Pharmacological inhibition of Rho-kinase signaling with Y-27632 blocks melanoma tumor growth

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Received October 26, 2009; Accepted November 27, 2009

DOI: 10.3892/or_00000708

Abstract. Primarily through *in vitro* studies, the Rho-family of small GTPases and their effector proteins have been implicated in mediating oncogenic properties of cancer cells. We sought to determine if pharmacological inhibition of the RhoA effector proteins known as Rho-kinases (ROCK) with the small molecule inhibitor Y-27632 could inhibit melanoma *in vitro* and *in vivo*. We demonstrate that Y-27632 treatment of a panel of melanoma cells alters cellular morphology leading to spindly cells with decreased lamellipodia and increased filopodia formation. Y-27632 treatment decreases invasion and alters cell survival of cultured melanoma cells. IP injection of Y-27632 in tumor-bearing mice resulted in a reduction in melanoma tumor volume compared to control treated mice. These findings suggest that ROCK inhibition can reduce melanoma tumorigenicity.

Introduction

While surgical removal of tumors leads to a very high 5-year survival rate for patients with early stage melanomas, current treatments for late stage metastatic melanoma are disappointing at best. Chemotherapy and radiation treatment of late stage metastatic ocular or skin melanoma demonstrates a success rate of 15-20%, however most responses are not sustained in the long term. Therefore, more effective therapeutics for metastatic melanoma are desperately needed to increase patient survival rates.

The RhoA small GTPase is a member of the Ras superfamily of proteins and plays an essential role in the rapid and dynamic reorganization of the actin cytoskeleton, leading to modulation of biological processes such as cell movement, shape change, cytokinesis, and adhesion. RhoA acts as a molecular switch cycling between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state. This cyclic regulation of RhoA is controlled by cytokine, growth factor, adhesion molecule, and G-protein coupled receptor modulation of GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) which inhibit RhoA, and guanine nucleotide exchange factors (GEFs) which activate RhoA (1-3). The most extensively studied RhoA effector is the serine/threonine Rho-associated kinase (ROCK), which controls actomyosin contractility via a direct phosphorylation of myosin light chain (MLC) and inhibitory phosphorylation of myosin phosphatase (4). These ROCK-mediated effects lead to cytoskeletal stress fiber formation, focal adhesion complex formation, smooth muscle contraction, and cell migration.

Numerous reports have implicated deregulation of the Rho-family signaling in cancer. Proteins involved in the RhoA-signaling pathway have been shown to be significantly elevated in a variety of tumors (5-9). Moreover, it has been proposed that increased expression of the Rho signaling proteins contributes to the metastatic behavior of some cancers (10). Because of their essential functions in regulating the cytoskeleton, Rho proteins and their effectors are promising targets for the development of novel anticancer drugs. Previous therapeutic approaches to target Rho-proteins include isoprenylation inhibitors, farnesyltransferase inhibitors, geranyltransferase inhibitors, and statins, and these compounds have been shown to reduce tumor progression and increase tumor cell death (11). One common drawback for each of these inhibitors is their promiscuity for multiple members of the Rho-family proteins, therefore more specific inhibitors of individual Rho proteins or their downstream signaling pathways could potentially be of great benefit for the treatment of cancer. Inhibition of ROCK proteins by specific pharmacological inhibitors has been used clinically to treat cardiovascular disease and cerebral vasospasm. Numerous reports utilizing *in vitro* models have suggested that ROCK inhibition blocks migration, invasion, proliferation, and survival of cancer cell lines, however only few studies in a very limited number of tumor types have demonstrated effectiveness of ROCK inhibition in effectively treating *in vivo*

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Key words: RhoA, ROCK, Rho-kinase, melanoma, Y-27632, small GTPase

tumor formation. For example, ROCK inhibition has been shown to block invasion of prostate tumors (12), increase apoptosis in glioma tumors (13), and, in combination with Cidofovir, block metastasis of human papilloma virus positive tumor cells to the lung (14). However, the effect of ROCK inhibition in most tumors and cancers is largely unknown. In this study, we determined the efficacy of Y-27632, a well established pharmacological inhibitor displaying a high specificity for ROCK proteins (K_i values are 0.14, 25, 26, and 250 μM for ROCK, protein kinase C, cAMP-dependent protein kinase, and myosin light-chain kinase, respectively), in inhibiting tumorigenic properties in a panel of skin and ocular melanoma cell lines and in blocking melanoma tumor formation in a rodent model. Our data indicate that Y-27632 treatment leads to changes in cellular morphology, decreased invasion, and alters survival in cultured skin and ocular melanoma cells. Moreover, we demonstrate that systemic delivery of Y-27632 significantly reduces melanoma tumor volume in mice.

