The Antiparasitic Drug, Potassium Antimony Tartrate, Inhibits Tumor Angiogenesis and Tumor Growth in Non-Small Cell Lung Cancer

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List of nonstandard abbreviations:

NSCLC non-small cell lung cancer cells
HUVECs human umbilical vein endothelial cells
PAT potassium antimony tartrate
VEGF vascular endothelial cell growth factor
FGF fibroblast growth factor
RTKs receptor tyrosine kinases
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>RTKIs</td>
<td>receptor tyrosine kinase inhibitors</td>
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<td>EGM-2</td>
<td>endothelial cell growth medium-2</td>
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**Recommended section assignment:** Drug Discovery and Translational Medicine
Abstract

Repurposing existing drugs not only accelerates drug discovery but rapidly advances clinical therapeutic strategies. In this article, we identified potassium antimonyl tartrate (PAT), an antiparasitic drug, as a novel agent to block angiogenesis by screening Food and Drug Administration-approved chemical drugs. By comparing the cytotoxicity of PAT in various non-small cell lung cancer cells (NSCLC) with that observed in primary cultured human umbilical vein endothelial cells (HUVECs), we found that HUVECs were much more sensitive to the PAT treatment. In *in vivo* tumor xenograft mouse models established either by PAT-resistant A549 cells or by patient primary tumors, PAT significantly decreased the tumor volume and tumor weight of NSCLC xenografts at dose of 40 mg/kg (i.p., daily) and, more importantly, augmented the antitumor efficacy of cisplatin chemotherapy. Remarkable loss of vascularization in the treated xenografts indicated the *in vivo* anti-angiogenesis property of PAT, which was well-correlated with its tumor growth inhibition in NSCLC. Furthermore, in the *in vitro* angiogenic assays, PAT exhibited dose-dependent inhibition of HUVEC proliferation, migration and tube formation in response to different stimuli. Consistently, PAT also abolished the vascular endothelial cell growth factor (VEGF)-induced angiogenesis in the Matrigel plugs assay.

Mechanistically, we found that PAT inhibited the activities of several receptor tyrosine kinases and specifically blocked the activation of downstream Src and focal adhesion kinases in HUVECs. Taken together, our results characterized the novel antiangiogenic and antitumor function of PAT in NSCLC. Further study of PAT in
anticancer clinical trials may be warranted.
Introduction

Angiogenesis, the generation of new blood vessels from the pre-existing vasculature, is responsible for the metabolic demands of all types of solid tumors (Hanahan and Folkman, 1996). Tumors secrete several proangiogenic factors stimulating endothelial cell migration, proliferation and capillary-like tube formation, thus satisfying the requirement for tumor growth and metastasis (Asahara et al., 1995; Niki et al., 2000). Inhibition of growth factor signals requisite for endothelial cells has become a therapeutic strategy (Algire et al., 1954; Folkman, 1971). In fact, no identical pattern of angiogenesis has been found in tumors, indicating that angiogenesis is comprehensively triggered and mediated by different pathways (Lund et al., 1998). Several regulatory and signaling molecules controlling angiogenesis have been characterized, including potent growth factors [e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and angiopoietin] (Ferrara and Kerbel, 2005; Presta et al., 2005; Saharinen et al., 2011), receptor tyrosine kinases (RTKs) (Jeltsch et al., 2013), and molecules involved in signaling cascades, such as Src kinase, focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), and AKT (Downward, 2003; McLean et al., 2005; Kim et al., 2009; Liu et al., 2009; Lemmon and Schlessinger, 2010). These molecules have been exploited for their potential as drug targets for anti-angiogenic therapy. It is generally agreed that multiple signaling pathways should be coordinately blocked in the treatment of solid tumors to achieve maximal angiogenesis inhibition.
Lung cancer, a type of heterogeneous solid tumors, is the leading cause of cancer-related deaths in both sexes worldwide (Siegel et al., 2013). Non-small cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancer cases, possesses several critical hallmarks, such as sustained angiogenesis, addiction to aerobic glycolysis and self-sufficiency in growth signals (Siegel et al., 2012). In 2006, the US Food and Drug Administration (FDA) approved bevacizumab, a VEGF-A monoclonal antibody, as the first anti-angiogenic agent for treatment of patients suffering from NSCLC (Ferrara et al., 2004; Sandler et al., 2006). However, toxicities, substantial cost and modest survival rates successively occurred (Ranpura et al., 2011). Recently, small molecule receptor tyrosine kinase inhibitors (RTKIs) and monoclonal antibodies targeting endothelial cells have shown promise in NSCLC treatment when combined with standard chemotherapy, but their efficacy remains to be decided (Sennino and McDonald, 2012). A second phase III trial of sorafenib, a multi-targeted antiangiogenic RTKI, combined with gemcitabine/cisplatin in a first-line setting in NSCLC patients has shown an improvement in progression-free survival rate but not in overall survival rate (Paz-Ares et al., 2012). More affordable and effective antiangiogenic agents are still greatly desirable.

