The Rho kinase inhibitor fasudil inhibits tumor progression in human and rat tumor models

Han Ying, Sandra L. Biroc, Wei-wei Li, Bruno Alicke, Jian-Ai Xuan, Rene Pagila, Yasuhiro Ohashi, Toshiya Okada, Yoichi Kamata, and Harald Dinter

1RBA Oncology and 2Research Center USA, Berlex Biosciences, Richmond, California; 3Nihon Schering Kabushiki Kaisha, Kobe, Japan; and 4Department of Laboratory Animal Medicine and University, Osaka, Japan

Abstract

The ability of cancer cells to undergo invasion and migration is a prerequisite for tumor metastasis. Rho, a Ras-related small GTPase, and the Rho-associated coiled coil-containing protein kinases (Rho kinases, ROCK1 and ROCK2) are key regulators of focal adhesion, actomyosin contraction, and thus cell motility. Inhibitors of this pathway have been shown to inhibit tumor cell motility and metastasis. Here, we show that fasudil [1-(5-isoquinolinesulfonyl)-homopiperazine], an orally available inhibitor of Rho kinases, and its metabolite 1-(hydroxy-5-isoquinoline sulfonyl-homopiperazine) (fasudil-OH) modify tumor cell morphology and inhibit tumor cell migration and anchorage-independent growth. In addition, we show that fasudil inhibited tumor progression in three independent animal models. In the MM1 peritoneal dissemination model, tumor burden and ascites production were reduced by >50% (P < 0.05). In the HT1080 experimental lung metastasis model, fasudil decreased lung nodules by ~40% (P < 0.05). In the orthotopic breast cancer model with MDA-MB-231, there were 3-fold more tumor-free mice in the fasudil-treated group versus saline control group (P < 0.01). Fasudil has been approved for the treatment of cerebral vasospasm and associated cerebral ischemic symptoms. In patients, fasudil is well tolerated without any serious adverse reactions. Therefore, the concept of Rho kinase inhibition as an antimetastatic therapy for cancer can now be clinically explored. [Mol Cancer Ther 2006;5(9):2158–64]

Introduction

Malignant tumors undergo extensive invasion and metastasis. The degree of invasion and metastasis negatively affects cancer patients prognosis and treatment benefit. In fact, tumor metastasis and associated complications are the primary cause for cancer mortality. Therefore, there is a great need to identify molecular pathways that are essential for tumor invasion and metastasis and to develop pharmacologic compounds that could effectively block these pathways.

Rho kinases, also termed Rho-associated coiled coil-containing protein kinases (ROCK1 and ROCK2), are protein serine/threonine kinases that are activated when bound to the GTP-bound form of the small GTPase RhoA or RhoC. Several substrates of Rho kinase have been characterized, including myosin light chain phosphatase, myosin light chain, ezrin-radixin-moesin proteins, GTPase-activating protein, neurofilament protein (NF-1), and LIM kinases (LIMK1 and LIMK2), many of which are involved in cell contractility and cytoskeleton assembly, suggesting an involvement of the Rho/Rho kinase pathway in cell migration, invasion, cell-cell adhesion, actomyosin contraction, cytokinesis, and mitosis (1–4).

Several lines of evidence indicate that the Rho/Rho kinase pathway may be important for cancer invasion, growth, and metastasis. Rho GTPases were required for Ras-mediated oncogenic transformation (5). Several members of the small GTPase Rho family, RhoA, RhoC, RhoH, Rac1, and CDC42, are overexpressed in several cancer types (6–9). In vitro studies, as well as animal experiments, suggest that interruption of Rho/Rho kinase pathway affects tumor invasion and metastasis. Rat hepatoma MM1 cells expressing a constitutively active mutant of ROCK enhanced the invasive activity of the cell. Conversely, expression of a dominant-negative, kinase-defective ROCK mutant significantly inhibited the invasiveness of the cell (10). A specific ROCK inhibitor, Y-27632, inhibited both Rho-mediated activation of actomyosin and invasive activity of these cells. Additionally, Y-27632 reduced the peritoneal dissemination of MM1 cells in rats (10). Furthermore, Y-27632 inhibited invasiveness of several other animal and human cancer cells, including carcinosarcoma Walker 256, neuroblastoma NIE-115, human hepatocellular carcinoma, and the human prostate cancer PC3 (11–14). Another Rho kinase inhibitor, Wa-536, inhibits metastatic invasion of human fibrosarcoma HT1080 and murine melanoma B16 cells (15, 16). These results indicate that Rho kinases play an essential part in tumor cell invasion and metastasis and suggest that the Rho kinases are potential therapeutic targets.

