

Afzelin exhibits anti-cancer activity against androgen-sensitive LNCaP and androgen-independent PC-3 prostate cancer cells through the inhibition of LIM domain kinase 1

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Abstract. Prostate cancer presents high occurrence worldwide. Medicinal plants are a major source of novel and potentially therapeutic molecules; therefore, the aim of the present study was to investigate the possible anti-prostate cancer activity of afzelin, a flavonol glycoside that was previously isolated from *Nymphaea odorata*. The effect of afzelin on the proliferation of androgen-sensitive LNCaP and androgen-independent PC-3 cells was evaluated by performing a water soluble tetrazolium salt-1 assay. In addition, the effect of afzelin on the cell cycle of the LNCaP and PC-3 prostate cancer cell lines was evaluated. Western blot analysis was performed to evaluate the effect of afzelin on the kinases responsible for the regulation of actin organization. Afzelin was identified to inhibit the proliferation of LNCaP and PC3 cells, and block the cell cycle in the G₀ phase. The anticancer activity of afzelin in these cells was determined to be due to inhibition of LIM domain kinase 1 expression. Thus, the *in vitro* efficacy of afzelin against prostate cancer is promising; however, additional studies on different animal models are required to substantiate its anticancer potential.

Introduction

Prostate cancer is the second most frequently diagnosed type of cancer and the sixth most common cause of cancer-associated mortality in males, worldwide (1). The typical treatment

strategies of chemotherapy and radiotherapy have not provided significant survival benefits for patients with advanced prostate cancer, and the majority of available strategies are only palliative (2). Therefore, there is requirement for the prompt identification of novel molecules to treat the increasing number of prostate cancer cases, particularly cases that are resistant to current chemotherapeutic agents (3,4).

Afzelin is a flavonol glycoside found in *Nymphaea odorata*, which has been identified to inhibit the growth of breast cancer cells by stimulating apoptosis (5). In addition, afzelin has been demonstrated to scavenge superoxide anion radicals in RAW264.7 cells (6). The structure of this compound is shown in Fig. 1.

In eukaryotic cells, the actin cytoskeleton is essential for mediating various biological functions, including providing the structural framework of the cell, and driving cellular motility and division. In particular, dynamic reorganization of the actomyosin cytoskeleton and remodelling of the extracellular matrix drive the multistep process of tumor cell metastasis. Tumor cells are able to cross tissue boundaries into the blood and lymphatic systems and migrate to distal regions of the body. Therefore, cancer therapy has recently focused on gaining a comprehensive understanding of the biological processes that regulate actin organization (7).

Rho GTPase family proteins are key regulators of the actin cytoskeleton and, with the aid of various target proteins, maintain the tight regulation of normal cell growth and differentiation (8-11). Eukaryotic cells exhibit a predisposition to rapid and uncontrollable growth following genomic alterations or carcinogenesis. For instance, increased expression levels of LIM domain kinase 1 (LIMK1) are observed in prostate cancer. The Rho GTPase myotonic dystrophy kinase-related Cdc42-binding kinase α (MRCK α), Rho-associated coiled-coil containing protein kinase (ROCK)1 and ROCK2 are responsible for the activation of LIMK1 (9,12). Previous studies have demonstrated the ability of ROCK inhibitors to reduce the invasive ability of tumor cells *in vitro* and prevent the spread of tumor cells *in vivo*, including melanoma and fibrosarcoma cells, as well as liver, breast, lung and prostate cancer tumor cells (13-17).

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Thus, inhibitors of LIMK1, MRCK α and ROCK1/2 are considered to restore normal cell proliferation and provide a key strategy for cancer treatment (18). Therefore, the aim of the present study was to evaluate the *in vitro* anti-prostate cancer activity of afzelin and its effect on prostate cancer-associated kinases.