Materials and methods

Cell lines and drug treatments. B16F1 mouse melanoma cells (ATCC, no. CRL-6323), UvMel 1.3, UvMel 1.5 and UvMel 270 human uveal melanoma cells (generous gifts from Dr Bruce Ksander at Harvard Medical School) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 60 U/ml of penicillin G, and 60 mg/ml of streptomycin. Cells were treated with either sham or 10 μM Y-27632 for the indicated time course. This concentration of Y-27632 was utilized in these experiments as it is the approximate IC_{50} of the drug based on changes in B16F1 cellular morphology (data not shown).

Detection of ROCK protein levels. To prepare whole cell lysates, cells were lysed in cell lysis buffer (50 mM Tris, 150 mM NaCal, 1 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate). The insoluble material was excluded by centrifugation. The resulting supernatant was mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and subjected to SDS-PAGE analysis. After electrophoresis, the proteins were transferred to a polyvinylidene diflouride (PVDF) membrane, and appropriate primary antibodies were utilized for protein detection. Following incubation with the primary antibody, the membrane was exposed to horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies, subjected to SuperSignal West Pico Chemiluminescent reagent (Pierce Biotechnology, Inc.), and exposed to film. Primary antibodies were use in the following dilutions: 1:50 anti-ROCK1 (sc-17794, Santa Cruz Biotechnology), 1:500 anti-ROCK2 (sc-5561, Santa Cruz Biotechnology), and 1:1000 anti-actin (sc-8432, Santa Cruz Biotechnology).

Morphological analysis. Drug-treated or control melanoma cells were grown for 48 h, at which time photographs were taken via image capture using a Nikon T150 inverted microscope and a Spot RT digital camera. Projection length was analyzed using Image-J software (150 projections measured per treatment). In the case of branching projections, the length of each branch was added to the total length of the primary projection.

For scanning electron microscope imaging of B16F1 cells, cells were grown on poly-D-lysine coated glass coverslips, fixed in 4% glutaraldehyde, stained with 1% osmium tetroxide, and dehydrated with subsequent ethanol washes. Samples were critical point dried, sputter-coated, and mounted on adhesive backing. Images were obtained using a JEOL JSM5600LV scanning electron microscope at magnification, x1000.

Migration/invasion assay. Melanoma cells were seeded onto 6-well plates, grown to 100% confluence, wounded with a sterile pipette tip to remove cells by linear scratches, and treated with sham or Y-27632. The progress of migration was digitally photographed immediately following injury and at 12 h after wounding with a SPOT camera attached to a Nikon Eclipse T150 inverted microscope.

Apoptotic index. Melanoma cells were challenged with the presence of either a 1-10,000 dilution of 3% H_2O_2 to mimic the free radical damaging effects caused by radiation or 10 μM cisplatin, a commonly utilized chemotherapeutic agent. At the same time, cells were treated with either sham or Y-27632. After 24 h of treatment, the cells were collected and prepared for cell cycle analysis with flow cytometry. Briefly, the culture media and trypsinized cells were centrifuged at 1,000 x g for 10 min and washed 2X with phosphate buffered saline (PBS). The samples were then fixed in a 70% ethanol: 30% PBS solution overnight at 4˚C. The samples were washed 2X in PBS, resuspended in PBS containing 50 μg/ml propidium iodide (Sigma no. P4170) and 100 μg/ml RNAse A (Sigma no. R6513), incubated overnight away from light at 4˚C, and analyzed the next day using a BD Biosciences FACSCalibur flow cytometer.

