Intrathecal Administration of Y-27632, a Specific Rho-Associated Kinase Inhibitor, for Rat Neoplastic Meningitis

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Abstract
The small GTP-binding protein Rho and its target Rho-associated kinase trigger an intracellular signaling cascade that controls actin cytoskeleton and plays an essential role in cell motility and adhesion. A specific Rho-associated kinase inhibitor, Y-27632, has been reported to inhibit cancer invasion. Clinically, disseminated tumor cells in the cerebrospinal fluid invade the intraparenchymal region, damaging the brain and nerves, resulting in fatal brain stem dysfunction, despite intrathecal chemotherapy. To expand therapeutic options for this devastating neoplastic meningitis, we evaluated the potential use of intrathecal Y-27632 administration by employing Walker 256 cells, a rat mammary cancer cell line. Y-27632 dose-dependently inhibited chemotactic and invasive activity of Walker 256 cells. Y-27632 also inhibited the phosphorylation level of regulatory myosin light chain in vitro, but the effect was temporary and was considerably diminished within 16 hours. Y-27632 induced striking morphologic changes in Walker 256 cells, as evidenced by decreased cell-matrix adhesion in culture dishes and three-dimensional collagen I gels, and slightly inhibited colony formation in soft agar. Nevertheless, this drug treatment did not affect Walker 256 cell growth rate. We were able to administer continuous delivery of this inhibitor using an osmotic pump and maintaining drug concentration of 10 μmol/L within the brain. Importantly, this concentration of Y-27632 showed minimal neurotoxicity both in vitro and in vivo. We found that an intrathecal therapy, combining 5-fluoro-2'-deoxyuridine with Y-27632, significantly increased the survival time of rats bearing meningeal carcinomatosis in comparison with animals treated with 5-fluoro-2'-deoxyuridine alone.

Taken together, our findings indicate that continuous intrathecal administration of Y-27632 could be a promising therapeutic method when combined with chemotherapy for treating human neoplastic meningitis. (Mol Cancer Res 2005;3(8):425–33)

Introduction
Meningeal dissemination of malignant tumors is generally treated by intrathecal administration of anticancer agents, topical application of radiation, or a combination of both. However, only a few anticancer drugs, including methotrexate, cytosine arabinoside, triethylene thiophosphoramide, and 5-fluoro-2'-deoxyuridine (FdUrd), can be injected intrathecally (1-5). The intrathecal administration of these anticancer agents achieves a therapeutic efficacy of 40% to 70%, and leads to temporary cancer regression for a short period in some cases, but, in other many cases, fails to improve the patient’s performance status. Acute neurotoxicity and retarded necrotizing encephalopathy, prevalent adverse effects of methotrexate, impose limitations on the use of doses necessary for satisfactory therapeutic effects (6, 7). In addition, patients cannot return to a satisfactory performance status even if intrathecal chemotherapy with these anticancer drugs is able to exert expected biological effects because cancer cells invade the brain, damaging normal brain function. Thus, protection against the invasion of cells disseminated in the subarachnoid space is critical and should be a prerequisite for therapy, as it leads to more beneficial chemotherapeutic effects, especially in the early stage of neoplastic meningitis.

Recently, signaling cascades by the family of small GTP-binding proteins, Rho, and attendant targets, Rho-associated kinases (8, 9), have been reported to be important in cell-to-substrate adhesion, stress fiber formation, and stimulation of actomyosin-based cellular contractility (10). We previously reported that Rho-associated kinase plays an essential role for hepatoma invasion (11). A specific Rho-associated kinase inhibitor, Y-27632 (11-13), has been reported to have no tumor cell killing effect but does inhibit tumor invasion (11, 14, 15). To evaluate the potential intrathecal use of Y-27632 for neoplastic meningitis, we first examined the biological effects of chemotaxis, chemoinvasion, morphologic changes in two-dimensional and three-dimensional collagen I gels, and colony formation and growth in soft agar and culture dishes) of this inhibitor on Walker 256 rat breast cancer cells in vitro. We then checked the neurotoxicity of this drug both in vitro and in vivo, and measured...
the concentrations of Y-27632 in various anatomic structures after continuous intrathecal administration. Finally, we investigated the combinative effects of Y-27632 and FdUrd intrathecal chemotherapy in vivo using a rat neoplastic meningitis model.

