Efficacy of PP121 in primary and metastatic non-small cell lung cancers

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Abstract. Tyrosine kinase inhibitors are a clinically standard treatment option for non-small cell lung cancers (NSCLCs), the leading cause of cancer-related deaths in the US. These targeted agents include first, second and third generation tyrosine kinase inhibitors; however, these lack clinical efficacy in the treatment of NSCLC due to intrinsic and acquired resistance. This resistance may be a result of genetic aberrations in oncogenic signaling mediators of divergent pathways. The present study aimed to investigate a novel dual tyrosine kinase and PI3K inhibitor, PP121, as a targeted agent in NSCLC cell lines. The present study co-cultured PP121 with healthy human astrocytes, a prevalent cell type located in the brain of NSCLC brain metastases. To date, few preclinical studies have examined the efficacy of PP121 as an anticancer agent, and to the best of my knowledge, no previous studies have previously evaluated its therapeutic potential in the treatment of NSCLC. To investigate the clinical heterogeneity of NSCLC, patient-derived adenocarcinoma (ADC) and squamous cell carcinoma (SCC) xenograft models were used, which exhibited epidermal growth factor receptor (EGFR) mutations and mesenchymal-epithelial transition (MET) factor amplifications. Notably, both EGFR and MET are known contributors to tyrosine kinase inhibitor resistance; thus, the aforementioned mutations and amplifications enabled the effects of PP121 to be evaluated in these solid tumors. In addition, a co-cultured model system using both NSCLC cells and astrocytes was employed to assess the effects of PP121 on the invasion of ADC and SCC cells in a multicellular environment. Results of the present study demonstrated that PP121 exerted an antitumorigenic effect in the aforementioned model systems via downregulation of pharmacodynamic targets.

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Introduction

Non-small cell lung cancers (NSCLCs) are the leading cause of cancer-related mortality in the United States, and are broadly comprised of two subtypes, including squamous cell carcinoma (SCC) and adenocarcinoma (ADC) (1,2). Patients with NSCLC have a poor prognosis, and this disease accounts for 85-90% of all lung cancers. Notably, NSCLC may be attributed to a number of genetic abnormalities, including genetic mutations, deletions and amplifications in receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), mesenchymal-epithelial transition (MET) factor and anaplastic lymphoma kinase, and activation of their downstream signaling mediators including Kirsten rat sarcoma viral oncogene homolog (KRAS), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), and phosphatidylinositol-3-kinase (PI3K). These genetic aberrations have brought about the clinical use of various kinase inhibitors as secondary targeted treatment strategies beyond surgery and radiation for patients diagnosed with NSCLC. Thus, numerous kinase inhibitors, such as erlotinib, gefitinib and crizotinib, have been used in clinical practice. Specifically, these inhibitors target EGFR, MET and ALK for the treatment of NSCLC (3,4). Despite the pharmacodynamic rationale for the treatment of NSCLCs using these tyrosine kinase inhibitors, the clinical management and overall survival of patients treated with these agents has not significantly improved. This may be due to high levels of acquired resistance to clinically used kinase inhibitors. These levels of resistance may be a result of increased activation of compensatory tyrosine kinases and downstream signaling mediators of the intended targets, which may inhibit the efficacy of first and second generation NSCLC kinase inhibitors (5-7). Notably, in preclinical studies, the use of combined treatment strategies that impair divergent signaling pathways have proven efficacious in the treatment of NSCLC resistant to EGFR inhibitors. More specifically, when apatinib, a vascular endothelial growth factor (VEGF) receptor-2 inhibitor, was combined with the EGFR inhibitor, gefitinib, it exerted an antitumorigenic effect in NSCLC as a consequence of independently downregulating VEGFR-2 and EGFR activity. This supported the notion that targeting multiple receptors and pathways may help overcome EGFR inhibitor resistance and treat NSCLC (8,9).

