ORIGINAL RESEARCH

CP-673451, a platelet-derived growth-factor receptor inhibitor, suppresses lung cancer cell proliferation and migration

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Abstract: Lung cancer is the leading cause of cancer mortality in the world. Although some advances in lung cancer therapy have been made, patient survival is still poor. The platelet-derived growth factor receptors (PDGFRs) and their ligands play critical roles in the regulation of many cancer cell processes, including cell survival and cell motility. Herein, we investigate the anticancer activities of CP-673451, a potent selective inhibitor of PDGFR kinase, in non-small-cell lung cancer (NSCLC) therapy. We found that CP-673451 is effective at suppressing cell viability, inducing cell apoptosis, and inhibiting cell migration and invasion by suppressing the PDGFR downstream signaling pathway in NSCLC cells. Furthermore, CP-673451 is effective at suppressing NSCLC tumor growth in vivo. In summary, our studies suggest that CP-673451 might be a promising therapeutic compound for NSCLC.

Keywords: CP-673451, platelet-derived growth factor receptors (PDGFRs), lung cancer, proliferation, migration

Introduction

Lung cancer is a leading deadly cancer and the most common cause of cancer death worldwide.[1–4] Non-small-cell lung cancer (NSCLC) is the most frequent type of lung cancer, accounting for about 85% of total lung cancer.[5–8] Although a lot of research has been done on lung cancer, the overall survival rate is still very low.[5,6]

The platelet-derived growth factor receptors (PDGFRs) and their ligands are involved in proliferation, migration, and invasion signaling in cells.[9–13] The ligand binding induces receptor dimerization, enabling autophosphorylation of some tyrosine residues and recruitment of a series of signaling molecules.[9,10] It is shown that inhibition of PDGFR signaling disrupts cancer cell survival.[13] Moreover, PDGFR overexpression has been observed in metastatic versus nonmetastatic cancer patient samples, and disruption of PDGFR function inhibited the metastatic potential of cancer cells.[14] In lung cancer, especially NSCLC, cytoplasmic PDGFR expression by tumors is a negative prognostic indicator.[15] Given its critical roles in cell survival and metastatic signaling, PDGFRs have become an attractive therapeutic target in a number of human malignancies, including lung cancer.

CP-673451 is a potent inhibitor of PDGFR kinase, being more than 450-fold more selective for PDGFRβ versus other receptors.[16] Although some research has been done to characterize the pharmacology of PDGFRβ inhibition by CP-673451 and its effect on tumor growth, its potential anticancer activity, especially on NSCLC, is far from clear. In this study, we investigated the potential anticancer activities of CP-673451 in NSCLC therapy.

Materials and methods
Materials
CP-673451 (Selleck Chemicals, Houston, TX, USA) was used in all experiments to evaluate its anticancer activities. Rabbit anti-phospho-Akt (Ser473; catalog number 4060), anti-phospho-p70S6K (Thr389; 9205), anti-phospho-S6 (Ser235/236; 2211), anti-phospho-GSK-3β (Ser9; 9336), anti-p70S6K (9202), anti-GSK-3β (9315), anti-PDGFRβ (28E1; 3169), anti-phospho-PDGRα (Tyr849)/PDGFβ (Tyr857 [C43E9]; 3170), anti-phospho-Bad (Ser136 [D25H8]; 4366), anti-β-actin (4967), and secondary horseradish peroxidase-conjugated antibody (7074) (all Cell Signaling Technology, Danvers, MA, USA) were used in Western blot analysis.

Cells and cell culture
Human lung cancer cell lines A549 and H1299 were purchased from the American Type Culture Collection (Manassas, VA, USA), and grown in Roswell Park Memorial Institute medium containing 10% heat-inactivated fetal bovine serum (FBS) with 2 mM L-glutamine and 100 U·mL^{-1} penicillin–streptomycin in a humidified incubator at 37°C in 5% CO_2.

