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Effect of Wf-536, a novel ROCK inhibitor, against metastasis of B16 melanoma

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Abstract *Purpose:* Rho-associated coiled-coil-forming protein kinase (ROCK) is pivotally involved in invasion by tumor cells and their evolution to metastasis. We have developed a novel inhibitor of ROCK, Wf-536 [(+)-(R)-4-(1-aminoethyl)-N-(4-pyridyl) benzamide monohydrochloride]. In the present study, we investigated its effect on in vitro invasion and in vivo pulmonary metastasis of B16 melanoma. *Methods:* The following were evaluated: the anti-invasive effect of Wf-536 against the motility of mouse B16BL6 melanoma cells through a culture insert layered with reconstituted basement membrane (Matrigel); the cytotoxic effect of Wf-536 in the same cell line; the antimetastatic effect of Wf-536, administered by osmotic pump, on spontaneous pulmonary metastasis following subcutaneous injection of B16BL6 melanoma in mice; and the inhibitory effect of orally administered Wf-536, alone or in combination with the antineoplastic drug paclitaxel, on pulmonary metastasis of intravenously injected B16F10 melanoma in mice. *Results:* Wf-536 inhibited in vitro invasion by B16BL6 cells significantly and in a concentration-dependent manner and displayed an anti-invasive effect under conditions of both chemotaxis and chemokinesis. No cytotoxic effect was observed at any of the concentrations used. In vivo, Wf-536 administration suppressed tumor colony formation on the lung surface in a dose-dependent manner (0.3–3 mg/kg per day), with a metastasis inhibition rate of 95% at 3 mg/kg per day. In experimental metastasis of B16F10 melanoma, oral

administration of Wf-536 significantly decreased tumor colony formation in the lung, with an inhibition rate of 41% at 3 mg/kg per day. The inhibition rate of paclitaxel (5 mg/kg per day) was 27%. The combination of Wf-536 and paclitaxel produced a synergistic effect on B16F10 metastasis and a 68% inhibition rate. Wf-536 administration at the doses used did not alter body weight, blood pressure or the health of treated animals as compared to vehicle-treated controls. *Conclusion:* The results suggest that Wf-536 is a potentially valuable drug for preventing tumor metastasis both in monotherapy and in combination with an antineoplastic drug.

Keywords Wf-536 · ROCK · Metastasis · Tumor invasion · Paclitaxel

Introduction

As well as robust proliferation, malignant tumors, including melanoma, are often accompanied by extensive invasion and metastasis, which greatly influence patient prognosis. Chemotherapy against this type of tumor therefore requires drugs which reduce systemic diffusion as well as cause regression of tumor colonies. In general, antineoplastic drugs affect the physiological functions of metastatic tumors, mainly through ‘cytotoxic’ activity. The antineoplastic drug paclitaxel (Taxol), which provides clinical benefit in the treatment of various tumors including melanoma, metastatic breast and ovarian tumors, and non-small-cell lung tumors [4, 22], decreases tumor volume and increases survival in treated melanoma-bearing mice [14, 28], but does not significantly reduce the incidence of metastasis in melanoma and carcinoma [11, 24]. To prevent tumor metastasis as well as growth, treatment with an effective antimetastatic drug in combination with an antineoplastic drug such as paclitaxel would therefore be useful.

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The signaling pathway consisting of Rho, a Ras-related small GTPase, and Rho-associated coiled-coil-containing protein kinase (ROCK) has been demonstrated to be pivotally involved in the invasive stage of tumor cell development [10, 25] and evolution to metastasis [12]. In a mouse model of Lewis lung carcinoma (LLC), Wf-536, a novel ROCK inhibitor, has shown a suppressant effect on metastasis [9] which involves inhibitory activity on the invasive motility of LLC cells and antiangiogenic activity [19].

In the present study, we investigated the effect of Wf-536 on B16 melanoma metastasis in order to clarify its potential as an antimetastatic drug. In addition to examining its inhibitory effect on *in vitro* invasion and *in vivo* spontaneous metastasis of B16BL6 cells, we investigated its antimetastatic effect in monotherapy and in combination with paclitaxel on experimental metastasis of B16F10, another type of B16 melanoma.

