7-Chloro-6-piperidin-1-yl-quinoline-5,8-dione (PT-262), a novel ROCK inhibitor blocks cytoskeleton function and cell migration

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1. Introduction

The reorganization of cytoskeleton plays important roles in the regulation of cancer cell migration and metastasis. The RhoGTPases are key regulators of cytoskeleton dynamics [1,2]. The RhoGTPase family members include RhoA, Rac1, and Cdc42 in mammalian cells. RhoA is involved in the regulation of cytoskeleton reorganization and focal adhesion, whereas Rac1 and Cdc42 work together at the regulation of cell leading edges to form lamellipodia and filopodia [3,4]. RhoA is a key family of RhoGTPases that participates in cancer migration and metastasis [5,6]. Overexpression of RhoA has been found in a variety of cancers including, lung, bladder, testicular, ovarian, colon, and breast [7]. ROCK (Rho-associated coiled-coil forming protein kinase) is a downstream effector of RhoA to mediate the phosphorylation of myosin light chain (MLC) and cytoskeleton reorganization. The RhoA–ROCK–MLC pathway has been shown to promote cancer cell migration and metastasis. Interestingly, PT-262 was more effective on inhibiting ROCK kinase activities than specific ROCK inhibitors Y-27632 and H-1152. PT-262 induced cytoskeleton remodeling and migration inhibition in A549 lung carcinoma cells. The total MLC and phosphorylated MLC proteins and stress fibers were blocked after treatment with PT-262. Nonetheless, the RhoA protein and GTPase activity were not altered by PT-262. A computational model suggests that PT-262 interacts with the ATP-binding site of ROCK protein. Together, these findings demonstrate that PT-262 is a new ROCK inhibitor.
migration, invasion and metastasis [15–17]. Y-27632 inhibits ROCK kinase activity to disrupt networks and remodeling of actin filaments in cancer cells [15,17]. Furthermore, Y-27632 reduces cancer invasion by reducing the levels of matrix metalloproteinases (MMPs) as MMP-2 and MMP-9 [17]. In addition, H-1152, (S)-(−)-2-methyl-1-[[4-methyl-5-isoquinoline]sulfanyl]-homopiperazine, is another specific ROCK inhibitor derived from isoquinoline–sulfonamide analogues similar to Y-27632 by binding to catalytic site of ROCK [10,18].

The 5,8-quinolinediones are useful precursors for producing multiple types of bioactive products [19]. The derivatives of quinolinediones have been shown to possess many biological activities including anti-tumor and anti-microbial actions. 6-Anilino-5,8-quinolinedione (LY83583) is an inhibitor of guanylyl cyclase that can reduce cancer cell proliferation [20]. 6-Chloro-7-(2-morpholin-4-ylamino)quinoline-5,8-dione (NSC 663284), an inhibitor of CDC25 protein phosphatases, induced cell cycle arrest by reducing CDC2 activation in cancer cells [21]. Lavendamycin is a bacterially derived quinolinedione that displays significant anticancer activities [22]. 7-Chloro-6-piperidin-1-ylquinoline-5,8-dione (designated as PT-262) is a new derivative of 5,8-quinolinedione, which induced apoptosis associated with inhibiting CDC2 and ERK phosphorylation [23]. In this study, we investigated a new ROCK inhibitor, PT-262, which inhibited cytoskeleton function, stress fiber formation, and migration in human lung carcinoma cells. In addition, PT-262 was more effective in inhibiting ROCK kinase activity and cancer cell migration than Y-27632 and H-1152. PT-262 can be developed as a novel ROCK inhibitor.

2. Materials and methods

2.1. Materials

The chemical synthesis of PT-262 was according to a previous study [23]. PT-262 was dissolved in 100% dimethyl sulfoxide (DMSO). The concentration of DMSO was <0.2% in the control and drug-containing media in each experiment. Hoechst 33258, propidium iodide (PI), 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), colchicine, paclitaxel, and the Cy3-labeled mouse anti-β-tubulin, gelatin, fibronectin and Y-27632 were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-ERK-2 (C-14) and anti-RhoA (sc-179) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). BODIPY FL phallacidin (B-607) and Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA). The Cy3-labeled goat anti-rabbit IgG was purchased from Amersham Pharmacia Biotech (Little Chalfont Buckinghamshire, UK). Anti-phospho-MLC (serine-19), anti-MLC, and anti-ROCK1 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). H-1152 was purchased from Merck Calbiochem (San Diego, CA).