Cell-viability assay
Cells were seeded in triplicate in 96-well plates, treated with CP-673451 for 24,48, or 72 hours, and analyzed using a CellTiter-Glo® assay kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions.

Transwell assay
Briefly, 1×10^5 cells in media supplemented with 1% FBS were plated into the upper chambers of the 24-transwell Boyden chamber wells (Costar®; Sigma-Aldrich, St Louis, MO, USA), and 650 μL media supplemented with 10% FBS was added into lower chambers to use as a chemoattractant. After treatment with CP-673451 for 12 hours, the cells were fixed and stained with 0.1% crystal violet. The nonmigrated cells were wiped off, and the migrated cells were photographed. Then, the cells were lysed with 10% acetic acid, and absorbance was measured at 595 nm.

Wound-healing assay
Cells were seeded in 96-well plates. Once 100% confluence was observed, the cell monolayer was scratched with a pipette tip. After being washed three times, the cells were treated with CP-673451 for 12 hours. Then, the scratches were photographed and the width of scratches measured.

Invasion assay
Briefly, the upper chamber of a 24-transwell Boyden chamber was coated with 1 mg/mL of Matrigel™ (BD Biosciences,

San Jose, CA, USA) for 4 hours at 37°C, and then 1×10^5 cells in media supplemented with 1% FBS were plated into the upper chambers of the 24-transwell Boyden chamber wells and 650 μL media supplemented with 10% FBS added into the lower chambers to use as a chemoattractant. After treatment with CP-673451 for 12 hours, the cells were fixed and stained with 0.1% crystal violet. The nonmigrated cells were wiped off, and the migrated cells were photographed. Then, the cells were lysed with 10% acetic acid and absorbance measured at 595 nm.

Fluorescent immunocytochemistry
Cells were seeded on glass coverslips, followed by treatments with compounds. Then, cells were fixed with 4% polyformaldehyde for 30 minutes, permeabilized with 0.1% Triton X-100 for 20 minutes, and blocked with 5% normal serum for 30 minutes. For detection of actin fibers, cells were incubated with tetramethylrhodamine isothiocyanate-labeled phalloidine for 30 minutes. For detection of nuclei, cells were incubated with Hoechst for 10 minutes. Nuclei images were taken with a fluorescent microscope.

Flow-cytometry analysis
Cells treated with CP-673451 were harvested, fixed, and stained with propidium iodide by using a Cycletest™ Plus deoxyribonucleic acid (DNA) reagent kit (BD Biosciences). Then, the cells were analyzed for DNA content with the use of FACSCalibur™ flow cytometry and CellQuest™ Pro software (BD Biosciences).

Western blot analysis
After treatment with CP-673451, the total proteins were extracted with radioimmunoprecipitation assay buffer (0.15 mM NaCl, 0.05 mM Tris-HCl, pH 7.5, 1% Triton, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, and 1% Nonidet P40). Then, the sample extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis separation, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked for 1 hour with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed and incubated with secondary antibody, and visualized by chemiluminescence.

Immunohistochemistry analysis
Tumor samples were harvested from mice, trimmed into 5 mm^3 pieces, and immediately fixed in 10% neutral buffered formalin, then transferred to 70% ethanol, embedded into paraffin blocks, and sectioned (5 mm thickness).

A terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling (TUNEL; Promega, Madison, WI, USA) assay was performed according to the manufacturer's instructions. The nuclei were stained with Hoechst for 20 minutes. The slides were photographed using a fluorescent microscope.