Materials and methods

Drugs

Wf-536 [(+)-(R)-4-(1-aminoethyl)-N-(4-pyridyl) benzamide monohydrochloride; Fig. 1] was synthesized and purified by Mitsubishi Pharma Corporation (Osaka, Japan). Paclitaxel was purchased from Sigma (St. Louis, Mo.).

Materials

The following materials were used: osmotic pump (Alzet model 1002, ALZA Corporation, Cupertino, Calif.); hydroxypropyl methylcellulose (HPMC; Mitsubishi Pharma Corporation, Osaka, Japan); Eagle's minimum essential medium (MEM; GIBCO, Life Technologies, Rockville, Md.); MEM non-essential amino acids (100×) and MEM vitamin mix (100×) (ICN Biomedicals, Costa Mesa, Calif.); fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah); fatty acid-free bovine serum albumin (Nacalai Tesque, Kyoto, Japan); cell culture inserts (8 µm pore size) and Matrigel (Becton Dickinson, Franklin Lakes, N.J.); Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan); and a Diff-Quik kit (International Reagents Corporation, Hyogo, Japan).

Cell culture

Mouse B16BL6 and B16F10 melanoma cells, aggressive variants of the B16 cell line, were kindly provided by Dr. T. Tsuruo (Center for Clinical Research and Drug Development, Japanese Foundation for Cancer Research, Tokyo, Japan), with the approval of Dr. I.J. Fidler (University of Texas MD Anderson Cancer Center, USA). B16BL6 and B16F10 cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air in MEM supplemented with 10% FBS, 1 mM sodium pyruvate, 1% MEM non-essential amino acid solution and 1% MEM vitamin solution.

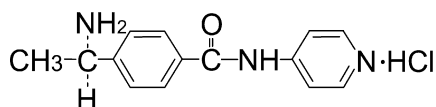


Fig. 1 Molecular structure of Wf-536

Invasion assay

In the chemotactic invasion assay, tumor cells (5×10^4) were suspended in 200 µl serum-free medium containing 0.5% bovine serum albumin and plated into cell culture inserts layered with 25 µg per insert of Matrigel, a reconstituted basement membrane matrix. A separate medium containing 10% FBS was dispensed into 24-well plates (700 µl per well) to produce chemoattractive motile activity. The inserts were then set on the 24-well plates and incubated for 18 h in the presence of the drug at various concentrations. After incubation, the cells on the upper surface of the insert were removed with a cotton swab and those on the lower surface of the insert and on the bottom of the well were fixed and stained using the Diff-Quik kit. The number of infiltrating cells per insert was determined by counting with a microscope the number of cells in ten 1-mm² areas on the lower surface of the insert and on the bottom of the well.

In the chemokinetic invasion assay, tumor cells were suspended in 200 µl medium containing 10% FBS in Matrigel-layered inserts. Other procedures were the same as for the chemotactic invasion assay.

Proliferation assay for cytotoxicity

B16BL6 cells (5×10^3) were plated in each well of a 96-well plate and incubated in the presence of the drug at various concentrations for 48 h. To each well of the culture was added 10 µl of the solution of the Cell Counting Kit-8 which was followed by incubation for 30 min. Absorbance at 450 nm (reference at 650 nm) was measured with a THERMOMax microplate reader (Molecular Devices, Menlo Park, Calif.). The data are expressed as the percentage in relation to control (vehicle-treated) cells.

Metastasis *in vivo*

All animal studies were performed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Mitsubishi Pharma Corporation, which complies with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. The protocols were approved by the Institutional Animal Care and Use Committee.