Materials and methods

Cell culture. Androgen-sensitive LNCaP (lymph node carcinoma of the prostate) and androgen-independent PC-3 (prostate cancer-3) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The LNCaP cells were maintained in Eagle's minimal essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, glutamine, 2% penicillin-streptomycin and 0.2% gentamicin (all obtained from Sigma-Aldrich). According to the manufacturer's instructions, the PC-3 cells were grown in Ham's F12K medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (90%) and 10% fetal bovine serum. The two cell lines were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell proliferation assay. LNCaP and PC-3 cells were seeded in 96-well plates at a density of 5×10^3 cells/well to a final volume of 100 μ l. At 24 h after seeding, the medium was removed and replaced with fresh medium, containing vehicle (dimethyl sulfoxide) or increasing concentrations of afzelin (0.1, 1.0 and 10.0 μ g/ml), in a final volume of 100 μ l. The cultures were prepared in quadruplicate for each afzelin concentration and time point, and maintained in a CO₂ incubator for three days. After 24, 48 and 72 h, 10 μ l water soluble tetrazolium salt (WST)-1 labeling solution (WST-1 cell proliferation assay kit; Roche Diagnostics, Indianapolis, IN, USA) was added to the cultures and the cells were returned to the CO₂ incubator for 2 h (19). The specific type of formazan product formed was detected by measuring its absorbance in a 96-well spectrophotometric plate reader (DiaSorin, Stillwater, MN, USA) at a wavelength of 420 nm, as described by the manufacturer (20).

Cell cycle analysis. Cell cycle analysis was performed as described previously (19). Briefly, cultured LNCaP and PC-3 prostate cancer cells were exposed to various concentrations of afzelin (0.1, 1.0 and 10.0 μ g/ml) for 24 h. Adherent cells were trypsinized (Sigma-Aldrich), pooled with the cells in suspension and washed three times with ice-cold phosphate-buffered saline (PBS). To determine cell viability, a fraction of the cells were stained with trypan blue (Sigma-Aldrich) and counted. The cultures were adjusted to a concentration of 1×10^6 cells/ml and fixed in a 2:1 ratio (v/v) of chilled methanol overnight. The fixed cells were subsequently stained with propidium iodide (Sigma-Aldrich) in the presence of RNase (Sigma-Aldrich). A minimum of 1×10^4 cells from each experimental group were analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA) to determine the cell cycle distribution and CellQuest cell cycle analysis software (version 5.1; BD Biosciences) to perform data analysis.

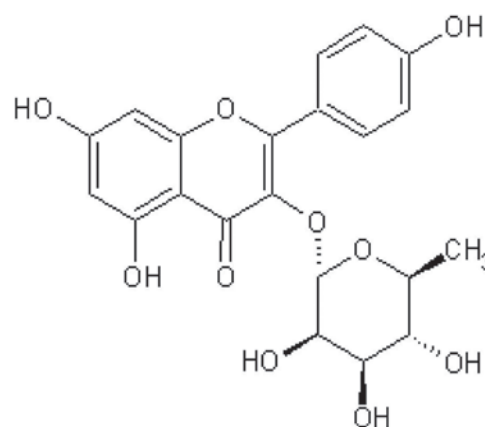


Figure 1. Molecular structure of afzelin, which has a molecular formula of 5,7-dihydroxy-2-(4-hydroxyphenyl)-3-[(2S, 3R, 4R, 5R, 6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one, and molecular mass of 432.38 g mol⁻¹.