Mouse model of tumor formation. B16F1 cells ($1x10^6$) were injected subcutaneously into the dorsolateral flank of 4-week old C57BL/6 mice (Charles River Laboratories). Mice were randomly assigned to sham (50% DMSO, 50% H_2O vehicle only; N=20 mice) or drug treatment groups (Y-27632 in 50% DMSO, 50% H_2O; N=20 mice). Y-27632 (10 mg/kg) was intraperitoneally injected weekly for three weeks, at which time tumors were excised and tumor volume was measured by water displacement.

Results

ROCK is expressed in multiple melanoma cell lines. In order to determine if pharmacological therapy against ROCK proteins is a viable option in melanoma tumors, it is imperative to confirm that the assumedly ubiquitous expression of ROCK proteins occurs in melanoma cells. ROCK1 and ROCK2 protein expression was detected via Western blotting in each cell line of a panel of melanoma lines including mouse B16F1 melanoma cells and three primary human uveal melanoma lines (Fig. 1).

Y-27632 treatment leads to altered morphology of melanoma cells. In order to determine if addition of Y-27632 affects the

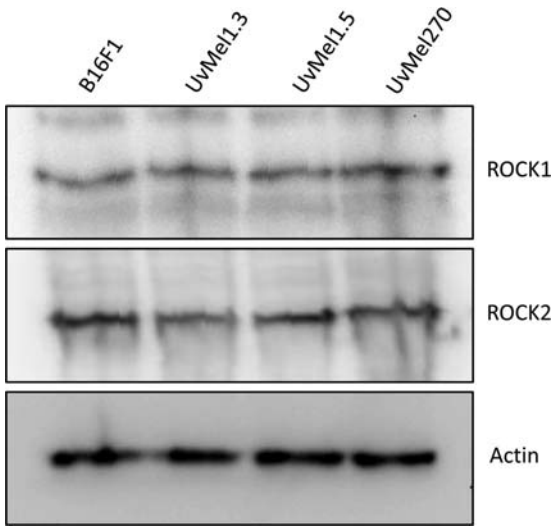

Figure 1. ROCK protein expression in melanoma cell lines. Western blot analysis was performed to confirm the expression of ROCK1 and ROCK2 in a panel of melanoma cells. The steady state protein level of actin was used as a loading control.

morphology of cultured melanoma cells, we treated B16F1 melanoma cells with either control or Y-27632 and analyzed cell morphology with scanning electron microscopy (SEM) and phase contrast microscopy. Using SEM at x1000 magnification, we observed that control cells display significant lamellipodia formation with a very limited number of filopodia (Fig. 2). In contrast, Y-27632 treated cells exhibit very limited lamellipodia formation, and instead display numerous elongated projections arising from the cell body and an abundance of filopodia arising from both the cell body and the extended projections. Quantification of projection length was determined on a panel of melanoma cells treated with sham control or Y-27632. Our findings demonstrate that addition of Y-27632 significantly increases projection length compared to the control in all melanoma cell lines tested (Fig. 3).

Y-27632 treatment reduces invasion of melanoma cells. To determine if Y-27632 treatment of melanoma cells results in alterations in the invasive properties of melanoma cells, we performed wound healing assays in which confluent monolayers of cells were scratch-wounded and allowed to migrate in order to close the resulting wound. Cells migrate to close the wound within a matter of several hours, mostly eliminating the contribution of proliferation to the assay. Y-27632 treatment resulted in a significant reduction in wound closure compared to the control in all cell lines tested (Fig. 4).

Y-27632 treatment results in decreased cell survival in melanoma cells. To determine if Y-27632 treatment of melanoma cells results in alterations in the rate of proliferation, we treated the panel of melanoma cells with control or Y-27632, and measured proliferation rates with an MTT assay 96 h later.