The *in vivo* spontaneous metastasis experiment was performed using B16BL6 cells according to a previously described method [3] with some modifications. B16BL6 cells (5×10^5) suspended in 50 µl phosphate-buffered saline were subcutaneously injected into the right hind footpad of 6-week-old male C57BL/6 mice (Charles River Japan, Shiga, Japan) under mild ether anesthesia. Wf-536 (0.3, 1, 3 mg/kg per day) was delivered continuously for 35 days from the day of tumor injection via an osmotic pump (pump fill volume 100 µl, infusion rate 0.25 µl/h) implanted in the peritoneal cavity. On day 21, the right leg bearing the tumor focus was amputated under mild ether anesthesia and on day 35 the mice were killed with ether, the lungs excised and fixed with Bouin's solution, and the number of pulmonary metastasized colonies counted microscopically.

The assay of experimental tumor metastasis was performed according to the methods of Fidler [5] and Neri et al. [20] with some modifications. Briefly, B16F10 cells (1×10^5) were intravenously injected into the tail of 8-week-old male C57BL/6 mice. The drugs were diluted with 0.5% (w/v) HPMC/dH₂O. Wf-536 (1, 3 mg/kg per day) was orally administered for 14 days from the day of tumor injection. In the combination experiment, 5 mg/kg of paclitaxel was administered intraperitoneally every other day for a total of six times. On day 14, the lungs were excised and the number of pulmonary metastasized colonies determined microscopically.

Statistical analysis

The results are presented as means ± SEM; *n* represents the number of experiments or the number of animals per group. Statistical analysis was performed using Dunnett's test. *P* values < 0.05 were

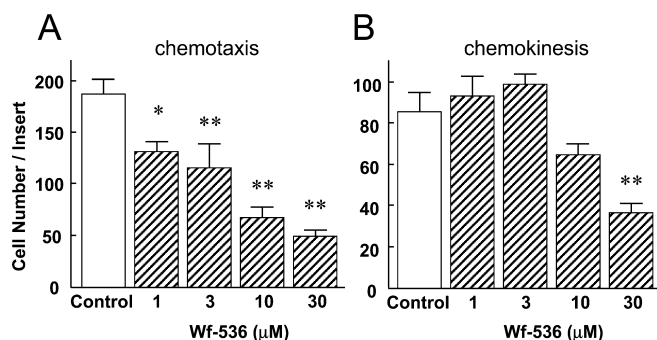


Fig. 2A, B Effect of Wf-536 on mouse B16BL6 melanoma cell invasion through a reconstituted basement membrane matrix under chemotactic (A) and chemokinetic (B) conditions. B16BL6 cells (5×10^4) were incubated on a culture insert layered with Matrigel for 18 h in the presence of the test drugs at the indicated concentrations. In chemotactic invasion, the medium in the upper compartment contained no FBS and the medium in the lower compartment 10% FBS. For the chemokinetic invasion experiment, both compartments contained medium containing 10% FBS. After incubation, the number of infiltrating cells per insert was determined. Each column represents the mean \pm SEM of four determinations from triplicate experiments. ** $P < 0.01$, * $P < 0.05$ vs control; Dunnett's test

accepted as significant. For experiments involving the simultaneous use of two drugs, the combination index for inhibitory effect was calculated by median effect analysis [13, 26].

Results

Inhibition of B16 melanoma cell invasion

Invasion by B16BL6 melanoma cells was observed under conditions of both chemotaxis and chemokinesis (Fig. 2), but with the number of cells infiltrating the Matrigel layer lower in the latter case. Wf-536 significantly decreased the number of B16BL6 cells invading through the Matrigel layer (Fig. 2). Inhibition by Wf-536 was observed under conditions of both chemotaxis and chemokinesis. In the former case (Fig. 2A), B16BL6 cell invasion was inhibited by Wf-536 at concentrations of 1–30 μ M in a concentration-dependent manner, with an inhibition rate between 30% and 74% of control. As shown in Fig. 2B, the inhibitory effect of Wf-536 on chemokinetic invasion also appeared to be concentration-dependent, but significant inhibition was observed only at 30 μ M, with an inhibition rate of 57%. Similar inhibitory activity was observed against invasion by B16F10 melanoma cells (data not shown).