**Results**

**In vitro Chemotaxis and Invasion Assay**

As shown in Fig. 1A, Y-27632 inhibited Walker 256 cell chemotaxis towards the serum-free conditioned medium in a dose-dependent fashion (5-40 μmol/L). These effects were comparable to those induced by two other actin target reagents, cytochalasin D and latrunculin B. Y-27632 also inhibited the chemoinvasion activity of Walker 256 cells as measured using Matrigel chamber in a dose-dependent fashion (5-40 μmol/L, Fig. 1B). The lowest concentration of Y-27632 used, 5 μmol/L, significantly inhibited both chemotaxis and chemoinvasion.

**Inhibition of Myosin Light Chain Phosphorylation**

To examine the biochemical effects of Y-27632 on Rho-associated kinase activity in Walker 256 cells, we analyzed the phosphorylation level of the 20-kDa regulatory myosin light chain, one of the substrates of Rho-associated kinase. As shown in Fig. 1C, Y-27632 inhibited the myosin light chain phosphorylation level in a dose-dependent fashion. The inhibitory effect was evident 30 minutes after treatment, with Y-27632 dosages higher than 10 μmol/L reducing the level of myosin light chain phosphorylation to less than half the value measured in untreated cells. This inhibitory effect declined in a time-dependent fashion to almost negligible levels at 16 hours for all but the 40 μmol/L dose of Y-27632, which maintained a significant inhibitory effect at this time point. Thus, the inhibitory effects of Y-27632 seem to be transient, imposing a strict requirement for maintaining drug concentrations to exert biological effects.

**Effect of Y-27632 on Morphology and Growth**

Next, we examined the effect of Y-27632 on the morphology and growth of Walker 256 cells. We used the 10 μmol/L dose of inhibitor because this concentration of Y-27632 effectively inhibited Rho-associated kinase activity, chemotaxis, and

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**FIGURE 1.** Effects of various Y-27632 doses (0-40 μmol/L) on chemotactic and chemoinvasion activities of Walker 256 cells, and the phosphorylation levels of regulatory myosin light chain in these cells. A. Chemotaxis assay using the serum-free conditioned medium as chemotacticant. Effects of two actin-target reagents [100 nmol/L of cytochalasin D (Cyt D) and 5 μg/mL of latrunculin B (Lat B)] were also examined. Columns, mean; bars, SE (n = 6-9). B. Chemoinvasion assay using Matrigel chamber. Columns, mean; bars, SE (n = 6). C. Phosphorylation levels of regulatory myosin light chain (substrate of Rho-associated kinase) were inhibited by Y-27632 treatment in a dose-dependent fashion, but this effect did not last longer than 16 hours. Points, mean; bars, SE (n = 3). Bar, 100 μm. *, P < 0.01, relative to control.
invasion in Walker 256 cells (Fig. 1), and could also be maintained in rat brain tissue using the osmotic pump delivery system (as shown in Table 1). Untreated Walker 256 cells adopted a spread morphology and, as seen by anti-vinculin antibody staining (Fig. 2A, green, top left), long striking focal adhesions were widely distributed in the bottom and peripheral regions of the cells. Rhodamine-phalloidin staining of actin filaments (Fig. 2A, red, top left) revealed cortical filamentous actin bundles in the peripheral region, but few stress fibers were seen. Treatment with 10 μmol/L of Y-27632 evoked drastic morphologic changes in Walker 256 cells (Fig. 2A, top right). Cell adhesiveness to the culture dishes was markedly weakened and gross cell morphology had changed, such that long process-like structures were found projecting from shrunken cell bodies (Fig. 2A, top right). Small focal adhesions were now visible only as dots in the peripheral region of the cells and filamentous actin stress fibers were completely gone (Fig. 2A, top right). Two other actin-targeting reagents, cytochalasin D and latrunculin B, also evoked distinct and striking morphologic changes (Fig. 2A, bottom). The cells became spread-out after treatment with cytochalasin D and fine focal adhesions were seen in the peripheral region of the cells. In the case of latrunculin B, most of the Rhodamine-phalloidin– and vinculin-stained features were gone, with filamentous actin bundles visible only as small dots in the cell body. All of these effects were reversible as cells regained normal morphology within 24 hours after washout of these reagents (data not shown). We further examined the morphologic changes in three-dimensional culture with collagen I gels. Y-27632 treatment inhibited cell scattering, allowing the cells to form typical colony-like structures within 24 hours of being added to the collagen I gel (Fig. 2B). Finally, we analyzed the effect of this inhibitor on the cellular growth of Walker 256 cells. Y-27632 treatment slightly inhibited Walker 256 cell colony formation in soft agar (Fig. 2C). In particular, the number of large-sized (diameter > 300 μm) colonies was reduced by Y-27632 treatment doses higher than 5 μmol/L (Fig. 2C). In contrast, Y-27632 did not affect the cellular growth of Walker 256 cells under usual two-dimensional culture conditions (Fig. 2D).

