) LTPC-A-2 and (C) NCI-H460 cells were transfected with pcDNA3.1-3xFlag, pcDNA3.1-3xFlag-PAK4WT or pcDNA3.1-3xFlag-PAK4KM and then incubated with 10 μ M of (-)- β -hydrastine. The MTT assay was performed after the indicated hours, and the results represent the mean $\pm$ SD of three experiments carried out in triplicate (* P <0.01 compared to the indicated hours of the 3xFlag/DMSO group). (D) A549 cells were transfected with shPAK4 and then treated with 10 μ M of (-)- β -hydrastine. The MTT assay was performed after the indicated hours (* P <0.01 compared to the Con/DMSO group, n =3). (E) A549 cells were treated with 10 μ M of (-)- β -hydrastine for 24 h following transfection with pcDNA3.1-3xFlag or pcDNA3.1-3xFlag-PAK4WT, and then proteins were extracted and subjected to western blot analysis. The membrane was probed sequentially with the indicated antibodies. (F) Proteins of the cells indicated in D were extracted to examine the expression of the indicated proteins by western blot analysis. (G) A549 cells were treated 10 μ M of (-)- β -hydrastine for 24 h, and proteins were extracted and examined with the indicated antibodies by western blot analysis. (H) A549 cells were transfected with shPAK4 and then treated with 10 μ M of (-)- β -hydrastine for 24 h, and proteins were extracted and examined with the indicated antibodies by western blot analysis.

All these results indicate that through inhibition of PAK4 kinase activity, (-)- β -hydrastine decreased the Bcl-2 level and

mitochondrial membrane potential, and subsequently induced the apoptosis of lung adenocarcinoma cells.

Figure 4. (-)- β -hydrastine induces the apoptosis of A549 cells via inhibition of PAK4 kinase activity. (A) A549 cells were pre-incubated with (-)- β -hydrastine for 24 h, and then the cells were treated using the Annexin V-FITC apoptosis detection kit and analyzed with FACS. The experiment was repeated three independent times. (B) A549 cells were pre-incubated with (-)- β -hydrastine for 24 h, and then the cells were stained with Hoechst 33258, and observed with a fluorescence microscope. (C) A549 cells were treated with (-)- β -hydrastine for 24 h and then examined for expression of indicated proteins by western blot analysis. A549 cells were transfected with (D) pcDNA3.1-3xFlag, pcDNA3.1-3xFlag-PAK4WT, pcDNA3.1-3xFlag-PAK4WT or (E) NC, siPAK4, and then the cells were treated using the Annexin V-FITC apoptosis detection kit after pre-incubation with 20 μ M (-)- β -hydrastine for 24 h. Apoptotic cells were detected by FACS assay (* P <0.05; ** P <0.01; n =3). A549 cells were transfected with (F) pcDNA3.1-3xFlag, pcDNA3.1-3xFlag-PAK4WT or (G) NC, siPAK4, and then total protein was extracted after treatment with 10 μ M (-)- β -hydrastine for 24 h and western blot assay was conducted using the indicated antibodies.

PAK4 regulates not only cancer cell proliferation, but also cancer cell migration. The inhibitory effects of (-)- β -hydrastine on cell migration and invasion of several lung

cancer cell lines were analyzed by Transwell assay (with or without Matrigel). The results showed that (-)- β -hydrastine significantly decreased the invasive and migratory potential

Figure 5. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was noted in the apoptotic lung cancer cells and was induced by (-)- β -hydrastine via PAK4. (A) A549 cells were pre-incubated with (-)- β -hydrastine for 24 h, and then the cells were stained with JC-1 and analyzed by flow cytometry. Cell percentages in Q4 indicating loss of $\Delta\Psi_m$ in three experiments were analyzed. (B) NCI-H460 cells were transfected with pcDNA3.1-3xFlag, pcDNA3.1-3xFlag-PAK4WT and pcDNA3.1-3xFlag-PAK4KM, and then stained with JC-1 after pre-incubation with 10 μ M (-)- β -hydrastine for 12 h. Cells were detected by flow cytometry, and data from three independent experiments were analyzed. (C) A549 cells alone or cells transfected with NC or shPAK4 were pre-incubated with (-)- β -hydrastine for 12 h, and then the cells were stained with JC-1 and analyzed using flow cytometry as above (* P <0.05; ** P <0.01 compared to the first column; n =3).