Cytotoxicity toward B16 melanoma

To check whether the inhibitory activity of Wf-536 on invasive functions could be due to a cytotoxic effect, the proliferation of B16BL6 melanoma cells was assessed in the presence of Wf-536 at 1–30 μ M (Fig. 3). No anti-proliferative effect on melanoma cells was observed at concentrations which produced significant inhibition of melanoma invasion.

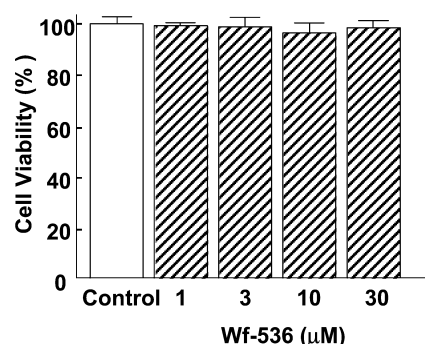


Fig. 3 Effect of Wf-536 on proliferation of B16BL6 melanoma cells. Each value represents the mean \pm SEM of three observations. Note that no significant change in B16BL6 cell proliferation was observed between 1 and 30 μ M of Wf-536

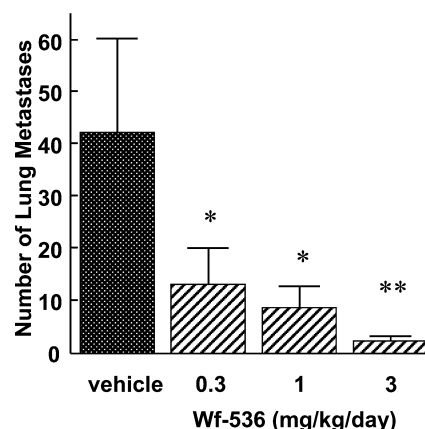


Fig. 4 Inhibitory effect of Wf-536 on melanoma B16BL6 spontaneous metastatic colony formation in mouse lung. Tumor-bearing mice were treated with Wf-536 for 35 days. Each column represents the mean \pm SEM ($n = 20$ –22). ** $P < 0.01$, * $P < 0.05$ vs vehicle-treated control; Dunnett's test

Inhibition of spontaneous metastasis in melanoma

We evaluated the effect of Wf-536 administration by osmotic pump on spontaneous tumor metastasis following subcutaneous injection of syngeneic mouse B16BL6 melanoma cells (Fig. 4). Wf-536 significantly reduced the number of B16BL6 pulmonary metastatic colonies at doses of 0.3–3 mg/kg per day, achieving an inhibition rate of 95% of control at 3 mg/kg per day. In this experiment, Wf-536 showed a slight inhibitory effect (of borderline significance) on primary tumor volume on day 21 (0.324 ± 0.031 cm³ with vehicle; 0.273 ± 0.018 cm³ with 0.3 mg/kg per day; 0.278 ± 0.018 cm³ with 1 mg/kg per day; 0.250 ± 0.016 cm³ with 3 mg/kg per day; $P < 0.05$, 3 mg/kg per day vs vehicle, $n = 20$ –22).

Inhibition of intravenous metastasis in melanoma

To clarify in detail the effect of Wf-536 on melanoma metastasis, Wf-536 was orally administered to mice

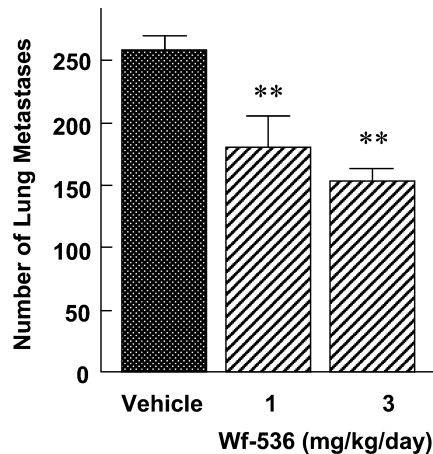


Fig. 5 Inhibitory effect of Wf-536 on melanoma B16F10 hematogenous metastatic colony formation in mouse lung. Tumor-bearing mice were treated with Wf-536 for 14 days. Each column represents the mean ± SEM ($n=8$). ** $P<0.01$ vs vehicle-treated control; Dunnett's test