Fasudil [1-(5-isoquinolinesulfonyl)-homopiperazine] is a well-described orally available Rho kinase inhibitor, which has been shown to modify myosin light chain phosphorylation in smooth muscle cells and thereby to...
regulate vasodilation (17, 18). Fasudil has been approved in Japan for the treatment of cerebral vasospasm following surgery for subarachnoid hemorrhage and associated cerebral ischemic symptoms (18). Following p.o. administration, fasudil is converted into the active metabolite 1-(hydroxy-5-isoquinoline sulfonyl-homopiperazine) (fasudil-OH). In patients, fasudil is well tolerated without any severe adverse reactions (19, 20). Recently, Yamaguchi et al. (21) showed that fasudil interacts with the phosphate-binding loop of Rho kinase and induces conformational changes that increases the surface complimentarity to the inhibitor, resulting in changes in catalytic activity of Rho kinase.

Although fasudil has been tested in cardiovascular disease models, its effect on cancer growth and dissemination has not been reported previously. Therefore, it is of great interest to investigate whether fasudil affects early events in tumor progression in vitro and in animal models and thus would have potential for the treatment of human cancer. Previous studies have provided a rationale for us to test the activity of fasudil on the peritoneal dissemination model that uses the rat hepatoma cell line MM1 and on the lung metastasis model using the human fibrosarcoma cell line HT1080. In addition, it has been shown that Rho proteins, such as RhoA, are overexpressed in breast cancer (6, 8, 9), which gives a rationale for selecting MDA-MB-231 for our studies. We found that fasudil and its in vitro metabolite, fasudil-OH, altered morphology of cells growing on plastic, inhibited in vitro cell migration and anchorage-independent growth in soft agar, and inhibited in vivo tumor progression in three independent tumor models.

Materials and Methods

Compounds

Fasudil and fasudil-OH were synthesized and purified by Asahi-KASEI Corp. (Osaka, Japan) and by Berlex Biosciences (Richmond, CA). Cell culture reagents were from Invitrogen (Grand Island, NY).

Cell Lines

Human breast carcinoma (MDA-MB-231) and human fibrosarcoma (HT1080) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in the medium containing MEMx plus 10% fetal bovine serum plus 0.1 mmol/L NEAA plus 1 mmol/L sodium pyruvate plus 2 mmol/L glutamine plus penicillin/streptomycin. Rat hepatoma (MM1) cells, isolated from parental rat ascites hepatoma (AH130) cells, and stably expressing constitutively active RhoA (Val14-RhoA) cells were a kind gift from Dr. Akedo (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan). These cells were grown in suspension in modified MEM supplemented with 10% fetal bovine serum. All cells were maintained at 37°C in a humidified atmosphere with 5% CO2.

Tumor Cell Morphology Study

MDA-MB-231 and HT1080 cells (8 × 10^4) were seeded in eight-well chamber slides (BD Biosciences, Mountain View, CA) with growth medium overnight at 37°C. The next day, cells were treated with 50 μmol/L fasudil for 3 hours. Cells were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 30 minutes, washed with PBS, and stored at 4°C overnight. For staining with the actin cytoskeleton staining kit (FAK 100, Chemicon International, Temecula, CA), cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature, washed with PBS, stained with TRITC-conjugated phalloidin (1:500 dilution), washed, and stained with 4,6-diamidino-2-phenylindole for 5 minutes (1:1,000 dilution). Slides were air dried and coverslipped with antifade mounting solution (DAKO, Carpinteria, CA). Fluorescence images were photographed using the ×10 or ×40 objective.