of the lung adenocarcinoma A549 (Fig. 6A and B) and LTP-A-2 (data not shown) cells in a dose-dependent manner, while (-)- β -hydrastine weakly decreased the invasive and migratory potential of the lung non-adenocarcinoma NCI-H292 and HCl-H460 (data not show) cells. LIMK/cofilin (2) and SCG10 (20) are substrates of PAK4 kinase and are associated with cell migration and invasion. Western blot analysis showed that exposure of A549 cells to (-)- β -hydrastine (10 μ M/l) for 24 h markedly decreased the levels of cofilin^{p-Ser3}, LIMK1^{p-Thr508} and SCG10^{p-Ser50} (data not shown). To further clarify the participation of PAK4 in (-)- β -hydrastine-induced cell migratory and invasive suppression, we examined the invasive or migratory capacity of PAK4-overexpressing or -silenced A549 cells following treatment with (-)- β -hydrastine. As a result, PAK4WT overexpression

enhanced the inhibitory effect of (-)- β -hydrastine on both the migration and invasion of A549 cells (Fig. 6C and D). Opposite results were obtained following the silencing of PAK4 in the A549 cells (Fig. 6E and F). At a concentration of 10 μ M/l, PAK4WT overexpression increased the inhibitory effect of (-)- β -hydrastine on expression of cofilin^{p-Ser3}, LIMK1^{p-Thr508}, SCG10^{p-Ser50} and MMP2, while silencing of PAK4 did not (Fig. 6G and 6H). These results clearly suggest that treatment of (-)- β -hydrastine, by targeting PAK4, exhibits anti-invasive effects in lung adenocarcinoma cells.

Discussion

It has been reported that *Hydrastis canadensis* extract exhibits chemopreventive effects on HeLa and A375 cells (2).

Figure 6. (-)- β -hydrastine inhibits the migratory and invasive potential of lung cancer cells via inhibition of PAK4 kinase activity and its downstream pathways. (A) A549 cells were pre-incubated with (-)- β -hydrastine for 24 h, and the Transwell assay (with or without Matrigel) was performed. (B) Cells were counted, and the results represent the mean $\pm$ SD of three experiments (* P <0.05; ** P <0.01 compared to the first column; n =3). (C and D) A549 cells were transfected with pcDNA3.1-3xFlag, pcDNA3.1-3xFlag-PAK4WT or pcDNA3.1-3xFlag-PAK4KM, and the Transwell assay [without (C) or with (D) Matrigel] was performed after treatment with 10 μM of (-)- β -hydrastine. The experiment was repeated 3 independent times (* P <0.05; ** P <0.01 compared to the first column; n =3). (E and F) A549 cells stably transfected with shPAK4 were used for the Transwell assay [without (E) or with (F) Matrigel]. The experiment was repeated three independent times (* P <0.05 compared to the first column; n =3). (G and H) The expression levels of LIMK1/LIMK1^{p-Thr508}, cofilin/cofilin^{p-Ser3}, SCG10/SCG10^{p-Ser50} and MMP2 in the PAK4-overexpressing cells (G) or the PAK4-silenced cells (H) were detected by western blotting, respectively.

In the present study, HEK-293, NCI-H460, NCI-H446, NCI-H292, A549 and LTEP-A-2 cells were used to detect

the anticancer effect of (-)- β -hydrastine, a main component of *Hydrastis canadensis*. As shown in the MTT assay,

(-)- β -hydrastine treatment inhibited the proliferation of A549 and LTP-A-2 cells in a concentration-dependent manner but had little effect on other lung cancer cells or gastric cancer cells. These data indicate that (-)- β -hydrastine may have selective activity on lung adenocarcinoma cells. In addition the mechanism of how (-)- β -hydrastine inhibits lung adenocarcinoma cell growth was investigated. The results showed that (-)- β -hydrastine arrested the A549 cells at the G₁ phase by decreasing the protein levels of cyclin D1/D3 and CDK2/4/6, which act as key regulators of the G₁-S checkpoint.

PAK4 is considered as a key regulator in cancer cell signaling. It was reported that LCH7749944 inhibits PAK4 kinase activity and gastric cancer cell proliferation (31). In the present study, the level of activated PAK4 in lung cancer cells was detected. The level of activated PAK4 (phospho-PAK4 Ser474) in the lung adenocarcinoma cells was higher than that in the non-adenocarcinoma cells, indicating that PAK4 may be involved in (-)- β -hydrastine-induced inhibition of lung adenocarcinoma cell proliferation. To confirm these findings, the inhibitory effect of (-)- β -hydrastine on PAK4 was examined. The activated PAK4 level was markedly decreased in the 10 μ mol/l (-)- β -hydrastine-treated A549 cells. The kinase activity of PAK4 was markedly decreased in a dose-dependent manner by (-)- β -hydrastine, and an IC₅₀ value of 28.05 μ mol/l by kinase Glo assay was clarified. All these results indicate that (-)- β -hydrastine is a novel, potent kinase inhibitor of PAK4. To further investigate whether (-)- β -hydrastine has a kinase inhibitory effect on other PAK family members, kinase assay was performed. The results showed weak or no inhibitory effect of (-)- β -hydrastine on PAK5 and PAK6, and also a weak inhibitory effect on PAK1, the most extensively studied member of group I PAKs. All of these data indicate that (-)- β -hydrastine may inhibit PAK4 kinase activity selectively and suppress lung adenocarcinoma cell proliferation via PAK4.