2.2. Cell culture

The A549 cell line (ATCC, #CCL-185) was derived from a non-small cell lung adenocarcinoma tumor. A549 cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1-glutamine (0.03%, w/v). The cells were maintained at 37 °C and 5% CO2 in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

2.3. Cytoskeleton staining and confocal microscopy

The cells were cultured on coverslips, which were kept in a 60-mm Petri dish for 16–20 h. At the end of treatment, the cells were washed with isotonic PBS (pH 7.4), and then the cells were fixed in 4% paraformaldehyde solution in PBS for 1 h at 37 °C. Subsequently, the coverslips were washed three times with PBS, and non-specific binding sites were blocked in PBS containing 10% FBS and 0.3% Triton X-100 for 1 h. Actin filament (F-actin) and β-tubulin were stained with 20 U/ml BODIPY FL phallacidin and anti-β-tubulin Cy3 (1:50) for 30 min at 37 °C, respectively. Finally, the nuclei were stained with 2.5 μg/ml Hoechst 33258 for 30 min. The samples were examined under a Leica confocal laser scanning microscope (Mannheim, Germany) that equipped with an UV laser (351/364 nm), an Ar laser (457/488/514 nm), and a HeNe laser (543 nm/633 nm). The fluorescence images were displayed through the frame store in the computer, and the cell length was calculated using a Leica confocal software (Ver. Lite).

2.4. Bio-atomic force microscopy (Bio-AFM)

To observe the cytoskeleton alteration and cell elongation by PT-262, the cells were analyzed by a BD CARV II confocal microscope combined a NanoWizard Bio-AFM (JPK Instruments, Berlin). Bio-AFM was mounted on an inverted microscope, TE-2000-U (Nikon, Japan). The silicon nitride non-sharpened cantilever was used by a nominal force constant of 0.06 N/m (DNP-20, Vecco). The images were scanner by using contact mode. Line scan rates are varied from 0.5 to 2 Hz.

2.5. Actin polymerization assay

Actin polymerization assays in vitro (BK003) were purchased from Cytoskeleton, Inc. (Denver, CO). After treatment with or without PT-262, the actin polymerization reaction was analyzed according to the manufacturer’s recommendations. Phallacidin was used as a positive control in actin polymerization. Briefly, the actin polymerization assays were based on the enhanced fluorescence of pyrene-conjugated actin that occurs during polymerization. The enhanced fluorescence were measured by pyrene monomer globular-actin (G-actin) formed polymer pyrene F-actin in a fluorometer at excitation wavelength 365 nm and emission wavelength 407 nm.

2.6. Boyden chamber analysis

The cell migration was examined by the Boyden chamber analysis. Boyden's chamber system was used two chambers separated by a collagen coated 8 μm pore size polycarbonate membrane. Briefly, the polycarbonate filter of 8 μm pore size was soaked in 0.5 M acetic acid overnight. The filter was washed with distilled water and then incubated for 16 h in a 100 g/ml gelatin solution and air dried. The gelatin coating filters were incubated again in a 10 μg/ml fibronectin solution for 2 h. The tested drugs were added to the bottom well of a Boyden chamber. Cells on the upper surface of the filters were carefully removed with a cotton swab. Cells on the lower surface were counted after stained with hematoxylin under a light microscope.

2.7. Wound healing assay

The cells were cultured in six-well plate for 24 h overnight, resulting in a monolayer that was more than 90% confluent. The cell monolayer was scraped with a 200 μl pipette tip to generate the wound of 6–7 mm. Thereafter, the cells were treated with or without PT-262, washed with PBS, and then re-cultured in fresh medium for various periods. Photographs were taken at 8 and 24 h on the same position of the wound.
2.8. ROCK kinase assay

A549 cells were treated with or without PT-262, Y-27632 or H-1152. After drug treatment, the cell lysates were diluted in kinase buffer and allowed to phosphorylate the bound substrate myosin binding subunit (MBS). The amount of MBS was calculated in a binding reaction with a horseradish peroxidase conjugate of AF20, an anti-phospho-MBS threonine-696 specific antibody, which catalyzes the conversion of the chromogenic substrate tetramethylbenzidine from a clear to a blue-colored solution. The color reaction product was quantified by measuring the absorbance at 450 nm using a VERSAmax microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA).