Cell Migration Assay

MDA-MB-231 and HT1080 cells were assessed for migration through a filter in a Transwell migration assay. Cells growing on plastic were treated with either 0.5% DMSO, 50 μmol/L fasudil, or 50 μmol/L fasudil-OH for 1 hour, trypsinized, counted, and added to the upper well of Transwell chambers (BD Biosciences, Mountain View, CA). DMSO (0.5%), fasudil (50 μmol/L), or fasudil-OH (50 μmol/L) was added to the upper chambers. Medium containing 10% FBS was added to the lower chambers and acted as the chemoattractant. After incubation for 6 hours at 37°C, cells in the lower chamber were fixed and stained with Syto-13. The fluorescence was quantified in a Wallac Victor II fluorescent plate reader (Perkin-Elmer, Wellesley, MA).

Soft Agar Assay

MDA-MB-231 cells were suspended in 0.5 mL of 0.3% low-melting agarose, seeded in a 35-mm Petri dish over a layer of 1 mL of 0.5% agarose at a density of 6,000 per plate, and then incubated for an additional 24 hours before compound treatment. The Rho kinase inhibitors were serially diluted into 0.3% agarose and 0.5 mL of each dilution was applied over the cell layer. Every 7 days, an additional 0.5 mL of 0.5% agarose containing the Rho kinase inhibitors was added. After 3 weeks of incubation (37°C; humidified, and 5% CO2), the colonies were stained with 1 mg/mL nitroblue tetrazolium, colony images were photographed on a Bio-Rad Imager (Bio-Rad, Hercules, CA), and the density and size of the colonies were analyzed by Image ProPlus. The relative potency of the compounds was described as percentage inhibition of number and size of colonies compared with the untreated control.

In vivo Animal Models

Based on pharmacokinetic studies done in-house (data not shown), we know that fasudil is rapidly cleared in rodents. Thus, a variety of dosing regimens was used in the animal efficacy studies to ensure maximal continuous systemic exposure of the drug. Fasudil was delivered by either i.v. thrice daily, in osmotic mini pump, in the drink, or p.o. gavage twice daily. In each case, fasudil was given at the maximum the animal could tolerate in a chronic dosing regimen. All studies were done under American Association for the Accreditation of Laboratory Animal Care guidelines for humane treatment of animals and adhered to all national and international standards.
For the syngeneic peritoneal dissemination model, $2 \times 10^7$ MM1 (rat hepatoma) cells were inoculated i.p. to male Donryu rats (body weight, 100 g) on day 0. Fasudil was given i.v. at 30 mg/kg/d (given in three injections spread 8 hours apart) from day 0 to 11. Eleven days after cell inoculation, the rats were euthanized, the peritoneal fluid was drained into a collection vessel, and the peritoneum was exposed. The ascites fluid was quantified and the tumor nodules were counted.

In the experimental lung metastasis model, HT1080 cells ($2 \times 10^6$) were injected into the tail vein of nude mice on day 0 to seed cancer cells to the lung. The mice ($n=18$ per group) were implanted s.c. with Alzet mini pumps (Durect Corp., Cupertino, CA) containing either 10% saline or fasudil to deliver 50 mg/kg/d (day 0–21). Twenty-one days after cell inoculation, the mice were euthanized and the lungs were fixed by injecting formalin into the tracheas followed by soaking with Bouin’s solution. The nodules were macroscopically counted and the presence of tumor cells in the nodules was confirmed microscopically.