Western blot analysis. To determine the effects of afzelin on the protein expression levels of MRCK α , LIMK1 and ROCK1, LNCaP and PC-3 prostate cancer cells were treated with varying concentrations of afzelin (0.1, 1.0 and 10.0 μ g/ml). The concentration of each protein was measured spectrophotometrically using a modified Lowry assay protocol (DC Protein assay; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Next, the lysates were electrophoresed through a 7.5-12.0% denaturing polyacrylamide gel. The resolved proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA) and incubated with blocking solution (PBS containing 0.05% Tween 20 and 5% skimmed milk) to block non-specific binding. Next, the membranes were incubated with monoclonal anti-rabbit MRCK α (1:1,000; cat. no. ab96659; Abcam, Cambridge, USA), LIMK1 (1:1,000; cat. no. 3842; Cell Signaling Technology, Inc., Beverly, MA, USA), p-LIMK1 (1:1,000; cat. no. 3841; Cell Signaling Technology, Inc.), ROCK1 (1:2,000; cat. no. ab45171; Abcam) and p-ROCK1 (1:1,000; cat. no. ab203273; Abcam) primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary rabbit antibodies (1:2,000; cat. no. G33-62G-1000; SignalChem, Richmond, BC, Canada) for 1 h at room temperature. The positive protein bands were visualized using enhanced chemiluminescence solution (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Statistical analysis. Data are expressed as the mean \pm standard deviation. Results were analyzed by one way analysis of variance and all data analysis was performed using SPSS version 21.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Afzelin decreases the growth of prostate cancer cells *in vitro*. The present study analyzed the effect of afzelin on the growth of two prostate cancer cell lines, PC-3 and LNCaP, by exposing the cells to increasing concentrations of afzelin (0.1, 1.0 and 10.0 μ g/ml) one day after seeding. As indicated in Fig. 2A and B, the viability of the PC-3 and LNCaP

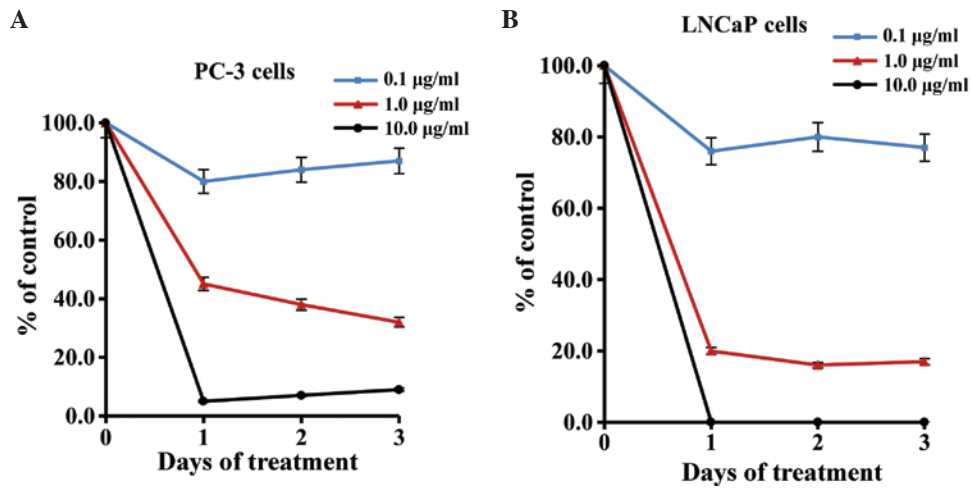


Figure 2. Cell proliferation assay. Inhibition of (A) androgen-independent PC-3 and (B) androgen-sensitive LNCaP prostate cancer cell line proliferation following afzelin treatment. Proliferation of the two cell lines was assessed in the presence of various concentrations of afzelin for 72 h. Absorbance was measured at a wavelength of 420 nm and expressed as a percentage of the absorbance of the control (untreated) cells. Values are expressed as the mean \pm standard deviation of four independent experiments.