In vitro Neurotoxicity

We then analyzed the neurotoxicity of Y-27632 employing primary mouse neuronal cell cultures. Notably, the total number of neuronal cells in cultures treated with 0.5 to 10 μmol/L of Y-27632 was higher than that of control cultures. In contrast, the number of cells in cultures treated with 40 to 100 μmol/L of Y-27632 was significantly less than that of the control group (Fig. 3A, left). Methotrexate-treated cultures showed a significant decrease in the number of neuronal cells at concentrations of 0.01, 0.1, 1.0, and 5.0 mmol/L (Fig. 3A, right). The number of cells in cultures treated at these methotrexate concentrations was comparable to those in cultures receiving 40 to 60 μmol/L of Y-27632. These findings suggested that Y-27632 concentrations less than 20 μmol/L would result in minimal neurotoxicity. Cells treated with Y-27632 and methotrexate showed quite different morphologic features (Fig. 3B). A number of neuronal cells with long thin projections were observed in the Y-27632–treated cultures, but not in the methotrexate-treated dishes (Fig. 3B). Y-27632 also did not affect the growth of primary culture of neonatal rat glial cells (data not shown).

In vivo Distribution of Y-27632 in Normal Rat Brain after Continuous Intrathecal Infusion

Employing the osmotic pump infusion system, we measured the concentrations of Y-27632 in different brain regions after delivery of the compound (Table 1). Although the drug concentrations varied quite widely among animals examined, within individual animals, there were corresponding concentrations in both the cerebellum and brain stem, as opposed to the cerebral cortex, where only trace amounts were detected. From the findings of the in vitro invasion assay, 5 to 10 μmol/L of Y-27632, corresponding to 1.710 to 3.420 ng/g Y-27632 (MW = 342), would essentially have been required for local brain tissue infusion. Thus, we believe that the two lower dosages tested (i.e., 1-10 mg/200 mL of Y-27632) were not sufficient and the higher doses of 50 to 100 mg/200 mL (corresponding to 0.74-1.47 mmol/L) of Y-27632 would be required to inhibit the invasion of tumor cells in vivo.

In vivo Neurotoxicity

Continuous intrathecal infusion of Y-27632 by the Alzet mini-pump caused the deaths of three of six rats, each of which received 100 mg of the drug in total. The three dead rats suffered severe hydrocephalus (data not shown). Continuous intrathecal administration of 50 mg/200 μL Y-27632 in the three surviving rats did not seem to have any adverse effects on brain tissue, as histologic examination of various brain regions (medulla, ventricle, front bases, and cistern) after continuous intrathecal administration of 50 mg/200 μL Y-27632 did not reveal any acute or chronic abnormalities (Fig. 3C).