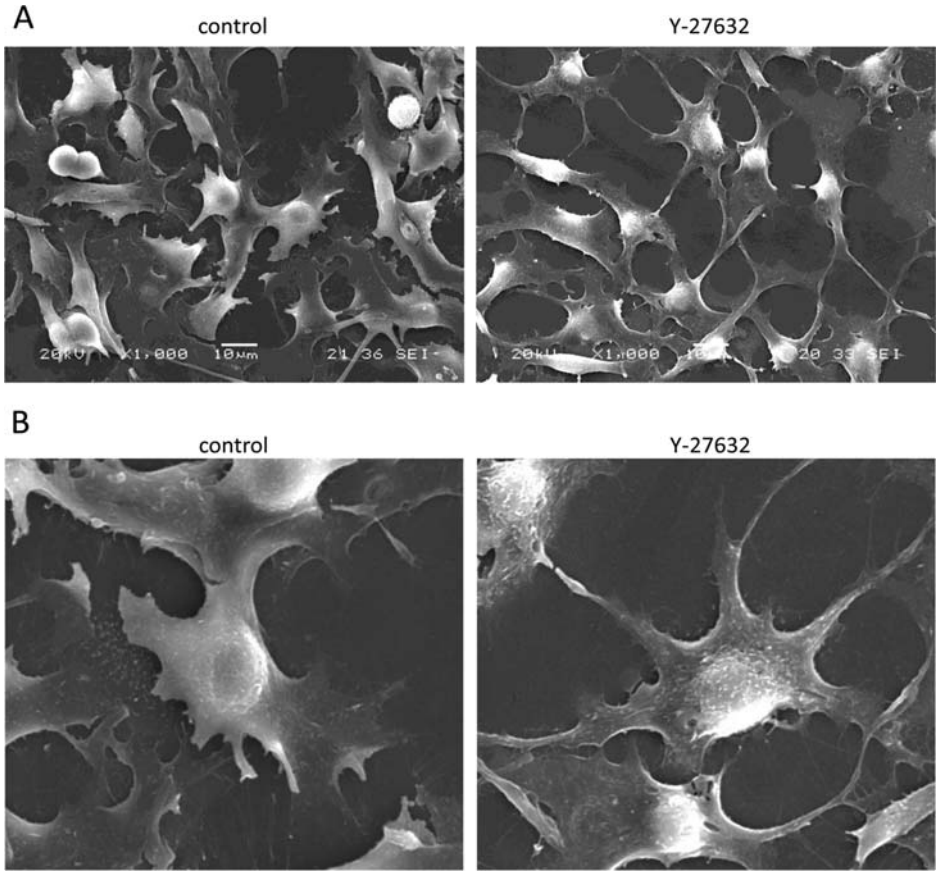

Figure 2. Scanning electron microscopy of control and Y-27632 treated melanoma cells. Scanning electron micrograph images of control or Y-27632 treated B16F1 cells at magnification, x1000 (A) and x2000 (B).

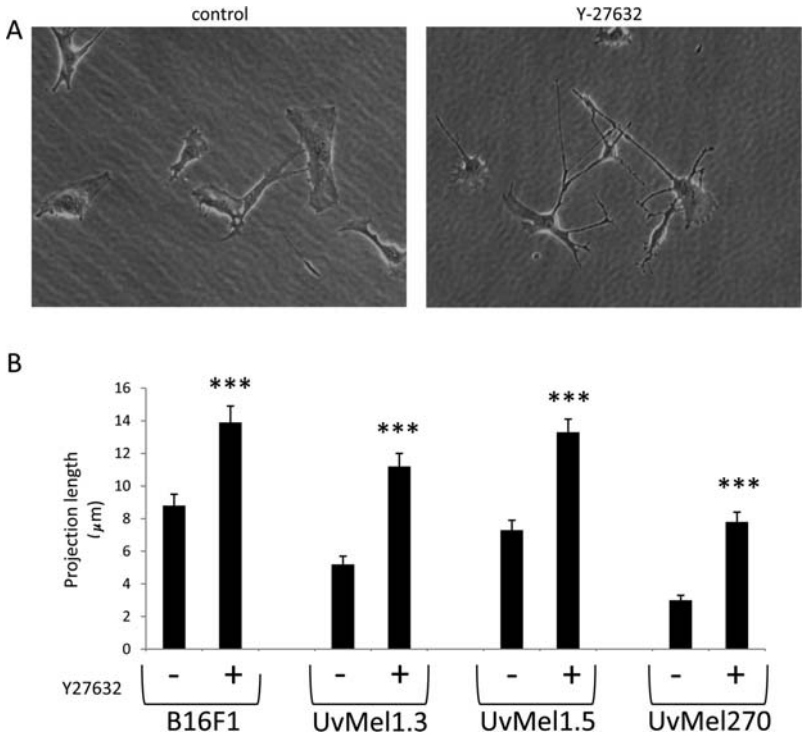

Figure 3. Morphological analysis of control and Y-27632 treated melanoma cells. Control or Y-27632 treated melanoma cells were cultured for 48 h and photographed. Photographs are of B16F1 cells. Cell projection length was quantified in both treatments and is represented with a scatter plot. *p≤0.0001.

