

Crenolanib, a PDGFR inhibitor, suppresses lung cancer cell proliferation and inhibits tumor growth in vivo

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Abstract: Platelet-derived growth factor (PDGF) and its receptors (PDGFR), including PDGFR α and PDGFR β , play important roles in tumorigenesis, tumor progression, and the regulation of stromal cell function. Constitutive activation of PDGFR signaling, gene rearrangement, and activating mutations of PDGFR have been identified in various types of human tumors and malignancies. PDGFR α and PDGFR β belong to the family of type III receptor tyrosine kinases and, upon stimulation, activate downstream signaling cascades. Crenolanib is a specific tyrosine kinase inhibitor that targets and inhibits the kinase activity of PDGFR and the FMS-related tyrosine kinase 3. Its clinical efficacy in several human tumors is currently under investigation in Phase II clinical trials. In this study, we examined the potential role of crenolanib in the treatment of non-small-cell lung cancer (NSCLC). Using A549 cells as a model system, we have shown that crenolanib is capable of suppressing proliferation and inducing apoptosis in a dose-dependent manner. Crenolanib-treated cells have reduced migratory activity in response to inducers of chemotaxis. Furthermore, the in vivo antitumor activity of crenolanib was confirmed in an NSCLC xenograft tumor model. Injection of crenolanib significantly inhibited the growth of tumor mass by inducing apoptosis in tumor cells. Our results provide strong evidence supporting the use of crenolanib as a potential therapeutic agent in treating NSCLC. This work sets a foundation for further development of targeted and personalized therapeutics for lung cancer.

Keywords: platelet-derived growth factor receptor signaling, receptor tyrosine kinase, tyrosine kinase inhibitor, non-small-cell lung cancer, chemotherapy, targeted therapy

Introduction

Platelet-derived growth factor receptors (PDGFRs), including PDGFR α and PDGFR β , belong to the family of cell surface type III receptor tyrosine kinases (RTKs).^{1,2} Upon binding of the ligands, platelet-derived growth factors (PDGFs), the receptor complex is activated and the cytosolic domains serve as docking sites for coactivators and subsequently initiate downstream signaling cascades such as MAPK, PI3K, and STAT3 pathways.³⁻⁶ PDGFR signaling regulates a variety of biological processes, including cellular growth, cellular differentiation, cell migration, and angiogenesis.^{1,7} Deregulated PDGFR signaling has been implicated in the pathogenesis of several human diseases and malignancies.⁸⁻¹¹ For example, in patients with gastrointestinal stromal tumors, chronic myelomonocytic leukemia, and glioblastoma multiforme, mutations have been identified in the genes encoding PDGFR, which results in constitutive activation of the kinase activity, overstimulation of signal transduction, interaction with adjacent stroma and vasculature, and eventually autocrine regulation of tumor cell growth.¹²⁻¹⁵ Therefore, the

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PDGFR pathway represents a potential therapeutic target in patients harboring activating mutations of PDGFR.

With PDGFR as a promising target in cancer therapeutics, efforts have been made to develop specific kinase inhibitors that disrupt the receptor activation of PDGFR. Crenolanib is a specific tyrosine kinase inhibitor that targets and inhibits the kinase activity of PDGFR and the FMS-related tyrosine kinase 3 (FLT3).^{16–18} In a completed Phase I clinical trial, its pharmacokinetics and safety were evaluated and the recommended dosage was well tolerated by patients.¹⁶ The clinical efficacy of crenolanib in several human tumors is currently under investigation in multiple Phase II clinical trials. Unlike other RTK inhibitors such as imatinib mesylate and motesanib, which have been previously studied and tested in clinical trials for various types of cancer, crenolanib preferentially targets the phosphorylated, thus active, form of PDGFR that is often derived from constitutively active mutations of genes encoding PDGFR.^{17,19,20} Therefore, crenolanib may provide targeted therapy to patients with PDGFR mutations and have reduced toxicity compared with other broad-spectrum multikinase inhibitors.