In vivo Antitumor Effects of Y-27632 on Rat Neoplastic Meningitis

We tested the in vivo antitumor effects of Y-27632 on a rat model of neoplastic meningitis. Although the group which received continuous intrathecal infusion of Y-27632 showed

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**Table 1. Distribution of Y-27632 in Anatomic Structure of Normal Rat Brain after Continuous Intrathecal Administration Using an Alzet Osmotic Mini-Pump**

<table>
<thead>
<tr>
<th>Y-27632 (mg/200 mL)</th>
<th>Rat No.</th>
<th>Cerebellum (ng/g)</th>
<th>Brain Stem (ng/g)</th>
<th>Cerebral Cortex (ng/g)</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>1</td>
<td>9,029</td>
<td>3,758</td>
<td>186</td>
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<tr>
<td></td>
<td>2</td>
<td>1,892</td>
<td>2,963</td>
<td>277</td>
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<tr>
<td>50</td>
<td>1</td>
<td>203</td>
<td>471</td>
<td>85.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,839</td>
<td>6,197</td>
<td>154.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>422</td>
<td>4,453</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>213</td>
<td>724</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>206</td>
<td>1,071</td>
<td>N.D.</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>23.2</td>
<td>117</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>63.8</td>
<td>26</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>66.4</td>
<td>226</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>426</td>
<td>203</td>
<td>N.D.</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>25.2</td>
<td>325</td>
<td>N.D.</td>
</tr>
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<td>2</td>
<td>156.3</td>
<td>37.4</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26.1</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

NOTE: N.D., not detected, <20 ng/g of tissue.
a longer mean survival period (mean days ± SE: 10.1 ± 0.7, n = 14) than the control group (8.7 ± 0.9, n = 9), the difference did not reach statistical significance (data not shown). Continuous intrathecal infusion ofFdUrd (200 μg/200 μL) significantly increased the survival time of treated rats (14.0 ± 0.7, n = 10) compared with that of the control group (11.7 ± 0.5, 11; P < 0.02; Fig. 4A), whereas animals which received the treatment regimen combining continuous intrathecal infusion ofFdUrd (200 μg/200 μL) with Y-27632 (50 mg/200 μL) showed significantly longer survival times (15.9 ± 1.0, 11) than did the group receiving continuous intrathecal FdUrd only (13.0 ± 0.7, 9; P < 0.02; Fig. 4B). The rats which received continuous intrathecal Y-27632 clearly showed minimal invasion of disseminated tumor cells into parenchymal tissues within the basal region of the midbrain and pons (Fig. 4C).

Discussion

Rho proteins, a family of small GTPases, together with one of the Rho effectors, Rho-associated coiled-coil-containing protein kinase, work as an intracellular molecular switch which is activated by the stimulation of several growth factors (16) and chemokines, including stromal cell–derived factor 1α (17). Stromal cell–derived factor 1α, secreted from normal stroma cells, vascular pericytes, and lymphocytes, binds and activates CXC chemokine receptor 4 (18, 19), one of the chemokine receptors overexpressed in glioblastoma (20, 21), which leads to Rho activation. The activated Rho/Rho-associated kinase signaling cascade regulates focal cell adhesion dynamics and cell motility through the reorganization of the actin cytoskeleton and the regulation of actomyosin-based contractility (22). Thus, Rho/Rho-associated kinase signaling is involved in both vascular contractility (22) and tumor invasion (11, 23).

A specific Rho-associated kinase inhibitor, Y-27632, was initially developed for the treatment of hypertension and vasospasm caused by subarachnoid hemorrhage (12). Although blood pressure rapidly declined when Y-27632 was injected i.v. in hypertensive rats (12), the serum half-life of Y-27632 averages only 12 to 16 hours (13), necessitating continuous administration of the drug to maintain its biological effect. Because this compound is both small (MW = 342) and hydrophilic, it exhibits whole body distribution and is also traced in the cerebrospinal fluid after being continuously administered. In the present study, we examined the potential effectiveness of an intrathecal administration of Y-27632 for the treatment of neoplastic meningitis.

