Rho Kinase Inhibitor Fasudil Suppresses the Vasculogenic Mimicry of B16 Mouse Melanoma Cells Both In Vitro and In Vivo

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Abstract

The aim of this study was to investigate the biologic role of the Rho kinase inhibitor fasudil in the vasculogenic mimicry (VM) of B16 mouse melanoma cells. It was previously reported that RhoA plays a critical role in angiogenesis by coordinating endothelial cell cytoskeleton remodeling and promoting endothelial cell motility. Although RhoA has been implicated in the regulation of angiogenesis, little has been described regarding its control of these tumor cell–lined channels. In this study, we established an in vitro model of VM using 3-dimensional cell culturing of mouse B16 melanoma cells and studied VM in vivo by transplanting B16 cells into C57/BL mice. Next, we explored the effect of RhoA and Rho-associated, coiled-coil containing protein kinase (ROCK) on VM formation using the Rho kinase inhibitor fasudil. We provide direct evidence that fasudil leads to reduced vascular-like channels in Matrigel. Additional experiments suggested that fasudil prevents both initial cellular architecture changes and cell migration in vitro. Finally, we provide in-depth evidence for the underlying mechanisms of fasudil-induced VM destruction using the Rho-GTPase agonist lysophosphatidic acid. In vivo studies revealed that fasudil reduced B16 melanoma cell xenograft tumor growth without causing significant toxicity in mice. Fasudil-treated tumors also displayed fewer VM channels. These results suggest that fasudil may be an emerging therapeutic option for targeting cancer VM. Mol Cancer Ther; 14(7); 1–9. ©2015 AACR.

Introduction

It has been well established that tumors require a blood supply for growth and hematogenous dissemination (1); consequently, researchers have focused on endothelial cells, which form the neovasculature of growing tumors. However, angiogenesis may not be the sole mechanism by which tumors acquire their microcirculation. In 1999, Maniotis and colleagues, for the first time, described a process by which tumor cells develop highly patterned vascular channels via reorientation of the F-actin cytoskeleton and matrix remodeling without the involvement of endothelial cells and fibroblasts (2). These vascular-like structures are composed of a basement membrane positive for periodic acid-Schiff (PAS) staining and participate in tumor perfusion (3). This process was defined as vasculogenic mimicry (VM). VM has been reported to be associated with more aggressive tumor behavior (2, 4–6). Theoretically, VM, without the barrier of endothelial cells, is a more convenient metastasis route (4, 6, 7). Furthermore, it was found that antiangiogenic agents, such as TNP470, anginex, and endostatin, were incapable of blocking the tube formation of aggressive uveal and cutaneous melanoma cells in vitro (8). Moreover, VM was also found to be linked to poor clinical outcomes, and this finding might partially explain why some tumors respond poorly to angiogenesis inhibitors (9). In addition, a recent in vitro study examined the role of VM in stem cell–driven spheroid formation (10). These findings prompted us to explore the therapeutic potential of targeting the tumor VM.

The functional plasticity of aggressive cancer cells is believed to be instrumental in VM tube formation (4, 9, 11). Mounting evidence suggests that Rho GTPases are essential for cell plasticity (12, 13). These kinases are found in nearly all eukaryotic cells, where they switch between the inactive GDP-bound and the active GTP-bound states. These kinases have been implicated in a wide array of cellular processes, including cytoskeletal dynamics, cell polarity, contraction, adhesion, migration, proliferation, and apoptosis (12, 13), all of which might be involved in carcinogenesis. Previous studies have indicated that, in cancer patients, the expression and activity of Rho GTPases tend to be elevated, and these kinases might play an important role in cancer growth, invasion, and metastasis. Currently, attention has focused on the small GTPase RhoA (14–16). Importantly, the Rho-associated, coiled-coil containing protein kinase (ROCK) family, a major downstream effector of RhoA, is a key factor in cytoskeleton remodeling, and it was reported that, upon ROCK activation by RhoA, endothelial cells may alter their cytoskeletal architecture, and neighboring cells may form vessels (17–20). Consistent with these findings, a pharmacologic study also demonstrated that a Rho pathway blocker could effectively inhibit angiogenesis (21).