Efficacy studies in mouse xenograft model in vivo

The nude mice were housed under specific pathogen-free conditions. The animal use and experimental protocol were reviewed and approved by the Animal Care and Use Committee of the First Affiliated Hospital of Xinxiang Medical University. A subcutaneous A549 xenograft model in nude mice was used to evaluate the anticancer activity of CP-673451. Briefly, A549 cells were injected into the axillary regions of mice (2×10^6 cells/mouse). When the tumor volumes reached 70 mm^3, the mice were randomly assigned to a control group and two CP-673451 groups (n=6 per group): low-dose (20 mg/kg) and high dose (40 mg/kg) groups (vehicle 10% 1-methyl-2-pyrrolidinone and 90% polyethylene glycol 300). These animals were administered intraperitoneally with CP-673451 (20 or 40 mg/kg/day) or with vehicle. During the treatment period, the implanted tumors were measured by caliper once a day in a blind fashion. The animal body weights were also measured at the same time. The tumor volume was calculated as follows: (width $\times$ width $\times$ length)/2. After treatment, the mice were killed, and the tumors were harvested and analyzed.
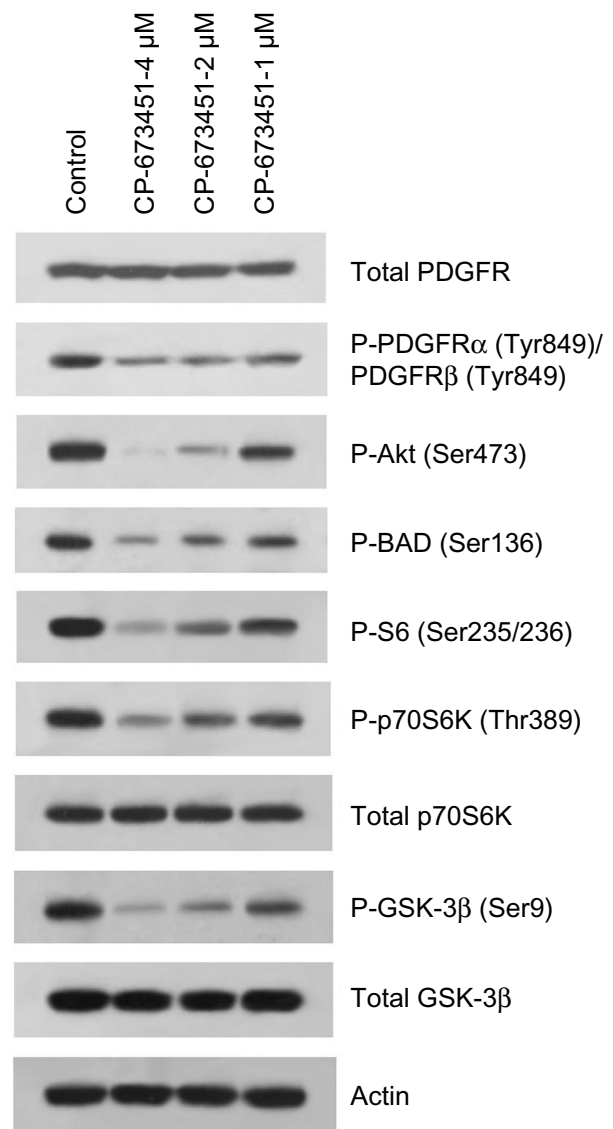
Statistics

Student's t-test and analysis of variance were performed using StatView (SAS Institute, Cary, NC, USA). The data shown are the mean values of triplicate measurements with error bars corresponding to standard deviation.
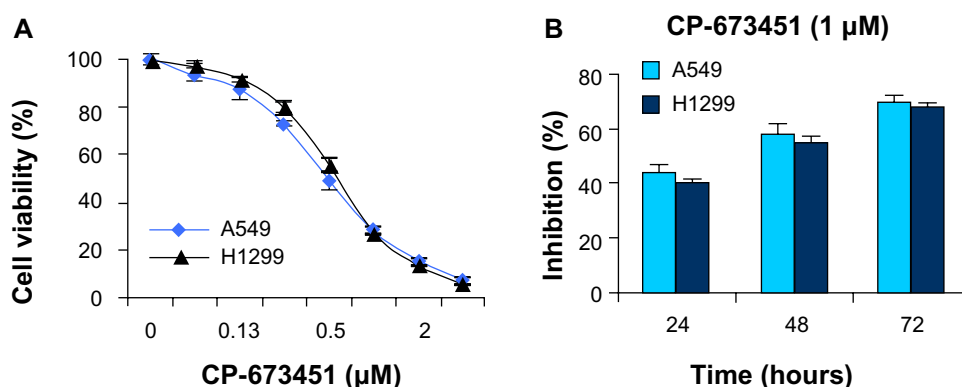

