Pazopanib diminishes non-small cell lung cancer (NSCLC) growth and metastases in vivo

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Keywords
Bioluminescence imaging; lung cancer; metastases; pazopanib.

Abstract
Background: Anti-angiogenesis has been demonstrated to have a critical role in lung cancer pathogenesis. Here, we characterized the effect of the small-molecule angiogenesis inhibitor pazopanib on non-small cell lung cancer (NSCLC) cells.

Methods: NSCLC cells were tested for viability and migration after incubation with varying concentrations of pazopanib. Further, the phosphorylation status of extracellular signal-regulated kinase, protein kinase B, and MEK were assessed in vitro.

For in vivo testing, mice grafted with NSCLC cell lines L9981 and A549 were treated orally with pazopanib.

Results: Pazopanib inhibits signaling pathways in tumor cells, thus blocking NSCLC cell growth and migration in vitro and inducing tumor cell arrest at G0/G1 phase. We show that pazopanib could inhibit tumor cell growth, decrease metastases, and prolong survival in two mouse xenograft models of human NSCLC.

Conclusion: These preclinical studies of pazopanib show the possibility of clinical application and, ultimately, improvement in patient outcome.

Introduction
Lung cancer is the leading cause of cancer mortality, resulting in approximately 1.6 million deaths annually around the world.1 In China, it is also the most commonly diagnosed cancer in men and the fourth most commonly diagnosed cancer and the second leading cause of cancer-related death in women.2 The one-year survival rate for lung cancer has been improved from 35% in 1975–1979 to 42% in 2002–2005.3 Even in patients with complete stage I, local or distant recurrence is commonly observed. Lung cancer will ultimately develop into metastases in 30 to 40% of patients, usually within 18–24 months of surgical resection.4,5

There is an urgent need for better treatment modalities for this disease. The dismal prognosis and limited tumor response to conventional cytotoxic agents pose a challenge to the scientific community to come up with alternative targets, heralding better clinical responses, as well as minimizing the adverse effects. Angiogenesis targeting agents were among the first to be recognized for potential benefit in non-small cell lung cancer (NSCLC).6 Angiogenesis is an important biological process and a relatively early event during lung cancer pathogenesis. An evolving understanding of tumor biology, specifically on selectively targeting anti-angiogenesis in NSCLC may bring us new targets for treating this malignancy.7

It is now known that vascular endothelial growth factor (VEGF) is one such key regulator of angiogenesis, and the role of the VEGF gene family in the regulation of angiogenesis has been intensively investigated in lung cancer.8 In humans, VEGFs and their receptors are expressed in lung carcinoma.9,10
Studies in NSCLC have reported that the overexpression of VEGF is associated with tumor progression and poor survival. Compelling evidence indicates that assembly and maturation of the vessel wall are highly complex processes requiring the coordinated action of platelet-derived growth factor B (PDGF-B) and other factors. Recent study has shown that platelet-derived growth factor A (PDGF-A) correlating with lymphatic angiogenesis, and the co-expression of PDGF-B and VEGFR-3 is strongly associated with poor survival in NSCLC patients. These findings suggest that combination therapies that target both VEGF and PDGF might be promising.

Pazopanib is an investigational, oral, once-daily, tyrosine kinase inhibitor. Its primary targets include vascular growth factor receptors (VEGFR-1, -2, and -3), platelet derived growth factor receptors (PDGFR-A, PDGFR-B), and c-KIT. The cell signalling pathways involving these molecules are important to the development of tumor and angiogenesis. The targets of pazopanib are widely accepted as cancers of the liver, lung, breast, kidney, bladder, ovaries, and colon. Consequently, the blockage of VEGFR, PDGFR, and KIT may prevent tumor growth and inhibit angiogenesis, thereby slowing or stopping the growth and spread of malignancies.

In previous studies, pazopanib demonstrated encouraging activity in patients with solid tumors, such as renal cell carcinoma (RCC), thyroid, breast, and cervical cancer. However, there are limited studies on the effects of pazopanib on NSCLC. Pazopanib was administered for a median of 16 days prior to surgery in patients with operable early stage NSCLC. Results showed that 86% of patients achieved a reduction in tumor volume. Therefore, these preliminary data have provided the evidence that pazopanib could be used effectively in patients with NSCLC. Our present study has determined the effects of pazopanib on different types of NSCLC, both in vitro and in vivo. We showed that pazopanib can be used effectively in patients with NSCLC through transcriptional regulation of certain cancer-related genes.