In this study, we aim to test the potential efficacy of crenolanib in treating non-small-cell lung cancer (NSCLC), a collective type of epithelial lung carcinoma. Previous research has suggested a strong correlation between deregulated PDGFR signaling and NSCLC tumor progression.^{21–24} Expression and activity of PDGF and PDGFR have been used as prognostic indicators in lung carcinoma. The traditional treatment for NSCLCs involves surgical resection followed by applications of antiangiogenic agents such as targeted cytotoxic drugs.^{25,26} A549 cells were derived from human lung carcinoma and express PDGFR α in high levels.²⁷ By inhibiting the PDGFR signaling, our data demonstrated that crenolanib treatment suppressed the proliferation and migration of A549 cells and inhibited tumor growth in an NSCLC xenograft tumor model. Our study provides strong evidence supporting the use of crenolanib as a personalized and effective second-line therapy for NSCLC patients.

Materials and methods

Reagents, cells, and mice

Crenolanib (CP-868596) was purchased from Selleck Chemicals LLC (Houston, TX, USA). Human NSCLC cell line A549 was purchased from the American Type Culture Collection (Rockville, MD, USA). A549 cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin–streptomycin in a humidified incubator at 37°C in 5% CO₂. The athymic nude mice were purchased from Slack Company (Shanghai, People's Republic of China). Care and

treatment of mice were in accordance with the Animal Care and Use Committee guidelines of Hebei Medical University (Shijiazhuang, Hebei, People's Republic of China).

Cell viability assay

Cells were seeded in 96-well plates in triplicate and treated with different doses of crenolanib for 24, 48, or 72 hours as indicated in Figure 1. Cell viability at the end of each treatment was measured using CellTiter-Glo Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, an equal volume of the CellTiter-Glo reagent (100 μ L) was added directly to the cell culture in 96-well plates and the plates were placed on an orbital shaker for 2 minutes followed by incubation at room temperature for 10 minutes. Luminescence was read in a luminometer (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry analysis

Cells treated with different concentrations of crenolanib were harvested, fixed, and stained with propidium iodide using a BD Cycletest Plus DNA Reagent Kit (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were collected and analyzed for deoxyribonucleic acid (DNA) content using FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest Pro flow cytometry analytic software (Becton Dickinson).

Nuclei staining

Cells were seeded on polylysine-coated glass coverslips and treated with different concentrations of crenolanib as indicated in Figure 2. After 48 hours, cells were fixed with 4% paraformaldehyde for 10 minutes and stained with Hoechst (Sigma-Aldrich, St Louis, MO, USA) for 10 minutes. Images were obtained and analyzed using an Olympus BX51 fluorescent microscope (Olympus America Inc., Melville, NY, USA).

Transwell assay

1 $\times$ 10⁵ cells in RPMI media supplemented with 1% fetal bovine serum were seeded into the upper compartments of the 24-transwell Boyden chamber (Costar, Bedford, MA, USA). Different concentrations of crenolanib were added to cells as indicated in Figure 3. Medium (650 μ L) supplemented with 10% fetal bovine serum was loaded into the lower chambers to be used as a chemoattractant. After incubation for 12 hours, the nonmigrating cells were wiped off from the upper surface, and the migrated cells on the lower side were fixed, stained with 0.1% crystal violet, and photographed using an Eclipse LV150L microscope (Nikon Corporation, Tokyo, Japan). For absorbance measurement, the migrated cells on the lower side of the chamber were lysed with 10% acetic acid and measured at 595 nm.

Efficacy studies in a mouse xenograft model in vivo

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 allocated to the control group, low-dose crenolanib group (10 mg/kg), or high-dose crenolanib group (20 mg/kg) ($n=6$ per group). The vehicle for crenolanib treatment consists of 10% 1-methyl-2-pyrrolidinone and 90% polyethylene glycol 300 (Sigma-Aldrich). The tumor size and mouse body weight were measured every other day for about 2 weeks. The tumor volume was calculated as follows: $(\text{mm}^3) = (\text{width} \times \text{width} \times \text{length})/2$. After treatment, the mice were euthanized using carbon dioxide, and the tumors were harvested and analyzed.