Figure 1 CP-673451 inhibits the PDGFR-mediated signaling pathway in non-small-cell lung cancer cells.
Note: A549 cells were treated with CP-673451 (1, 2, and 4 µM) in the presence of serum for 3 hours, followed by Western blot analysis with the indicated antibodies.
Abbreviations: PDGFR, platelet-derived growth factor receptor; P, phospho; GSK, glycogen synthase kinase.

Results

CP-673451 inhibits PDGFR-mediated signaling pathway in A549 cells

PDGFRs have been suggested as potential targets in cancer therapy. CP-673451 is a known specific inhibitor of PDGFR.[16] To explore the roles of CP-673451 in therapeutics for NSCLC, we first examined the effect of CP-673451 on PDGFR-mediated signaling in A549, an NSCLC cell line. As shown in Figure 1, CP-673451 efficiently suppressed the PDGFR downstream signaling pathway. It inhibited phosphorylation of Akt, GSK-3β, p70S6, and S6 in A549 cells in a concentration-dependent manner. In contrast, no significant changes were observed in total protein levels after treatment.

CP-673451 is effective at suppressing NSCLC cell viability

PDGFRs and their downstream PI3K/Akt signaling pathways play crucial roles in promoting cancer cell survival.[17–23] We next observed the effect of CP-673451 on NSCLC cell viability. The results in Figure 2A show that CP-673451 significantly reduced the viability of NSCLC cell lines A549 and H1299, with a half-maximal inhibitory concentration of 0.49 and 0.61 µM, respectively. Figure 2B shows that CP-673451 reduced the cell viability of these cell lines in a time- and concentration-dependent manner.

Figure 2 (**A** and **B**) CP-673451 suppresses non-small-cell lung cancer cell viability.
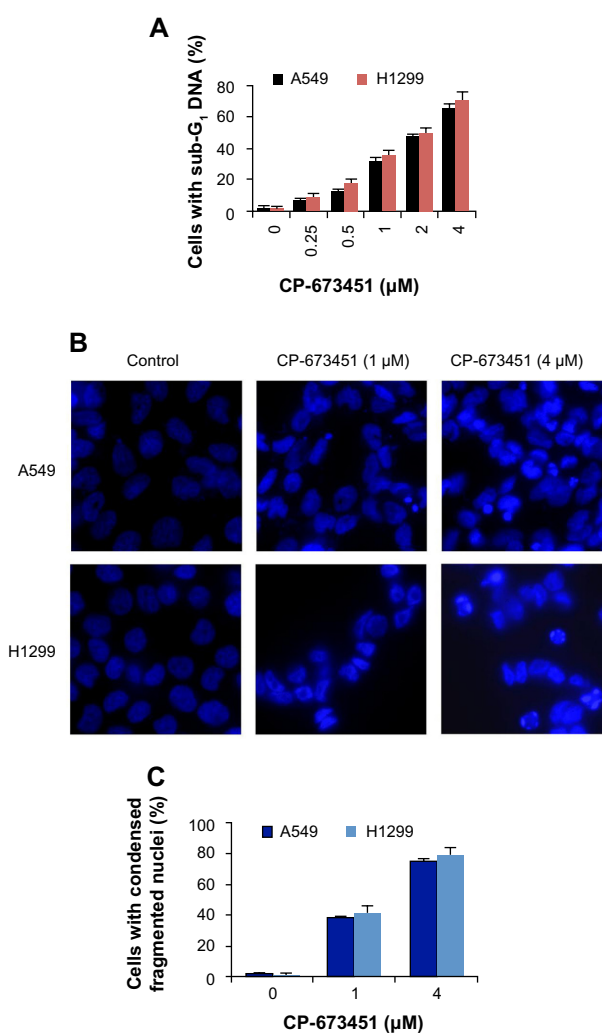**Notes:** (**A**) A549 and H1299 cells were treated with CP-673451 (0.0625–4 μM) in the presence of serum for 72 hours, followed by measurement with a cell-viability assay.
(**B**) A549 and H1299 cells were treated with CP-673451 (1 μM) in the presence of serum for 24, 48, or 72 hours, followed by measurement with a cell-viability assay.