We showed marked dose-dependent inhibitory effects of Y-27632 on the invasive activity of Walker 256 cells, as assessed by chemotaxis and chemoinvasion assays and reduced levels of
myosin light chain phosphorylation in these cells. However, the effects were transient and declined in a time-dependent fashion. This was consistent with our previous report on Y-27632 activity using rat hepatoma MMI cells (11, 23), further emphasizing the need for continuous delivery methods to maintain optimal drug concentrations in vivo. Y-27632 also induced drastic morphologic changes in Walker 256 cells cultured in two-dimensional and three-dimensional collagen gels, comparable to those induced by two actin-target reagents, cytochalasin D and latrunculin B, which have limited in vivo use due to their severe cytotoxicity. Of note, Y-27632 inhibited the colony formation of Walker 256 cells in collagen gels and suppressed cell scattering. These effects may be due to decreased cell adhesiveness to the extra cellular matrix (collagen I in this case) as shown in the two-dimensional culture, suggesting a novel mechanism for inhibiting cancer invasion in vivo.

In terms of neurotoxicity, high doses of Y-27632 (40-100 μmol/L) significantly decreased the number of surviving mouse neuronal cells compared with the control-treated group. In contrast, low doses of Y-27632 (0-5 μmol/L) significantly increased the number of neuronal cells, consistent with previous reports in which Y-27632 stimulated axon regeneration by neurite extension after central nervous system injury (24, 25).

We also examined the effects in rats, in vivo, of continuous intrathecal Y-27632 administration into the major cistern using an Alzet mini-pump. Three of the five rats (60%) that received a high dose of Y-27632 (100 mg/200 μL) died within a few days from severe hydrocephalus, probably due to hematoma caused by trauma from the tip of the catheter being placed in the major cistern, or as a result of disturbed cerebrospinal fluid circulation. We also speculate that the neurotoxicity may have been caused

FIGURE 3. Neurotoxicity of Y-27632 and methotrexate in a primary culture of mouse ED14 neurons. A. The number of cells in cultures treated with methotrexate was less than those in cultures treated with Y-27632. *, P < 0.01, relative to control. B. Morphologic differences between cells treated with Y-27632 and methotrexate. The cells treated with methotrexate were more slender and had longer projections than those treated with Y-27632. Bar, 100 μm. C. Histologic evaluation of acute and chronic influences on normal rat brain after continuous intrathecal administration of Y-27632 (50 mg/200 μL) into the major cistern. Tissue sections were examined after H&E and Klüver Barrera stainings. No abnormalities were found.
by a direct effect of the catheter on the medulla oblongata. Therefore, placing the tip of the catheter into the lateral ventricle instead of the major cistern might help to avert such traumas and produce better results.

The survival study experiments using the rat neoplastic meningitis model showed the potential antitumor effects of Y-27632. Although rats which received continuous intrathecal administration of Y-27632 alone survived slightly longer than did control-treated rats, this difference did not reach statistical significance. On the other hand, animals treated with continuous intrathecal infusions of both FdUrd and Y-27632 survived significantly longer than the group receiving continuous intrathecal FdUrd only. Moreover, rats that received continuous intrathecal Y-27632 displayed less tumor cell invasion in parenchymal tissues within the pons and midbrain. In conclusion, our results suggest that continuous intrathecal Y-27632 may be a promising therapeutic method for treating neoplastic meningitis, particularly when combined with intrathecal FdUrd chemotherapy.

Materials and Methods

Animals

Seven-week-old female Wistar rats and eight-week-old female C3H/He mice (SLC, Shizuoka, Japan) were housed under specific pathogen-free conditions. All animal experiment protocols were approved by the local animal ethics committee in the Osaka Medical Center for Cancer and Cardiovascular Diseases.
Reagents
Y-27632 was supplied by Mitsubishi Pharma Co. (Tokyo, Japan) and anti-phospho-myosin light chain antibody was a generous gift from Dr. Fumio Matsumura (Rutgers University, New Brunswick, NJ). All other chemicals used were commercial products. Both Y-27632 and methotrexate were first dissolved in PBS and then diluted to required concentrations in the culture media.

Cell Culture
Walker 256 rat carcinoma cells (Cancer Research Funds, Tokyo, Japan) were cultured in DMEM (Invitrogen, Tokyo, Japan) with 10% FCS (Equitech-Bio, Kerrville, TX). Conditioned medium and serum-free conditioned medium were prepared from DMEM incubated with Walker 256 cells for 24 hours. For growth in three-dimensional collagen I, collagen gels were prepared according to the protocol of the manufacturer (Chemicon, Temecula, CA) and as previously described (26). Cells (1 × 10^5 cells) were added to a collagen I solution with or without 10 μmol/L Y-27632. The culture was incubated at 37°C to allow the collagen I solution to gel and then overlaid with culture media with or without 10 μmol/L Y-27632.