2.9. Western blot analysis

Western analyses of RhoA, ERK-2, MLC, phospho-MLC, and ROCK1 were performed using specific antibodies. At the end of the treatment, the cells were lysed in ice-cold whole cell extract buffer containing protease inhibitors as described [24]. Briefly, equal amounts of proteins in samples were subjected to electrophoresis using 10–12% sodium dodecyl sulfate-polyacrylamide gels. After...
electrophoretic transfer of proteins onto polyvinylidene difluoride membranes, they were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Finally, the protein bands were visualized using the enhanced chemiluminescence detection system (NEN, Boston, MA).

2.10. RhoA GTPase activity assay

Activated GTP-RhoA proteins were detected by a pull-down assay kit (Upstate Biotechnology, Lake Placid, NY) for Rho–GTP bound to the Rho-binding domain (RBD) of Rhotekin, a downstream effector of Rho. Rho activation kit used the Rhotekin-RBD fused with GST to affinity precipitate cellular Rho–GTP. Briefly, the cells were treated with or without PT-262. At the end of treatments, the cell lysates were collected and then added equal volumes to incubate with GST–Rhotekin–RBD and glutathione–sepharose beads according to the manufacturer's instructions. Activated GTP–RhoA proteins bound to beads in cell lysates were precipitated and then subjected to Western blot using specific anti-RhoA antibody. The levels of active RhoA were determined by normalizing with the total RhoA proteins present in the cell lysates.

2.11. Transfection

Control siRNA (5'-UUCUCCGAACGUGUCACGU-3') and RhoA siRNA (5'-CGGAUGAUGACGACACAA-3') were purchased from Qiagen Inc. (Valencia, CA) that were used for transfection by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations.

Table 1
The actions of various cytoskeleton blockers.

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<th>Compounds</th>
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<td>Microtubule polymerization</td>
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<tr>
<td>Paclitaxel</td>
<td>+</td>
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<td>Colchicine</td>
<td>-</td>
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<td>Cytochalasin B'</td>
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<td>Phallacidin</td>
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a Induction of mitotic arrest by inhibiting spindle function but not inducing cell elongation.
b Induction of mitotic arrest by inhibiting spindle function but not inducing cell elongation.
c A cytokinesis inhibitor induces actin depolymerization.
d Cytochalasin B inhibits actin polymerization and cytokinesis.
e Cell elongation by inducing direct binding to F-actin.
f Cell elongation by inducing abnormal actin polymerization but did not directly bind to F-actin.
2.12. Statistical analysis

All results were obtained from at least three separate experiments. Data were analyzed by one-way or two-way analysis of variance (ANOVA), and further post hoc tests using the statistic software of GraphPad Prism 4 (GraphPad software, Inc. San Diego, CA). A p value of <0.05 was considered as statistically significant in each experiment.

3. Results

3.1. PT-262 induces the cytoskeleton alteration and cell elongation in lung carcinoma cells

As shown in Fig. 1A, treatment with 2 μM PT-262 dramatically induced cytoskeleton alteration in A549 lung cancer cells. The cells were elongated and formed an abnormal cytoskeleton following

![Chemical structures of PT-262, Y-27632, and H-1152](image)

Fig. 3. Effect of PT-262 on the ROCK kinase activity, phospho-MLC and total-MLC expression, and stress fibers in lung carcinoma cells. (A) Chemical structures of PT-262, Y-27632, and H-1152. (B) A549 cells were treated with or without PT-262, Y-27632 and H-1152. At the end of the treatments, the cell lysates were subjected to ROCK kinase activity assays. The amount of phosphorylated MBS was calculated in a binding reaction with a horseradish peroxidase conjugated an anti-phospho-MBS threonine-696 specific antibody, which catalyzes the chromogenic substrate tetra-methylbenzidine to blue-color. Results were obtained from 3 separate experiments. The bar represents the mean ± S.E. *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant difference between control and inhibitor treated samples. #p < 0.05 indicates a significant difference between PT-262 and ROCK inhibitor (Y-27632 or H-1152) treated samples. (C) and (D), A549 cells were treated with or without 2–10 μM PT-262, Y-27632, and H-1152. Representative Western blot data of the protein levels of ROCK1, phospho-MLC, and total MLC were shown from one of three separate experiments with similar findings. (E) F-actin proteins were stained with BODIPY FL phallacidin, which displayed a green color. The nuclei were stained with Hoechst 33258, which displayed a blue color. The arrows indicate the location of stress fibers. (For interpretation of the references to color in this sentence, the reader is referred to the web version of the article.)
exposure to PT-262. To view the elaborate structure of cell morphology, the cells were observed by confocal microscope combined Bio-AFM. The cytoskeleton alteration and cell elongation were clearly induced by PT-262 by comparison with the untreated sample (Fig. 1B). The green color exhibited by F-actin formed an elongated shape after treatment with 2 μM PT-262 for 24 h. The magnified pictures of the circle marks in Fig. 1B show the fork shape in the elongated cell (Fig. 1C). Cell length elongated to ~160 μm following treatment with 2 μM PT-262 (Fig. 1D). Moreover, PT-262 increased the formation of F-actin concentrated spikes (Fig. 1D, arrows). The average cell length was extended from 39.15 to 65.30 μm following treatment with PT-262 by calculating the long diameter (Fig. 1E).