Fasudil, the only clinically available ROCK inhibitor (22), has been used for the treatment of cerebral vasospasms following
subarachnoid hemorrhage (23). Because of fasudil’s reasonable safety profile, it has been clinically tested in several trials on cardiovascular diseases, including angina pectoris, hypertension, pulmonary hypertension, stroke, and heart failure (24). Moreover, fasudil has been shown to inhibit malignant tumor progression in human subjects and rodent models (16) and significantly suppress the viability and invasive ability of lung carcinoma-conditioned endothelial cells and cancer cells (25–27). In addition, Yin and colleagues reported that fasudil can inhibit VEGF-induced angiogenesis both in vitro and in vivo (28). However, to our knowledge, the effect of fasudil on VM has not been reported. On the basis of these studies, we hypothesized that this drug might inhibit VM through a similar mechanism by which it affects angiogenesis, i.e., by impacting the cytoskeletal structure of tumor cells, inhibiting their motility, and ultimately blocking the formation of a tumor cell–lined network. To better decipher the underlying mechanism, we also tested whether the effect of fasudil on VM would be attenuated by lysophosphatidic acid (LPA), a well-characterized Rho GTPase activator (29–32).

In this study, we observed the effect of fasudil on VM, examined the possible roles of Rho kinase inhibition and Rho GTPase activation, and explored the potential of using fasudil for cancer treatment.

Materials and Methods

Cell culture

The mouse melanoma cell line B16 was obtained from the dermatology department, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), in March 2013 and stored in the Union Hospital Cancer College, Huazhong University of Science and Technology (Wuhan, China). The cells were resuspended and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS at 37°C. B16 cells were not authenticated because there is no public reference database to match the genetic profile of mouse-origin cells at present. Log-phase cells were used in the experiments described below.

Cell proliferation assay

Cell proliferation was measured using an MTT assay. B16 cells were seeded in 96-well plates (6 × 10^3 cells/well) and exposed to different doses (0, 10, 20, 40, 80, 160 μmol/L) of fasudil hydrochloride (Tianjin Hongri Pharmaceutical Co. Ltd.) for 24 hours. The results are expressed as absorbances at 490 nm. The final IC_{50} value was calculated using GraphPad Prism 5. The results represent the mean of 3 separate experiments.

Capillary-like structure formation by B16 cells: VM

A previously reported technique was used to observe VM (33). Briefly, 2 × 10^5 B16 cells per well were seeded onto Matrigel (BD Biosciences) in 96-well plates. Before seeding, fasudil was added to the cell suspensions at concentrations of 0, 10, 30, or 60 μmol/L. The formation of capillary-like tubes was analyzed 6 hours after plating. The well images were captured under an inverted light microscope at 100× magnification. The corresponding areas were measured using the NIH ImageJ software package.

PAS staining of vasculogenic-like networks in vitro

After 3-dimensional cell culturing, the tubular networks formed in vitro were visualized by staining with the PAS method (8).

Scanning electron microscopy

Cover slides were placed on each well of the 24-well plates. Then, 150 μL of Matrigel was added onto each slide and incubated at 37°C for 1 hour until the gel solidified. B16 cells in the control and fasudil-treated (30 μmol/L) groups were cultured on the cover slips for 6 hours. After rinsing with PBS (pH 7.4), the cells were fixed with 2% glutaraldehyde for 2 to 4 hours at 4°C. The cells were subsequently washed with PBS 3 times, buffered in 1% OsO_4 for 2 hours, and dehydrated in ethanol. The samples were dried and sprayed with carbon and gold (34). Images were captured using a scanning electron microscope (VEGA 3 LMU, TESCAN).

Rhodamine-phalloidin immunofluorescence

The actin filaments in B16 cells were cytochemically stained using a rhodamine-labeled anti-phalloidin antibody (cytoskeleton). Briefly, fasudil-treated or untreated B16 cells were seeded onto sterile cover slips, fixed in 4% formaldehyde for 10 minutes at room temperature, and incubated in 0.1% Triton X-100 in PBS for 15 minutes to permeabilize the cells before addition of 50 μL of 100 nmol/L rhodamine phalloidin. The images were captured and analyzed using a Zeiss confocal photomicroscope (Olympus FluoView FV1000).