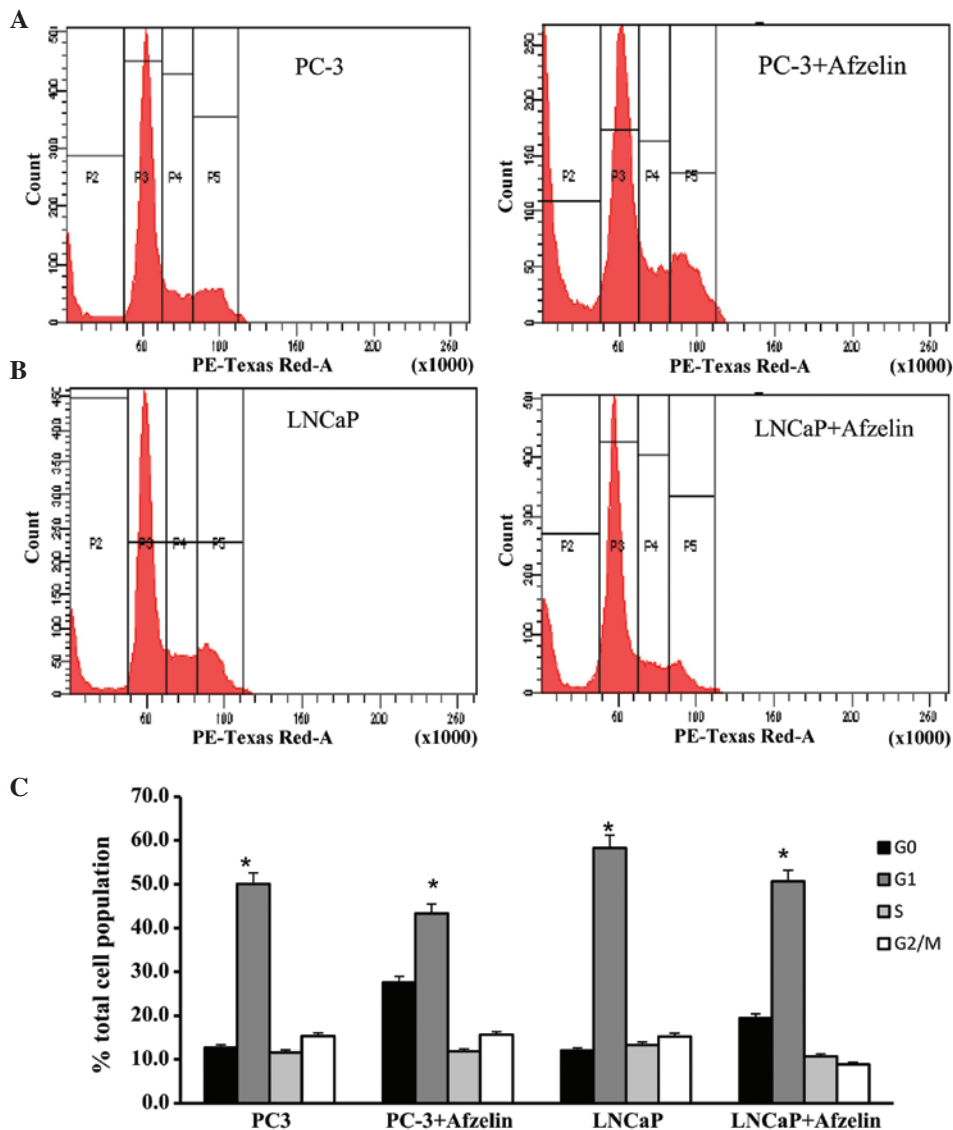


Figure 3. Cell cycle analysis of (A) PC-3 and (B) LNCaP cells treated with 10.0 μ g/ml afzelin or vehicle alone (0.1% DMSO) for 24 h. Cell cycle distribution was analyzed on a minimum of 1×10^4 cells for each experimental condition and data analysis was performed using CellQuest cell cycle analysis software. (C) Data from the cell cycle analysis were quantified into a graph to illustrate the percentage of cells in each phase of the cell cycle. P2, P3, P4 and P5 in Fig. 3A correspond to the G₀, G₁, S and G₂/M phases of the cell cycle in Fig. 3B, respectively. *P<0.05 vs. G₀.