3.2. Various cytoskeleton inhibitors induce cytoskeleton remodeling and morphological alteration

To investigate the cytoskeleton alteration by PT-262, A549 cells were compared with various cytoskeleton blockers, including paclitaxel, colchicine, and phallacidin. β-tubulin and F-actin were displayed by staining with Cy3-labeled mouse anti-β-tubulin and BODIPY FL phallacidin, respectively. Treatment with 50 nM paclitaxel for 24 h increased the red fluorescence intensity of β-tubulin by inducing the microtubule polymerization (Fig. 2). In contrast, colchicine (50 nM, 24 h) reduced the red intensity of β-tubulin by the inhibition of microtubulin polymerization (Fig. 2). Phallacidin (0.5 U/ml, 24 h) increased the green fluorescence

![Image](image-url)
3.3. PT-262 inhibits ROCK kinase activity and stress fiber formation

The chemical structures of PT-262, Y-27632, and H-1152 are shown in Fig. 3A. Treatment with 2–10 μM PT-262 inhibited ROCK kinase activities via a concentration-dependent manner in A549 cells (Fig. 3B). Furthermore, PT-262 was more effective on inhibiting ROCK kinase activities than Y-27632 and H-1152 (Fig. 3B, p < 0.05). The IC_{50} value (the concentration of 50% kinase activity inhibition) was around 5 μM by PT-262; the IC_{50} values of Y-27632 and H-1152 were >10 μM. Nonetheless, PT-262, Y-27632, and H-1152 did not reduce ROCK protein expression (Fig. 3C). ERK-2 protein has been used as an internal control [24,26]. The protein level of ERK-2 was not altered by PT-262 (Fig. 3C). In addition, treatment with 5–10 μM PT-262 decreased the levels of phospho-MLC and total MLC proteins (Fig. 3D). Y-27632 and H-1152 also decreased the MLC protein expression. The formation of stress fibers was found in the PT-262-un-treated cells (Fig. 3E, arrows); in contrast, PT-262 or Y-27632 can block the stress fiber formation completely (Fig. 3E).

3.4. PT-262 inhibits cancer cell migration

To examine the effect of PT-262 on cancer cell migration, the cells were examined by Boyden chamber analysis. Treatment with 2–10 μM PT-262 for 6 h significantly blocked the cell migration in a concentration-dependent manner (Fig. 4A and C). The negative control of cell migration was used by incubating in serum free medium. Moreover, PT-262 was more effective in inhibiting cell migration than Y-27632 and H-1152 (Fig. 4B and Fig. 4D). The migration inhibition of PT-262 was verified by the wound-healing assay. As shown in Fig. 5A, Fig. 5, the wound width by the cell migration was observed under a phase contrast microscope following treatment with 2 μM PT-262 for 8 h or 24 h. PT-262 significantly increased the wound width than untreated samples (Fig. 5B).

3.5. Blockage of RhoA expression inhibits cancer cell migration

Transfection with 20–80 nM RhoA siRNA for 48 h decreased RhoA gene expression and protein levels in A549 cells (Fig. 6A and C). The blockage of RhoA expression inhibited cell migration via a concentration-dependent manner (Fig. 6B). Furthermore, the phospho-MLC and total MLC proteins also were reduced by transfection with RhoA siRNA (Fig. 6B). The ROCK protein expression was not altered by RhoA siRNA (Fig. 6C). The effect of PT-262 on RhoA GTPase activity was further analyzed by RhoA pull-down assays. However, treatment with PT-262, Y-27632 and H-1152 did not significantly alter the RhoA GTPase activity and protein expression (Fig. 6D and E).