An additional contributor to the therapeutic resistance and high mortality rates associated with NSCLCs are brain

metastases, estimated to occur in 20-40% of diagnosed cases, with a ~100% mortality rate (9-11). Thus, the biology of lung cancer brain metastasis and the underlying molecular mechanisms remain poorly understood. However, the receptor tyrosine kinases, EGFR and MET, are implicated in the propagation of lung cancer brain metastases. MET exerts signaling capacity in cancer cells via activation and stimulation of the PI3K/AKT/mTOR pathway, and is expressed in 44% of NSCLC brain metastatic tissues. In addition, activating mutations in EGFR may induce DNA synthesis and tumor cell proliferation via signal transduction activation of MAPK, AKT and JNK (12). Collectively, these results demonstrate that a network of signaling kinases not only contribute to the survival of primary NSCLC, but also impact the progression of NSCLC brain metastases. Identification of novel therapeutic agents is urgently required to overcome these clinical resistance mechanisms of NSCLCs, to improve the management and overall survival outcomes of patients diagnosed with these tumors.

PP121 is a novel dual kinase inhibitor that targets tyrosine kinases and PI3K. It is often used in a single agent approach to simultaneously target multiple pro-tumorigenic signaling mediators in primary NSCLC and NSCLC that metastasizes to the brain. Notably, results of preclinical studies have demonstrated the antitumorigenic properties of PP121 in the inhibition of esophageal and brain cancer cell proliferation, and the impaired migration of anaplastic thyroid carcinoma cells (13-15). In addition, results of a previous study demonstrated that PP121 inhibited ovarian cancer metastasis (16). Mechanistically, downregulation of PI3K-mTOR signaling mediators and inhibition of MEK have been associated with the suppression of tumor cell proliferation and metastatic progression of these solid tumors (17-19). These results further demonstrate the potential specificity of PP121 in targeting divergent receptor tyrosine kinase molecular signaling pathways. Collectively, these results support the hypothesis that PP121 may exhibit potential as a single agent-targeted strategy to overcome multilateral compensatory resistant mechanisms in primary NSCLC and NSCLC that metastasizes to the brain.

Materials and methods

Cell culture conditions and reagents. ADC (NCI-H1975; CRL-5908) and SCC (NCI-H2170; CRL-5928) NSCLC cells were purchased from the American Type Culture Collection. All cell lines were cultured in RPMI medium containing 10% FBS and penicillin-streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. PP121 was purchased from Tocris Bioscience. Healthy human astrocytes (product no. 1800) were purchased from ScienCell Research Laboratories, Inc., and cultured in astrocyte media (ScienCell Research Laboratories, Inc.).

Crystal violet cell proliferation assay. For dose-response experiments, cells were plated in 12-well plates with 1, 5 and 10 μM and 500 nM PP121, and incubated for 48 h at 37˚C with 5% CO_2. Vehicle controls were treated with dimethyl sulfoxide (DMSO). Subsequently, tissue culture medium was removed, the cell monolayer was fixed with 100% methanol for 5 min at room temperature (22˚C) and stained with 0.5% crystal violet in 25% methanol for 10 min at room temperature. Cells were washed three times using distilled water for 5 min each time to remove excess dye. Subsequently, cells were left to dry overnight at room temperature. The incorporated dye was solubilized in 0.1 M sodium citrate (Sigma-Aldrich; Merck KGaA) in 50% ethanol. In total, 100 μl of treated and control samples were transferred to 96-well plates and optical densities were read at 540 nm using an X-mark microplate absorbance spectrophotometer (BioRad Laboratories, Inc.).

Patient-derived xenograft organoids (PDXOs). NSCLC-PDXO experiments were carried out in collaboration with Crown BioScience. NSCLC-PDXOs (LU6471B-SCC and LU5162B-ADC) were generated from histopathologically identified patient-derived xenografts. Written informed consent was obtained from patients and ethics approval was obtained from the Integreview-Advarra ethical review board (Columbia, USA; approval no. MRL01). Organoids were processed using Matrigel for subsequent screening and size determination. Organoids were collected, plated in triplicate at a density of 250 NSCLC-PDXOs/well and treated with PP121 (0.0078-2 μM) or vehicle controls for 5 days. Cell viability was assessed as an endpoint using the CellTiter-Glo® Luminescent assay (cat. no. G9683; Promega Corporation).

Western blotting. Cells were plated and treated with PP121 or DMSO for 3 h, rinsed with PBS and lysed using CelLytic M Cell lysis reagent (Sigma-Aldrich; Merck KGaA). Protein concentrations were subsequently determined using Bradford reagent. Proteins (30 μg) were separated via SDS-PAGE in 8% polyacrylamide gels and transferred to PVDF membranes. Membranes were incubated with primary antibodies at 1:500 against phosphorylated (p)-Akt (product no. 4060L), Akt (product no. 4691), p-S6 ribosomal protein (p-RPS6; product no. 4858S), S6 ribosomal protein (product no. 2317), and cyclophilin B (product no. 43603; all from Cell Signaling Technology, Inc.) overnight at 4˚C. Following primary incubation, membranes were incubated with an HRP-conjugated secondary antibody (product no. 7074S; Cell Signaling Technology, Inc.) at 1:1,000 for 1 h at room temperature, and visualized using an enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Inc.), and a UVP BioSpectrum imaging system (Analytik Jena AG).