Immunohistochemistry analysis

Tumor samples were harvested from mice, trimmed into 5 mm^3 pieces, and immediately fixed in 10% neutral buffered formalin. After fixation, the samples were transferred to 70% ethanol, embedded into paraffin blocks, and sectioned in 5 mm thicknesses. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using FragEL kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, slides were stained with TUNEL reagent for 1 hour followed by incubation with an Alexa Fluor 488-conjugated antibody for 30 minutes. The nuclei were stained with Hoechst for 20 minutes. The slides were photographed using an Olympus BX51 fluorescent microscope (Olympus America Inc.).

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. $P < 0.05$ was considered statistically significant.

Results

Crenolanib decreases NSCLC cell viability

PDGFR signaling promotes cell survival through activation of various downstream signal transduction cascades, including MAPK, PI3K, and STAT3 pathways. To determine whether inhibition of PDGFR signaling affects cellular proliferation, the A549 NSCLC cells were treated with crenolanib with a series of concentrations for 72 hours, and the cell viability was measured. We observed a dose-dependent inhibition of

cancer cell viability (Figure 1A). To find out the onset of action for crenolanib, A549 cells were treated with crenolanib (50 nM) for 24, 48, or 72 hours, followed by measurement of cell viability. The inhibitory effect of crenolanib was observed as early as 24 hours after treatment (Figure 1B).

Crenolanib induces apoptosis in NSCLC cells

PDGF is a potent mitogenic factor that enhances proliferation and antagonizes apoptosis. We further tested whether treatment of crenolanib was capable of inducing apoptosis in cancer cells. After treatment with crenolanib for 48 hours, the sub-G1 DNA content of A549 cells was measured as an indicator of late stage apoptosis.²⁸ Similar to the previous observation in cell viability, crenolanib caused increased sub-G1 DNA content in a dose-dependent manner, indicating that inhibition of PDGFR signaling promoted cells to undergo apoptosis (Figure 2A). Apoptosis-induced condensed and fragmented DNA is visualized in Figure 2B. Consistently, the number of cells

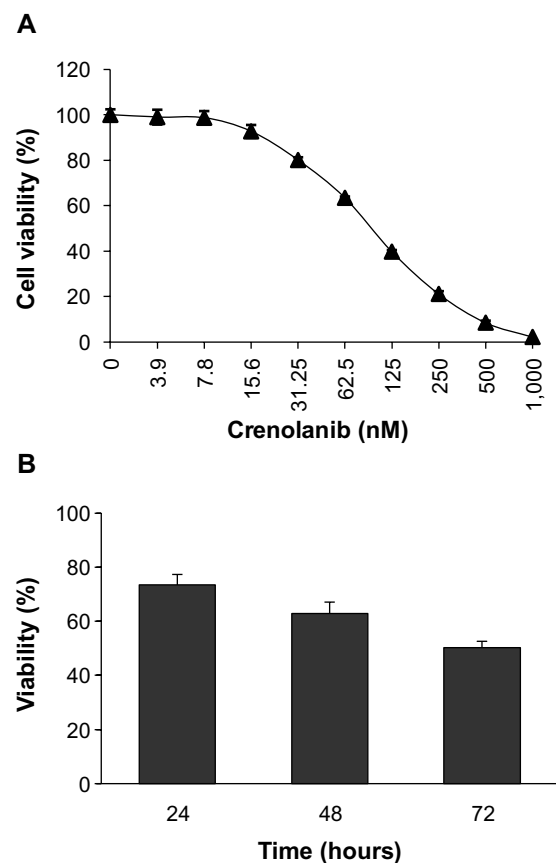


Figure 1 Crenolanib decreases non-small-cell lung cancer cell viability.