4. Discussion

The RhoA-ROCK pathway has been shown to promote cancer cell migration, invasion and metastasis. The RhoA-ROCK pathway is a potential target for cancer chemotherapy. In this study, we provide a newly synthesized compound, PT-262, that markedly
The cytoskeleton proteins of microtubules and F-actin are potential targets for cancer chemotherapy [2,28]. The disruption of F-actin has been found in malignant transformed cells [2]. Actin polymerization and remodeling play a critical role in the morphologic and phenotypic events in cancer cells [2,28]. Chemotherapeutic drugs that target cytoskeleton remodeling can prevent malignant transformation and metastasis processes. Paclitaxel is an effective anticancer drug that acts by stabilizing microtubules and inducing formation of microtubule bundles to block mitosis [29,30]. The vinca alkaloids and colchicine induce mitotic arrest by inhibiting microtubule polymerization and disassembling the mitotic spindle [28,31]. Cytochalasins bind to the plus end of F-actin, reduce F-actin mass, and prevent actin polymerization [28,32]. Malignant cells are reported to be more sensitive to cytochalasin B than normal cells [28,33]. Phalloidins can bind to F-actin to inhibit actin depolymerization [32]. Among these cytoskeleton blockers, we found paclitaxel and colchicine induced microtubule polymerization and depolymerization, respectively. However, phallacidin promoted actin polymerization by stabilizing F-actin to induce the cell elongation in lung cancer cells. Interestingly, PT-262 increased cell elongation in a manner comparable to phallacidin treatment, indicating that PT-262 may act in a similar fashion, promoting F-actin stability. Nonetheless, PT-262 did not bind to F-actin directly using actin polymerization in vitro assays. Therefore, we suggest that PT-262 blocks cytoskeleton functions indirectly by inhibiting ROCK signaling.

The ROCK1 protein crystal structure has been reported [10]. It has been shown that four crystal structures contain ATP-competitive inhibitors of ROCK1 in the third pocket site [10]. Fig. 7A shows a computational structure model of the interaction of PT-262 and ROCK1 protein. The 3D structures of ROCK inhibitors on the binding site of ROCK1 were utilized for docking analysis [10]. The coordinates of binding-pocket atoms were taken from the PDB for the docking tools such as GEMDOCK [34,35]. Furthermore, the potential binding sites were predicted by using Q-SiteFinder, which was an energy-based method for the prediction of protein–ligand binding sites [36,37]. We have further compared the binding of PT-262, H-1152, and Y-27632 to ROCK1 (Supplementary Fig. 1).

The nitrogen atoms on the quinoline group of PT-262, the pyridine group of H-1152, and the isoquinoline group of Y-27632 all form hydrogen bonds with the nitrogen on the main chain of M156. Three functional groups occupy the same space as the adenine of ATP occupies (Supplementary Fig. 1). In addition, the chlorine and quinoline moieties of PT-262 contact with the MET153 amino acid.
residue, and the chloride and piperidin moiety of PT-262 contact with ALA215 amino residue, which may determine the selectivity at the ROCK inhibition. The chloride of PT-262 may be the reason more effective on inhibition of ROCK activity than Y-27632 and H-1152. Accordingly, the computational model supports the findings that PT-262 blocks the ROCK kinase activities by binding on ATP-binding site.

The derivatives of quinolinediones have been shown to possess many biological activities including anti-tumor actions. For example, 6-anilino-5,8-quinolinedione (LY83583) is an inhibitor of guanylyl cyclase that can prevent cancer cell proliferation [20]. In addition, 6-chloro-(2-morpholin-4-ylethylamino) quinoline-5,8-dione (NSC 663284) is an inhibitor of CDC25 protein phosphatases that mediates cell cycle arrest by reducing CDC2 activity [21]. In this report, we provide a novel function of quinolinedione derivatives that is a ROCK inhibitor.

In conclusion, we presented a model of the inhibition of ROCK and its downstream regulation of cell migration by PT-262 (Fig. 7B). The model provides a novel mechanism of cell migration inhibition by the 5,8-quinolinedione derivative through the irreversible effects of cytoskeleton alteration and cell elongation in blocking ROCK pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.01.009.

References