Wound-healing assay

B16 cells were seeded onto a six-well culture plate and cultured to a subconfluent state in complete medium. Cell monolayers were linearly scraped with a P-200 pipette tip (250-μm width). Cells detached from the bottom of the well were mildly aspirated and incubated in serum-free medium containing different concentrations of fasudil (0, 10, 30, 60 μmol/L) for 24 hours. The width of the scratch was microscopically monitored at various time points and quantified in terms of the difference between the original width of the wound and the width after cell migration. The percentage of wound closure [(original width – width after cell migration)/original width] was calculated. The width of the wound was measured using Image-Pro Plus 6.0.

Transwell migration assay

A complementary transwell migration assay was performed by employing a modified Boyden chamber (Corning Costar) containing a gelatin-coated polycarbonate membrane filter (pore size, 8 μm; ref. 6). A total of 2 × 10^4 cells in 500 μL of culture medium containing various concentrations of fasudil were added to the upper chamber, and the lower chamber contained culture medium with 20% FBS to stimulate cell migration. The migration assays were incubated for 24 hours at 37°C in 5% CO_2 and then the cells were stained with crystal violet. Cells on the undersides of the filters were observed under a microscope at a magnification of 200× magnified and counted. Cell counting was performed using Image-Pro Plus 6.0.

RNA isolation and reverse-transcription PCR

B16 cell RNA was extracted from whole cell lysates and reverse transcribed to cDNA using TRlzlol (Invitrogen) and a reverse-transcriptional kit (TaKaRa Bio, Inc.). RT-PCR was performed in triplicate with the SYBR Prime Script RT-PCR Kit (TaKaRa Bio, Inc.) on the Step One Plus Real-Time PCR system (Applied Biosystems). The following sequences were used: RhoA forward primer 5’-CCTCCGGAGATGAGCACACGAC-3’ and reverse primer 5’-AGATGGGCACCCAGACTTT-3’; ROCK1 forward primer 5’-ACCCACCATCTGGCATTG-3’ and reverse primer

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samples were divided randomly into 7 groups, with 3 mice approximately 2 to 3 mm was observed at the site of injection, and the tumor width was measured by daily caliper measurement, and the tumor volumes were calculated using the formula: tumor volume = (width)² × length × 0.5.

Six days after inoculation, a tumor mass with a diameter of 2 to 3 mm was observed at the site of injection, and the tumor volume was calculated using the formula: tumor volume = (width)² × length × 0.5.

Cell extracts and Western blots

Cultured cells were washed twice with ice-cold PBS, solubilized in a lysis buffer containing 100 mm/L phenylmethanesulfonyl fluoride (PMSF) on ice, and quantified using the bicinchoninic acid method. Cell lysate protein samples (50 μg) were separated by SDS-PAGE under reducing conditions using Tris-glycine running buffer and were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) buffer (10 mmol/L Tris-HCl, pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20) for 2 hours. Protein expression was detected using primary antibodies incubated overnight at 4°C (antibodies directed against RhoA, ROCK1, ROCK2, and GAPDH 1:200 dilution; Santa Cruz Biotechnology). Thereafter, membranes were washed and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase–conjugated secondary antibody. After the membranes were washed with TBS buffer 6 times, the proteins were visualized using an enhanced chemiluminescence reagent (Beyotime).

Cotreatment of B16 cells with fasudil and LPA

Oleoyl-LPA was purchased from Sigma-Aldrich. B16 cells were treated with fasudil (30 μmol/L), LPA (5 μmol/L), or a combination of fasudil (30 μmol/L) and LPA (5 μmol/L). Next, the VM activity, cytoskeleton remodeling, and downstream protein expression profiles of the cells in each group were examined.

Xenograft murine model

Zhang and colleagues demonstrated that VM, as an adaptation to rapid tumor growth, is the dominant blood supply mode for early stage tumors. When the tumor mass expands, endothelium-dependent vessels eventually replace VM to become the major blood supply architecture (35). To confirm their findings, we repeated their work.

Male C57/BL6 mice (6-week-old) were procured from the Experimental Animal Center of Wuhan University (Wuhan, China). All experiments were performed in accordance with the recommendations of the Policy on Humane Care and Use of Laboratory Animals. Tumors were implanted into the right hind limb of each animal by subcutaneously injecting 5 × 10⁶ B16 melanoma cells in 100 μL of serum-free media. Tumor dimensions were recorded by daily caliper measurement, and the tumor volume was calculated using the following formula: length × width² × 0.5.