Radius cell migration assays. Radius motility assays were established by placing inserts into 12-well plates and seeding $5x10^4$ NSCLC cells via openings at the top of the inserts (Cell Biolabs, Inc.) and incubated for 24 h at 37˚C with 5% CO_2. Subsequently, inserts were removed and NSCLC cells were treated with 500 nM PP121 for 96 h. At the end of the incubation period, cells were stained with crystal violet as previously described, and the cell-free zone was quantified using ImageJ [1.52a; Java 1.8.0_112 (64-bit) National Institutes of Health]. For radius cell migration assays using cell co-culturing, NSCLC cells were plated as previously described, followed by the plating of $1x10^5$ healthy human astrocytes.

Immunofluorescence. Immunofluorescence labeling was performed 5 days after cells were treated with 500 nM PP121. Cells were rinsed in PBS and fixed in 4% paraformaldehyde

for 5 min at room temperature. Subsequently, cells were rinsed with PBS, permeabilized in 0.075% Triton X-100/PBS for 5 min, rinsed again with PBS, and blocked with 3.0% bovine serum albumin and 1.5% horse serum (Vector Laboratories, Inc.) in PBS for 1 h at room temperature. Cells were incubated overnight at 4˚C with primary antibodies (1:500) against thyroid transcription factor 1 (product no. 12373) and glial fibrillary acidic protein (product no. 3656; Cell Signaling Technology, Inc.). Samples were subsequently rinsed three times with PBS, incubated with an Alexa 488 goat anti-mouse-conjugated secondary antibody at 1:1,000 (product no. 4408; Cell Signaling Technology, Inc.) for 1 h in the dark, rinsed again and examined using an Olympus IX53 fluorescence microscope (Olympus Corporation).

P-glycoprotein (P-gp) assay. A P-gp Glo assay kit was purchased from Promega Corporation and the P-gp assay was performed following the manufacturer's instructions. Untreated controls (Pgp-Glo assay buffer), positive controls (0.05 mM Na_3VO_4 and 0.1 mM verapamil) and 50 μM PP121 were prepared following the manufacturer's instructions. P-gp membranes were added to wells containing untreated controls, 0.05 mM Na_3VO_4, 0.1 mM verapamil or 50 μM PP121 and incubated at 37˚C for 5 min. Reactions were subsequently initiated following the addition of 5 mM magnesium adenosine triphosphate (MgATP), and samples were incubated for 40 min at 37˚C. Luminescence was initiated following the addition of ATP detection reagent, followed by incubation at room temperature for 20 min. Subsequently, luminescence was read using a luminometer.

Statistical analysis. Cell viability, radius cell migration and Pg-p activity experiments were performed at least three times using duplicate or triplicate samples. Unpaired Student's t-tests and one-way ANOVA followed by Tukey's post hoc analysis were performed using GraphPad Prism 8.0 (GraphPad Software, Inc.) to determine the statistical significance between groups. The results are presented as the average means ± standard error of means. Coexpression analyses of mRNA expression were performed using Spearman's correlation test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PP121 reduces NSCLC cell viability and migratory invasion. To the best of my knowledge, PP121 has not been evaluated as a targeted agent for the treatment of lung cancers, specifically NSCLC. To determine the use of PP121 as a novel single drug agent for the treatment of NSCLC, an expression analysis of the receptor tyrosine kinase, MET was carried out. The potential association between MET and PIK3CA was determined using The Cancer Genome Atlas database. Results of the present study revealed a positive association between PIK3CA and MET (Table I and Fig. 1). As drug doses differ between experiments and clinical practice, a range of PP121 concentrations (500 nM-10 μM) were evaluated to establish the lowest doses that exerted antiproliferative effects on SCC and ADC cells.

Results of the present study demonstrated that PP121 decreased NSCLC cell viability in a dose-dependent manner,

Table I. Co-expression of MET and PIK3CA in NSCLC.