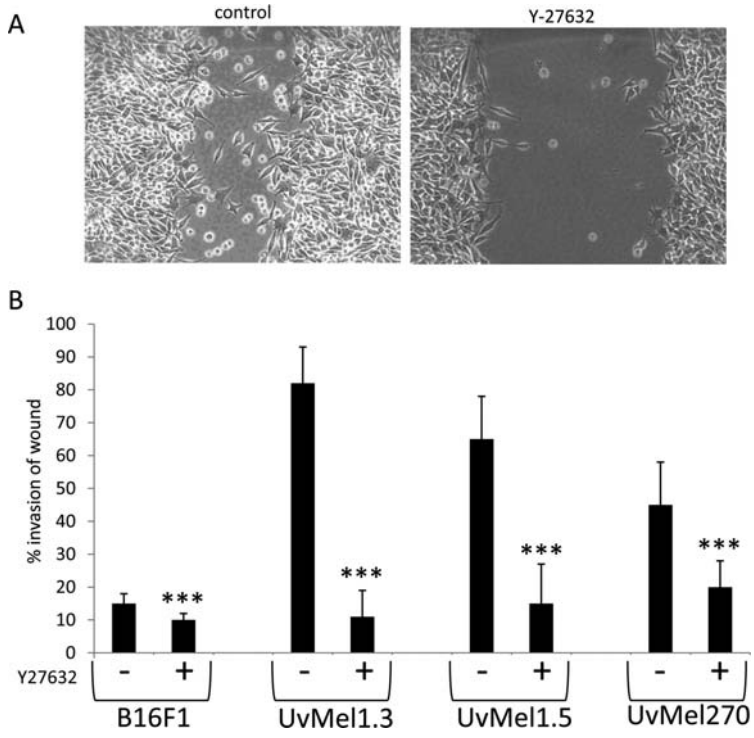

Figure 4. Effect of Y-27632 on invasion in melanoma cells. Melanoma cells were grown to 100% confluence, wounded with a sterile pipette tip, and treated with sham or Y-27632. Photographs were taken (magnification, x40) at 12 h after the injury. Photographs are of UvMel 1.3 cells (A). The wound closure of at least three wells was quantified and is reported as mean percentage invasion ± standard deviation (B). *p<0.01 as demonstrated by Student's t-test.

No significant difference in proliferation rate was observed between treatments for any cell line (data not shown). To determine if Y-27632 alters survival in melanoma cells, the panel of melanoma cell lines was treated with either H_2O_2 (to mimic the free radical damage which occurs during radiotherapy of tumors) or cisplatin (a commonly utilized platinating chemotherapeutic agent), and either sham or Y-27632. Cells were collected 24 h post-treatment and the apoptotic index