Six days after inoculation, a tumor mass with a diameter of approximately 2 to 3 mm was observed at the site of injection, and the tumor formation rate was 95% among the animals. All 21 C57/BL6 mice were divided randomly into 7 groups, with 3 mice in each group. From day 11 to day 17, one randomly selected group (3 mice) was sacrificed. For histopathologic analysis, tumor samples were fixed in 4% paraformaldehyde immediately after harvesting and then paraformaldehyde-embedded. Sections were hematoxylin and eosin (H&E) stained. To confirm the presence of VM and endothelial-dependent vessels, the sections were doubly stained with a base membrane marker (PAS) and an endothelial cell-specific marker (CD31).

Evaluation of the effects of fasudil on VM in vitro

The in vitro impact of fasudil on VM was assessed using the subcutaneous tumor xenograft model as discussed previously. Similarly, 6-week-old male C57/BL6 mice received inoculations with 5 × 10⁶ B16 melanoma cells by subcutaneous injection. When the tumors grew to a diameter of 2 to 3 mm, the mice were randomly allocated to a treatment group receiving fasudil (diluted in 0.9% normal saline vehicle solution, 50 mg/kg, daily for 5 days) and a control group, which was given 0.9% normal saline of the equivalent volume alone intraperitoneally (5 mice in each group). Five days later, the tumors were excised. Some tumors were frozen in liquid nitrogen for subsequent RT-PCR and Western blot analysis, and others were fixed in 4% paraformaldehyde for histopathologic examination (H&E staining and CD31-PA double staining).

CD31 and periodic acid-Schiff double staining

Briefly, formalin-fixed, paraffin-embedded tissue specimens (4-μm thickness) were deparaffinized in three successive xylene baths for 15 minutes. The slides were subsequently hydrated in a series of ethanol solutions (100%, 90%, 75%, and 50%). Endogenous peroxide activity was blocked with 3% hydrogenperoxide for 10 minutes at room temperature. The slides were washed in PBS. After pretreatment with citrate buffer (0.01 mol/L citric acid, pH 6.0) for 20 minutes at 100°C in a microwave oven, the slides were allowed to cool to room temperature and washed in PBS 3 times. Then, the slides were incubated for 30 minutes with 5% BSA to block nonspecific antibody binding. Rabbit monoclonal anti-CD31 protein IgG (ab28364; Abcam, dilution 1:50) was added, and the samples were incubated at 4°C overnight. After rinsing with PBS again, the sections were incubated with goat anti-rabbit secondary antibody (Zhongshan Goldenbridge Biotechnology Co. Ltd.) for 20 minutes at 37°C, visualized with 3,3-diaminobenzidin (DAB), and stored in a dark chamber at room temperature for 1 minute. After washing with distilled water, the sections were incubated with periodic acid for 15 minutes. Then, the sections were rinsed with water for 5 minutes and incubated with the Schiff reagent in a dark chamber for 10 minutes. Finally, the sections were counterstained with hematoxylin. Tumor xenograft microvessels were counted under a light microscope. For each section, the average number of VM channels (PAS positive) and endothelium-dependent vessels (CD31 positive) on each slide was determined in areas without necrosis in 5 randomly selected fields (400×).

Statistical analyses

All in vitro experiments were performed in triplicate. All data are presented as the mean ± SDs. The Student t test (2-sided, unpaired) was used for significance evaluation. All comparisons were made relative to untreated controls, and a P value < 0.05 was considered significant.

Results

Inhibitory effect of fasudil on B16 cell proliferation in vitro

B16 cells were seeded in 96-well plates and incubated with various concentrations of fasudil for 24 hours. The cell inhibitory
rate was measured using an MTT assay. Fasudil inhibited B16 cell proliferation in a dose-dependent manner (Supplementary Fig. S1), and the IC50 for 24 hours of fasudil in B16 cells was 71 \text{m}\text{mol/L}. Thus, we used 10, 30, and 60 \text{m}\text{mol/L} fasudil to optimize the conditions of subsequent in vitro experiments.