Type of cancer	Spearman's correlation	P-value
SCC	0.19	$2.65e^{-5}$
ADC	0.16	$2.08e^{-4}$

MET, mesenchymal-epithelial transition; PIK3CA, phosphatidyl inositol 3 kinase; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; ADC, adenocarcinoma.

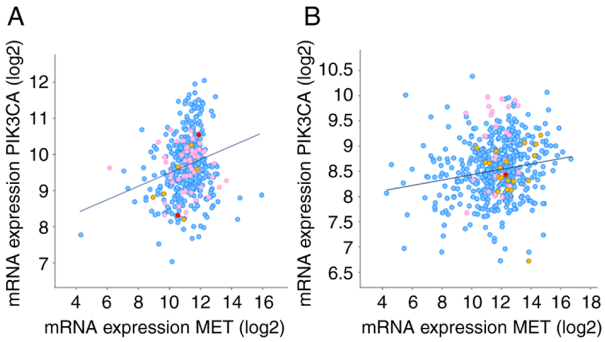

Figure 1. Co-expression of PIK3CA and MET in non-small cell lung cancer. (A) Adenocarcinomas and (B) squamous cell carcinomas. MET mutated (yellow); PIK3CA mutated (pink); both mutated (red); neither mutated (blue).

and that exposure to concentrations as low as 500 nM significantly decreased cell viability by 75 and 70% in SCC and ADC cells, respectively (Fig. 2). The observed decrease in NSCLC cell viability following treatment with PP121 was consistent with previous efficacy experiments, demonstrating that PP121 treatment reduced glioblastoma and breast cancer cell proliferation (14). Notably, the antiproliferative effect of PP121 was demonstrated in NCI-1975 cells possessing mutations in EGFR and PIK3CA.

To assert a more clinically relevant approach for evaluating the response of NSCLC to PP121, translational applicable model systems were used. Specifically, PDXOs were used to further determine the preclinical antitumorigenic effects of PP121 (Fig. 3). Notably, PDXOs are 3D *in vitro* models developed from *in vivo* patient-derived xenografts. These are useful preclinical models that maintain the genetic heterogeneity of clinical tumors. Results of the present study demonstrated that PP121 significantly decreased the proliferative capacity of NSCLC-PDXOs, compared with vehicle controls (Fig. 3).

NSCLC brain metastasis is a major contributor to NSCLC disease progression, as a consequence of therapeutic evasion and resistance. To recapitulate NSCLC brain metastases, NCI-H1975 ADC cells were co-cultured with healthy human astrocytes. Results of the present study demonstrated that NCI-H1975 ADC cells migrated and penetrated healthy human astrocytes (Fig. 4A-C). However, treatment with PP121 inhibited SCC cell migration and the invasion of healthy human astrocytes (Fig. 4D and E). In addition, the results of radius cell migration studies demonstrated that PP121 decreased the migration of ADC and SCC cells, consequently increasing the cell-free zone by 28 and 52%, indicative of reduced NSCLC

