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The Effect of Ras Homolog C/Rho-Associated Coiled-Protein Kinase (Rho/ROCK) Signaling Pathways on Proliferation and Apoptosis of Human Myeloma Cells

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Manuscript Preparation E
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Background: The aim of this study was to explore the impact of Ras homolog C/Rho-associated coiled-protein kinase (Rho/ROCK) signaling pathways intervention on biological characteristics of the human multiple myeloma cell lines RPMI-8226 and U266 cells, and to investigate the expression of RhoC, ROCK1, and ROCK2 in RPMI-8226 and U266 cells.

Material/Methods: RPMI8226 and U266 cell lines were treated by 5-aza-2-deoxycytidine (5-Aza-Dc), trichostatin A (TSA), RhoA inhibitor CCG-1423, Rac1 inhibitor NSC23766, and ROCK inhibitor fasudil. Cell proliferation was examined by Cell Counting Kit-8 (CCK-8) assay and clone formation. Cell apoptosis was examined by flow cytometry and TUNEL assay. The mRNA and protein expressions of RhoC, ROCK1, and ROCK2 were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot, respectively.

Results: CCG-1423, NSC23766, and fasudil could significantly inhibit the proliferation of RPMI8226 and U266 cells. The inhibitory effect was dose- and time-dependent within a certain concentration range ($P<0.05$). After treatment with CCG-1423, NSC23766, and fasudil for 24 hours, the apoptosis rates of RPMI8226 and U266 cells were significantly higher than those of the control group, which were dose-dependent ($P<0.05$). Compared with the control group, the mRNA and protein expressions of RhoC, ROCK1, and ROCK2 in RPMI8226 and U266 cells were significantly decreased with single 5-Aza-Dc or TSA treatment. However, the effects were obviously stronger after combined treatment of 5-Aza-CdR and TSA ($P<0.05$).

Conclusions: We found that 5-Aza-Dc and TSA can effectively decrease the mRNA and protein expressions of RhoC, ROCK1, and ROCK2. Furthermore, Rho and ROCK inhibitors significantly inhibit cell growth and induce cell apoptosis in the human multiple myeloma cell lines RPMI-8226 and U266.

MeSH Keywords: Multiple Myeloma • Population Characteristics • rho-Associated Kinases

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Background

Multiple myeloma (MM) is a malignant tumor of terminally differentiated B lymphocytes and plasma cells. A large number of clonal proliferation and abnormal immunoglobulin generation are observed in MM patients. Extensive infiltration of malignant plasma cells and deposition of M protein leads to multiple osteolytic damage, recurrent infections, anemia, hypercalcemia, hyper-viscosity syndrome and renal damage. These clinical complications can eventually cause serious adverse consequences [1]. The incidence of MM on a worldwide scale gradually increases, which is more observed in younger population [2]. So far, MM is still an incurable disease. The pathogenesis of MM is extremely complex, involving a variety of cellular factors, adhesion molecules, signal transduction pathways, cytogenetic abnormalities, and bone marrow microenvironment. Researches have shown that the occurrence and development of MM is related to genetics, immunology, and cellular factors.

Reticular activating system (Ras) superfamily is an important class of functional proteins in human, most of which are oncogenes. Recent research has suggested that Ras signaling transduction pathway is involved in the occurrence and development of multiple cancers by promoting cell proliferation and inhibiting cell apoptosis [3]. Madanle et al. [4] identified a new family of Ras in 1985, namely Ras homolog (Rho) subfamily. As a member of the Rho family, Ras homolog C (RhoC) is an important signal transduction molecule in cells. It is located in the cytoplasm, containing 193 amino acids. Meanwhile, it is also a GTP binding protein, whose gene is located on 1p13-p21 [5]. The occurrence, development, invasion and metastasis of malignancies are related to RhoC downstream effector Rho associated kinase (ROCK). RhoC and its downstream molecules are important signaling pathways, which play an important role in the growth, metastasis, invasion, and apoptosis of liver cancer cells [6,7]. As an oncogene, RhoC protein plays a vital role in the invasion and metastasis of solid tumors, including liver cancer, pancreatic cancer, and breast cancer. Rosenthal et al. [8] demonstrated that RhoC is differentially expressed in primary tumor and metastatic tissues. In addition, RhoC plays a key role in the migration process of tumor cells.