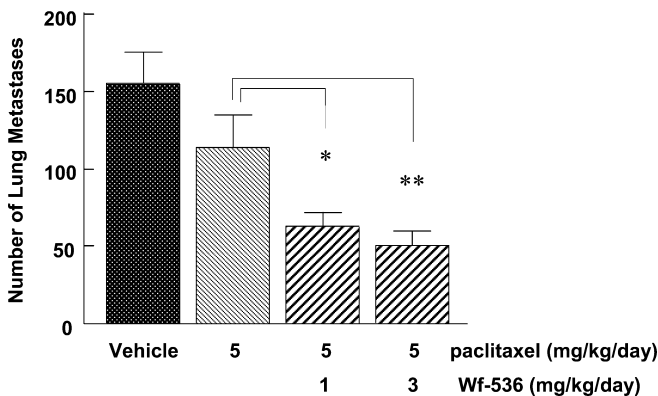


Fig. 6 Synergistic effect of Wf-536 in combination with paclitaxel on melanoma B16F10 hematogenous metastatic colony formation in mouse lung. Tumor-bearing mice were treated for 14 days with paclitaxel alone or Wf-536 combined with paclitaxel. Each column represents the mean ± SEM ($n=8$). ** $P<0.01$, * $P<0.05$ vs paclitaxel-treated group; Dunnett's test

following intravenous injection of B16F10 melanoma cells, another B16 cell line with less spontaneous metastatic potency [8]. Wf-536 showed significant inhibition of B16F10 melanoma pulmonary metastasis at doses of 1 and 3 mg/kg per day (Fig. 5). The inhibition rates versus control were respectively 30% and 41%, which appeared to be lower than in spontaneous metastasis at the same dose (Fig. 4).

This antimetastatic effect of Wf-536 in monotherapy suggested that it would have utility in combination regimens. We therefore subsequently examined the effect of Wf-536 in combination with the antineoplastic drug paclitaxel. In preliminary experiments, the effects of different doses of paclitaxel (1, 2.5, 5, 10, 20 mg/kg per day) were examined (data not shown). The sub-plateau effective dose (i.e. the dose significantly inhibiting tumor metastasis) was 5 mg/kg per day, with an inhibition rate of 27% (Fig. 6), a result similar to those of previous

studies [24]. In the present model, combined administration of Wf-536 (1 or 3 mg/kg per day) and paclitaxel (5 mg/kg per day) produced a statistically significant decrease in the number of melanoma lesions appearing in the lung in comparison with paclitaxel alone (Fig. 6). The inhibition rates with the combined administration with paclitaxel were 60% (Wf-536 1 mg/kg per day) and 68% (3 mg/kg per day), and the combination indices were 0.0182 and 0.0028, respectively. The values of less than 1.0 indicated synergism of these drugs in preventing tumor metastasis [13, 26].

No general toxicity, loss of weight, or hypotensive effect was observed in any of the treated animals during the treatment period (data not shown).

Discussion

The present study demonstrated that Wf-536 inhibits melanoma invasion through a reconstituted basement membrane layer under both chemotactic and chemokinetic conditions. Administration of Wf-536 significantly prevented the pulmonary metastasis of melanoma in a spontaneous metastasis model. In an experimental metastatic model, as well as significant inhibition in monotherapy, Wf-536 showed synergistic inhibition of melanoma metastasis in combination with paclitaxel.

A relationship between degree of motility and metastatic potential has been noted in some studies [16, 18, 21]. In general, the motile ability of tumor cells can be divided into directional motility, or chemotaxis, and non-directional motility, or chemokinesis. In tumor metastasis, chemotaxis is observed when a chemoattractant, such as a migration factor or a growth factor, affects tumor cells and produces directional motility toward the origin of the chemoattractant. In addition, tumor cells with high metastatic potency show greater motility toward blood vessels in the primary tumor than do non-metastatic control cells, suggesting the importance of chemotactic invasion for tumor cell intravasation in metastasis [27]. Besides chemotaxis, chemokinesis, the unordered and random diffusion of tumor cells, may be a necessary factor in the evolution of tumor metastasis, for example in order to avoid attack by host lymphocytes and reach the sites most conducive to survival.