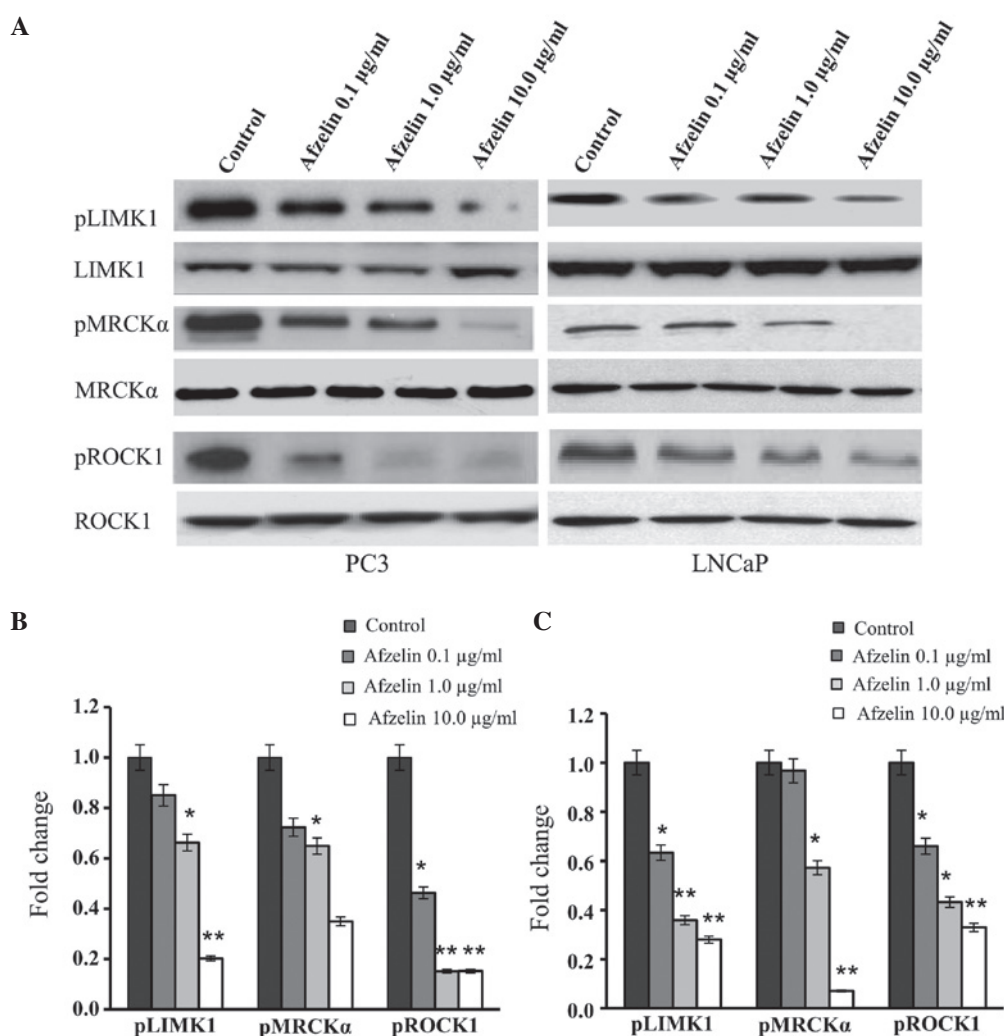


Figure 4. (A) Western blot analysis of the effect of afzelin on the protein expression levels of members of the Rho GTPase family in PC-3 and LNCaP cells. Following treatment with increasing concentrations of afzelin (0.1, 1.0 and 10.0 $\mu\text{g/ml}$) for 24 h, the expression levels of LIMK1 and its downstream proteins, MRCK α and ROCK1, were evaluated in the two cell lines. Afzelin significantly attenuated the expression of all three proteins in a dose-dependent manner, with a maximum decrease observed at 10.0 $\mu\text{g/ml}$ in the two cell lines. (B) PC-3 and (C) LNCaP cell blots were quantitatively analyzed using densitometry. All blots are representative of 3 independent experiments. * $P < 0.05$ and ** $P < 0.01$, vs. control. pLIMK, phosphorylated LIM domain kinase 1; pMRCK α , phosphorylated myotonic dystrophy kinase-related Cdc42-binding kinase α ; pROCK, phosphorylated Rho-associated, coiled-coil-containing protein kinase 1.

cells, respectively, was monitored for 72 h using a WST-1 cell proliferation assay. The dose response curves are presented as the percentage of cell growth in the control groups on the corresponding day, and each value represents the mean of four independent experiments. The results indicate that exposure to afzelin at concentrations of 1.0 and 10.0 $\mu\text{g/ml}$ markedly inhibited the growth of PC-3 and LNCaP cells at 24, 48 and 72 h. Although afzelin was a potent inhibitor of cell growth in the LNCaP and PC-3 cell lines ($P < 0.01$), no growth inhibition was apparent in the vehicle (0.1% DMSO)-treated control cells.

