Rho-associated kinase inhibitor, Y-27632, inhibits the invasion and proliferation of T24 and 5367 bladder cancer cells

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Abstract. The serine/threonine kinases, Rho-associated protein kinase I and II (ROCK I and II), regulate the cytoskeleton by acting downstream of the small GTPase, Rho, and have been implicated in tumorigenesis and cancer metastasis. Inhibition of ROCK signaling has been shown to suppress the invasion and migration of several types of cancer cells. In this study, the effect of the ROCK inhibitor, Y-27632, on the proliferation and invasion of T24 and 5637 bladder cancer cells was investigated. In the proliferation assays, the cells were exposed to 0, 10, 25, 50, 75, 100, 125 or 150 µmol/l Y-27632 and proliferation was determined using Cell Counting kit-8 after 24, 48 and 72 h. In the invasion assays, the cells were placed in the upper chamber of transwell plates and subjected to 0, 25, 50 or 75 µmol/l Y-27632 for 24 h, after which invasion was measured. Y-27632 significantly suppressed the cell proliferation of T24 and 5637 cells in a concentration- and time-dependent manner. Y-27632 also inhibited the invasion of T24 and 5637 cells in a concentration-dependent manner (P<0.001). In addition, Y-27632 suppressed myosin light chain kinase (MLCK) phosphorylation in T24 and 5637 cells, confirming that it is also a downstream effector of the Rho/ROCK pathway in T24 and 5637 bladder cancer cells. In conclusion, the Rho/ROCK/P-MLCK pathway may be important in tumor cell metastasis in bladder cancer.

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Introduction

There is significant morbidity and mortality resulting from urothelial bladder cancer (UBC) globally; it is the seventh most common type of cancer in males and the seventeenth in females (1). Smoking and occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons are the most important risk factors for UBC (1,2). There is significant global variability in the incidence of UBC with the highest rates occurring in Europe, Egypt and the United States, while Asia, South America and Sub-Saharan Africa have the lowest rates (3). Global incidence appears to coincide with the distribution of risk factors (3). Although the majority of cases of UBC are non-invasive at the time of diagnosis, up to 25% of cases do show different degrees of invasiveness at the time of diagnosis (1). Therefore, understanding the mechanism by which UBCs acquire invasive properties may aid in the identification of novel therapeutic targets.

The Rho/Rho-associated protein kinase (ROCK) pathway has been shown to effect the proliferation and invasion of several types of cancer cells (1-4). Specifically, ROCK is an important molecule in metastasis (4). In addition, the Rho protein is a small GTPase that exhibits a key biological role in cell division and proliferation through its downstream molecules. Furthermore, members of the Rho family of small GTPases regulate microfilament network organization, intercellular contact and malignant transformation (5). They also regulate cytoskeletal activity, and are consequently involved in cellular invasion and migration via epithelial to mesenchymal transition (6). ROCK is the most significant downstream effector of Rho (7), and myosin light chain kinase (MLCK) is a downstream effector of ROCK. The Rho/ROCK/MLCK pathway is important in cell morphology, motility, invasion, adhesion, polarity formation and mitosis, and thus participates in the pathogenesis of cancer (8). Therefore, it is hypothesized that the Rho/ROCK/MLCK pathway may have a role in cancer progression by regulating the reorganization of the actin cytoskeleton. Moreover, ROCK inhibitors, such as Y-27632 (a 4-amino pyridine) have been shown to inhibit the invasion of tumor cells (9,10).

The expression of RhoA and RhoC is significantly higher in bladder cancer cells, suggesting a role in tumorigenesis and invasiveness (11). In 5637 and UM-UC-3 bladder cancer cells, Rho/ROCK signaling increased proliferation and migration,

Abbreviations: BSA, bovine serum albumin; FBS, fetal bovine serum; MLCK, myosin light chain kinase; ROCK I and II, Rho-associated protein kinase I and II; SD, standard deviation; UBC, urothelial bladder cancer