The inhibitory effect of fasudil on B16 melanoma cell VM

The effect of fasudil on VM was studied by seeding B16 cells onto Matrigel and culturing them for 6 hours to allow for the development of VM structures. The cells treated with the vehicle alone, serving as negative controls, underwent a conspicuous rearrangement and quickly formed a vasculogenic network within 6 hours. In contrast, cells treated with fasudil showed a significant dose-dependent destruction of VM structures (Fig. 1A). The cells were PAS-stained to highlight the matrix-associated vascular channels. The total tube area of each group was determined using ImageJ (Fig. 1B). Scanning electron microscopy revealed that B16 cells had numerous pseudopods that intertwined with each other. However, the protrusions were decreased after fasudil treatment (Fig. 1C), confirming the findings observed under contrast microscopy.

Disruption of actin stress fibers by the Rho kinase inhibitor fasudil

It is well known that Rho kinase plays a crucial role in the formation of actin stress fibers. To confirm the effect of fasudil, a Rho kinase inhibitor, on cytoskeletal structures, we added 0, 10, 30, or 60 \text{m}\text{mol/L} fasudil to B16 cells for 1 hour. The cells were subsequently stained with rhodamine-phalloidin. Figure 2 shows that, compared with the control cells, treatment with fasudil caused the breakage and fracture of stress fibers in B16 cells, indicating that Rho kinase is crucial for cytoskeletal actin filament integrity.

Inhibitory effect of fasudil on B16 cell motility in vitro

It has been reported that cell migration begins with an initial protrusion of the plasma membrane at the leading edge of the cell and is driven by the polymerization of a network of cytoskeletal actin filaments (36). The in vitro migration ability of B16 cells was evaluated using wound-healing and transwell migration assays. After cells were treated with fasudil at different concentrations, a significant inhibition was observed. Cells in the control group quickly migrated into the available space and nearly filled the wound gap over 24 hours. In contrast, cells treated with fasudil underwent morphologic changes due to cytoskeletal disruption, and few cells migrated (Fig. 3A). The wound-healing effect was quantified, and the data are shown in Fig. 3B. In agreement with the wound-healing assay, treatment with fasudil significantly suppressed B16 cell migration in a dose-dependent manner in the transwell migration assay (Fig. 3C and D).
Effect of fasudil on B16 cell RhoA/ROCKs expression

To examine the effect of fasudil on RhoA kinase expression, RT-PCR and Western blotting were performed to analyze the expression of RhoA and ROCK at the mRNA and protein levels in B16 cells. The amplification specificity was verified by sequencing, and the fold changes relative to the negative control are given in Fig. 4A. The results showed that, in B16 cells, the mRNA expression of RhoA, ROCK1, and ROCK2 was significantly inhibited by fasudil in a dose-dependent manner. Of note, when the concentration of fasudil reached 60 μmol/L, the expression of these three mRNAs was inhibited more than 50%. These results were in agreement with the Western blots (Fig. 4B).

Extinction effect of LPA on fasudil’s role in B16 cell VM activity, cytoskeletal disruption, and downstream protein expression

Firm, tube-like structures were observed in the control and LPA-stimulated groups; the latter group formed larger tubular structures within 4 hours (2 hours earlier than the control group). VM formation was significantly decreased in the cells incubated with fasudil; however, the coadministration of LPA reversed these effects (Fig. 5A). Similar effects were observed in F-actin polymerization (Fig. 5B). The phosphorylation of the myosin light chain (p-MLC) has been reported as the downstream molecular mechanism of RhoA/ROCKs actin-myosin force generation (32, 37, 38) and angiogenesis (18). Thus, changes in ROCK1, ROCK2, and p-MLC were measured using Western blot analysis to determine RhoA activity (Fig. 5C). ROCK1 expression in the presence of fasudil and LPA was similar to that of the control group. However, while comparable results were obtained for ROCK2 and p-MLC after the addition of LPA, the inhibitory effect of fasudil on ROCK2 and p-MLC expression was blocked with the addition of 5 μmol/L LPA. These observations further indicate that fasudil-induced inhibition of VM activity and F-actin polymerization is RhoA/ROCK dependent.

