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

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Received July 24, 2014; accepted October 28, 2014

ABSTRACT

Repurposing existing drugs not only accelerates drug discovery but rapidly advances clinical therapeutic strategies. In this article, we identified potassium antimony tartrate (PAT), an antiparasitic drug, as a novel agent to block angiogenesis by screening US Food and Drug Administration–approved chemical drugs. By comparing the cytotoxicity of PAT in various nonsmall-cell lung cancer (NSCLC) cells 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 dosage 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 antiangiogenesis property of PAT, which was well correlated with its tumor growth inhibition in NSCLC cells. 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–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 cells. 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 (Aligire 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, such as 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 antiangiogenic 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 tumor, is the leading cause of cancer-related deaths in both sexes worldwide.
(Siegel et al., 2013). Nonsmall-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 antiangiogenic agent for treatment of patients suffering from NSCLC (Ferrara et al., 2004; Sandler et al., 2006). However, toxicities, substantial costs, and modest survival rates successively occurred (Ranpura et al., 2011). Recently, small-molecule RTK inhibitors 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, 2019). The human RTK phosphorylation antibody array was purchased from RayBiotech. For Western blotting analysis, anti-VEGF receptor 2 (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 nonradioactive cell proliferation kit was purchased from Promega (Madison, WI). All other chemicals used in this study were ACS reagents.

Materials and Methods

Reagents

PAT (purity >99%) was obtained from Sigma-Aldrich (St. Louis, MO), and PAT stock solutions were prepared with dimethylsulfoxide (Sigma-Aldrich) for in vitro assays. For in vivo experiments, PAT and cisplatin (Sigma-Aldrich) were diluted in phosphate-buffered saline (PBS). Recombinant human VEGF165 and bFGF146 were a gift from the Experimental Branch of the National Institutes of Health (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 calcine-AM was obtained from Sigma-Aldrich. The human RTK phosphorylation antibody array was purchased from RayBiotech. For Western blotting analysis, anti-VEGF receptor 2 (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 nonradioactive 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 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) and nonradioactive cell proliferation kit was 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 × 10^4 cells/well) were first starved and then treated with various concentrations of PAT in either EGM-2 or angiogenesis stimulation medium for 72 hours. A549, PC9, NCI-H460, NCI-H441, NCI-H522, NCI-H661, WI-38, and MRC-5 cells (4–6 × 10^5 cells/well) were directly exposed to indicated concentrations of PAT in full growth medium for 72 hours. To determine cell viability, we used a nonradioactive cell proliferation kit containing a tetrazolium compound (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and a VERSAMax microplate reader (Molecular Devices, Sunnyvale, CA). The 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 this 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-hour light/dark cycle. All treatments were administered according to the National Institutes of Health standards established in the Guidelines for the Care and Use of Experimental Animals.
Human NSCLC Tumor Xenograft Mouse Model

Cultured NSCLC Cell Xenograft Mouse Model. The cultured NSCLC cell xenograft mouse model was constructed as previously described (Pang et al., 2009b). A549 cells (5 × 10^6 cells/mouse) were injected subcutaneously 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:

\[ V = \left( \frac{L}{2} + \frac{W}{2} \right)^2 \times 0.52, \]

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 days, and the body weight was measured every other day. At the end of the experiment, mice were euthanized. 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-naive 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. Briefly, surgically removed tumor tissues were cut into fragments of approximately 15 mm^3 and implanted using a trocar needle subcutaneously into two to three male NOD/SCID mice within 2 hours. 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 serial passage to other immunodeficient mice. After three consecutive mouse-to-mouse passages, the xenograft was considered to be "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 previously described (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 performing 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 minutes for immunohistochemistry and 1 hour for immunofluorescence). Sections were then visualized by the streptavidin–biotin-peroxidase complex method with 3,3′-diaminobenzidine and counterstained with hematoxylin (immunohistochemistry) or 4',6'-diamidino-2-phenylindole (immunofluorescence) as the chromogen. Each group had at least six samples, and sections from the same group were processed in parallel from at least three samples. Images were taken using a Leica DM4000B photo microscope (200× magnification; Leica, Solna, Germany).