Materials and methods

Drugs

Pazopanib was provided by GlaxoSmithKline through a Material Collaborative Research and Development Agreement with NIH. For in vitro experiments, pazopanib was reconstituted in dimethyl sulfoxide (DMSO) and stored at −80°C. For in vivo experiments, pazopanib was suspended in 0.5% hydroxypropylethycellulose with 0.1% Tween 80 (vehicle). Protein kinase B (AKT), extracellular signal-regulated kinase (ERK) and MEK inhibitors were purchased from Cell Signaling Technology.

Cell lines

A549, YTLMC-90, and L9981, including luc-cells, were obtained from the Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment. All of these cells were cultured at 37°C in a 5% CO2, 95% air environment in humidified incubators. Standard procedures were used for MTT assay, Western blot analysis, scratch motility and invasion assay, and cell cycle and viability assays.

MTT assay

NSCLC cells were incubated with increasing concentrations of pazopanib or vehicle for 96 hours. Cells were assayed for viability using MTT. Data are represented as mean ± standard error of the mean (SEM) of three independent experiments.

Cell cycle analysis

Cells were treated with 5 μM pazopanib or vehicle (DMSO) for 72 hours and then analyzed by flow cytometry. The data shown are representative of three separate experiments.

Western blot assay

NSCLC cells were serum starved overnight and subsequently treated with 5 μM pazopanib, 10 μM pazopanib or DMSO for 24 and 48 hours. After treatment, the effects on signaling proteins were assayed. “p-” refers to phosphorylation of the residue(s) in parentheses. The data shown are representative of three separate experiments.

Scratch motility assay

NSCLC cells were seeded in 24-well plates and incubated for 12 hours. A line was scratched with a sterilized pin to wipe off the adherent cells in this line to create a wound. Cells were treated with 5 μM pazopanib or vehicle. Twenty-four hours later, the migration of NSCLC cells were assessed using an inverted light microscope at 50× magnification; the distances of NSCLC cells migrated were measured.

Invasion assay

Transwell chamber inserts (Gorning Inc, Corning, NY) with filter membrane pore size of 8 μm were used for invasion assay. NSCLC cells, at the concentration of 5 × 105/mL serum-free Dulbecco’s Modified Eagle’s Medium (DMEM), were incubated with five and 10 μm/L pazopanib or vehicle at the upper chamber. DMEM containing 10% fetal bovine serum was added to the lower compartment. Seventy-two hours later, cells that migrated through the permeable membrane were fixed in paraformaldehyde, stained with Giemsa,
and counted under an inverted light microscope at 100× magnification. Each assay was done at least three times.

**Animal experiments**

We used luc-cells for animal experiments, which were screened with G418 and could stably express the luciferase reported gene. Adult nude mice were injected in the right dorsal flank with 1 × 10⁷ luc-cells. Mice (n = 10) were anesthetized with isoflurane balanced with oxygen for five minutes and injected intraperitoneally with D-luciferin. Animal weights and tumor volumes (V = L × W² × π/6) were measured every other day and the tumor growth was monitored using bioluminescence imaging (BLI). All mice were housed in an isolated animal facility with free access to food and water. All procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee guideline.

**Statistical analysis and microarray data analysis**

Statistical significance of differences between pazopanib-treated and vehicle-treated control cultures was determined by means of an unpaired student t test. The minimal level of significance was P < 0.05. Overall survival (OS) in animal studies was measured using the Kaplan–Meier method, and results are presented as the medianOS, with 95% confidence intervals.