Y-27632 (mmol/l)	T24 cell proliferation (%)			
	24 h	48 h	72 h	
0	100	100	100	
10	97.39±1.1	95.3±1.38ª	93.74±2.44ª	
25	90.72±1.55 ^{a,b}	$87.7 \pm 0.98^{a,b}$	$84.35 \pm 4.27^{a,b}$	
50	84.27±1.11 ^{a-c}	$67.88 \pm 0.9^{\text{a-c}}$	73.32±2.11ª-c	
75	$76.25 \pm 1.89^{a-d}$	$61.73 \pm 1.02^{a-d}$	60.31±2.81 ^{a-d}	
100	50.83±1.23 ^{a-e}	47.29±1.21 ^{a-e}	45.07±0.9 ^{a-e}	
125	45.97±2.33 ^{a-f}	43.12±2.03 ^{a-f}	41.26±1.43 ^{a-e}	
150	40.38±1.38 ^{a-g}	38.81±1 ^{a-g}	35.22±1.04 ^{a-g}	
P-value	<0.001	<0.001	< 0.001	
P<0.05, significantly different fr	om cells treated with ^a 0, ^b 10, ^c 5, ^d 50, ^c 75,	^f 100 and ^g 125 mmol/l Y-27632. n=6 per	group.	

Table I. T24 cell proliferation in concentrations of Y-27632 ranging from 0 to 150 mmol/l at 24, 48 and 72 h.

and this was inhibited by treatment with the ROCK-specific inhibitor, fasudil (HA-1077) (12). In a similar study, inhibition of the Rho/ROCK pathway by *Clostridium difficile* toxin B, HA-1077 and Y-27632, inhibited the migration of T24 and J8 bladder cancer cells (13). Also, the inhibition of RhoC by microRNA-493 decreased T24 and J82 bladder cancer cell migration (14). Notably, high expression of Rho/ROCK in patients with bladder cancer is associated with poor tumor differentiation, muscle invasion, and lymph node metastasis (15).

Elements of the Rho/ROCK/MLCK pathway may therefore be therapeutic targets. However, relatively few studies have investigated the effect of the Rho/ROCK pathway on bladder cancer. In the present study, the ROCK inhibitor, Y-27632, was used to investigate the effect of the Rho/ROCK pathway on the proliferation and invasion of T24 and 5634 bladder cancer cells.

Materials and methods

Cell proliferation assay. T24 and 5637 bladder cancer cell lines were purchased from the Shanghai Institute Cell Bank (Shanghai, China). Cells (1x10⁴ cells per well) were seeded in 96-well plates in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both from Gibco-BRL, Carlsbad, CA, USA). Cells were treated with 0, 10, 25, 50, 75, 100, 125 or 150 μ mol/l Y-27632 (Tocris, Bristol, UK) for 24, 48 and 72 h at 37°C with 5% CO₂. At each time point, 10 μ l Cell Counting kit-8 solution (Dojindo, Kumamoto, Japan) was then added to each well, and the cells were further incubated for 2 h at 37°C with 5% CO₂. Three independent assays were conducted, and the average was taken.

Cell invasion assay. Transwell chambers (5- μ m pore size; Corning, NY, USA) were pre-coated with BD MatrigelTM (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The cells were harvested and resuspended in media containing FBS, and 200 μ l of the suspension containing 5x10⁴ cells was added to the upper chamber. The cells were then treated with 0, 25, 50 or 75 μ mol/l Y-27632, and incubated for 24 h at 37°C. The transwells were removed and stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and the cells in the lower partition were counted under a light microscope (GX41; Olympus, Tokyo, Japan) at x200. The percentage of inhibition was calculated according to the following formula: Percentage inhibition = (control group penetrated cells-experimental group penetrated cells) / control group penetrating cells x 100.

Western blot analysis. Cells were treated with various concentrations of Y-27632 then harvested and lysed in lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). After 50 μ g of protein was separated by 7.5% SDS-PAGE (7.5% Mini-Protean TGX[™] Precast Protein gels; Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA), the membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline for 60 min and washed three times. The membranes were then incubated with monoclonal rabbit antihuman antibodies against P-MLCK (1:1,000; cat. no. ab76092; Abcam, Cambridge, UK) or polyclonal rabbit anti-human antibodies against β-actin (1:1,000; cat. no. A2668; Sigma-Aldrich) at 4°C overnight, and then with polyclonal goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) at room temperature for 60 min. NIH Image J analysis software V.1 (National Institutes of Health, Bethesda, MD, USA) was used to detect the optical density of P-MLCK bands, and was normalized to the values obtained for β -actin to determine the relative expression of P-MLCK.