In vivo inhibition of tumor growth and VM formation by fasudil

We repeated the study by Zhang and colleagues and observed similar findings (data not shown). A B16 xenograft model was successfully established (Supplementary Fig. S2). Because the amount of VM inversely relates to tumor volume, and the Rho kinase pathway is hypothesized to be active in the early stages of cancer progression (16), fasudil treatment began 6 days after B16 cell inoculation. The mice were anesthetized on day 11, and the tumors were measured. This study demonstrated that fasudil treatment significantly reduced tumor growth compared with the control group (82.17 ± 21.91 mm3 in control animals vs. 22.27 ± 9.62 mm3 in fasudil-treated animals; P < 0.05; Fig. 6A). VM density was examined under a light microscope (Olympus). Five independent areas were randomly selected at a low magnification (100×) and then counted at high power (400×) to improve accuracy. Immunohistochemical staining revealed rich blood sinoids in xenografts (Supplementary Fig. S2B). However, VM density was significantly reduced after fasudil treatment (1.80 ± 0.37 vessels/field) compared with saline treatment (3.48 ± 0.46 vessels/field; P < 0.05; Fig. 6B–D). Moreover, fewer VM channels and larger areas of necrosis and bleeding were observed in the fasudil-treated group (Fig. 6C and D).

Finally, RNAs and proteins were extracted from freshly frozen tissues, and RT-PCR and Western blotting were used to explore the possible mechanisms of fasudil VM inhibition in xenografts. Consistent with in vitro findings, the in vivo study showed that RhoA, ROCK1, and ROCK2 were significantly downregulated in fasudil-treated tumors (Fig. 6E and F).

Discussion

VM was initially discovered in aggressive melanomas in 1999 (2); therefore, melanoma is an ideal model system to study this phenomenon. Notably, recent studies have indicated that tumor cells might act as the progenitors for tumor vasculature (39, 40). It was also reported that VM vessels supply nutrients and oxygen for tumors in the early stage, and then, endothelial cells grow into the space made by tumor cells and consequently induce angiogenesis and vasculogenesis (35, 41). Thus, these vasculogenic tumor cells are reasonable targets for novel anticancer therapy.

We found that fasudil, a Rho kinase inhibitor, could inhibit the formation of VM via the RhoA/ROCK signaling pathway both in vitro and in vivo in B16 melanoma cells. It is generally accepted that the organization of tumor cells into a capillary-like structure is a hallmark of tumor VM (42). Using 3-dimensional culturing, which has gained popularity due to its close mimicry of the in vivo environment (43), the tube-formation capacity of B16 melanoma cells was confirmed. We observed that when these cells were exposed to fasudil, the number and the total area of the vasculogenic networks were decreased, which agreed with the previous finding that Rho kinase inhibitors prevented the formation of tubular structures on Matrigel by endothelial cells (44). For the first time, we showed that the Rho kinase inhibitor fasudil also inhibited the capillary-like tubes formed by B16 cells, and this
finding laid the foundation for our additional studies. It has long been noted that Rho kinase works as an effector that mediates actomyosin cytoskeletal rearrangements and thereby regulates cell migration, which is exactly the mechanism by which Rho kinase acts in angiogenesis. In our study, B16 cells became morphologically flattened within an hour of fasudil treatment. Confocal photomicroscopy revealed that the stress fibers were incapable of reorganization. Moreover, B16 cell migration was dramatically inhibited by fasudil during wound healing and transwell migration. These findings further support the pivotal role of fasudil in the disruption of cytoskeletal formation and migration. From these results, we were led to theorize that the attenuated tube formation of B16 cells might be induced by fasudil. We then investigated whether the effects of fasudil were regulated through the RhoA/ROCK signaling pathway. Our findings suggest that fasudil effectively inhibited the expression of RhoA and its downstream effectors ROCKs at the mRNA and protein levels compared with their normal controls. Moreover, we found that the inhibitory effect of fasudil on ROCKs (especially ROCK2) and the downstream expression of p-MLC was attenuated under the addition of the Rho GTPase activator LPA, which is consistent with B16 cell functional changes (VM activity and F-actin polymerization). Thus, our data strongly suggest that the Rho kinase inhibitor fasudil suppresses the VM of B16 melanoma cells in vitro, and such an effect is most likely mediated through RhoA/ROCK pathway inhibition.