**Results**

**Pazopanib inhibits lung cancer cell growth**

The impact of pazopanib on NSCLC cell growth and survival was investigated on A549, YTLMC-90, and L9981 cells. The cells were treated with 10 μM pazopanib or vehicle for 96 hours. Pazopanib decreased growth and survival in all NSCLC cell lines tested, including A549, YTLMC-90, and L9981 cells. MTT results showed consistently decreased survival with an IC50 ranging between 4–6 μmol/L pazopanib in all cell lines (Fig 1A). Cell cycle analysis was performed using vehicle or 5 μM pazopanib. After 48 hours of pazopanib treatment, YTLMC-90, A549, and L9981 cells were induced into complete cell cycle arrest in G0/G1 with 26.6%, 13.2%, and 12.4%, respectively. The proportion of S phase was diminished in A549, H460, and 9981 cells by 12.9%, 7.1%, and 8.4%, respectively (Fig 1B, C). However, the cell cycle did not show that pazopanib triggered apoptosis in both cell lines (data not shown). These results suggest that pazopanib induced a cell cycle arrest, but not apoptosis in NSCLC cell lines.

**Pazopanib inhibits lung cancer cell migration and invasion in vitro**

Wound-healing and transwell cell invasion assays determined that pazopanib inhibited NSCLC cell migration and invasion. The migration of A549 was inhibited by pazopanib in a dose-dependent manner, detected by wound-healing assay (Fig 1D). Invasion through the Transwell chamber was significantly inhibited by pazopanib at the concentration of 10 μM in A549, YTLMC-90, and L9981 cells (Fig 1E).

**Pazopanib prolongs mouse survival in xenograft mouse models by inhibition of tumor growth and angiogenesis**

We transfected pGL4.17 (luc2/neo) plasmid into NSCLC cell lines of A549 and L9981, and used NSCLC xenograft mouse models to evaluate the efficacy of pazopanib in an in vivo imaging system. Immune-deficient beige-nude mice were inoculated in the flank with 1 × 10⁷ of two types of NSCLC cells (A549-luc, L9981-luc), which are cell lines stably expressing a luciferase reporter gene. When the tumors reached a palpable size, mice were randomized into a treated group (pazopanib 100 mg/kg) and a control group. Pazopanib or vehicle was orally administered daily. Tumor growths in the treated groups were significantly delayed compared with the control groups (Fig 2A–C, Fig 3A–B), and pazopanib also reduced the number of metastases in the xenograft mice (Fig 2D). Pazopanib prolonged the mouse survival in the treated group, and the mean OS was days 46.1 and 50.4 in the treated group of A549 and L9981 mice, versus days 55.3 and 56 in the control groups (Fig 2E, Fig 3C). The weight of the mice in the pazopanib treated and control groups were not affected by the tumor or pazopanib, indicating a low adverse drug reaction. Pazopanib also reduced the number of metastases in the xenograft mice, which was detected by BLI (Fig 2E). Above all, pazopanib inhibited NSCLC tumor growth and was associated with prolonged survival.

**Pazopanib selectively inhibits vascular endothelial growth factor (VEGF)-triggered phosphorylation and activation of downstream signaling molecules**

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), RAF/MEK/extracellular signal-regulated kinases (ERK) mitogen-activated protein kinase (MAPK) transmit signals from receptor tyrosine kinases to downstream effector networks regulating cell growth, metabolism, survival, and proliferation. We tested whether pazopanib could abrogate VEGF-induced tyrosine phosphorylation in NSCLC cells. We confirmed that Pazopanib inhibited both VEGF-triggered
tyrosine phosphorylation and activation of downstream signaling molecules (AKT, ERK, and MEK for pazopanib) (Fig 4). These results showed that pazopanib inhibits the VEGF-triggered pathway in NSCLC cells.

Discussion

NSCLC is a particularly aggressive cancer. Combination chemotherapy remains the standard therapy for patients with advanced or metastatic disease. However, despite the available treatment options for patients who progress beyond first-line therapy, prognosis remains poor. Angiogenesis is a tightly regulated process controlled by a delicate balance between pro- and antiangiogenic factors and their receptors; tumors induce angiogenesis by disrupting this balance and secreting various growth factors. Inhibition of tumor-related angiogenesis has become an attractive target for anticancer therapy. Antiangiogenic strategy includes monoclonal antibodies against VEGF and VEGF receptors and small molecule inhibitors of VEGF tyrosine kinase activity (tyrosine kinase inhibitors). Tyrosine kinase inhibitors are orally active, small molecules that represent a new class of drugs with a relatively high safety profile.