In vitro Chemotaxis and Chemoinvasion Assays
The capacity of cells to migrate was assayed using a Transwell chamber (8 μmol/L pore size; Becton Dickinson Labware, Bedford, MA). Walker 256 cells (4.76 × 10^4 cells/cm^2) were applied to the upper chamber in DMEM containing 0.1% bovine serum albumin, with or without Y-27632. Serum-free conditioned medium served as a chemotactant and was added to the lower chamber. After 16 hours, the migrating cells on the lower side of the filter were fixed with 70% methanol following the protocol previously described (27, 28). The chemoinvasion assay was carried out as previously described (29). Briefly, Walker 256 cells (2.17 × 10^5 cells/cm^2) were seeded onto the upper chamber of a Matrigel-coated polyethylene terephthalate membrane (8 μmol/L pore size; 100 μg/cm^2 of Matrigel, BIOCATH, Becton Dickinson) in DMEM, containing 0.1% bovine serum albumin with or without added Y-27632. Conditioned medium served as a chemointractant and was added to the lower chamber. After 20 hours, the migrating cells on the lower side of the filter were fixed with 70% methanol and counted.

Estimation of Phospho-Myosin Light Chain Levels
Walker 256 cells (2 × 10^5 cells) were plated in culture media for 24 hours. Cells in the control group were subsequently treated with serum-free conditioned medium whereas the experimental group cells were treated with Y-27632 diluted in serum-free conditioned medium for the indicated times. Medium was removed, and the cells were lysed in Laemmli’s sample buffer (30). Immunoblotting analysis was done as previously described (11, 27, 31). For estimation of phospho-myosin light chain levels, the blot was probed with anti-phospho-myosin light chain polyclonal antibodies (1:100). The relative phosphorylation levels of myosin light chain were estimated by normalizing the phospho-myosin light chain signal to the myosin light chain signal, which was obtained by immunoblotting with MY21 monoclonal antibodies (Sigma, Tokyo, Japan) on a duplicate blot. Protein levels were measured and analyzed using NIH image software (version 1.62) on a Macintosh personal computer (Apple, Tokyo, Japan). The graph data represent the results of three different experiments.

Immunofluorescence
We followed a previously described protocol (27). Briefly, cells were cultured with or without 10 μmol/L Y-27632 on uncoated or type I collagen-coated two-well chamber slides (Becton Dickinson). After 24 hours, cells were fixed with 1% paraformaldehyde in PBS and then permeabilized with 0.2% Triton X-100 in PBS. The primary antibodies used were anti-vinculin monoclonal antibodies (1:100; Sigma) and secondary antibodies were Alexa-488 anti-mouse immunoglobulin G (1:1,000; Molecular Probes, Eugene, OR). These cells were then stained with rhodamine phalloidin (1:100; Molecular Probes). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (300 nmol/L; Molecular Probes; refs. 27, 28).

Soft-Agar Colony Formation Assay
Soft agar (0.36%; SeaPlaque, FMC Bioproduct, Rockland, ME) was inoculated with 3 × 10^5 cells with or without Y-27632 in 60 mm dishes as previously described (32). The plates were incubated for 14 days and then photographed under a microscope.

Cell Proliferation Assay
Cell proliferation analysis was done as previously described (28). Briefly, Walker 256 cells (5 × 10^5 cells) were plated into 24-well plates and cultured in DMEM with 10% FCS for 24 hours. Then the cells were treated with Y-27632 for 7 days. The culture media was changed every 2 days. Cell numbers were measured every 24 hours by the WST-8 (2-(2-methoxy-4-phenylindole (300 nmol/L; Molecular Probes), Cell nuclei were stained with 4',6-diamidino-2-phenylindole (300 nmol/L; Molecular Probes; refs. 27, 28).