Cell Migration Assays

Endothelial Cell Migration Assay. HUVECs were allowed to grow to full confluence in six-well plates precoated with 0.1% gelatin (Sigma-Aldrich). To inactivate cell proliferation, HUVECs were first synchronized with EGM-2 basal medium containing 0.5% FBS for 6 hours. The cells were then wounded with pipette tips and washed with PBS followed by exposure to EGM-2 medium containing PAT (0–10 μM) or dimethylsulfoxide. After 8–10 hours of migration, cells were labeled with 2 mg/ml calcein-AM for 30 minutes and visualized using an inverted microscope (64× magnification; Olympus, Tokyo, Japan). Migrated cells in four random fields were quantified by manual counting. Three independent experiments were performed in triplicate.

Endothelial Cell Transwell Migration Assay. The chemotactic motility of HUVECs was determined using a Transwell migration assay (BD Biosciences) with 6.5-mm diameter polycarbonate filters (8-mm pore size) as previously described (Pang et al., 2009a). Briefly, the filter was coated with gelatin. Angiogenesis stimulation medium (500 μl) was placed into the bottom chambers. HUVECs (4 × 10^4) were incubated with indicated concentrations of PAT in EGM-2 containing 0.5% FBS for 30 minutes at 37°C before seeding into the upper chambers. After 8–10 hours of incubation, nonmigrated 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 (160× magnification). 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 previously described (Lee et al., 1999). Briefly, each well of a 48-well plate was coated with 80 μl 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 hour at 37°C before seeding. Cells were then plated onto the Matrigel layer at a density of 4–6 × 10^4 cells per well for 4–6 hours. Cells with tube networks were visualized with 2 μg/ml calcein-AM using an Olympus fluorescence microscope (64× magnification). Three independent experiments were performed.

In Vivo Matrigel Plug Assay

The Matrigel plug assay was performed as previously described (Pyun et al., 2008). Briefly, 0.5 ml Matrigel in the presence or absence of 60 ng VEGF, 20 U heparin, and indicated concentrations of PAT (15 and 50 μM) was subcutaneously injected into the ventral area of 6-week-old C57BL/6 mice (n = 6). After 1 week, the mice were euthanized 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 antibody (Chemicon, Temecula, CA) was used for immunohistochemistry. Images of neovascularization were taken using a Leica DM 4000B photo microscope (400× magnification).

Phospho-RTL 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 hour with gentle shaking. Cells were harvested using a cell scraper and lysed in modified radioimmuno-precipitation buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na4P2O7, and 1 mM
phenylmethylsulfonyl fluoride) supplemented with a phosphatase and protein inhibitor cocktail (Calbiochem, San Diego, CA). The lysates were gently rocked at 4°C for 30 minutes and centrifuged at 14,000g for 15 minutes. 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 hours and then pretreated with or without various concentrations of PAT followed by stimulation with angiogenesis stimulation medium for 2–30 minutes. The whole-cell extracts were prepared in radioimmunoprecipitation 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-Aldrich). 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 (Microsoft Corporation, Redmond, WA) and GraphPad Prism Software (version 5.0). Comparisons between groups were performed using one-way analysis of variance followed by the t test. Data were presented as means ± S.D. P values less than 0.05 were considered statistically significant.