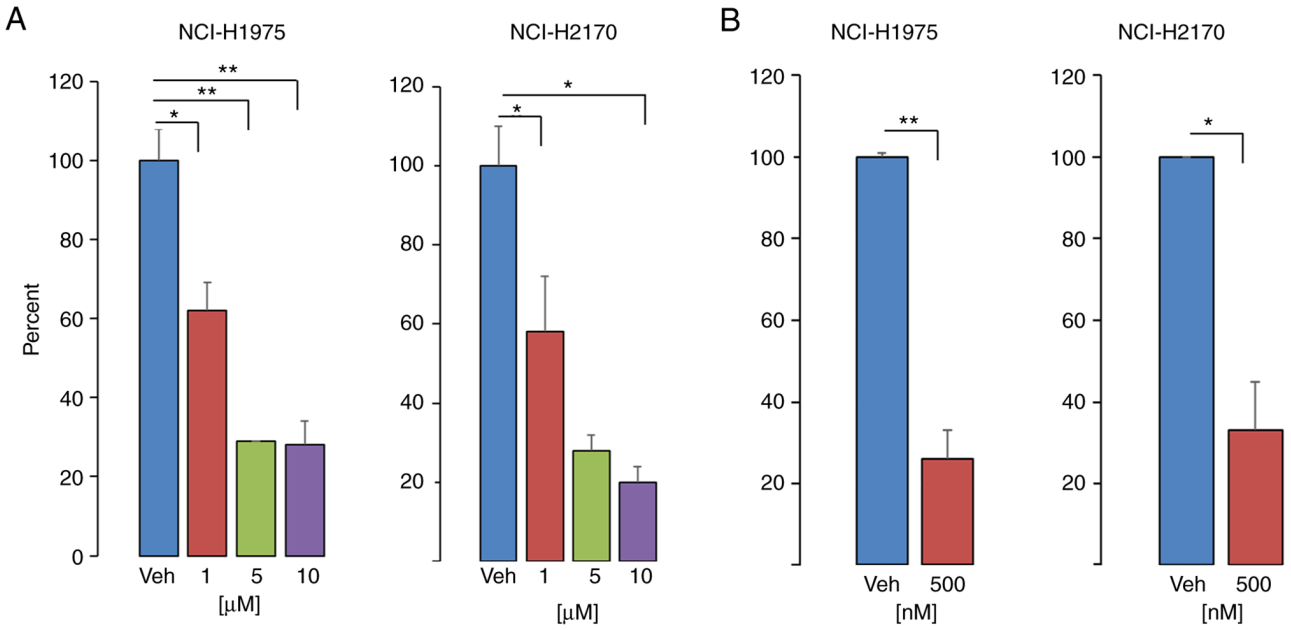

Figure 2. PP121 reduces the cell viability of non-small cell lung cancer. (A) ADC (NCI-H1975) and SCC (NCI-H2170) cells were treated with 1-10 μM PP121 for 48 h. (B) ADC and SCC cells were treated with 500 nM PP121 for 5 days. The data shown are representative of three independent experiments. *P<0.05 and **P<0.01. Error bars ± SEM. ADC, adenocarcinoma; SCC, squamous cell carcinoma.

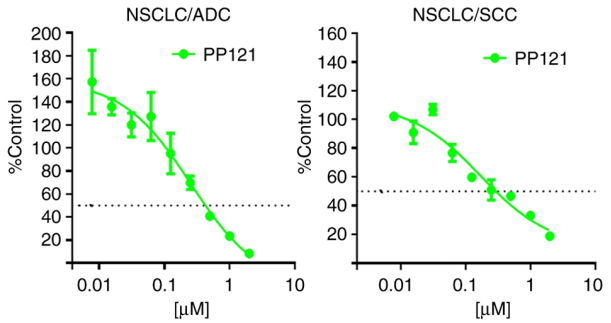

Figure 3. Efficacy of PP121 in adenocarcinoma and squamous cell carcinoma patient-derived xenograft organoid models, 5 days after treatment. Experiments were performed in triplicate and the data shown are representative of two independent experiments. Error bars ± SEM. NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SCC, squamous cell carcinoma.

cell migration (Fig. 4F and G). Moreover, the impact of PP121 on NSCLC cell migration was evaluated in a co-cultured model system comprised of NSCLC cells and healthy human astrocytes, a resident glial cell located in the mammalian brain (Fig. 4). Results of the present study demonstrated that clinically relevant concentrations of PP121 markedly reduced NSCLC cell migration in both SCC and ADC cells (Fig. 4). Although few studies have previously examined the efficacy of PP121 in the inhibition of cancer cell migration, the capacity of PP121 to suppress NSCLC cell migration is supported by its inhibition of anaplastic thyroid carcinoma cell migration and invasion (15).

Downregulation of pharmacodynamic and kinetic targets of PP121. PP121 antagonizes PI3K and tyrosine kinases (12). Results of the present study demonstrated that the protein expression levels of p-RPS6, a downstream effector of PI3K and mTOR, were markedly reduced following treatment with PP121 in SCC and ADC cells (Fig. 5A), but the levels of unphosphorylated RPS6 were not reduced (Fig. 5B). Additionally, PP121 decreased p-Akt protein expression in SCC cells but it did not downregulate unphosphorylated Akt (Fig. 5C). The reduced expression of these signaling mediators as a mechanistic response to PP121 in NSCLC is comparable with previous findings in esophageal cancer cells, glioblastoma, and thyroid carcinoma. These results demonstrated reduced p-Akt and p-RPS6 protein expression following treatment with this targeted agent without decreasing total Akt and RPS6 protein levels in these solid cancers treated with PP121 concentrations as high as 10 μM (13-15). In addition, the effects of PP121 on P-gp, a drug efflux transporter that plays a role in drug metabolism, clearing and drug resistance were determined (20,21). Notably, P-gp is expressed at high levels in the human brain for protection against cytotoxic agents (22). Moreover, P-gp contributes to the resistance of drugs used to treat diseases of the brain (23) such as NSCLC brain metastases. Therefore, inhibition of P-gp function enhances drug bioavailability and the subsequent therapeutic efficacy. Using an activity assay approach, the results of the present study demonstrated that PP121 decreased P-gp activity, compared with the activity of the vehicle and positive controls, sodium orthovanadate and verpamil (Fig. 6).