For the orthotopic model with MDA-MB-231 cells, 6-to 8-week-old female, athymic mice (nu/nu) were obtained from Simonsen (Gilroy, CA). Mice were placed under isoflurane anesthesia and 40 $\mu$L of $2 \times 10^6$ MDA-MB-231 cells mixed 1:1 with Matrigel (BD Biosciences, Bedford, MA) were injected into the left inguinal mammary fat pad on day 0 ($n=30$). Tumor volume (mm$^3$) was estimated by caliper measurement in two perpendicular directions and calculated using the formula: (shortest diameter)$^2 \times$ (longest diameter)$ \times 0.5$. Immediately after cell implantation, the p.o. gavage treatments began. The control group received water in the bottle and water by gavage twice daily (day 0–57). The ‘‘gavage-only’’ group received fasudil by gavage (100 mg/kg) twice a day (200 mg/kg/d) from day 0 to 57. The ‘‘dual administration’’ group received fasudil in the drinking water (1 mg/mL) from day 6 to 57 and by gavage (100 mg/kg) once a day from day 0 to 57 (228 mg/kg/d based on mice drinking 3.2 mL/d). Mice exhibited a visible red flush and were lethargic for about an hour after giving the gavage dose (as expected from a drug inducing vasodilation), indicating the drug had systemic exposure. Tumors became palpable 4 weeks after cell implantation. Mice in the control group drank ~4.2 mL/d; in the dual administration group, mice drank ~3.2 mL/d. The health of the mice was not affected by the smaller liquid consumption. On day 57, all mice were euthanized and the tumors were excised and weighed.

**Statistical Analysis**

Statistical analyses were done with JMP 5.1 (SAS Institute, Inc., Cary, NC). Significance was determined by the Student’s unpaired $t$ test, the Kruskal-Wallis, the nonparametric Wilcoxon test (Mann-Whitney $U$ test), and the one-way ANOVA with pair-wise comparison by Bonferroni method. The Fisher’s exact test was applied to incidence of tumor formation. $P$s <0.05 were considered significant.
plastic surface as shown in Fig. 1A and C for MDA-MB-231 and Fig. 1E and G for HT1080 cells. After 3 hours of compound treatment, however, the cell body became elongated (MDA231 cells, Fig. 1B and D; HT1080 cells, Fig. 1F and H), less spread with a narrowing of the cell body shape (MDA231 cells, Fig. 1B and D; HT1080 cells, Fig. 1F and H). When cells were treated with either fasudil (10 μmol/L) or fasudil-OH (10 or 50 μmol/L), they had similar appearance (data not shown). The change in morphology was not due to toxicity because MDA-MB-231 or HT1080 cells are able to withstand up to 100 μmol/L fasudil or 100 μmol/L fasudil-OH in an ATP metabolism-based (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay (data not shown). These results imply that fasudil and fasudil-OH can modify the cytoskeleton via Rho kinase–dependent pathways leading to altered morphology.

**Fasudil and Fasudil-OH Inhibits Tumor Cell Motility In vitro**

To investigate the effects of fasudil and fasudil-OH on the motility of tumor cells, MDA-MB-231 or HT1080 cells were tested in the Transwell migration assay. Cells growing on plastic were treated with drug for 1 hour and then harvested and seeded in serum-free medium in the upper chamber containing the drug substance. After 6 hours of incubation, the cells that had migrated into the serum-containing lower chamber were quantified. Treatment with 50 μmol/L fasudil-OH significantly inhibited migration of MDA-MB-231 (P = 0.029) and HT1080 (P = 0.024) cells by ~50% (Fig. 2). In contrast, 50 μmol/L fasudil inhibited HT1080 migration by only ~26% (P = 0.275) and it did not inhibit MDA-MB-231 migration, suggesting that fasudil is slightly less potent than fasudil-OH in inhibiting motility of tumor cells.