Statistical analysis. Continuous variables was presented as the mean \pm standard deviation. Repeated measurement analysis of variance (ANOVA) with Bonferroni post hoc tests were performed to compare the differences between different time points at each concentration of Y-27632 in T24 and 5637 cell proliferation assays. One-way ANOVA with Bonferroni post hoc tests were performed to compare the differences among the different concentrations of Y-27632 in T24 and 5637 cell proliferation, cell count, and inhibition rate at each

Y-27632 (mmol/l)	5637 cell proliferation (%)			
	24 h	48 h	72 h	
0	100	100	100	
10	97.7±1.33	95.39±3.68	92.37±0.71ª	
25	92.36±1.16 ^{a,b}	90.49±3.51 ^{a,b}	85.3±0.63 ^{a,b}	
50	86.9±2.27 ^{a-c}	80.64±3.01 ^{a-c}	78.72±0.57 ^{a-c}	
75	75.02±1.47 ^{a-d}	69.32±2.8 ^{a-d}	66.3±0.53 ^{a-d}	
100	49.8±0.86 ^{a-e}	43.06±1.23 ^{a-e}	41.2±0.27 ^{a-e}	
125	$40.32 \pm 1.49^{a-f}$	38.63±1.83 ^{a-e}	36.12±0.48 ^{a-f}	
150	35.2±1.36 ^{a-g}	33.4±1.18 ^{a-g}	31.56±0.56 ^{a-g}	
P-value	<0.001	<0.001	<0.001	

Table II. 5637 cell proliferation in concentrations of Y-27632 ranging from 0 to 150 mmol/l at 24, 48, and 72 h.

P<0.05, significantly different from cells treated with ^a0, ^b10, ^c25, ^d50, ^c75, ^f100, and ^g125 mmol/l Y-27632. n=6/group.



Figure 1. Effect of Y-27632 on bladder cancer cell proliferation. (A) T24 and (B) 5637 cell proliferation was determined using the Cell Counting kit-8 assay. *P<0.05, compared with 48 h or *P<0.05, compared with 72 h.

time point. P<0.05 was considered to indicate a statistically significant difference. SPSS 17.0 statistics software (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

Results

Effect of Y-27632 on T24 and 5637 cell proliferation. T24 and 5637 bladder cancer cells were subjected to Y-27632 concen-



Figure 2. Comparison of the rate of inhibition of T24 and 5637 cell proliferation by different concentrations of Y-27632. *P<0.05, compared with 0 μ mol/l; *P<0.05, compared with 25 μ mol/l and *P<0.05, compared with 50 μ mol/l.

tration-response and time course assays. Y-27632 significantly suppressed the cell proliferation of T24 and 5637 cells in a concentration-dependent manner (Fig. 1, Tables I and II). At 24 h, the proliferation of T24 cells significantly decreased from 0 to 150 μ mol/l Y-27632 (P<0.001). Similar decreases in T24 cell proliferation were observed at 48 and 72 h (Table I). In addition, T24 cell proliferation decreased significantly with time from 0 to 72 h (Fig. 1A). At 24 h, the proliferation of 5637 cells decreased significantly from 0 to 150 μ mol/l (P<0.015). Similar decreases in 5637 cell proliferation were observed at 48 and 72 h (Table I).

Effect of Y-27632 *on* T24 *and* 5637 *cell invasion*. Y-27632 inhibited the invasion of T24 and 5637 cells in a concentration-dependent manner (P<0.001; Fig. 2). At 24 h, the inhibition of cellular invasion increased significantly with increasing concentrations of Y-27632. Representative images from the invasion assays are shown in Fig. 3.

Suppression of P-MLCK expression by Y-27632 in T24 and 5637 cells. MLCK is a known downstream effector of the Rho/ROCK pathway in several cells. Therefore, P-MLCK



Figure 3. Effect of Y-27632 on bladder cancer cell invasion. Invasive T24 cells (top panels) and 5637 cells (bottom panels) were stained with crystal violet after they were treated with 0 (Aa and Ab), 25 (Ba and Bb), 50 (Ca and Cb) and 75 (Da and Db) mmol/l Y-27632. x200 magnification.