Over the past decade, an effective approach to accelerate drug development has been proposed and gained considerable attention (Chong and Sullivan, 2007). This approach is termed “drug repurposing”, “drug repositioning”, “drug re-profiling” or “indication switching”. Due to established drugs possessing well-known safety and pharmacokinetic profiles, identification of new applications of approved drugs could
be rapidly evaluated in Phase II and III clinical studies. Recently, many existing non-cancer drugs have entered into clinical trials for cancer-related treatment, such as thalidomide (D’Amato et al., 1994), nonsteroidal agents (Jones et al., 1999) and rapamycin (Guba et al., 2002), in which promising clinical results have been achieved. Following the hypothesis of drug indication switching, we performed the screening of existing drugs against tumor angiogenesis using a panel of NSCLC cells and primary cultured human umbilical vein endothelial cells (HUVECs). We found that the cytotoxicity of potassium antimonyl tartrate (PAT), a long-term use antiparasitic, specifically targeted HUVECs, rather than NSCLC cells, suggesting its potential antiangiogenic activity.

PAT is a trivalent antimonial salt that has previously been applied as an antiparasitic agent in the treatment of leishmaniasis and schistosomiasis for more than 100 years. PAT interferes with phosphofructokinase and the thiol redox potential of the parasite (Schulert et al., 1966). Initially reported by Lecureur and colleagues, PAT, which is similar to As₂O₃, was found to be cytotoxic towards acute promyelocytic leukemia (Lecureur et al., 2002a; Lecureur et al., 2002b). However, a more complete characterization of its anticancer role is still limited, especially in solid tumors. In this article, we evaluated the putative antiangiogenic activities of PAT in a series of in vitro and in vivo tumor preclinical models. Our data revealed that PAT notably retards tumor growth and potentiates the efficacy of standard chemotherapy in human NSCLC by blocking tumor angiogenesis.
Materials and Methods

Reagents

PAT (purity > 99%) was obtained from Sigma (St. Louis, MO), and PAT stock solutions were prepared with dimethyl sulfoxide (DMSO; Sigma) for in vitro assays. For in vivo experiments, PAT and cisplatin (Sigma) were diluted in phosphate buffered saline (PBS). Recombinant human VEGF165 and bFGF146 were gifted from the Experimental Branch of the NIH (Bethesda, MD), and Angiogenin-1 (Ang-1) was purchased from RayBiotech (Norcross, GA). Growth factor–reduced Matrigel was purchased from BD Biosciences (San Jose, CA), and calcein-AM was obtained from Sigma. The human RTK phosphorylation antibody array was purchased from RayBiotech. For Western blotting analysis, anti-VEGFR2, anti-pTyr1175-VEGFR2, anti-pTyr996-VEGFR2, anti-Tie2, anti-pTyr416-Src, anti-Src, anti-pTyr397-FAK, anti-FAK, anti-pThr202/Tyr204-ERK, anti-ERK1/2, anti-pSer473-AKT, anti-AKT, and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). For the immunofluorescence and immunohistochemistry analyses, the antibody against CD31 was purchased from Abnova (Taipei, Taiwan), and the Ki-67 antibody was purchased from Cell Signaling Technology. The non-radioactive cell proliferation kit was purchased from Promega (Madison, WI). All other chemicals used in this study were ACS reagents.

Cell lines and cell culture

Primary human umbilical vascular endothelial cells (HUVECs) were kindly provided...
by Dr. Xinli Wang (Baylor College of Medicine, Houston, TX) in 2008 and cultured in endothelial cell growth medium-2 (EGM-2; Lonza, Basel, Switzerland) according to the manufacturer's instructions. Angiogenesis stimulation medium was defined as EGM-2 basal media containing 0.5% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and supplemented with the following growth factors: VEGF (50 ng/mL), bFGF (12 or 50 ng/mL) or Ang-1 (200 ng/mL). NSCLC cell lines (A549, PC9, NCI-H460, NCI-H441, NCI-H522 and NCI-H661) were obtained from the American Type Culture Collection (Manassas, VA) in 2010 and cultured in RPMI 1640 medium containing 10% FBS. Human fetal lung fibroblast cells (WI-38) and human pulmonary fibroblast cells (MRC-5) were obtained from the National Platform of Experimental Cell Resource for Sci-Tech (Shanghai, China) in 2012 and cultured in Eagle’s Minimal Essential Media containing 10% FBS and 1% penicillin/streptomycin, kanamycin sulfate and glutamax. All cell lines have been tested and authenticated by DNA (short tandem repeat genotyping) profiling. The last test was performed in March 2013. All of these cells were grown at 37°C under a humidified 95:5 (%; v/v) mixture of air and CO₂.

Cell proliferation assays

HUVECs (2-5×10³ cells/well) were first starved and then treated with various concentrations of PAT in either EGM-2 or angiogenesis stimulation medium for 72 h. A549, PC9, NCI-H460, NCI-H441, NCI-H522, NCI-H661, WI-38 and MRC-5 cells (4-6×10³ cells/per well) were directly exposed to indicated concentrations of PAT in full growth medium for 72 h. To determine cell viability, we used a non-radioactive
cell proliferation kit containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and a VERSAmax microplate reader (Molecular Devices; Sunnyvale, CA). The half inhibitory concentration (IC₅₀) values were calculated by Graphpad Prism software (GraphPad Software, La Jolla, CA). Each assay was performed at least three times and each treatment had four repeats.