**Fasudil and Fasudil-OH Inhibit Anchorage-Independent Growth of Tumor Cells**

Although the molecular mechanism underlying tumor cell growth in soft agar is unknown, tumor cells in soft agar reside and interact with the extracellular matrix, proliferate in response to signals in the environment, and invade and penetrate the matrix to form colonies. It is conceivable that to some extent this process mimics the molecular events that are involved in tumor metastatic dissemination. Therefore, soft agar assays were done to determine the antimetastatic potential of fasudil and fasudil-OH. MDA-MB-231 cells were incubated for 3 weeks in the presence of fasudil or fasudil-OH at various concentrations. As shown in Fig. 3, 1 μmol/L fasudil inhibited ~40% colony formation, whereas 1 μmol/L fasudil-OH inhibited ~80% colony formation. Fasudil or fasudil-OH inhibited both number and size of MDA-MB-231 colonies. In additional experiments (data not shown), the IC50 of fasudil and fasudil-OH on MDA-MB-231 was determined to be ~1 μmol/L or ~30 nmol/L, respectively. This study suggests that inhibition of Rho kinases decreases the capacity of colony formation of tumor cells in vitro.

**Fasudil Inhibits Tumor Early Establishment In vivo**

The hypothesis that fasudil affects invasion and metastasis was tested in vivo with the MM1 (rat), MDA-MB-231 (human), and HT1080 (human) tumor models. The pharmacokinetic variables of fasudil (short serum half-life, rapid conversion to metabolite) dictated the use of a variety of dosing regimens, each designed to provide maximum continuous systemic drug exposure. The animal models were run in prevention mode because the Rho kinase pathway is hypothesized to be active in early stages of cancer progression.

In the peritoneal dissemination model, cancer cells are introduced by i.p. injection whereupon the cells attach to internal organs and form tumors. The tumors shed cells, which give rise to a swollen abdomen from ascites accumulation. Rat MM1 cells were inoculated i.p. to male Donryu rats. Then, fasudil was given i.v. at 30 mg/kg/d (given in three injections spread 8 hours apart) for 11 days. As shown in Table 1, the number of peritoneal tumor nodules was significantly reduced in the fasudil-treated mice compared with the saline-treated control group (4.2 ± 1.5 versus 9.9 ± 0.8; P = 0.004). In addition, the amount of ascites fluid was significantly reduced in the fasudil-treated rats compared with the control group (7.0 ± 2.4 versus 16.0 ± 2.2; P = 0.013; Table 1).

![Figure 2](Image)

**Figure 2.** Fasudil and fasudil-OH inhibit the motility of cancer cells in a Transwell migration assay. HT1080 (A) or MDA-MB-231 (B) cells were allowed to migrate from serum-free medium (upper chamber) toward serum-containing medium (lower chamber). DMSO (0.5%), fasudil (50 μmol/L), or fasudil-OH (50 μmol/L) was added to the upper chambers. After 4 h, cells in the lower chamber were stained with Syto-13 and the fluorescence was measured. Columns, average of triplicate wells; bars, SD. Experiment was repeated with similar results. FU, fluorescence units.
In the experimental lung metastasis model, HT1080 tumor cells were injected into the tail vein of nude mice. Fasudil was given continuously for 3 weeks by Alzet mini pump at 50 mg/kg/d. As shown in Table 1, the number of tumor nodules was significantly reduced in fasudil-treated mice compared with the vehicle-treated control group (45.9 ± 5.5 versus 74.1 ± 10.0, P = 0.017).