**Results**

**PAT Exerts Cytotoxicity Directly toward HUVECs.** Chong et al. (2007) successfully repurposed the antifungal drug, itraconazole, as a potent angiogenesis inhibitor. There are currently 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 four 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 antiproliferative activity toward 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 μM, 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 because their IC_{50} values were not measurable below the maximum tested concentration of 100 μM. 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 mutant) 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 because 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 version; Gly to Asp). Mice bearing established progressive tumor xenografts were administered PAT at a dosage of 40 mg/kg i.p. daily. Our results showed that administration of PAT significantly decreased the tumor growth rates of both xenografts (Fig. 1). 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 with that of cisplatin monotherapy. Compared with either single-agent therapy, the addition of PAT to the cisplatin regimen resulted in a dramatic tumor growth inhibition. The combination 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 because 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

**Table 1**

The cytotoxicity of PAT is directed toward endothelial cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC_{50} Value μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human endothelial cells</td>
<td></td>
</tr>
<tr>
<td>HUVECs (EGM-2)</td>
<td>4.32</td>
</tr>
<tr>
<td>HUVECs (VEGF)</td>
<td>7.55</td>
</tr>
<tr>
<td>HUVECs (bFGF)</td>
<td>6.05</td>
</tr>
<tr>
<td>Human lung fibroblast cells</td>
<td></td>
</tr>
<tr>
<td>WI-38</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MRC-5</td>
<td>49.72</td>
</tr>
<tr>
<td>Human lung cancer cells</td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NCI-H441</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>42.72</td>
</tr>
<tr>
<td>PC9</td>
<td>72.52</td>
</tr>
<tr>
<td>NCI-H522</td>
<td>44.68</td>
</tr>
<tr>
<td>NCI-H661</td>
<td>67.87</td>
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showed that no obvious differences were found in mouse body weight among the PAT-treated groups and the control group. By 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 because 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). 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). Compared 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 cisplatin and PAT in vivo. Given the synergistic effect of cisplatin and PAT in antiangiogenesis 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 Fig. 1). These data were 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 with 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 μM (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 μM PAT led to significant inhibition of endothelial cell migration, which was much less than its IC50 value of 2.5 μM, indicating that cell motility was the primary target of PAT. During the treatment periods of migration assays, PAT even at 10 μM had no obvious toxic effect on endothelial cell viability (Supplemental Fig. 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 two-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). However, tubular structure formation was inhibited by PAT 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 mM.

**PAT Inhibits VEGF-Associated Angiogenesis In Vivo.** To better characterize the inhibitory function of PAT on VEGF-induced angiogenesis in vivo, we used the mouse Matrigel plug assay. After being embedded subcutaneously into 6-week-old C57BL/6 mice for 7 days, plugs containing VEGF alone appeared dark red (Fig. 5A), indicating that infiltrating neovascularation had formed inside the Matrigel via angiogenesis. By 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 μM PAT were dramatically pale, indicating maximal inhibition of blood vessel formation.

Immunohistochemistry with the von Willebrand factor 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 Proangiogenic 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, FGF receptor 1 (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 Fig. 6B, PAT at the same concentration strongly inhibited VEGF-activated VEGFR2 phosphorylation (at both sites of Tyr 1175 and Tyr 996). To test whether PAT blocked the activation of the RTKs by interfering with their total protein levels, we used 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 hours (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), bFGF (50 ng/ml), and Ang-1 (200 ng/ml) in HUVECs at concentrations ranging from 2.5 to 5 μM (Fig. 6Di–iii, respectively). In addition, PAT inhibited the activation of the Src/FAK pathway in a time-dependent manner (Supplemental Fig. 2).
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 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. Because 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

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**Fig. 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, or 10 μM PAT. Migrating cells were stained by calcein-AM and quantified by manual counting. (B) PAT inhibited migration of stimulated HUVECs in a Transwell assay. HUVECs (4 to 5 × 10^4) were pretreated with PAT in EGM-2 containing 0.5% FBS for 30 minutes 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. Columns are the mean from three different experiments with duplicates, and bars are the S.D. *P < 0.05; **P < 0.001 versus the DMSO group (EGM-2 alone, VEGF alone, or bFGF alone). magnification, 64× in (A); 160× in (B). DMSO, dimethylsulfoxide.