Then, we ascertained whether the (-)- β -hydrastine-induced growth inhibition of lung adenocarcinoma cells was through inhibition of PAK4 kinase activity. PAK4WT/KM overexpression or PAK4 knockdown in lung cancer cells before (-)- β -hydrastine treatment demonstrated that (-)- β -hydrastine suppressed lung adenocarcinoma cell proliferation by inhibiting the expression of cyclin D1/D3 and CDK2/4/6, leading to cell cycle arrest at the G₁ phase in a PAK4-dependent manner. It has been reported that PAK4 is involved in the apoptosis signaling pathway by phosphorylation of Bad in prostate cancer cells (30). In addition, PAK4 affects Bcl-2 by targeting CREB in prostate cancer cells (32). In the present study, our data confirmed that PAK4 decreased the apoptosis of lung cancer cells by maintaining the Bcl-2 level and mitochondrial potential in lung adenocarcinoma cells. We found that (-)- β -hydrastine promoted lung adenocarcinoma cell apoptosis by decreasing the Bcl-2 level and by increasing caspase 3 and 8 levels. In the PAK4-overexpressing lung cancer cells, (-)- β -hydrastine decreased the Bcl-2 level and increased the caspase 3 level to a greater degree when compared with that in the control cells. In contrast, the change in caspase 8 induced by (-)- β -hydrastine was almost not affected by overexpression or silencing of PAK4. All of these findings indicate that (-)- β -hydrastine induces lung cancer cell apoptosis through the mitochondrial apoptosis pathway in a PAK4 kinase-dependent manner and

through the receptor apoptosis pathway independent of PAK4 kinase activity.

In addition to the effect on cell proliferation, we also demonstrated the inhibitory effects of (-)- β -hydrastine on the migration and invasion of lung adenocarcinoma cells. It has been reported that PAK4 promoted prostate cancer cell migration through the HGF/PAK4/LIMK1/cofilin pathway (19). It was also reported that PAK4 increased the migration of gastric cancer cells via phosphorylation of SCG10 (20). Moreover, PAK4 promoted ovarian cancer cell migration and invasion through the MEK1/ERK1/2/MMP2 pathway (25). Our results showed that (-)- β -hydrastine significantly suppressed the migratory and invasive ability of lung cancer cells in parallel with downregulation of LIMK1^{p-Thr508}/cofilin^{p-Ser3}, SCG10^{p-Ser50} and MMP2. Transfection of PAK4WT enhanced the inhibitory effect of (-)- β -hydrastine on A549 cell migration and invasion, in parallel with cofilin^{p-Ser3}, LIMK1^{p-Thr508}, SCG10^{p-Ser50} and MMP2 protein levels, while transfection of PAK4KM did not, compared to the control cells.

At present, several compounds which inhibit PAK4 activity, such as PF-3758309 (33), staurosporine (34), LCH-7749944 (31) and various CDK inhibitors have been identified. However, it is still difficult to identify specific PAK4 inhibitors among the PAK family members. However, (-)- β -hydrastine which we identified as a novel inhibitor of PAK4 with a kinase inhibitory IC₅₀ value of 28.05 μ mol/l showed a weaker inhibitory effect on PAK1/5/6. Thus, it is expected that (-)- β -hydrastine may offer a novel therapeutic strategy for advanced metastatic lung cancer and may be a better selective PAK4 inhibitor after its structural modification.

In summary, (-)- β -hydrastine was identified to be capable of inhibiting PAK4 kinase activity and its downstream signaling pathways and suppressing PAK4-mediated lung cancer cell behaviors including growth, migration and invasion. Although these results warrant further research using experimental models *in vivo*, considering the selective inhibitory effect of (-)- β -hydrastine among the PAK family, the present findings do support the concept that (-)- β -hydrastine may be a more effective PAK4 kinase inhibitor after various modifications to decrease its IC₅₀ value. The results presented in the present study have broadened the scope of the exploitation and application concerning PAK4 kinase inhibitors and may offer a novel therapeutic strategy for advanced metastatic lung cancer.

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