Discussion

Pro-tumorigenic signaling cascades that promote cancer cell survival and recurrence enable the resistance of NSCLC to clinical therapeutic approaches. Kinase inhibitors have been used as a primary strategic approach to prevent disease progression. Kinase inhibitors targeting EGFR have demonstrated efficacy in wild-type or mutant EGFR NSCLC cells, but NSCLC was not cured and metastatic disease was not prevented (24). Notably, small molecule inhibitors activate

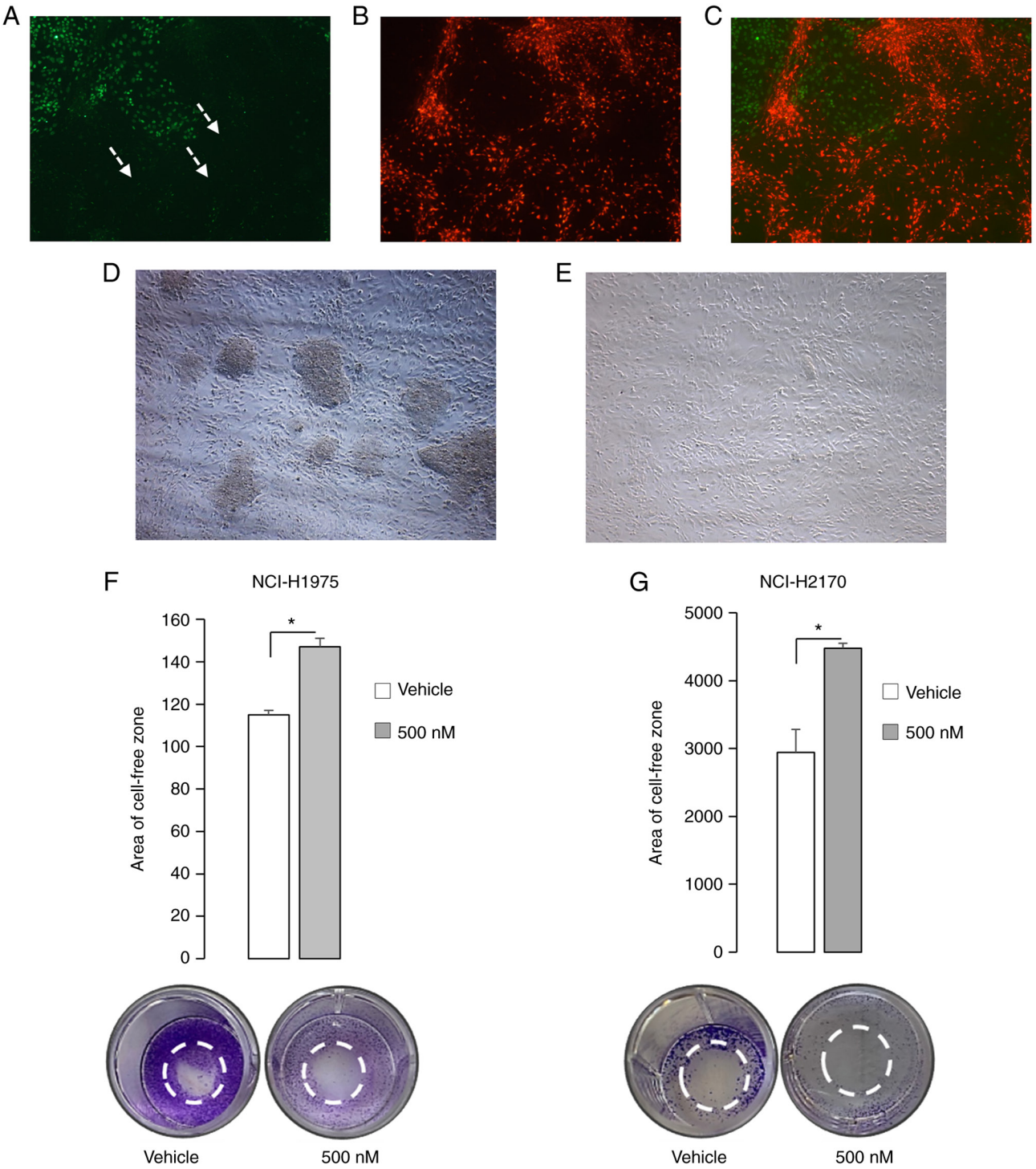

Figure 4. PP121 reduces non-small cell lung cancer migratory invasion. (A) Thyroid transcription factor 1-positive ADC cells (NCI-H1975) penetrate (B) glial fibrillary acidic protein-labeled healthy human astrocytes. (C) Merged images of ADC and healthy human astrocyte cells. (D) Co-cultured NCI-H2170 SCC and healthy human astrocyte cells treated with vehicle control. (E) Co-cultured NCI-2170 SCC and healthy human astrocyte cells treated with 1 μM PP121. Radius cell migration of (F) ADC cells and (G) SCC cells 96 h post-PP121 treatment. White arrows, migrating cells (A); dashed white circles, cell migration zone (F and G). Total magnification, x200 (A-E); x25 (F and G). Data shown are representative of three independent experiments. *P<0.05. Error bars ± SEM. ADC, adenocarcinoma; SCC, squamous cell carcinoma.

compensatory pathways that contribute to disease evolution and resistance. Thus, an improved targeted therapeutic approach, such as the use of single agents capable of simultaneously targeting multiple signaling pathways, is required. Results of the present study demonstrated that PP121, a dual inhibitor of tyrosine kinases and PI3K, decreased SCC and ADC cell viability, comparable with the anti-tumorigenic effects of anlotinib and famitinib in Phase III clinical trials and *in vivo* mouse studies, respectively (25,26). Notably, NSCLC cells demonstrated an increased sensitivity to PP121 exposure compared with famitinib, which required higher concentrations to reduce cell viability. This is likely attributed