**Animal studies**

BALB/cA nude mice, immunodeficient NOD/SCID mice and C57BL/6 mice used in the present study were purchased from National Rodent Laboratory Animal Resources (Shanghai, China) and maintained in a laminar airflow cabinet under specific pathogen-free conditions and a 12 h light–dark cycle. All treatments were administered according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals, and all the protocols were approved by East China Normal University.

**Human NSCLC tumor xenograft mouse model**

*Cultured NSCLC cell xenograft mouse model.* The cultured NSCLC cell xenograft mouse model was constructed as described previously (Pang et al., 2009b). A549 cells (5×10⁶ cells/mouse) were injected s.c. into the right flank of 5- to 6-week-old male BALB/cA nude mice. Tumor size was evaluated every other day by caliper measurements, and the approximate volume of the tumor mass was calculated using the following formula: \( V = (L + [W])^2 \times 0.52 \); where \( L \) is the longest diameter of the
tumor; and W is the shortest diameter of the tumor. Tumor-bearing mice with an average tumor volume exceeding approximately 150 mm$^3$ were randomly divided into the following groups: vehicle control (PBS; i.p.; daily); PAT (40 mg/kg; i.p.; daily); cisplatin (4 mg/kg; i.p.; weekly); or a combination of PAT and cisplatin ($n=8$ each group). All groups were continuously administered for 34 d, and the body weight was measured every other day. At the end of the experiment, mice were sacrificed. Solid tumors were removed, weighed and processed for immunofluorescence and immunohistochemistry.

**Primary NSCLC tumor xenograft mouse model.** The primary mouse model was established as previously described (Migliardi et al., 2012). Surgical samples of patient primary NSCLC presenting adenocarcinoma histology were obtained from treatment-naïve patients at the Shanghai Changzheng Hospital (Shanghai, China). Prior written informed consent was obtained from all patients, and the study protocol was approved by the local hospital ethics committee. In brief, surgically removed tumor tissues were cut into fragments of approximately 15 mm$^3$ and implanted using a trocar needle s.c. into 2-3 male NOD/SCID mice within 2 h. Transplanted mice bearing xenografts were observed weekly. When tumor volume reached approximately 1000 mm$^3$, primary xenografts at the exponential growth phase were removed by serially passage to other immunodeficient mice. After three consecutive mouse-to-mouse passages, the xenograft was considered to be stabilized and submitted to mutation analyses to verify that it consistently maintained clinical NSCLC molecular features. “Hot spot” mutations in $EGFR$ (exons 18, 19 and 21),
KRAS (exons 2 and 3), PIK3CA (exons 9 and 20), and BRAF (exon 15) were screened by direct sequencing. Numerous samples from early passages were immediately stored in liquid nitrogen and used for further experiments. Xenografts at passage 3 were used in this study. Mice were treated using the same conditions as in the cultured NSCLC cell xenograft mouse model. On day 28, the solid tumors were harvested, weighed and analyzed for indicated protein markers.

**Immunofluorescence and immunohistochemistry**

As described previously (Qian et al., 2013), the solid tumors were fixed, dehydrated, and embedded in paraffin. Serial sections (4 μm thick) were cut, mounted on adhesion microscope slides, dewaxed in xylene and rehydrated in gradient alcohols. After quenching the endogenous peroxidase activity and antigen retrieval, the sections were blocked with 1% bovine serum albumin and incubated with primary antibody overnight. For tissue localization of Ki-67 and CD31, sections were incubated with secondary antibody at room temperature (20 min for immunohistochemistry and 1 h for immunofluorescence). Sections were then visualized by the streptavidin-biotin-peroxidase complex method with 3,3′-diaminobenzidine and counterstained with hematoxylin (immunohistochemistry) or DAPI (immunofluorescence) as the chromogen. Each group had at least six samples, and sections from the same tumor were processed in parallel from at least three samples. Images were taken using a leica DM4000B photo microscope (magnification of 200x).
Cell migration assays

**Endothelial cell migration assay.** HUVECs were allowed to grow to full confluence in 6-well plates precoated with 0.1% gelatin (Sigma). To inactivate cell proliferation, HUVECs were first synchronized with EGM-2 basal medium containing 0.5% FBS for 6 h. The cells were then wounded with pipette tips and washed with PBS followed by exposure to EGM-2 medium containing PAT (0-10 μmol/L) or DMSO. Following 8-10 h of migration, cells were labeled with 2 mg/mL calcein-AM for 30 min and visualized using an inverted microscope (Olympus; magnification of 64×). Migrated cells in four random fields were quantified by manual counting. Three independent experiments in triplicate were performed.

**Endothelial cell Transwell migration assay.** The chemotactic motility of HUVECs was determined using a Transwell migration assay (BD Biosciences; San Jose, CA) with 6.5-mm diameter polycarbonate filters (8-mm pore size) as described previously (Pang et al., 2009a). Briefly, the filter was coated with gelatin. Angiogenesis stimulation medium (500 μL) was placed into the bottom chambers. HUVECs (4-5×10^4) were incubated with indicated concentrations of PAT in EGM-2 containing 0.5% FBS for 30 min at 37°C before seeding into the upper chambers. After 8-10 h of incubation, non-migrated cells in the upper chamber were removed using a cotton swab. The migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an Olympus inverted microscope (magnification of 160×). Migrated cells in four random fields were quantified by manual counting. Three independent experiments were performed.
Endothelial cell capillary-like tube formation assay

The tube formation assay was conducted as described previously (Lee et al., 1999). Briefly, each well of a 48-well plate was coated with 80 μL of growth factor reduced-Matrigel and incubated at 37°C for polymerization. HUVECs were trypsinized and suspended in either EGM-2 medium or angiogenesis stimulation medium. Various concentrations of PAT were added to the cells for 1 h at 37°C before seeding. Cells were then plated onto the Matrigel layer at a density of 4-6×10⁴ cells per well for 4-6 h. Cells with tube networks were visualized with 2 μg/mL calcein-AM using an Olympus fluorescence microscope (magnification of 64×). Three independent experiments were performed.