Afzelin induces apoptosis in LNCaP and PC-3 cells. Flow cytometric analysis was used to determine the cell cycle distribution of the LNCaP and PC-3 cells in the absence and presence of afzelin for 24 h. As indicated in Fig. 3A, a broad peak of cells equivalent to 27.6% of the total cell population accumulated in the G_0 population of the afzelin-treated (10.0 $\mu\text{g/ml}$) PC-3 cells, compared with only 12.7% of the control cells. In addition, 43.3% of the cells

accumulated in the G_1 phase of the cell cycle. Thus, an increased proportion of PC-3 cells localized in the G_0 region following treatment with afzelin. Notably, similar results were obtained in the LNCaP cells, where 12.0% of the control cell population localized in the G_0 phase compared with 19.4% of the afzelin-treated (10.0 $\mu\text{g/ml}$) LNCaP cells (Fig. 3B). Furthermore, 50.7% of cells accumulated in G_1 phase. The elevated population of G_0 cells observed subsequent to afzelin treatment indicates the occurrence of extensive apoptosis or an ongoing cytotoxic response. The results indicated that afzelin exhibits a selective effect on the two cell types.

Afzelin decreases the expression of Rho GTPase family proteins. To investigate the molecular effects of afzelin on the LNCaP and PC-3 prostate cancer cell lines, an analysis was conducted to determine the effect of afzelin treatment on the expression levels of specific molecules that are critical in cell cycle progression and apoptosis. Western blot analysis was performed to determine the effect of increasing concentrations of afzelin on the expression levels of members of

the Rho GTPase family proteins, including LIMK1, MRCK α and ROCK1. The results identified that phosphorylation of these kinases progressively diminished in the LNCaP and PC-3 cells as the dose of afzelin was increased (Fig. 4A). However, densitometry evaluation indicated that this suppression was more significant in the PC-3 cells (Fig. 4B).

Discussion

Invasion and metastasis are critical events in cancer progression, which often lead to patient mortality. Metastatic cells undergo a sequence of changes that result in loss of contact inhibition and enhanced motility, which subsequently promotes cell migration from the primary tumor site, leading to invasion and angiogenesis (21). At present, the treatments available for patients with metastatic tumors are limited. Thus, it is critical to identify and develop compounds that target the molecular events involved in cell invasion and metastasis in cancer therapy.

The contractility of the actin-myosin complex is vital for cell motility and plays an important role in tumor cell invasion and metastasis. Myosin II, a critical component of the cytoskeletal contractile machinery, is regulated by the phosphorylation of myosin II light chain proteins (MLC) at Thr18 and Ser19 (22). Furthermore, the members of the Rho GTPase family are involved with the regulation of actin cytoskeleton organization and dynamics, mediating the development of focal adhesions and stress fibers (23-25).

Rho A and C proteins promote the actomyosin contractile force generation via ROCK. ROCK1 and 2 cause the phosphorylation of several downstream target proteins, including MLC, the myosin binding subunit of MLC phosphatase, LIMK1 and LIMK2 (12,26). These target proteins catalyze various processes involved in cancer progression, such as changes in structural arrangement, alterations in cellular polarity, proliferation, migration, invasion, transformation and cytokinesis (26,27).

The phosphorylation of the LIMKs is also mediated by MRCK α and p21-activated kinase, acting downstream of the Rho/Rac/Cdc42 signaling pathway (28-31). In addition, LIMK1 and 2 have been shown to exhibit a crucial role in cancer progression, angiogenesis and metastasis (32). Previous studies have identified increased LIMK1 expression in human breast cancer cell lines; increased expression of LIMK1 increased tumor invasion, whereas the inhibition of LIMK1 expression, or blockade of LIMK1 activity reduced the aggressive behavior of human MDA-MB-231 and MDA-MB-435 breast cancer cell lines (33,34). Thus inhibitors of LIMK1, ROCK1 and MRCK α may inhibit tumor invasion and metastasis effectively.