CP-673451 induces dramatic apoptosis in NSCLC cells

Since the PDGFRs and their downstream signaling pathways play important roles in the suppression of apoptosis,[17–23] we next observed CP-673451-induced apoptosis in NSCLC cells. Treatment with CP-673451 caused a dose-dependent increase in sub-G_1 DNA content in A549 cells, as measured by flow cytometry ($P < 0.01$). CP-673451 induced 50% apoptotic cell death in A549 and H1299 cell lines at concentrations of 2.4 and 2.1 μM, respectively (Figure 3A). Consistently, CP-673451 treatment dose-dependently induced condensed and fragmented nuclei in A549 cells, suggesting that CP-673451 could significantly induce apoptosis in NSCLC cells (Figure 3B and C).

CP-673451 is effective at inhibiting migration and invasion of NSCLC cells by suppression of lamellipodia formation

It is known that PDGFRs and their downstream signaling pathways contribute remarkably to cancer cell migration and invasion by inducing lamellipodia formation at the leading edge of cells.[24–26] We next observed the effect of CP-673451 on the migration and invasion of A549 cells. As shown in Figure 4A–D, CP-673451 concentration-dependently inhibited migration of A549 cells in both transwell and wound-healing assays. CP-673451 (25 nM) exerted an inhibition of 56.34%. Furthermore, CP-673451 also efficiently suppressed cancer cell invasion in a dose-dependent manner in a Matrigel-coated transwell model (Figure 4E and F). Due to the significant roles of lamellipodia in cell motility, we next analyzed the influence of CP-673451 on lamellipodia formation, and found CP-673451 dramatically suppressed lamellipodia formation in A549 cells, which at least partially