In vivo Matrigel plug assay

The Matrigel plug assay was performed as described previously (Pyun et al., 2008). Briefly, 0.5 mL of Matrigel in the presence or absence of 60 ng of VEGF, 20 units of heparin and indicated concentrations of PAT (15 μmol/L and 50 μmol/L) was s.c. injected into the ventral area of 6-week-old C57BL/6 mice (n=6). After one week, the mice were sacrificed and the plugs were removed. The plugs were then fixed and embedded. For identification of the infiltration of newly formed endothelial cells, a von Willebrand factor (vWF) antibody (Chemicon, Temecula, CA) was used for immunohistochemistry. Images of neovasculature were taken using a Leica DM 4000B photo microscope (Solms, Germany; magnification of 400×).

Phospho-receptor tyrosine kinase analysis
The human RTK phosphorylation antibody array was used according to the manufacturer’s protocol. Briefly, membranes with 71 distinct RTKs were blocked for 1 h with gentle shaking. Cells were harvested using a cell scraper and lysed in modified radioimmunoprecipitation (RIPA) buffer (20 mmol/L Tris, 2.5 mmol/L EDTA, 1% TritonX-100, 1% deoxycholate, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L Na$_4$P$_2$O$_7$, and 1 mmol/L phenylmethylsulfonyl fluoride) supplemented with a phosphatase and protein inhibitor cocktail (Calbiochem; San Diego, CA). The lysates were gently rocked at 4°C for 30 min and centrifuged at 14,000 × g for 15 min. The sample protein concentrations were quantified using a micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Lysates with 500 μg of total protein were subjected to the membranes at 4°C. Membranes were then incubated with biotin-conjugated antibodies and exposed to an enzyme horseradish peroxidase-conjugated streptavidin. The signals were visualized by the Li-Cor Odyssey Infrared system (LI-COR Biosciences, Lincoln, Nebraska).

**Western blot analysis.**

To determine the effects of PAT on the angiogenesis signaling cascade, HUVECs were first starved in serum-free EGM-2 for 10 h and then pretreated with or without various concentrations of PAT followed by stimulation with angiogenesis stimulation medium for 2-30 min. The whole-cell extracts were prepared in RIPA buffer. Approximately 40-50 μg of cellular protein from each treatment was applied to 6-8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Billerica, MA). Membranes were incubated overnight with primary antibodies
followed by incubation with fluorescent secondary antibodies (1/10,000; Sigma).

After several washes, the signals were detected by

the Li-Cor Odyssey Infrared system.

**Statistical analysis**

The statistical tests were carried out using Microsoft Excel and GraphPad Prism

Software (version 5.0). Comparisons between groups were performed using one-way

ANOVA followed by Student’s *t* test. Data were presented as means ± standard

deviation. *P*-values less than 0.05 were considered statistically significant (*P*<0.05; **P*<0.01; ***P*<0.001).
Results

PAT exerts cytotoxicity directly towards HUVECs

Chong and colleagues successfully repurposed the antifungal drug, itraconazole, as a potent angiogenesis inhibitor (Chong et al., 2007). Currently, there are several active clinical trials evaluating itraconazole as a cancer therapeutic agent (Nacev et al., 2011). Here, we applied a systematical screening by comparing differential sensitivities of endothelial cells and various NSCLC cells to 1280 chemical compounds approved by the FDA. We found that HUVECs were extremely susceptible to 4 drugs, including PAT, dactinomycin, thimerosal and phenylmercuric acetate compared with NSCLC cells (data not shown). In this article, we selected the antiparasitic agent, PAT, as a potential antiangiogenic candidate for further evaluation.

PAT selectively inhibited the growth of endothelial cells, but it had little direct anti-proliferative activity towards a panel of NSCLC cells with distinct molecular characteristics, including A549, PC9, NCI-H460, NCI-H441, NCI-H522, and NCI-H661 cells. As shown in Table 1, the IC$_{50}$ of PAT in endothelial cells was approximately 4.32 µmol/L, which was at least 10-fold less than that in NSCLC cells, suggesting that PAT possessed relatively specific and directional cytotoxicity toward endothelial cells. To further evaluate the specificity of the inhibitory activities of PAT on activated endothelial cells, we examined its efficacy under three types of angiogenic stimuli, including EGM-2 (containing multiple growth factors), VEGF and
bFGF. Our results showed that PAT exhibited equipotent inhibition of endothelial cell growth under these stimuli conditions, thus indicating that the agent might target the common growth factor-mediated angiogenesis signaling pathway.