Notes: (A) A549 cells were treated with crenolanib (3.9–1,000 nM) for 72 hours followed by measurement with cell viability assay. (B) A549 cells were treated with crenolanib (50 nM) for 24, 48, or 72 hours followed by measurement with cell viability assay. In both experiments, viability of cells without crenolanib treatment was set to 100%.

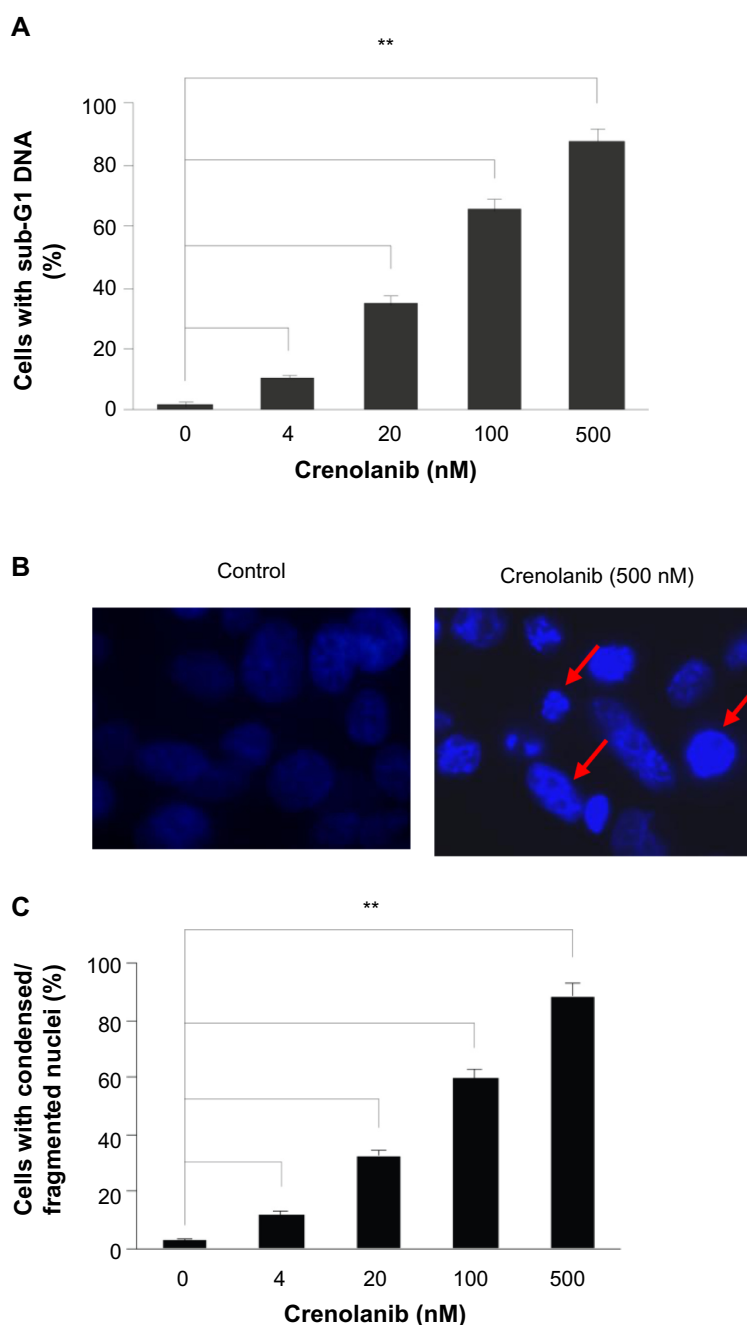


Figure 2 Crenolanib induces apoptosis in non-small-cell lung cancer cells.

Notes: (A) A549 cells were treated with crenolanib at the indicated concentrations for 48 hours followed by propidium iodide staining and flow cytometry analysis for sub-G1 deoxyribonucleic acid (DNA) content. (B) A549 cells were incubated with crenolanib (500 nM) for 48 hours. The nuclei were stained with Hoechst and analyzed using a fluorescent microscope. The representative images are shown. Red arrows indicate apoptotic cells with condensed or fragmented DNA. (C) The number of cells with condensed/fragmented nuclei was quantitated by counting in five random fields. Percentage was calculated and the averaged results are shown.