Figure 3 (**A–C**) CP-673451 induces apoptosis in non-small-cell lung cancer cells.
Notes: (**A**) A549 cells were treated with CP-673451 at the indicated concentrations in the presence of serum for 48 hours, followed by propidium iodide staining and flow-cytometry analysis. (**B**) A549 cells were incubated with CP-673451 in the presence of serum for 48 hours. The nuclei were stained with Hoechst, and analyzed using a fluorescent microscope. The representative images are shown. (**C**) The number of cells with condensed/fragmented nuclei was quantitated by counting in six random fields.
Abbreviation: DNA, deoxyribonucleic acid.
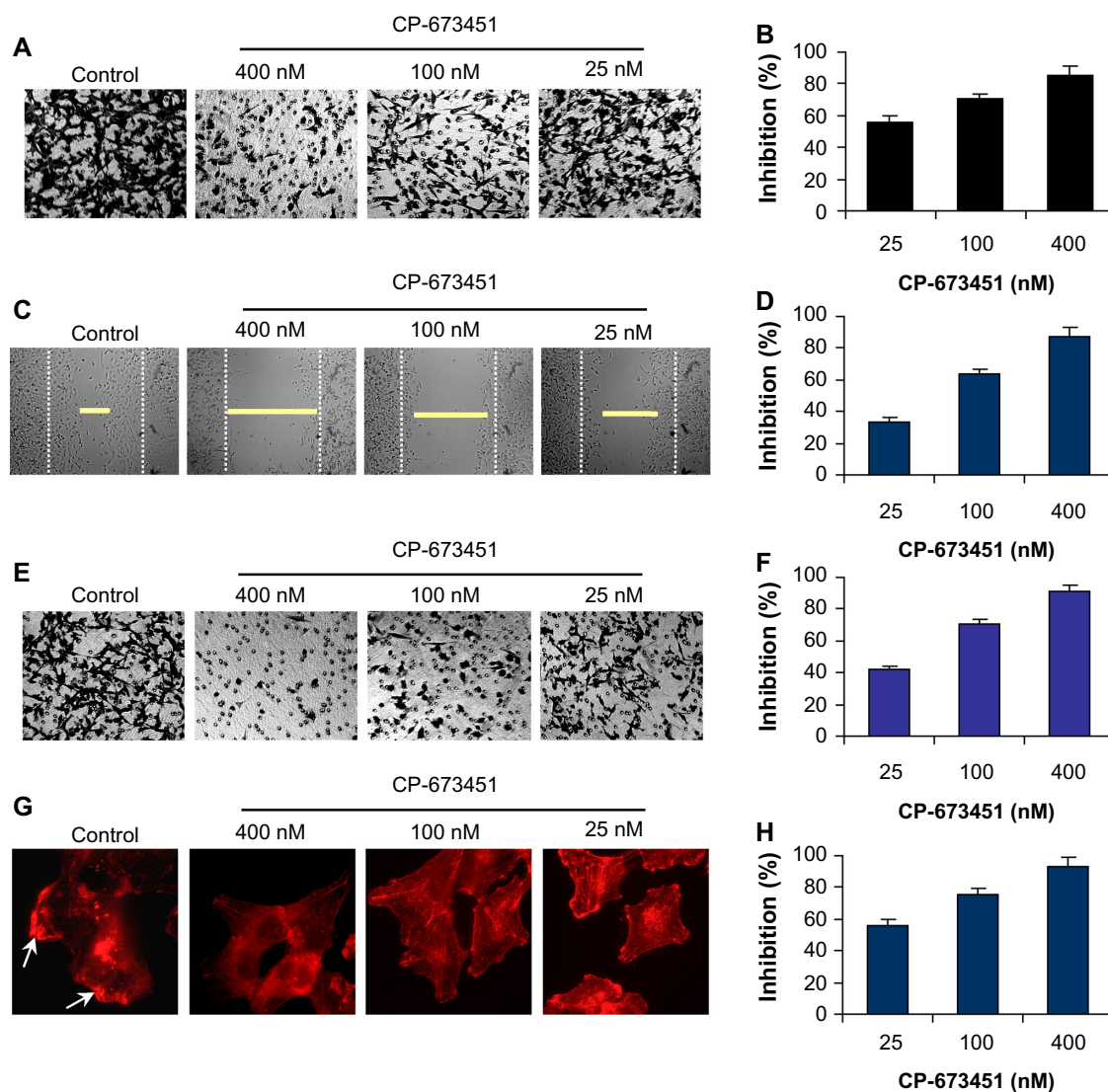
Figure 4 (**A–H**) CP-673451 inhibits lamellipodia formation and cell migration and invasion in non-small-cell lung cancer cells.
**Notes:** (**A**) A549 cells were treated with CP-673451 (25, 100, or 400 nM) in the absence of serum for 12 hours. The nonmigrated cells on the upper surface of the filter were removed, and the migrated cells on the lower side were stained and photographed. The representative images are shown. Then, cells were lysed and colorimetric determination was made at 595 nm. (**B**) Quantitation of the inhibition from transwell assay. (**C**) A scratch was introduced into a monolayer of A549 cells, followed by treatment with CP-673451 (25, 100, or 400 nM) in the absence of serum for 12 hours. The width of wounded cell monolayers was measured in seven random fields, and representative images are shown (white dashed lines show the original wound width, yellow lines show the final wound width). (**D**) Quantitation of the inhibition from the transwell assay. (**E**) A549 cells were seeded on a Matrigel-coated transwell membrane, and the treatment and analysis were similar to the transwell assay. (**F**) Quantitation of the inhibition from the invasion assay. (**G**) A549 cells were treated with CP-673451 (25, 100, or 400 nM) in the presence of serum for 12 hours. The actin fibers were stained and analyzed using a fluorescent microscope. The representative images are shown (the lamellipodia are indicated by white arrows). (**H**) The number of cells with significant lamellipodia was quantitated by counting in six random fields, and the inhibition was calculated.

accounted for the motility-inhibitory activity of CP-673451 (Figure 4G and H).