The A549 and NCI-H441 cancer cells were highly resistant to PAT as their IC₅₀ values were not measurable below the maximum tested concentration of 100 µmol/L. To avoid the concern of direct inhibition of PAT on tumor cells in vivo, we conducted a tumor xenograft mouse model using PAT-resistant A549 lung cancer cells.

**PAT inhibits the growth of NSCLC cells either as a single agent or in combination with cisplatin chemotherapy**

To investigate the in vivo anticancer effects of PAT, we established two types of xenograft mouse models. One type was constructed by PAT-resistant A549 (KRAS mut) lung cancer cells, and the other type used patient primary tumors. It is generally agreed that the patient-derived primary xenograft mouse model is the most favorable preclinical model for drug discovery as the explants of these models are biologically stable. The primary NSCLC tumors we used in this study represented adenocarcinoma with a KRAS mutation in codon 12 (a GGT→GAT transversion; Gly to Asp). Mice bearing established progressive tumor xenografts were i.p. administered daily with PAT at a dose of 40 mg/kg. Our results showed that administration of PAT significantly decreased the tumor growth rates of both xenografts (Fig. 1A and Fig. 1B). Compared with the vehicle group at the end of the treatment, single-agent therapy with PAT in A549 tumors and primary tumors resulted in 46.8 and 42.2%
inhibition of tumor volume \((P < 0.05)\) as well as 49.4 and 43.0\% inhibition of tumor weight \((P < 0.05)\), respectively. Such inhibitory effect of PAT was comparable to that of cisplatin monotherapy. When comparing with either single-agent therapy, addition of PAT to the cisplatin regimen resulted in a dramatic tumor growth inhibition. The combinational regimen led to 68.1\% inhibition of tumor volumes in A549 tumors \((P < 0.001)\) and 82.71\% inhibition of tumor volumes in patient primary tumors \((P < 0.001)\), suggesting a potent and synergistic anticancer action. The effect of combination therapy was quiet durable as it was maintained throughout the therapy period.

To further evaluate the health status of mice treated with PAT, the average body weight of mice was monitored every other day throughout the entire experiment. Our results showed that no obvious differences were found in mouse body weight among the PAT-treated groups and the control group. In contrast, the body weight of mice treated with cisplatin was marginally decreased \((P>0.05)\). Furthermore, no adverse effects in other gross measures, such as skin ulcerations or toxic death, were observed in PAT-treated groups. These data provided an indication that PAT is safe at the tested dose and that the inhibition of tumor growth by PAT is not attributable to systemic toxicity.

**PAT inhibits tumor angiogenesis in vivo**

CD31 has been used as a biomarker of angiogenesis as it is constitutively expressed on the surface of the vascular endothelium (Takahama et al., 1999). Analysis of CD31 immunofluorescence staining of the tissue sections showed a
significant reduction of tumor microvessel density in PAT-treated A549 and patient primary tumors (Fig. 2A). When compared with therapy involving cisplatin alone, addition of PAT to a cisplatin regimen (4 mg/kg every 7 days) resulted in enhanced inhibition of the tumor vasculature indicated by CD31-positive endothelial cells (green).

To further investigate the antiproliferative activity of PAT on lung cancer tumors in vivo, we directly analyzed the expression of Ki-67 protein by immunohistochemistry (Fig. 2B). In comparison with the vehicle treatment, PAT monotherapy led to 40.9 and 46% inhibition of Ki-67-positive cells in A549 tumors and in patient tumors, respectively. Addition of PAT to the combinational regimen resulted in the reduction of Ki-67 expression to 31.4% ($P<0.001$) and 19.5% ($P<0.001$) in A549 tumors and in patient primary tumors, respectively, suggesting a synergistic effect of CDDP and PAT in vivo. Given the synergistic effect of CDDP and PAT in anti-angiogenesis in vivo, we also evaluated the combinational effect of PAT and cisplatin in HUVECs in vitro. The results showed that the combination indexes under 50%, 75% and 90% effective dose were all less than 1, indicating a synergistic effect between these two drugs (Supplemental Figure 1). This data was well consistent with the potent and synergistic anticancer action of PAT and cisplatin in the tumor xenograft models.

**PAT potently inhibits chemotactic migration of HUVECs in vitro**

Endothelial cell migration is an important process in angiogenesis and
functionally differs from proliferation (Lamalice et al., 2007). The effects of PAT on
the chemotactic motility of HUVECs were assessed by wound-healing migration and
Transwell assays. Compared to basal medium, EGM-2 with multiple growth factors
triggered cell motility, but this effect was dose-dependently inhibited by PAT. The
minimal effective action of PAT was observed at an approximate concentration of 1
µmol/L (Fig. 3A). In the Boyden chamber assay, we applied different stimuli to
evaluate the inhibitory effects of PAT on HUVEC migration. PAT exhibited equipotent
suppression of endothelial cell motility in EGM-2-, VEGF- and bFGF-stimulated
conditions (Fig. 3B). In the Boyden chamber assay, 1 µmol/L PAT led to significant
inhibition of endothelial cell migration, which was much less than its IC₅₀ value of 2.5
µmol/L, indicating that cell motility was the primary target of PAT. During the
treatment periods of migration assays, PAT even at 10 µmol/L had no obvious toxic
effect on endothelial cell viability (Supplemental Figure 2)

**PAT inhibits capillary-like structure formation of endothelial cells**

Although angiogenesis is a complex process, tube formation is one of the key
steps (Montesano et al., 1983). To investigate the potential effects of PAT on HUVEC
tube formation, we conducted a 2-dimensional Matrigel assay. When HUVECs were
placed on the growth factor–reduced Matrigel, elongated, cross-linked and robust
tube-like structures were formed in the presence of VEGF, bFGF, and EGM-2 stimuli
(Fig. 4). Incubation with indicated concentrations of PAT, tubular structure formation
was inhibited in a dose-dependent manner. PAT exhibited a similar degree of potency
for inhibition across all stimuli as shown in the HUVEC proliferation and chemotactic
migration assays. The most effective concentration of PAT was around 2.5 µmol/L.