**Fig. 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 hours, the tubular networks were visualized with calcein-AM and photographed. Original magnification, 64×. DMSO, dimethylsulfoxide.
the past years (Gupta et al., 2013). Through genomics, proteomics, and informatics technologies, researchers have characterized many noncancer drugs for their anticancerous activities, and most of these repurposed drugs show promising anticancer efficacy in the clinic. The successful reprofiling 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 validated and approved for use in multiple myeloma and other malignancies.

Fig. 5. PAT inhibits VEGF-stimulated angiogenesis in vivo. Six-week-old C57BL/6 mice were injected with 500 μl Matrigel containing indicated concentrations of PAT, 60 ng VEGF, and 20 U 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. The Matrigel plugs were fixed, sectioned, and stained with the vWF antibody. vWF, von Willebrand factor. Original magnification, 400× in (B).

Fig. 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 μM PAT or with DMSO for 24 hours. 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 (Di), 50 ng/ml bFGF (Dii), or 200 ng/ml Ang-1 (Diii)] for 2–30 minutes. Proteins from different groups were analyzed by Western blotting analysis using specific antibodies. DMSO, dimethylsulfoxide.
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 because the agent showed modest effects at even higher concentrations on NSCLC cells harboring different molecular characteristics. On the basis of this observation, we postulate that vascular endothelial cells may be the primary target of PAT rather than the surrounding tumor cells in tumor microenvironments. In addition, at concentrations of 1–2.5 μM, 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 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 μM 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 non-RTK 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 proangiogenic 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. In addition, Zhang et al. (2011) recently stated that Src is a common signaling node downstream of multiple trastuzumab-resistant pathways, and Src-targeting regimens are proposed as a 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 in vitro (Lecueur et al., 2002a,b). In this study, we report for the first time that PAT also exerted anticancer properties in solid tumors. In addition to the in vitro assays, we conducted two representative tumor xenograft models to illustrate the antitumor efficacy of PAT in vivo. Our 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 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 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 μM, and such concentrations are likely to be achievable in vivo in humans.

In the current cancer treatment of NSCLC, epidermal growth factor receptor inhibitors (e.g., gefitinib and erlotinib), have been applied into clinics as first-line drugs. However, tumors harboring KRAS mutations inevitably result in intrinsic resistance to epidermal growth factor receptor inhibitor-based therapy (Massarelli et al., 2007). Unfortunately, no effective drug has been developed to treat this type of cancer with a KRAS mutation genotype. Importantly, the in vivo tumors we used in this study are all 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 KRAS mutant NSCLC.

Authorship Contributions

Participated in research design: Wang, Yu, Guo, Liu, Pang.

Conducted experiments: Wang, Yu, Guo, Jiang.

Performed data analysis: Wang, Pang.

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

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

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Supplemental Fig.1. The synergistic effect of PAT and CDDP in HUVECs. HUVECs were directly treated with indicated concentrations of PAT and CDDP alone or combined for 72 h in endothelial cell growth medium. Cell viability was determined by MTS assay. Combination Index (CI) was calculated by the CalcuSyn software (Version 2; Biosoft). Fa means the fraction affected by the dose. CI values for the combination of PAT and CDDP were calculated; CI <0.7 means synergism. ED50, ED75 and ED90 represented 50%, 75% and 90% effective dose, respectively. Columns, mean; dots, standard deviation.
Supplemental Fig. 2. PAT had no obvious toxic effect on cell viability in HUVECs after 12-h exposure. HUVECs were grown in EGM-2 and directly treated with indicated concentrations of PAT for 12 h. Cell viability was determined by MTS assay. *$, P<0.05$ versus control group.
Supplemental Fig.3. PAT inhibited the activation of the Src/FAK pathway in a time-dependent manner. HUVECs were pretreated with or without various concentrations of PAT for 5 and 10 hours during starvation. Those treated cells were then subjected to angiogenesis stimulation medium containing 50 ng/mL of bFGF for about 10 minutes. Proteins from different groups were analyzed by Western blotting analysis using specific antibodies.