Figure 4. Effect of Y-27632 on MLCK phosphorylation. The ratio of P-MLCK/ β -actin, in (A) T24 cells and (B) 5637 cells after incubation with different concentrations of Y-27632. MLCK, myosin light chain kinase; P-MCLK, phosphorylated-MCLK.

protein levels were assessed in response to various concentrations of Y-27632 by western blot analysis. As shown in Fig. 4, Y-27632 suppressed P-MLCK protein expression in T24 and 5637 cells, confirming that it is also a downstream effector of the Rho/ROCK pathway in T24 and 5637 bladder cancer cells.

Discussion

Evidence from several studies indicates that signaling pathways downstream of Rho GTPases are vital in tumor development and progression (16,17). Consequently, there has been considerable interest in the possibility that specific proteins in the Rho/ROCK signal transduction pathway could represent potential cancer therapeutic targets. Relatively little investigation has been conducted, however, regarding the transduction mechanisms of the Rho/ROCK pathway in bladder cancer. The Rho/ROCK signal transduction mechanisms shown in other types of cancers could be common to all types of cancer or idiosyncratic to the cancer types investigated. It is, therefore, important to study these mechanisms in bladder cancer to generate a knowledge base for the potential development of bladder cancer-specific, Rho pathway-derived, therapeutic targets. In the present study, it was demonstrated that Y-27632 inhibited T24 and 5637 cell proliferation and invasion in a concentration-dependent manner. It also suppressed MLCK phosphorylation in T24 and 5637 cells.

In this study, the specific ROCK inhibitor, Y-27632, was used to compare proliferation and invasion of bladder cancer cells when the Rho/ROCK pathway was inhibited by increasing concentrations of Y-27632. Cell proliferation of the T24 and 5637 cancer cell lines was inhibited by Y-2763 in a concentration-dependent manner. The inhibition of bladder cancer cell

proliferation by Y-27632 is in contrast to what has been reported of non-tumor cells. For example, Horani *et al* (18) reported an increase in airway epithelial basal cell proliferation upon Rho inhibition, and Yu *et al* (19) also reported an increase in astrocyte proliferation upon inhibiting ROCK with Y-27632. The fact that inhibition of the Rho/ROCK pathway inhibits bladder cell proliferation while sparing or enhancing the proliferation of normal tissues renders the proteins in this pathway an attractive therapeutic target in bladder cancer. However, further studies are required to assess the mechanism by which Y-27632 alters bladder cancer proliferation as well as assess its effects *in vivo*.

ROCK is a serine/threonine protein kinase that is an important downstream target of Rho. It is involved in several biological activities, including cell adhesion, mitosis, cytoskeletal reorganization, muscle cell contraction, and invasion of tumor cells (20). It was also demonstrated that inhibition of ROCK by Y-27632 suppressed T24 and 5637 cell invasion. The inhibition of invasion was concentration-dependent and in agreement with the findings of other groups. For example, Huang *et al* (21) reported the inhibition of TSGH urothelial cancer cell invasion upon inhibiting ROCK with Y-27632, and Imamura *et al* (10) reported a similar effect with ascites hepatoma cells.

ROCK performs these biological functions by phosphorylating a variety of downstream substrates, including MLCK, connexin and LIM kinase (22-24). Therefore, MLCK was investigated as a possible downstream effector. Y-27632 inhibited the phosphorylation of MLCK in a concentration-dependent manner, indicating that MLCK is a downstream effector of the Rho/ROCK pathway for the enhancement of proliferation and invasion in these bladder cancer cells. This expands the number of potential bladder cancer therapeutic targets in the Rho/ROCK pathway. However, further studies are required to analyze whether the inhibition of bladder cancer cell proliferation and invasion by Y-27632 was mediated by its effect on MLCK phosphorylation.

This study was limited in its scope as it investigated the effects of Y-27632 on *in vitro* cell proliferation and invasion only. Thus, further *in vivo* studies are warranted to confirm these findings as well as uncover the underlying mechanism. In addition, the expression of these proteins may be analyzed in bladder cancer samples isolated from patients to determine the prognostic value of analyzing this pathway.

In conclusion, this study showed that T24 and 5637 bladder cancer cell proliferation and invasion were inhibited with the Rho kinase inhibitor, Y-27632. Furthermore, Y-27632 suppressed P-MLCK protein expression in T24 and 5637 cells. Thus, the Rho/ROCK/P-MLCK pathway may be important in tumor cell metastasis in bladder cancer.

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