**PAT inhibits VEGF-associated angiogenesis in vivo**

To better characterize the inhibitory function of PAT on VEGF-induced angiogenesis in vivo, we utilized the mouse Matrigel plug assay. After being embedded s.c. into 6-week-old C57BL/6 mice for 7 days, plugs containing VEGF alone appeared dark red (Fig. 5A), indicating that infiltrating neovasculature had formed inside the Matrigel via angiogenesis. In contrast, the addition of different concentrations of PAT significantly inhibited neovascular formation. As indicated by the color of the plugs, the Matrigel plugs containing VEGF plus 10 µmol/L PAT were dramatically pale, indicating maximal inhibition of blood vessel formation.

Immunohistochemistry with the vWF antibody showed that infiltrating endothelial cells in PAT-treated groups were much less than those in the control group (embedded with equal amount of PBS) (Fig. 5B). These results suggested that PAT dramatically suppresses angiogenesis in vivo.

**PAT inhibits the activation of key pro-angiogenic kinases**

We further delineated the underlying mechanism of the antiangiogenesis effects of PAT. As shown in Fig. 6A, analysis of phospho-RTKs in treated HUVECs revealed that PAT suppressed the activation of multiple critical receptors primarily contributing to angiogenesis, including VEGFR2, FGFR1, FGFR2, Tie2 and ErbB2. None of the other RTKs present on the protein array were similarly affected. To validate this result, we further confirmed the inhibition of PAT on the activation of VEGFR2. As shown in
figure 6B, PAT at the same concentration strongly inhibited VEGF-activated VEGFR2 phosphorylation (at both sites of Tyr 1175 and Tyr 996). To test if PAT blocked the activation of the RTKs by interfering with their total protein levels, we utilized the whole cellular protein from the treated HUVECs in Western blotting analyses. As indicated, PAT had little effect on the expression levels of VEGFR2 and Tie2 in HUVECs even when the HUVECs were exposed to PAT for 48 h (Fig. 6C).

We further examined whether PAT successively inhibited the intracellular signaling molecules downstream of the RTKs, and found that PAT dose-dependently suppressed the phosphorylation of Src and FAK triggered by VEGF [50 ng/mL; (i)], bFGF [50 ng/mL; (ii)] and Angiogenin-1 [200 ng/mL; (iii)] in HUVECs at concentrations ranging from 2.5 to 5 µmol/L (Fig. 6D). In addition, PAT inhibited the activation of the Src/FAK pathway in a time-dependent manner (Supplemental Figure 3). Notably, these effects were specific because the phosphorylation of AKT and ERK was not detectably affected by PAT under the same treatment.
Discussion

Although targeted therapy for cancer has been significantly advanced, cancer remains to be one of the leading causes of mortality worldwide. During the last decade, pharmaceutical industries have increased their research spending. However, it is estimated that only 0.01% of prospective anticancer agents receive FDA approval and that only 5% of oncology drugs entering into Phase I clinical trials are ultimately approved. As various diseases stem from a common molecular basis and share similar targets of the cells, repurposing established drugs is rationally considered and has gained attention over the past years (Gupta et al., 2013). Through genomics, proteomics, and informatics technologies, researchers have characterized many non-cancer drugs for their anti-cancerous activities, and most of these repurposed drugs show promising anticancer efficacy in the clinic. The successful re-profiling of thalidomide is an example. Thalidomide, which was originally used as a sedative hypnotic and withdrawn from the market due to teratogenesis induction, exhibits potent properties against several malignancies through regulating numerous cancer-related signaling pathways. This drug was finally approved by the FDA for treating patients with multiple myeloma because of its antiangiogenic (D'Amato et al., 1994) and oxidative DNA-damaging properties (Parman et al., 1999).

Cancer-associated angiogenesis contributes to the growth and progression of solid tumors and remains a primary target for anticancer drug development (Kerbel, 2008; Cook and Figg, 2010). Based on the hypothesis of clinical drug indication switching, we performed screening of existing drugs against tumor angiogenesis. Our screening
strategy by analyzing the cytotoxicity of established drugs in various NSCLC cells and HUVECs differed from the reported workflow (Chong et al., 2007) in that only endothelial cells were applied to the initial screening. By comparison of the IC50 values, we described PAT as a novel agent with antiangiogenic potential. Using in vitro and in vivo angiogenesis assays, we reported for the first time that PAT effectively inhibits angiogenesis and tumor growth of NSCLC.