To further investigate the effect of fasudil on tumor progression, an orthotopic model, in which MDA-MB-231 cells were implanted into the mammary fat pad, was used. In one group, fasudil was given p.o. twice daily at 200 mg/kg/d and a second group received fasudil p.o. once daily by gavage and continuously in the drink for ~228 mg/kg/d. At the end of the study, plasma levels of fasudil and fasudil-OH were present above 1 μmol/L at 4 hours after the final dose. This level should be sufficient to inhibit Rho kinase because Hattori et al. (23) showed that a plasma concentration of ~0.4 μmol/L reduced phosphorylation of client proteins (ezrin-radixin-moesin family) in cardiac tissue. As shown in Fig. 4, the number of tumor-bearing mice was dramatically reduced in the fasudil-treated groups. Whereas only 6 of 29 (21%) mice were tumor free in the control group, 16 of 27 (59%; P < 0.003) of the dual administration mice and 16 of 26 (62%; P < 0.003) of the gavage-only mice were tumor free. However, the tumors that formed had similar size in vehicle control and fasudil groups. Thus, fasudil treatment does not suppress growth of established tumors but greatly reduces the incidence of tumor formation.

**Discussion**

In this study, we report the antitumor activities of the Rho kinase inhibitors fasudil and its metabolite fasudil-OH in vitro and in animal tumor models. We first showed that both fasudil and fasudil-OH induce dramatic morphologic changes in MDA-MB-231 and HT1080 cells. Already, within an hour of treatment, the cell bodies became elongated, suggesting that the cells were losing their attachment points to the plastic surface. This effect was even more pronounced at 3 hours. During prolonged exposure to fasudil (7 hours), many cells became rounded and some cells detached from the plastic surface (data not shown). These results agree with published studies, showing that the Rho kinase inhibitor Y-27632 causes morphologic changes of PC3 prostate cancer cells as well as endothelial cells (14, 24). Therefore, our study further supports the pivotal role of Rho/Rho kinase pathway in regulating reorganization and assembly of the cytoskeleton.

The functions of the Rho/Rho kinase pathway in cell migration and invasion have been shown by several studies. The Rho kinase inhibitor, Wf-536, reduced both

### Table 1. Fasudil reduced tumor burden in two animal models of cancer

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<th>In vivo model</th>
<th>Treatment</th>
<th>n</th>
<th>No. tumor nodules in lung or peritoneum</th>
<th>P*</th>
<th>Ascites volume (mL)</th>
<th>P*</th>
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<tr>
<td>Peritoneal dissemination</td>
<td>Saline</td>
<td>10</td>
<td>9.9 ± 0.8</td>
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<td>16.0 ± 2.2</td>
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<td></td>
<td>Fasudil</td>
<td>10</td>
<td>4.2 ± 1.5</td>
<td>0.004</td>
<td>7.0 ± 2.4</td>
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<tr>
<td>Lung seeding</td>
<td>Saline</td>
<td>18</td>
<td>74.1 ± 10.0</td>
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<tr>
<td></td>
<td>Fasudil</td>
<td>18</td>
<td>45.9 ± 5.5</td>
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*Fasudil treated compared with control group using the one-way ANOVA with pair-wise comparison by Bonferroni method. Ps < 0.05 were considered significant.

*Rat MM1 cells (2 × 10⁶) were injected i.p. into Donryu rats on day 0. Fasudil was given daily at 30 mg/kg/d i.v. for 11 days. The animals were euthanized on day 12, the ascites fluid was quantified, and the tumor nodules were counted.

*Nude mice were inoculated by i.v. tail vein injections with 2 × 10⁶ HT1080 cells on day 0 (n = 18). The mice were implanted s.c. with Alzet mini pumps to deliver either 10⁻¹ saline or Fasudil at 50 mg/kg/d from day 0 to 21. On day 21, the animals were euthanized, the lungs were fixed with formalin and Bouin’s solution, and the tumor nodules were counted.
invasion and migration in Lewis lung carcinoma cells and it inhibited invasion, migration, and the formation of capillary-like tubes by endothelial cells on Matrigel (25).

In a separate study, the authors showed that WI-536 inhibited migration of murine melanoma B16BL6 cells and displayed anti-invasive effects under conditions of both chemotaxis and chemokinesis (16). Similar results were reported with Y-27632, which inhibited lysophosphatidic acid–induced migration and invasion of Caov-3 and OVCAR-3 ovarian cancer cells (26). These data are corroborated by our results, which show inhibition of tumor cell migration and invasive growth in soft agar by the Rho kinase inhibitor, fasudil.