Note: ** $P < 0.01$.

with condensed/fragmented DNA increased proportionally with crenolanib treatment (Figure 2C).

Crenolanib inhibits cell migration in NSCLC cells

PDGF is a critical chemoattractant that binds to its cell surface receptor and regulates cell proliferation and chemotaxis.²⁹

Malignant tumor cells are capable of migrating and invading to a second site for tumorigenesis. PDGFR signaling plays an important role in this process and the following angiogenesis to contribute to the metastasis of tumors. Therefore, PDGF/PDGFR becomes an attractive target for cancer therapeutics.

We next investigated the effect of PDGFR inhibition on cancer cell migration. As shown in Figure 3A and B, treatment

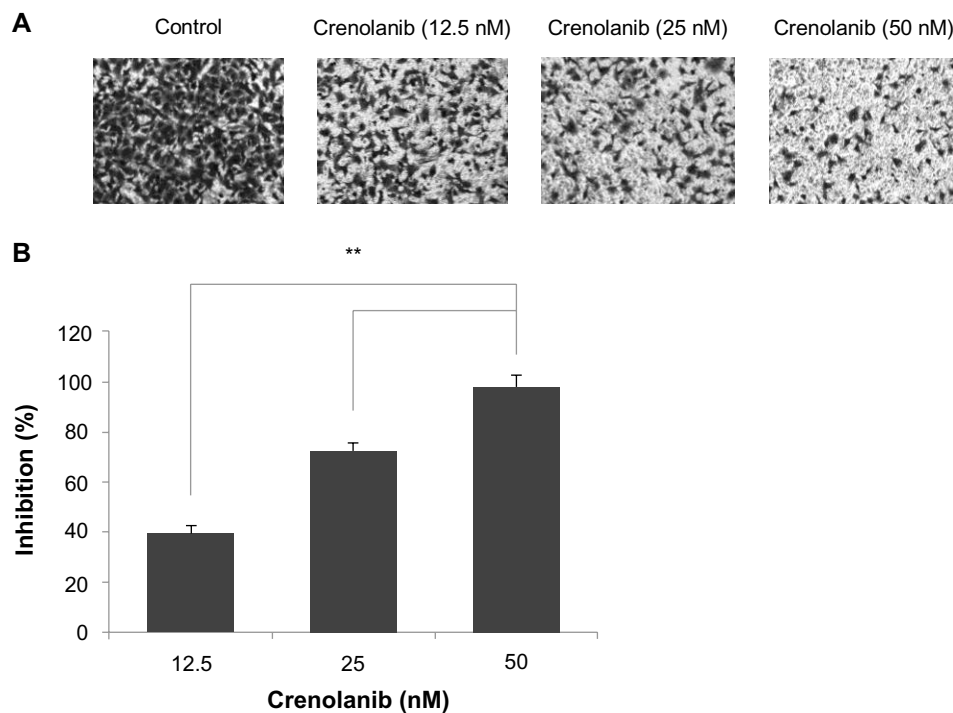


Figure 3 Crenolanib inhibits cell migration in non-small-cell lung cancer cells.

Notes: (A) A549 cells were treated with crenolanib (12.5, 25, or 50 nM) for 10 hours. The nonmigrated cells on the upper chamber of the transwell were removed and the migrated cells on the lower side were stained and photographed. The representative images are shown. (B) The migrated cells were lysed and colorimetric determination was made at 595 nm. Summary of the quantitation of the inhibition from transwell assay is shown.

Note: ** $P < 0.01$.

with crenolanib inhibited the migration of A549 cells in a dose-dependent manner. This inhibition is unlikely due to the cytotoxicity of crenolanib, as the duration of treatment was significantly shorter and the concentrations were significantly lower compared with the conditions used in Figure 1.