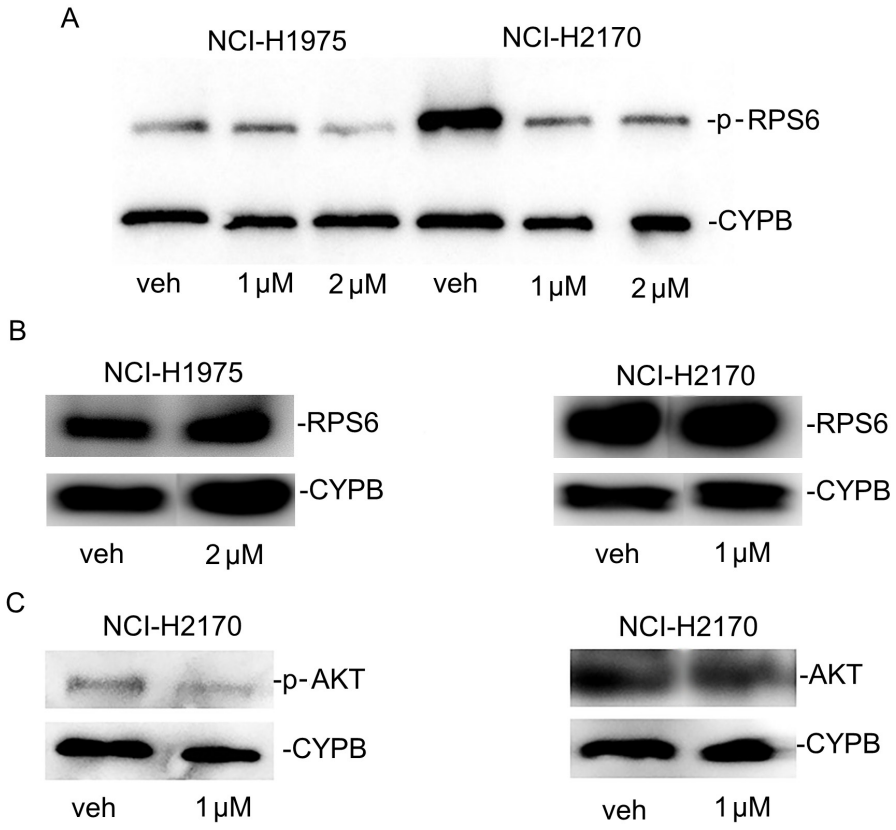

Figure 5. Downregulation of mTOR signaling effector proteins. PP121 decreased the expression of (A) p-RPS6 but not (B) unphosphorylated RPS6 in NCI-H1975 adenocarcinoma and NCI-H2170 SCC cells treated for 3 h. (C) p-Akt expression was downregulated in NCI-H2170 SCC cells after PP121 treatment but not unphosphorylated Akt. Cyclophilin B was used as a loading control. Data shown are representative of three independent experiments. p-, phosphorylated; RPS6, S6 ribosomal protein; SCC, squamous cell carcinoma.

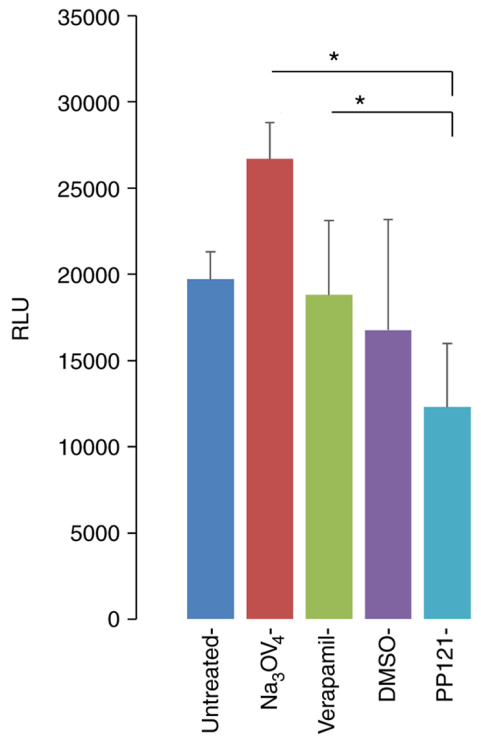

Figure 6. PP121 decreases P-glycoprotein activity. Na_3OV_4 (0.05 mM) and verapamil (0.1 mM) were used as positive controls. Samples were treated with PP121 (0.05 mM) and DMSO-vehicle control. *P<0.05. Error bars ± SEM. DMSO, dimethyl sulfoxide.

to the differential targets, including VEGF receptor 2/3, stem cell factor receptor and platelet-derived growth factor receptor.

A major therapeutic consideration for individuals diagnosed with NSCLC is the treatment of brain metastases, which occur in ~40% of cases. NSCLC brain metastases cause a median survival rate of 3-6 months, highlighting an urgent and unmet requirement for the identification of novel treatment strategies for this progressive disease (27). Astrocytes, a type of glial cell located in the brain, has been described as an inducer and cultivator of NSCLC brain metastasis, acting as the soil for NSCLC cells, as part of the seed and soil hypothesis (28-31). Results of the present study demonstrated that PP121 inhibited NSCLC migratory invasion in an astrocytic environment, and decreased P-gp activity. Notably, high expression levels of P-gp are present at the blood brain barrier and P-gp also plays a role in drug efflux functions (22). These factors may contribute to therapeutic resistance and reduce drug accessibility to tumors residing in the brain (23). Collectively, these findings provide pre-clinical experimental evidence that PP121 may be an effective strategy for the treatment of NSCLC brain metastasis, as a consequence of enhanced bioavailability and distribution.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate the anti-tumorigenic capacity of PP121 in NSCLC, and the subsequent ability to impede NSCLC brain metastases which contribute to high mortality rates. Moreover, the novelty of these findings provides insight into the pharmacokinetic properties of this dual kinase

inhibitor. Future experimental studies should evaluate the pharmacokinetics of PP121 *in vivo*, to further determine the bioavailability of PP121. More specifically, further pharmacokinetic parameters will be examined in a murine model system, including the half-life, time of maximum plasma concentration, area under the curve, peak concentration and elimination rates. In addition, further *in vivo* studies should assess the effects of PP121 on NSCLC brain metastases.

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Availability of data and materials

The author will make reagents and data available upon requests.

Authors' contributions

QQ was responsible for all aspects of this study that included conceptualization, experimentation, and data analysis. QQ confirms the authenticity of all the raw data. QQ read and approved the final manuscript and agrees to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that there are no competing interests.

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