How the Rho/Rho kinase pathway affects tumor migration, invasion, and dissemination is yet to be understood. Tumor cell migration requires extensive reorganization of the cytoskeleton and can be dependent on external stimulatory signals (e.g., from the extracellular matrix). Previous studies indicate that the Rho/Rho kinase pathway is involved in stress fiber assembly, the formation of actin-rich spikes called filopodia, which are thought to sense tactic signals, and the establishment of actin-rich ruffles called lamellipodia, which are formed at the leading edge of the cell. Furthermore, the pathway plays a role in the rapid actin transport at the leading edge of the cell (4, 6, 7). All these events contribute to the contractility of the cell body and the formation of integrin-based cell-extracellular matrix contacts. Therefore, interfering with these events may inhibit migration and invasion of tumor cells.

More importantly, we showed the antitumor efficacy of fasudil in tumor early establishment in three independent animal models: fasudil can (a) reduce dissemination of cancer in the peritoneal cavity, (b) reduce blood borne metastasis to the lung, and (c) prevent early establishment of breast tumors in the mammary fat pad. Considering similar efficacy data obtained with Y-27632 and WI-536, it is evident that interruption of the Rho/Rho kinase pathway by Rho kinase small-molecule inhibitors could potentially prevent tumor dissemination and metastasis (10–16). Because fasudil has been approved for clinical use, the concept of Rho kinase inhibition for cancer therapy can now be clinically explored.

When introduced in vivo, the majority of fasudil is converted to fasudil-OH in the liver. It is therefore important to ascertain the potency of fasudil-OH against Rho kinases. We have measured the potency of fasudil and fasudil-OH against purified recombinant ROCK1. Fasudil-OH was slightly more potent than fasudil at constant ATP concentration.6 We have analyzed the potency of fasudil and fasudil-OH in migration assays. Fasudil-OH was slightly more potent than fasudil in inhibiting motility of MDA-MB-231 and HT1080 cells. We also showed that 1 μmol/L fasudil inhibited ~40% colony formation of MDA-MB-231 in soft agar assays, whereas 1 μmol/L fasudil-OH inhibited ~80% colony formation. We further showed that both fasudil and fasudil-OH inhibited Rho kinase activities in MDA-MB-231 and HT1080 lysates and live cells. In these assays, fasudil and fasudil-OH showed similar potency. Taken these together, we have shown that fasudil-OH is at least as potent in cell-based assays. The differential effect of fasudil and fasudil-OH on MDA-MB-231 in soft agar assay is yet to be understood.

Although fasudil showed potent antitumor activities in the three prevention models, it did not suppress growth of already established MDA-MB-231 fat pad tumors, indicating that neither fasudil nor fasudil-OH has antiproliferative activity. Indeed, fasudil or fasudil-OH did not inhibit proliferation of MDA-MB-231 or HT1080 cells when grown in culture dishes (data not shown), mirroring data obtained with Y-27632 in M1M and neuroblastoma cells (10, 12). Furthermore, Y-27632 inhibited chemotaxis and invasion and induced morphologic changes in Walker 256 cells but did not affect the growth rate of the cell (27). The compound prolonged survival in the Walker 256 animal model when combined with a cytotoxic anticancer drug, 5-fluorouracil (27). This reinforces the concept that fasudil exerts its antitumor activity by affecting tumor dissemination and metastasis rather than by directly affecting cellular proliferation. Therefore, in the clinical setting, Rho kinase inhibitors, which act as antimetastatic compounds, should be combined with antiproliferative agents.

Our results provide compelling evidence on the role of Rho/Rho kinase pathway in tumor establishment. The fact

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6 In preparation.
that fasudil is approved for human use and is tolerated without serious adverse reactions makes it an attractive anticancer drug candidate for prevention of cancer metastasis.

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