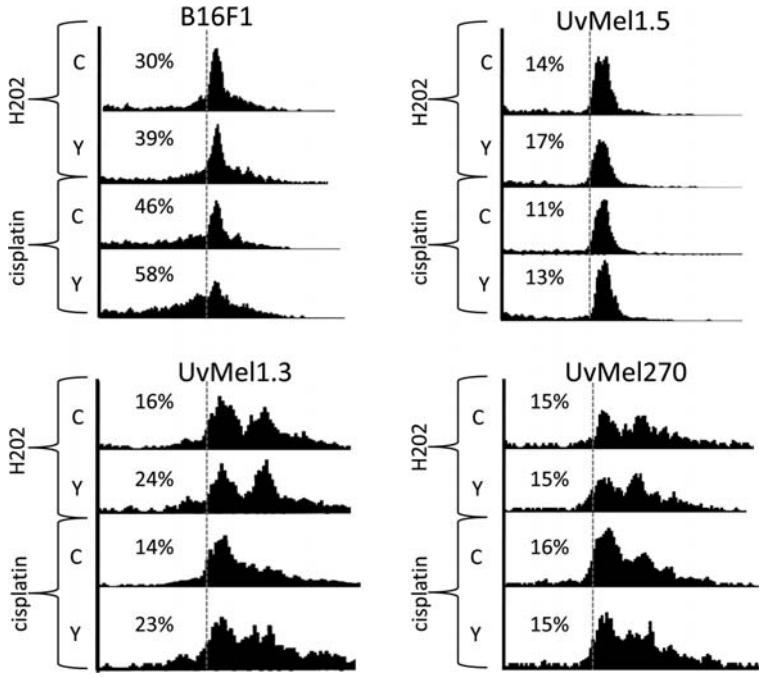

Figure 5. Effect of Y-27632 on survival in melanoma cells. Control or Y-27632 treated melanoma cells were cultured in the presence of H_2O_2 or cisplatin. After 24 h, the cells were collected and analyzed for propidium iodine staining using flow cytometry. Sub-G1 populations (counts left of the dashed vertical line) represent apoptotic cells.

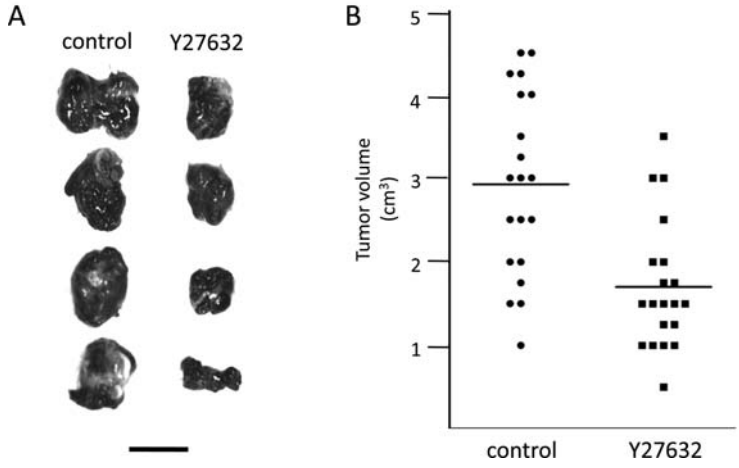

Figure 6. Y-27632 effects on *in vivo* melanoma tumor formation. B16F1 cells ($1x10^6$) were subcutaneously injected into mice. Sham or Y-27632 was delivered intraperitoneally on a weekly schedule. (A) After three weeks, tumors were removed from the animals and photographed. Scale bar represents 1 cm. (B) Tumor volume was measured using water displacement and is represented by a scatter plot. ($p \leq 0.05$).

was quantified using propidium iodide staining of the cellular DNA by flow cytometry (Fig. 5). When treated with either H_2O_2 or cisplatin, both B16F1 and UvMel 1.5 cells largely arrested in the G1 phase of the cell cycle, while UvMel 1.3 and UvMel 270 appeared to continue progression through the cell cycle even in the presence of the cytotoxic agents. Y-27632 addition significantly increased the sub-G1 population of B16F1 and UvMel 1.3 cells treated with either H_2O_2 or cisplatin; however compared to cells grown in the absence of Y-27632, no difference in apoptotic index was observed by ROCK inhibition in either UvMel 1.5 or UvMel 270 cells. These findings suggest that Y-27632 addition to challenged melanoma cells increases cell death; however this occurs in a cell type specific manner.

Intraperitoneal injection of Y-27632 results in decreased in vivo tumor formation. To test if systemic treatment with Y-27632 affects melanoma tumor formation *in vivo*, we subcutaneously injected $1x10^6$ B16F1 cells into the dorsolateral flank of C57BL/6 mice (Charles River Laboratory) followed by intraperitoneal (IP) injection of empty vehicle or 10 mg/kg Y-27632. Subsequent IP injection of vehicle or Y-27632 was performed weekly thereafter. After 3 weeks, the tumors were dissected from the mice, photographed (Fig. 6A) and tumor volume was calculated using standard water displacement (Fig. 6B). While tumor volume varied within each group, a significant reduction in the average tumor volume was observed in tumors collected from Y-27632 treated mice (mean ± SEM=1.7±0.2) compared to control treated mice

(mean ± SEM=2.9±0.2), suggesting that Y-27632 inhibits tumor formation *in vivo*. All animal research conformed to IACUC guidelines and approved protocols.