In the present study it was demonstrated that afzelin, a flavonol glycoside found in *Nymphaea odorata* (35), exhibits significant anti-prostate cancer activities in androgen-sensitive LNCaP and androgen-independent PC-3 prostate cancer cell lines. Afzelin has been reported to exhibit antioxidant, DNA-protective, ultra-violet radiation-absorbing and anti-inflammatory properties (36). In addition, a recent study revealed that afzelin inhibits breast cancer cell proliferation by stimulating apoptosis (5). Notbaly, afzelin attenuated asthma in a murine model of asthma (37). In the present

study, afzelin inhibited cell proliferation in the two prostate cancer cell lines and blocked the cell cycle in the G₀ phase. Furthermore, afzelin attenuated the expression of a number of kinases involved in the maintenance of the actin cytoskeleton.

The effect of afzelin on cell growth in different cell lines was evaluated in the current study, with afzelin identified to markedly inhibit the growth of the prostate cancer cell lines, LNCaP and PC-3. Furthermore, the effect of afzelin on cell cycle progression was evaluated in the LNCaP and PC-3 cells. The results revealed that afzelin (10.0 μ g/ml) caused a markedly increased population of PC-3 and LNCaP cells to accumulate in the G₀ phase when compared with the corresponding control cells. To improve current understanding of the inhibitory effect of afzelin on cell growth and cell cycle progression, the expression levels of specific kinases were evaluated in the PC-3 and LNCaP cell lines upon treatment with increasing concentrations of afzelin. Afzelin was found to attenuate the protein expression levels of LIMK1 in the LNCaP and PC-3 cells. Elevated expression of LIMK1 has been previously identified in prostate cancer cell lines; therefore, afzelin represents a novel strategy for prostate cancer treatment. Furthermore, LIMK1 is activated by MRCK α and ROCK1 (9,12); thus, the effect of afzelin on the expression of these kinases was also evaluated. The results of the present study revealed that afzelin inhibited the protein expression levels of MRCK α and ROCK1, clarifying that downregulation of LIMK1 by afzelin is due to its inhibitory effect on MRCK α and ROCK1, upstream in its signaling pathway. Previous studies have demonstrated that ROCK inhibitors appear to reduce the invasive ability of tumor cells *in vitro* and prevent the dissemination of tumor cells *in vivo* in different types of cancer, particularly in prostate cancer (10,13-17,38). Furthermore, LIMK1, MRCK α and ROCK1 are proteins of interest in the development of novel cancer therapies due to their involvement in regulating actin organization (7) and, in combination with other proteins, maintaining the tight regulation of normal cell growth and differentiation (8,9).

Increased MRCK α expression has been reported in a number of cancer types, including adenocarcinoma (39), cutaneous squamous cell carcinoma (SCC) (40), in oral, hypopharyngeal, head and neck SCC, oral cavity and tongue carcinoma (41-47). In addition, increased MRCK α expression has also been identified in *in vitro* studies using U937 histiocytic lymphoma, MDA-MB 231 breast cancer, A549 lung cancer and PLB-985 myelocytic leukemia cell lines (46,47). Thus, the inhibition of MRCK α may present an effective strategy to inhibit cancer. Furthermore, Lourenço *et al* (48) revealed that LIMK2 expression was decreased in the intestinal tumors of cancer-prone genetically modified mice, as well as in human colorectal cancer cell lines and tumors. In the present study, afzelin was found to inhibit the expression of LIMK1, ROCK1 and MRCK α , indicating that their inhibition may prevent tumor cell invasion and metastasis. These results indicate that afzelin may effectively reduce tumor progression, however, further studies are required to investigate the association between afzelin and the expression of LIMK1, ROCK1 and MRCK α as afzelin may also have upstream targets, such as RhoA, Rac1a and Cdc42 that may effect the activation and signal transduction downstream of LIMK1, ROCK1 and MRCK α .

Conventional therapies, including radiotherapy and chemotherapy, exhibit certain limitations in the treatment of hormone-refractory prostate cancer; therefore, the development of novel treatment strategies is required to enhance the number of positive treatment outcomes. The results of the present study indicate that afzelin may be an important candidate in prostate cancer therapy. However, additional studies are required to evaluate its therapeutic properties, particularly in animal models.

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