In tumor-bearing mice, we detected tumor cell–lined blood vessels using PAS staining for the vascular basement membrane. At an early stage in tumor development (6 days after B16 cell inoculation in our model), abundant PAS-positive lumens were...
observed. As the tumor grew, these lumens were replaced by endothelial cell–lined vessels. These results agreed with the previously published finding that VM is an adaptation to the early internal environment (35). On the basis of these findings, fasudil was administered at an early stage, and the drug showed potent antitumor and anti-VM activity. Importantly, fasudil, as an

Figure 5.
LPA attenuates the inhibitory effects of fasudil on VM activity, cytoskeletal stability, and VM-related protein expression in B16 cells. B16 cells were treated with fasudil (30 μmol/L), LPA (5 μmol/L), or a combination of fasudil (30 μmol/L) and LPA (5 μmol/L). Then, the formation of vessel-like structures and actin stress fibers was observed using a tube-formation assay (magnification, 100×; A) and rhodamine-phalloidin immunofluorescence (magnification, 1,000×; B). C, the protein levels of RhoA, ROCK1, ROCK2, MLC, and p-MLC in each group were determined by Western blot analysis.

Figure 6.
Fasudil reduces the growth of B16 cell tumor xenografts in C57/BL mice and attenuates the formation of PAS-positive vessels. A, the tumor volume was 82.17 ± 21.91 mm³ in control animals versus 22.27 ± 9.62 mm³ in fasudil-treated animals; P = 0.005. B, quantification of PAS-positive microvessels from five random fields of each tumor with the 40× objective. C and D, representative images of VM (vascular-like structures, composed of PAS-positive tumor cells, in which blood cells were located) in each group. The arrows in this picture represent areas of necrosis (yellow arrow) and hemorrhagic focus (red arrow). E and F, relative expression levels of RhoA, ROCK1, and ROCK2 in tumor tissues, which were measured by RT-PCR and immunoblotting. The data shown are the mean ± SD of five mice in each group. *, P < 0.05.
attractive antitumor drug candidate, has already been well characterized (16, 45, 46). However, this study demonstrated for the first time that fasudil might affect VM in vivo. RNAs and proteins were extracted from freshly frozen tissues and analyzed by RT-PCR and Western blotting. As expected, the expression of RhoA, ROCK1, and ROCK2 in tumor tissues was suppressed after fasudil treatment, which was consistent with the in vitro findings.

Our results possessed good reproducibility but still suffered from certain limitations. First, although the study showed that both ROCK1 and ROCK2 expressions were inhibited by fasudil, the addition of LPA only upregulated ROCK2. A previous study indicated that VEGF-driven angiogenesis is largely mediated by inhibition of ROCK1, ROCK2, or both through ROCK2, rather than ROCK1 (47). Therefore, whether the effects are mediated by inhibition of ROCK1, ROCK2, or both remains unclear. Further research is needed to clarify this issue by transfecting cells with ROCK1 shRNA or siRNA, employing isoform-specific inhibitors or using ROCK1- and ROCK2-knockout mouse models. Second, the upstream and downstream components of the RhoA/ROCK signaling pathways were not examined in this study. Further investigation of sema4D and myc light chain (MLC) phosphatase (MYP1T1), MLC2, LM kinases, and ezrin/radixin/moesin (ERM) is warranted (18, 48). Moreover, the importance of other Rho-GTPases, such as RhoC, in VM should be further investigated. Third, the dose of fasudil used in mice was determined by consulting the literatures (49–52). However, the serum concentration of fasudil during the in vivo treatment was unknown; the low volume of mouse blood (1–2 mL) was an obstacle to identifying the serum concentration of fasudil in our study. Finally, all experiments were performed using mouse melanoma cells, and future studies should be conducted in human cancer cell lines to confirm and extend our findings.

In conclusion, we described a new mechanism of VM in mouse melanoma in this study. For the first time, the results of the present study provide experimental evidence that fasudil suppresses mouse melanoma-induced VM. This finding may lead to new alternatives in cancer treatment by targeting both angiogenesis and VM using the Rho kinase inhibitor fasudil. Moreover, the fact that fasudil is approved for human use and is well tolerated makes it an attractive anticancer drug candidate. In the future, we expect that these findings may translate to clinical use to accelerate the development of alternative and more selective cancer treatment strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: F. Zhu, G. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Xia, X.-Y. Cai, J.-Q. Fan, F. Zhu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-Q. Fan, G. Wu
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X.-Y. Cai, L.-L. Zhang, J.-H. Ren, J. Chen, G. Wu

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References
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