Crenolanib suppresses NSCLC tumor growth in vivo

We then went on to investigate the antitumor effect of crenolanib in vivo. A549 cells were injected into the axillary regions of athymic nude mice. After successful inoculation, crenolanib (10 mg/kg and 20 mg/kg) was administered, and the tumor volumes were measured every other day. Crenolanib significantly inhibited the growth of tumor mass, and the strongest inhibitory effect was observed with 20 mg/kg treatment (Figure 4A). Consistent with the in vitro studies, crenolanib induced massive apoptosis in tumor cells, which was demonstrated by significantly increased DNA fragmentation detected in both TUNEL assay and nuclei staining (Figure 4B). Furthermore, the dosage of crenolanib applied was well tolerated by recipient mice. No weight loss was observed during the course of treatment (Figure 4C). Taken together, these results indicate that crenolanib can

effectively suppress tumor growth in vivo by inducing apoptosis of tumor cells.

Discussion

Activating mutation in genes encoding critical signaling molecules has been identified in patients with different types of tumors, including NSCLC. For example, gene rearrangements and point mutations in the gene encoding the epidermal growth factor receptor have been found in a subset of NSCLC patients, and patients with such mutations had significantly increased response to treatment of gefitinib, a specific RTK inhibitor targeting the epidermal growth factor receptor.^{30–33} In another study, mutations in *PDGFA*, the gene encoding for PDGF α , were identified in 13% of NSCLC patient tumor samples.³⁴ Therefore, by selecting the RTK inhibitors with specific targeting capacity, effective chemotherapy can be directed to a targeted population.

Aberrant PDGFR signaling due to overexpression of the ligand PDGF or activating mutations of PDGFR has been suggested to be a critical player in tumorigenesis and angiogenesis of a variety of tumors. Blocking the PDGFR signaling by either specific neutralizing antibodies that target the receptors or RTK inhibitors that disrupt the kinase activity

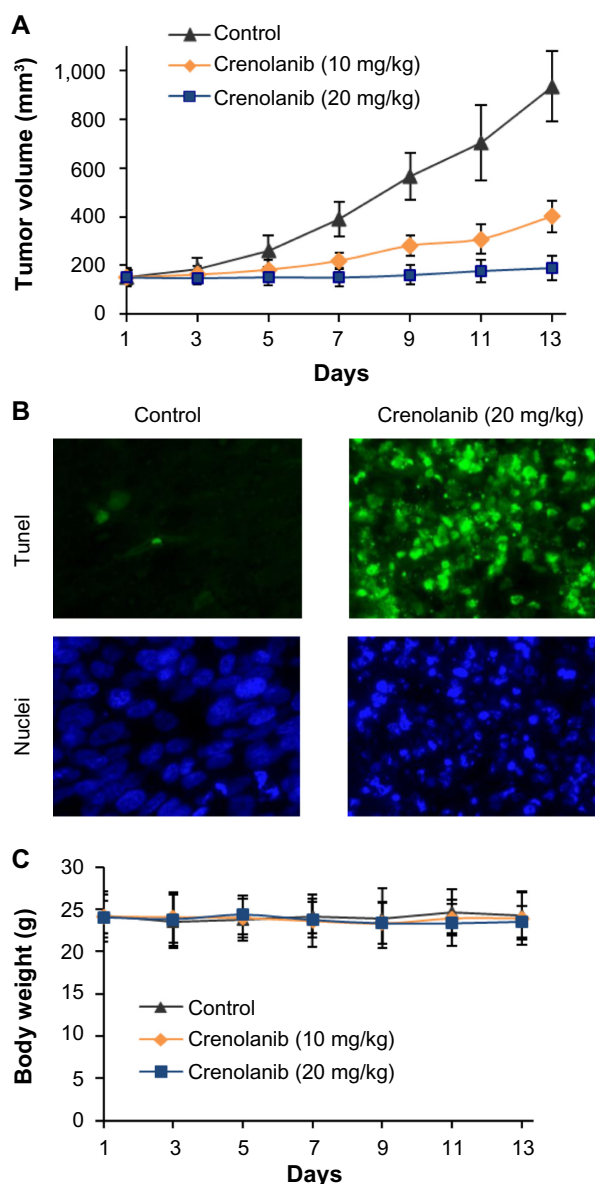


Figure 4 Crenolanib suppresses non-small-cell lung cancer tumor growth in vivo and induces tumor cell apoptosis.