Discussion

ROCK protein inhibition has been shown to inhibit invasion and metastasis in a limited number of *in vivo* tumor studies (12-14); however the effect of blocking ROCK signaling in most tumor types, including melanoma, is largely unknown. Using both *in vitro* and *in vivo* studies our data suggest that pharmacological inhibition of ROCK proteins with the well-established Y-27632 reduces invasion, alters survival of cultured melanoma cells, and blocks melanoma tumor formation in a mouse tumor model.

Our data demonstrate that ROCK is expressed in multiple melanoma cell lines. Overexpression of ROCK proteins has been demonstrated in testicular cancer, bladder cancer, and hepatocellular carcinoma at the protein level (6,15,16). Because of the essential role of ROCK proteins in mediating key cellular processes such as cytoskeletal rearrangements, and the fact that ROCK proteins are overexpressed in many cancers, development of effective inhibitors of ROCK signaling potentially could inhibit tumor growth and metastasis.

We demonstrate that Y-27632 treatment leads to drastic alterations in melanoma cell morphology including extended projections from the cell body, decreased lamellipodia formation, and increased filiopodia number. Indeed, similar results have been observed in neurons where ROCK inhibition increases neurite outgrowth (17). Changes in cell morphology in Y-27632 treated cells are not surprising given that ROCK proteins have been shown to modulate the actin cytoskeleton via control of myosin regulatory light chain, cofilin, and LIM-kinase (18,19), and ROCK inhibition in fibroblasts reportedly results in loss of central stress fibers and focal adhesions (20). Y-27632 treatment resulted in a marked decrease in migration/invasion capabilities of cultured melanoma cells, likely due to alterations in the morphological properties of the cells. The RhoA/ROCK signaling pathway is considered the master regulator of cellular migration, and inhibition of this pathway logically disrupts cell migration. For instance, inhibition of RhoA signaling reportedly results in the formation of multiple competing lamellipodia that ablates cell migration, as well as upregulation of integrin-mediated focal adhesions accompanied by increased phosphotyrosine signaling through Pyk-2 and paxillin (21,22).

Our data demonstrate that Y-27632 treatment results in cell type specific alterations in cell survival in melanoma cells subjected to H_2O_2 or cisplatin. We do not understand the molecular details that dictate how ROCK inhibition can be pro-apoptotic in one cell type, yet have no effect on cell survival in another cell type. However, the role of ROCK signaling in cell survival remains a very controversial issue, with activation of ROCK being pro-apoptotic in many cell types (23-25) and anti-apoptotic in neural, glial, and embryonic stem cells (26,27). It has been suggested that ROCK protein-mediated regulation of cell survival/apoptosis occurs at least in part through modulation of c-Jun amino terminal kinase (JNK) (28) and Fas receptor (29).

IP injection of Y-27632 in tumor-bearing mice resulted in a significant reduction in melanoma tumor volume compared to control treated mice. These results are very promising in light of our findings that suggest blocking ROCK signaling affects cell survival in some melanoma cells. For instance, tumor instability and increased apoptosis as a result of chemotherapy or radiation may be synergistically enhanced through combination treatment with Y-27632. Moreover, pharmacological inhibition of ROCK with hydroxyl-fasudil, a clinically approved oral therapeutic used in Japan for cardiovascular disease and cerebral vasospasm, leads to very few side effects and is considered very safe, therefore combinations of chemotherapy/radiation and pharmacological ROCK inhibitors could result in lower effective doses needed to treat patients. At present our laboratory is examining if combination therapies with Y-27632 results in enhanced efficacy compared to chemotherapy/radiation alone.

Acknowledgements

This study was funded by minigrants and departmental funding awarded to BAB through Worcester State College. We would like to thank Dr P. Bradley (Worcester State College) for his assistance with SEM imaging and Ms. L. Emmons (Worcester State College) for aiding with tumor cell and drug injections into mice. We would also like to thank Dr B. Ksander (Harvard Medical School) for the generous gift of the uveal melanoma cell lines.

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