Angiogenesis is the summation of multiple cellular and biologic processes to propagate existing blood vessels. In this study, we evaluated the effect of PAT on multiple steps of endothelial cell function. In HUVEC viability assays, PAT consistently exhibited potent antiproliferative activities, and the potency was comparable in response to different stimuli. Moreover, the growth inhibition of PAT was cell-type specific as the agent showed modest effects at even higher concentrations on NSCLC cells harboring different molecular characteristics. Based on this observation, we postulate that vascular endothelial cells may be the primary target of PAT rather than the surrounding tumor cells in tumor microenvironments.

Additionally, at concentrations of 1-2.5 μmol/L, PAT was sufficient to inhibit endothelial chemotaxis and tube formation, but the effective inhibitory concentration of PAT on endothelial cell proliferation was relatively higher, thereby suggesting that the molecules involved in cell motility, skeleton and differentiation were the principal targets of PAT. Novel angiogenesis therapies are being developed to target receptor tyrosine kinases (RTKs) (Wozniak, 2012). Evaluating the phosphorylation status of RTKs showed that PAT had the capacity to suppress the activation of multiple critical
receptor kinases (VEGFR2, FGFR1, FGFR2, Tie2 and ErbB2) involved in angiogenesis. We confirmed that this inhibition mediated by PAT was not associated with the reduction RTK protein levels, which differed from itraconazole (antifungal drug) that inhibited VEGFR2 glycosylation and trafficking (Nacev et al., 2011). PAT at a lower concentration of 2.5 μmol/L effectively inhibited the RTK-triggered Src and FAK activation in endothelial cells, but had little effect on the activation of AKT and ERK, thereby indicating the specificity of PAT on cellular signaling intervention. The nonreceptor tyrosine kinase, Src, directly interacts or cooperates with RTKs, including epithelial growth factor receptor and VEGFR2, as well as G protein–coupled receptors, integrins, actins, GTPase-activating proteins and FAK. These interactions are mainly involved in the regulation of cell shape, motility and differentiation. In agreement with the above results, the motility of endothelial cells triggered by different stimuli was significantly inhibited by PAT at lower concentrations, suggesting that Src/FAK signaling is a rational molecular target of PAT in angiogenesis. The repression of Src/FAK signaling leads to a downregulation of growth factor expression (Kanda et al., 2007; Kim et al., 2009). Thus, we assumed that PAT might also block the production of pro-angiogenic molecules released from tumor cells. Considering the potency of PAT on cell motility by blocking Src and FAK kinases, its direct action on tumor metastasis should be further studied. Additionally, it has been addressed recently that Src is a common signaling node downstream of multiple trastuzumab-resistant pathways (Zhang et al., 2011), and Src-targeting regimens are proposed as the novel strategy to treat patients with breast cancer.
suffering from metastasis. Given that PAT specifically affects the Src/FAK complex, this agent may be a constituent in combination with trastuzumab in the treatment of tumor metastasis.

Initial study has demonstrated that PAT, like As2O3, may inhibit acute promyelocytic leukemia \textit{in vitro} (Lecureur et al., 2002a; Lecureur et al., 2002b). In this study, we reported for the first time that PAT also exerted anticancer properties in solid tumors. In addition to the \textit{in vitro} assays, we conducted two representative tumor xenograft models to illustrate the antitumor efficacy of PAT \textit{in vivo}. Our \textit{in vivo} results strengthened the antiangiogenesis data for PAT as evidenced by CD31 immunofluorescence. In addition to A549 tumors, we additionally set up patient-derived primary tumor xenograft models, which have explants that are biologically stable when passaged in mice in terms of global gene expression patterns, mutational status, tumor architecture, and drug responsiveness. In both of the \textit{in vivo} models, PAT presented significant inhibition of tumor volume and tumor weight as a single agent. When combined with the chemotherapy drug, cisplatin, PAT contributed to a durable cytostatic tumor growth response, thereby suggesting a synergistic property. As one of the first chemotherapeutic agents used in the treatment of leishmaniasis, PAT was abandoned because of its toxicity. In our preclinical cancer \textit{in vivo} models, PAT did not show any gross toxicity at the tested dosage, suggesting that PAT may be clinically safe in NSCLC treatment. Importantly, PAT concentrations acting on endothelial cells were less than 10 µmol/L, and such concentrations are likely to be achievable \textit{in vivo} in humans.
In the current cancer treatment for NSCLC, EGFR inhibitors (e.g., gefitinib and erlotinib), have been applied into clinics as first-line drugs. However, tumors harboring \textit{KRAS} mutations inevitably result in intrinsic resistance to EGFR inhibitor-based therapy (Massarelli et al., 2007). Unfortunately, no effective drug has been developed to treat this type of cancer with a \textit{KRAS} mutation genotype.

Importantly, the \textit{in vivo} tumors we used in this study are all \textit{KRAS} mutant NSCLC, and PAT effectively inhibited the tumor growth by blockade of tumor angiogenesis. Our findings have critical clinical implications for potential use of PAT as an efficient agent for \textit{KRAS} mutant NSCLC.
Authorship Contributions

Participate in research design: Wang, Yu, Guo, Lu, Liu, Pang

Conducted experiments: Wang, Yu, Guo, Jiang

Performed data analysis: Wang, Pang

Wrote or contributed to the writing of the manuscript: Wang, Pang
References


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Footnotes

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Universities [Grant 78260029].
Legends for Figures

Figure 1. PAT potently retards tumor growth of NSCLC in vivo.