In the present study, we demonstrated that Wf-536 inhibits both motile functions of tumor cells (Fig. 2). The concentration dependency of Wf-536's action in inhibition of melanoma B16BL6 invasion reflected that seen in its inhibition of mouse LLC cell invasion. A positive correlation has been observed between the inhibitory effect on enzymatic ROCK and mouse LLC cell invasion [19]. Moreover, Wf-536 inhibited the invasion of tumor cells but did not show a cytotoxic effect (Fig. 3). These findings suggest that the inhibitory action of Wf-536 on melanoma invasion is dependent on ROCK inhibition and that its anti-invasive action is involved in its suppressant effect on B16BL6 melanoma metastasis (Fig. 4).

In a spontaneous metastasis assay, tumor cells are implanted intramuscularly or subcutaneously to form a local tumor, and metastases to distant organs are measured. Alternatively, in an experimental metastasis assay, tumor cells are directly injected into the circulation. The latter assay is thought to mimic only the latter half of hematogenous metastatic spread, in that the tumor cells are not subjected to the initial steps required to escape from the primary tumor site into the circulation [15]. In the present study, Wf-536 was effective in suppressing spontaneous B16 melanoma metastasis, with subcomplete inhibition with administration of 3 mg/kg per day (Fig. 4), whereas in the experimental metastasis assay its inhibitory effect was less than 50% at the same dose (Fig. 5). In addition, although it inhibited metastasis potently, Wf-536 showed only a slight reductive effect on the primary tumor volume in mice. These findings suggest that one of the significant actions of Wf-536 is in modifying the metastatic function(s) of tumor cells in the stages from the shedding of cells from the primary to intravasation. The pathophysiological role of motile function in the multistep process of tumor metastasis has, however, not been adequately clarified, and needs to be investigated further as a specific and important index for additional clarification of the action mechanism(s) of Wf-536 in prevention of metastasis.

In the present study, Wf-536 succeeded in inhibiting both spontaneous and hematogenous metastasis of B16 melanoma. Also, the IC_{50} values for invasion of several different human tumor cell lines are almost the same [9]. Furthermore, even in lymphatic metastasis models, such as B16F10 or C1300, Wf-536 displays an antimetastatic effect [6, 9]. These findings suggest that Wf-536 has potential activity in various types of tumor and metastasis.

Paclitaxel, a potent cytotoxic diterpene effective against a wide range of tumors, binds to and stabilizes microtubules, preventing depolymerization and resulting in G_2/M arrest, microtubule binding, and tumor cell death [23]. While the complete molecular mechanism contributing to paclitaxel-induced apoptosis remains unclear, it has additionally been reported to cause hyperphosphorylation and activation of raf-1 kinase [2], inducing apoptosis via phosphorylation of Bcl-2 protein accompanied by loss of Bcl-2 function [7]. These potencies may be associated with the action of paclitaxel in causing regression of primary tumors and its less notable suppressant effect on tumor metastasis. Logically, Wf-536 and paclitaxel, which have different action mechanisms and action specificities, in combination might yield a synergistic effect in preventing tumor metastasis by making up for each other's shortcomings.

Wf-536 has an inhibitory effect on angiogenesis and tumor cell infiltration through the cell layer [19], whereas paclitaxel also shows antiangiogenic activity [17] and inhibits endothelial cell migration [1]. Their inhibitory effect on tumor metastasis may therefore be additionally associated with these specific effects. The precise mode of action of the combination of Wf-536 and paclitaxel in preventing tumor metastasis is still under investigation.

In the future, the full mechanism of the synergistic effect of these drugs on tumor metastasis and their action in increasing survival time will need to be clarified.

In conclusion, our experiments raise the possibility of the clinical development of Wf-536 as an antimetastatic drug which could be used not only in monotherapy but also in combination with an antineoplastic drug such as paclitaxel.

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