Rho-associated coiled-protein kinase (ROCK) has serine/threonine protein kinase activity. It is a Rho-binding protein associated with apoptosis, which is also the main molecule of the Rho family [9]. ROCK has 3 subtypes, including ROCK1 and ROCK2, which are encoded by 2 different genes [10,11]. ROCK1 and ROCK2 are direct cleavage products for activated caspase-3 and caspase-2 or granzyme B. The 2 molecules are involved in caspase-mediated apoptosis [12,13]. ROCK2 is mainly highly expressed in heart and brain tissues. ROCK1 is mainly expressed in lung, liver, spleen, and kidney tissues. However, no significant difference is found on their functions [14]. As an effect molecule of the Rho GTP enzyme, ROCK is widely involved in a large number of cellular functions, such as cell contraction, adhesion, migration, proliferation, differentiation, apoptosis, and immune cell chemotaxis.

In the most recent 10 years, Rho/ROCK signaling pathway has attracted great attention, mainly in the areas of cardiovascular system, central nervous system, embryonic development, and cancer. Some researchers have demonstrated that Rho/ROCK signaling pathway plays an important role in tumor cell proliferation [15]. Rho/ROCK signaling pathway is also very important in cancer occurrence, development, invasion, and metastasis. As a negative regulator of Rho GTP enzyme, Rho GAP family member DLC-1 acts as a tumor suppressor gene. Meanwhile, its effect is achieved through Rho/ROCK signaling pathway. DLC-1 promoter hyper-methylation leads to loss or decline of its expression in most human solid tumors. Expression or activity of Rho and ROCK family members increases in tumor cells. However, expression of some members is significantly downregulated. This condition can lead to tumorigenesis and enhanced invasion and metastasis. Epigenetic studies have found that DNA methyltransferase (DNMTS) and histone deacetylase (HDAC) can synergistically regulate gene transcription, resulting in silencing of tumor suppressor genes and loss of effect in inhibiting tumor development. As DNA methyltransferase inhibitor and histone deacetylase inhibitor, 5-aza-2-deoxycytidine (5-Aza-Dc) and trichostatin A (TSA) can reverse epigenetic changes to a certain extent. This may lead to silent O6-methylguanine-DNA methyltransferase (MGMT) and other tumor suppressor gene re-expression [16–19]. However, the effect and mechanism of DLC1 hyper-methylation and Rho/ROCK signaling pathway is rarely reported in multiple myeloma. Our previous study found that DLC-1 gene displayed aberrant DNA hyper-methylation in the multiple myeloma cell line RPMI8226. The expression of DLC-1 gene was significantly decreased. However, 5-Aza-CdR and TSA could effectively reverse DLC-1 gene expression, significantly inhibit cell proliferation and migration, and induce cell apoptosis. Moreover, the expression of RhoA and Rac1 was significantly reduced in RPMI-8226 cells [20]. This effect may be related to Rho/ROCK signaling pathway inhibition. Therefore, in this study, upstream and downstream specific signaling inhibitors were transfected into RPMI-8226 and U266 cells. Moreover, the expressions of Rho/ROCK pathway signaling molecules RhoC, ROCK1, and ROCK2 and the changes of biological characteristics were observed in MM cells.

Material and Methods

Experimental materials

RPMI-8226 and U266 cell lines were obtained from the cell bank of Center Laboratory, the Affiliated Hospital of

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Qingdao University; 5-Aza-CdR and TSA were purchased from Sigma-Aldrich (St. Louis, MO, USA); Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA); Cell Counting Kit-8 (CCK-8) was purchased from Yiyuan Biotechnologies (Yiyuan, China); Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis double staining kit was purchased from Becton Dickinson (Franklin Lakes, NJ, USA); TRIzol, reverse transcription kit, and polymerase chain reaction (PCR) kit were purchased from TaKaRa (Tokyo, Japan); RhoC antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), ROCK1 and ROCK2 antibody were purchased from Abcam (Cambridge, MA, USA); RhoC, ROCK1/ROCK2 primers and internal reference were synthesized by Shanghai Shenggong Bioengineering (Shanghai, China).

Experimental groups

RPMI-8226 and U266 cells in the exponential growth phase were divided into 2 groups: control group and experimental group. Cells in the control group were not treated with drugs. However, cells in the experimental group was divided into 1) 5-Aza-CdR (0.5, 1, 2, 5, and 10 μmol/L); 2) TSA (0.2, 0.6, 1, 1.6, and 2 μmol/L); 3) combined-drug group (5 μmol/L 5-Aza-CdR and 0.2, 0.6, 1, and 2 μmol/L TSA); 4) CCG-1423 (5, 10, 20, 40, and 80 μmol/L); 5) NSC23766 (5, 10, 20, 40 and 80 μmol/L); and 6) fasudil (1, 5, 10, 50, and 100 μmol/L) groups. All cells were cultured in a 37°C, 5% CO_2 incubator. Each group was cultured in triplicate.