Pre-studies have shown that pazopanib, which is an oral small-molecule VEGF receptor inhibitor, was generally well tolerated and demonstrated single-agent activity in some patients with early-stage NSCLC. There have been validating target-specific responses to pazopanib, indicating that pazopanib treatment has persistent effect on lung cancer tissue. Therefore, we have characterized the effect of pazopanib on NSCLC, including A549, YTLMC-90, and L9981 cells. The result indicate that pazopanib inhibit tumor cell growth and migration of NSCLC, which is consistent with previous experiment results of RCC, myeloma, and lymphocytic leukemia. It was found that model substances with an inhibitory effect on VEGF receptor tyrosine kinases could induce cell cycle arrest and in vitro, which suggest that pazopanib induced a cell arrest, but not apoptosis. Moreover, we identified that pazopanib plays a role in the inhibition of tumor cell proliferation, accompanied by the inhibition of...
Figure 2. Pazopanib inhibits tumor growth and metastases and prolongs survival in L9981 xenograft mouse models. (a) Image of primary tumor. Color indicates presence of tumor in the L9981 xenograft model and pazopanib inhibits primary tumor growth. (b) Primary tumor volume of L9981. Tumor volume was measured every other day by using a caliper \((\text{calculated volume} = (4\pi/3) \times (\text{width}/2)^2 \times \text{(length}/2))\). Tumor volume is presented as means \(\pm\) SE. *<0.05 versus control group. (c) Bioluminescence imaging (BLI). Result are reported as mean relative light units (RLU) \(\pm\) standard deviation (SD) for the primary tumor of L9981 xenograft model. *<0.05 versus control group. (d) Image and number of metastases. The number of metastases detected using BLI were counted. Results are reported as mean \(\pm\) SD numbers of metastases. *<0.05 versus control group. (e) Survival curve of L9981. Survival was evaluated by using Kaplan–Meier curves and log-rank analysis.
MEK, AKT and ERK pathways. These data confirm the significance of pazopanib in NSCLC by inhibiting the PI3K/AKT and MARK pathway and preventing tumor development.

In vivo experiments reveal that pazopanib inhibits tumor growth, decreases metastases, and prolongs host survival in xenograft models of lung cancer cell lines, which can be continuously assessed by BLI. In our study, we constituted xenograft animal models using A549-luc and L9981-luc cells and a BLI system for monitoring the tumors by luciferase expressing cancer cells. The therapeutic effects of pazopanib can be assessed without having to sacrifice the mice because luc-expressing cells can be monitored frequently, quantitatively, and non-invasively with ease in real time. We observed the effects of pazopanib on tumor invasion and metastasis using a non-invasive method. Daily treatment with

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**Figure 3** Pazopanib inhibits tumor growth and prolongs survival in xenograft mouse models. (a) Image of primary tumor. Color indicates presence of tumor in the L9981 xenograft model and pazopanib inhibits primary tumor growth. (b) Primary tumor volume of A549. Tumor volume was measured every other day using a caliper (calculated volume = \(4\pi r^3/3\) \times (width/2)^2 \times (length/2)). Tumor volume is presented as means ± standard error (SE). *<0.05 versus control group. (c) Survival curve of A549. Survival was evaluated using Kaplan–Meier curves and log-rank analysis.
Pazopanib at a dose of 100 mg/kg can effectively inhibit tumor growth of two different human NSCLC xenograft models and prolong the life span of tumor-bearing nude mice, which may be attributed to the antiangiogenic effects of pazopanib. Between the treatment and control groups, there were no significant weight changes in vivo, which suggest that the drug may be well tolerated.

**Conclusion**

Pazopanib exerted a selective effect and justifies the expectation of a good risk-benefit ratio in humans. The availability of oral pharmaceutical forms of the drug is probably associated with good compliance. In conclusion, our observations show cytotoxicity of pazopanib in NSCLC cell lines and provide evidence that selective inhibition of the VEGF signaling pathway, indicating that pazopanib could be a promising method for the further improvement of NSCLC therapy.

**Acknowledgments**

We thank Zhihao Wu, Xuebing Li for the luc-cells. The authors thank YiLu for data and helpful discussions.

**Disclosure**

No authors report any conflict of interest.

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