A. PAT inhibited A549 tumor growth as measured by tumor volume (left) and solid tumor weight (middle) and had little effect on the mouse body weight (right) at the tested dose. Mice bearing A549 tumors were treated with PBS, PAT (40 mg/kg; i.p.; daily), cisplatin (CDDP; 4 mg/kg; i.p.; once a week), or a combination of PAT and cisplatin (n=8 per group). B. PAT suppressed the growth of patient primary NSCLC tumors. The patient primary NSCLC tumor xenograft mouse model was set up according to the protocol described in the Materials and Methods. Individual groups of mice bearing patient primary tumors were subjected to different treatments. Dots, mean; bars, standard deviation; *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. the vehicle control group.

Figure 2. PAT inhibits angiogenesis and tumor cell proliferation in human NSCLC xenografts.

A. Antiangiogenic effects of PAT, CDDP, and a combination of PAT and CDDP as assessed by immunohistochemical analysis of CD31 (green) in mice implanted with A549 cells or human primary tumors. B. Immunohistochemical analysis of Ki-67 staining in tumors (brown) from two types of human NSCLC xenograft mouse models. Representative CD31 or Ki-67 staining of lung tumors viewed at 200× magnification. Quantitative number of CD31-positive and Ki-67-positive cells was determined by manual counting in three random fields from six solid tumors of each group. Columns,
mean; * bars, standard deviation; **, $P < 0.01$ vs. control.

**Figure 3.** PAT potently inhibits endothelial cell migration and chemotaxis *in vitro.*

* A. PAT inhibited HUVEC migration in a wound-healing assay. HUVECs were first synchronized by starvation. Confluent cells were then scratched and treated with EGM-2 media containing DMSO or 1, 2.5, 5, 10 μmol/L PAT. Migrating cells were stained by calcein-AM and quantified by manual counting. Magnification of 64×. *B.* PAT inhibited migration of stimulated HUVECs in a Transwell assay. HUVECs ($4 \times 10^4$) were pretreated with PAT in EGM-2 containing 0.5% FBS for 30 min before seeding into the upper chambers. The bottom chamber was filled with EGM-2 basic media supplemented with EGM-2, 10 ng/mL VEGF, and 12 ng/mL bFGF. The HUVECs that migrated through the membrane were quantified. Magnification of 160×. *Columns,* mean from three different experiments with duplicates; *bars*, SD. *, $P < 0.05$; ***, $P < 0.001$ vs. the DMSO group (EGM-2 alone, VEGF alone or bFGF alone).

**Figure 4.** PAT inhibits the capillary-like structure formation in endothelial cells.

HUVECs stimulated with EGM-2, 10 ng/mL VEGF, or 12 ng/mL bFGF were allowed to spontaneously form tube networks after treatment with DMSO or indicated concentrations of PAT. After 4-6 h, the tubular networks were visualized with calcein-AM and photographed (magnification of 64×).
Figure 5. PAT inhibits VEGF-stimulated angiogenesis in vivo.

Six-week-old C57BL/6 mice were injected with 500 μL of Matrigel containing indicated concentrations of PAT, 60 ng of VEGF, and 20 units of heparin into the ventral area (n=6 per group). After a week, the skin of mice was pulled back to expose the intact Matrigel plugs. A. Matrigel plugs with different treatments were photographed. B. Representative vWF staining of Matrigel plugs viewed at 400× magnification. The Matrigel plugs were fixed, sectioned and stained with the vWF antibody (magnification of 400×).

Figure 6. PAT inhibits the activation of RTKs and the Src/FAK pathway in endothelial cells.

A. PAT suppressed the expression of multiple RTKs in HUVECs. Cell lysates were generated from EGM-2–stimulated HUVECs treated with 15 μmol/L PAT or with DMSO for 24 h. Cell lysates was then centrifuged and hybridized to a human RTK phosphorylation antibody array. B. PAT dramatically suppressed VEGF-mediated activation of VEGFR2. Proteins from different treatments were analyzed by Western blotting assay. C. PAT had little effect on the expression levels of VEGFR2 and Tie2. D. PAT inhibited the activation of the Src/FAK pathway but had little effect on the activation of AKT and ERK. Starved HUVECs were pretreated with or without various concentrations of PAT followed by stimulation with angiogenesis stimulation medium (50 ng/mL VEGF, 50 ng/mL bFGF or 200 ng/mL Ang-1) for 2-30 min. Proteins from different groups were analyzed by Western blotting analysis using...
specific antibodies.
### Tables

#### Table 1. The Cytotoxicity of PAT is Direct towards Endothelial Cells

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*Note.* Growth inhibition of HUVECs, a panel of NSCLC cells and normal lung fibroblast cells by PAT as determined by MTS assay. The half inhibitory concentration (IC<sub>50</sub>) values were shown.
Figure 1

A

A549 xenografts

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Figure 2

A

Control | PAT | CDDP | PAT+CDDP

A549 xenografts

Primary tumor xenografts

B

Control | PAT | CDDP | PAT+CDDP

A549 xenografts

Primary tumor xenografts

Graphs showing microvessel density and Ki-67 positive nuclear expression for A549 xenografts and primary tumor xenografts under different treatments.