CP-673451 suppresses NSCLC tumor growth in vivo

We next examined the effect of CP-673451 on the growth of A549 tumors in nude/nude mice. The results showed that low-dose CP-673451 (20 mg/kg) led to a medium suppression of tumor growth, while high-dose CP-673451 (40 mg/kg) strongly inhibited tumor growth

in vivo (Figure 5A). Doses of 20 and 40 mg/kg CP-673451 exerted an inhibition of 42.56% and 78.15% at day 10, respectively. The hematoxylin and eosin staining results showed that CP-673451 induced significant apoptosis in tumors, as shown with a great number of condensed nuclei in the CP-673451-treated group (Figure 5B). This effect was further confirmed by using TUNEL and nuclei-staining assays. In the TUNEL assay, the fluorescent signal in the CP-673451 group was much stronger than in the control group. Nuclei staining showed that CP-673451 induced
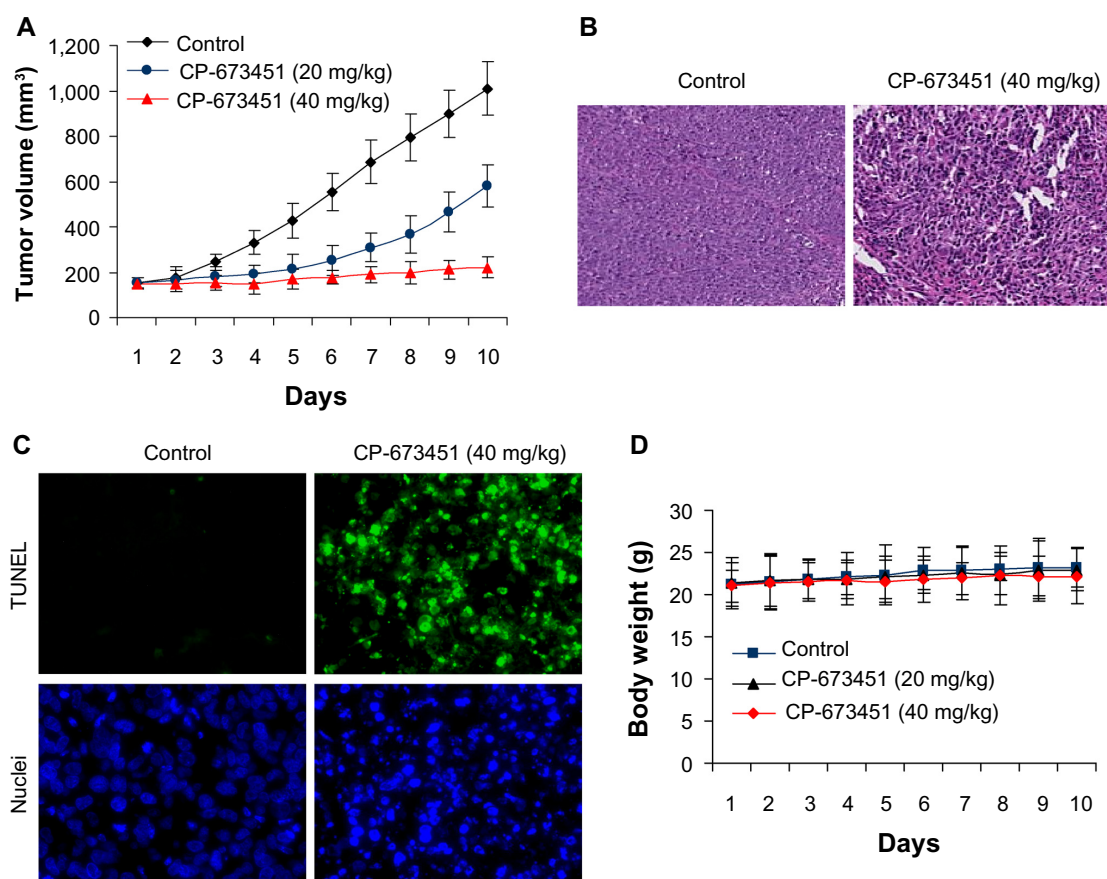
Figure 5 (**A–D**) CP-673451 suppresses non-small-cell lung cancer tumor growth in vivo.
Notes: (**A**) After inoculation of A549 cells, CP-673451 (20 and 40 mg/kg) was injected into mice every day. The tumors were measured every day, and the tumor volumes are shown. (**B**) Hematoxylin and eosin staining of tumor sample slides. (**C**) CP-673451 induced apoptosis of A549 tumor cells in vivo measured with a terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling (TUNEL) assay (green), and the nuclei were stained with Hoechst (blue). (**D**) CP-673451 had no significant cytotoxic effects on the body weight of mice during the treatments.