Notes: (A) After inoculation of A549 cells, crenolanib (10 mg/kg and 20 mg/kg) was injected into the nude mice. The tumors were measured every other day for about 2 weeks and the tumor volumes are shown. (B) After treatment of crenolanib for 2 weeks, tumors were harvested. Crenolanib induced apoptosis of tumor cells measured with terminal deoxynucleotidyl transferase dUTP nick end labeling assay (green) and nuclei staining with Hoechst (blue). (C) Body weight of mice during the course of treatment was measured every other day. Crenolanib had no significant cytotoxic effects on the body weight of mice during the treatments.

has proven to have clinical efficacy in treating a number of human malignancies, including NSCLC.^{35–38} Criteria for selecting candidate RTK inhibitors in cancer therapy include minimal toxicity and higher affinity with mutated forms of the kinase targets that are frequently present in cancer patients. Crenolanib, a potent RTK inhibitor with narrow and selective reactivity against both wild-type and mutated FLT3 and PDGFR (including PDGFR α and PDGFR β), represents

the first anticancer RTK inhibitor with predictably minimal toxicity. Previous research and clinical trials have validated the therapeutic efficacy of crenolanib in gastrointestinal stromal tumors and human acute myeloid leukemia.^{16,18–20} Our results provide additional evidence to demonstrate that crenolanib can yield clinical benefit to NSCLC patients with deregulated PDGFR signaling.

In this study, we found that treatment with crenolanib inhibited growth and induced apoptosis of NSCLC cells in a dose- and time-dependent manner, which was consistent with a previous study in other tumor cell lines.¹⁷ Treating tumor cells with crenolanib resulted in decreased phosphorylation and thus reduced activity of downstream signaling molecules, including AKT3, MAPK, and STAT5,¹⁷ which we believe led to inhibition of cell proliferation. However, the mechanism of crenolanib-induced apoptosis has not been elucidated. It will be informative to examine the status of apoptosis marker molecules before and after crenolanib treatment. Caspase-3 cleavage, ratio of anti- and proapoptotic proteins, level of HSP90 client proteins, and other apoptosis-related pathways should be thoroughly assessed. In addition, crenolanib-treated NSCLC cells displayed reduced migratory activity in response to inducers of chemotaxis, which implies impaired angiogenesis. Although this hypothesis is tempting, solid data from in vitro and/or in vivo angiogenesis assays are required. Furthermore, injection of crenolanib significantly inhibited the growth of NSCLC tumor cells in vivo. This is the first report that documents the antitumor activity of crenolanib in NSCLC cells. This work could set a foundation for further development of targeted and personalized therapeutics for lung cancer.

The role of PDGFR signaling in interaction between tumor cells and stroma has also been suggested by previous studies.^{11,39,40} Stromal fibroblasts are recruited to the site of the tumor and contribute to the growth of tumor mass and the angiogenic process. In a mouse xenograft model of lung carcinoma, highest expression and activity of PDGFR α have been detected in the tumor-surrounding stroma, and when the PDGFR signaling is blocked by neutralizing soluble recombinant PDGFR, tumor growth and tumor invasion are significantly inhibited.¹¹ These findings indicate that suppressing PDGFR signaling has an antitumor effect on both tumor cells and the microenvironment. Further investigation can be performed to assess the effect of crenolanib in tumor stroma and fibroblast recruitment. If crenolanib could affect both tumor survival and the angiogenic process, it may lead to great clinical benefit in patients with advanced or metastasized NSCLC.

Disclosure

The authors declare no conflicts of interest in this work.

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