In vitro Neurotoxicity
Primary neuronal cultures were prepared after harvesting cortical neurons from the cerebral cortices of 14-day-old C57BL/6 mouse embryos (ED14). The separated cell suspensions were plated at 106 cells/cm^2 in 24-well plates (2 cm^2/well) coated with poly-l-lysine, and cultured with DMEM supplemented with 10% fetal bovine serum, penicillin (200 IU/ml), and streptomycin (200 μg/mL). Twenty-four hours later, the medium was replaced with Neurobasal Medium (Invitrogen) with G-5 medium supplement for neurons (Invitrogen). The next day, the cells were treated with various doses of Y-27632 in quadruplicate. The number of viable cells in each well were counted by the trypan blue dye exclusion method (34). To assess the neurotoxicity of Y-27632 more precisely, we compared the effect of this compound with that of methotrexate, the most commonly used intrathecal anticancer drug for the clinical treatment of neoplastic meningitis. Various concentrations of methotrexate (0.01, 0.1, 1.0, and 5.0 nmol/L) were used for comparing the neurotoxicity of Y-27632. These concentrations were estimated from clinical intrathecal doses.
In vivo Continuous Intrathecal Infusion of Y-27632

A tip of the silicon tube was placed in the major cistern of each rat at a depth of 2.5 mm with a silicon stopper plate fixed on the overlying dura mater with Aron α A (Sankyo, Tokyo, Japan). This tube was then connected to the osmotic Alzet minipump (model 2002, Durect Co., Cupertino, CA; total volume of the pump is 200 μL, and 12 μL per day of the drug was infused into the major cistern), one of which was placed s.c. in the back of each rat.

In vivo Y-27632 Distribution in the Brain

The instruments for continuous intrathecal infusion of Y-27632 were surgically implanted into 27-week-old female Wistar rats, such that each five rats received dosage levels set at either 1, 10, 50, or 100 mg/200 μL. The rats were sacrificed for tissue sampling on the 12th day and the cerebellum and pons were removed. The rats were anesthetized with ethyl ether and perfused via the heart with cold saline until discoloration of the liver was observed (after first opening the abdominal cavity and cutting the large abdominal vein). They were then decapitated, and the brains were quickly removed and stored at −80°C until assayed. Determination of the Y-27632 levels in the cerebellum and brain stem (pons and medulla oblongata) was done by high-performance liquid chromatography with a detection limit of 20 ng/g (13).

In vivo Neurotoxicity

We evaluated the in vivo neurotoxicity of continuous intrathecal administration of Y-27632 using the Alzet minipump in normal Wistar rats. Two weeks (defined as acute neurotoxicity) and 2 months (defined as chronic neurotoxicity) after the commencement of drug infusion, all the rats were sacrificed and brain tissues were collected. Tissue sections were then examined after H&E and Klüver Barrera stainings.

In vivo Therapeutic Experiments

Eight-week-old female Wistar rats weighing ~200 g were used. We followed a previously described protocol (35) to establish a rat model of neoplastic meningitis. Briefly, the procedure involves puncturing the rat cisterna magna with a 26-gauge needle under general anesthesia with ethyl ether, and injecting 0.1 mL (5 gauge needle under general anesthesia with ethyl ether, and the procedure involves puncturing the rat cisterna magna with a 26-gauge needle). We followed a previously described protocol (35) to establish a rat model of neoplastic meningitis. We inoculated tumor cells into the brain parenchyma, especially basal regions. These evaluations were based on microscopic pathologic examinations done independently by two pathologists. The results described here represent concordant findings by both pathologists.

Statistical Analysis

All statistical analyses were carried out using a software package JMP 5.1.1J (SAS Institute, Inc., Cary, NC). Data are expressed as mean ± SE. For the in vitro studies, all statistical comparisons were done using the unpaired t test. P < 0.01 was considered statistically significant. Statistical analyses of results from the animal survival studies were done using the Kaplan-Meier Method (log-rank test). P < 0.02 was considered statistically significant.

Acknowledgments

We thank Mitsubishi Pharma Co. for the generous gift of Y-27632. We also thank T. Murozono for the measurement of Y-27632 concentrations.

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Molecular Cancer Research

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