condensed/fragmented nuclei (Figure 5C). Moreover, CP-673451 showed no significant toxicity in mice, and there was no significant loss of weight ($P>0.05$) (Figure 5D).

Discussion

Lung cancer is a leading cause of cancer death worldwide, with NSCLC accounting for the majority of all lung cancer cases.[1–8] The high mortality rate associated with lung cancer has prompted numerous exhaustive efforts to identify novel therapeutic targets and treatment modalities.[5,6] The resistance of many types of cancer to conventional chemotherapies is a major factor undermining successful cancer treatment. It is well known that PDGFRs and their ligands play critical roles in cancer cell migration and proliferation.[17–23] One important downstream signaling pathway of PDGFR is the PI3K/Akt pathway, which plays crucial roles in promoting cell survival, inhibiting cell apoptosis, and suppressing cell motility. PDGFR activation and overexpression contributes not only to tumorigenesis and tumor metastasis but also to resistance acquisition to chemotherapy.[27,28] Therefore, PDGFR

inhibition is a promising therapeutic strategy for cancer, including NSCLC.

Here, we investigated the potential anticancer activities of CP-673451, a PDGF-specific inhibitor, in NSCLC therapy. Our studies show that CP-673451 could efficiently suppress NSCLC cell viability, induce cell apoptosis, and inhibit lamellipodia formation, cell migration, and invasion. All these effects are at least partially attributed to the inhibition of PDGFR and subsequently its downstream signaling pathway in NSCLC cells by CP-673451 treatment. Importantly, CP-673451 is effective at suppressing NSCLC tumor growth in vivo.

The anticancer activity of CP-673451 has been poorly reported, and the detailed underlying anticancer mechanisms are far from clear. The antiangiogenic activity of CP-673451 was previously reported.[1] However, there have been no reports about its antimigration and anti-invasion activities so far. Here, for the first time, we report that CP-673451 exerted strong inhibition on actin reorganization and lamellipodia formation, a driving resource for cell motility. Subsequently, we found

CP-673451 has strong antimigration and anti-invasion activities in NSCLC cells. We found CP-673451, as a specific PDGF specific inhibitor, could strongly inhibit PDGFR-mediated signaling pathways, which can be used to explain, at least partially, its activities of cell-viability suppression, cell-apoptosis induction, lamellipodia inhibition, and cell migration and invasion suppression. Our study is also a systemic study of anticancer activities of CP-673451, including its antiproliferation, antimotility, and apoptosis-induction activities in NSCLC cells, a high-mortality cancer type worldwide. In summary, our studies suggest that CP-673451 might be a potential therapeutic agent in NSCLC, and might shed new light on NSCLC therapy.

Disclosure

The authors report no conflicts of interest in this work.

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