Cell culture

After successful resuscitation of frozen RPMI-8226 and U266 cells, they were maintained in RPMI 1640 culture medium containing 10% FBS and 1% penicillin and streptomycin in a saturated humidity incubator at 37°C and 5% CO_2. RPMI8226 cells were half passed every 48 hours, and U266 cells were half passed every 72 hours. Cells in logarithmic growth phase were used for subsequent experiments. Each experiment was repeated 3 times.

Plate cloning experiment

Log-phase RPMI-8226 and U266 cells were harvested, and the cell suspension concentration was adjusted to 1×10^4 cells/L. Then 2000 cells were plated in a 6-well plates. After culturing in a 37°C incubator for 3 to 4 days, cells were fixed with 4% paraformaldehyde for 30 minutes then stained with 0.1% crystal violet for 30 minutes. The numbers of clones per well were counted and photographed.

Detection of cell proliferation by Cell Counting Kit-8 (CCK-8) assay

CCK-8 assay was employed to determine the number of viable cells. RPMI-8226 and U266 cells were adjusted to $(1.0–2.0)\times10^5$/mL density, and were seeded into 96-well culture plates at 100 μL per well according to volume. 10 μL of CCG-1423, NSC23766, and fasudil with different concentrations were added to the experimental group. Cell-free RPMI 1640 culture solution was added to blank zero-setting wells. Six wells were used for each group. Subsequently, 96-well plates were incubated at 37°C in a 5% CO_2 incubator for 12, 24, and 48 hours, respectively. Then 10 μL of CCK-8 solution was added to each well, followed by incubation at 37°C in a 5% CO_2 incubator for 3 hours in the dark. Optical density at 450 nm (OD 450) was measured by an enzyme-linked immunometric meter. Finally, cell viability rate was calculated.

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay

RPMI-8226 and U266 cells were fixed in 4% formaldehyde and then washed with phosphate-buffered saline (PBS) containing 2% hydrogen peroxide at room temperature. Two drops of TdT enzyme buffer (Beyotime Institute of Biotechnology, Haimen, China) were then added to the cells and allowed to react at room temperature for 1 hour prior to termination. The cells were incubated in TdT buffer for 1 hour at 37°C. After washing with PBS for 3 times, cells were incubated with the peroxidase-labeled anti-digoxigenin antibody (1: 200, cat. no. ab150155, Abcam, Cambridge, MA, USA) in a wet box at room temperature for 30 minutes. TUNEL staining was observed and recorded under an optical microscope (IX70; Olympus, Tokyo, Japan).

Detection of cell apoptosis by flow cytometry

RPMI-8226 and U266 cells were seeded into 6-well plates at a density of 1.0×10^6 cells/well. Cells were treated with different concentrations of CCG-1423, NSC23766, and fasudil, followed by incubation at 37°C in a 5% CO_2 incubator for 24 hours. Then the cells were harvested and washed with ice-cold PBS twice. After re-suspension with 300 μL Binding Buffer, 5 μL annexin V-FITC was added to cells and mixed lightly to allow the staining reaction at room temperature for 15 minutes in the dark. Subsequently, 5 μL PI was added to lucifugal reaction in the dark, followed by incubation at room temperature for 5 minutes. Then 200 μL binding buffer was added, and cell apoptosis was detected by flow cytometry within 1 hour. Annexin V-FIFC-negative/PI-negative denotes living cells, Annexin V-FIFC-positive/PI-negative staining denotes early apoptotic cells, Annexin V-FIFC-positive/PI-positive staining denotes late apoptotic cells. Finally, the rate of cell apoptosis was calculated.

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Table 1. RhoC, ROCK1/ROCK2 gene primer's sequence for qRT-PCR.

Names	Primer's sequence	AT (°C)
GAPDH	F: 5'-AGAAGGCTGGGGCTCATTTG-3'	60
	R: 5'-AGGGGCCATCCACAGTCTTC-3'	
RhoC	F: 5'-AAGTGGACCCCAGAGGTGAAG-3'	60
	R: 5'-TTATTCCCCACCAGGATGATG3'21	
ROCK1	F: 5'-AAAAATGGACAACCTGCTGC-3'	60
	R: 5'-GGCAGGAAAATCCAAATCAT-3'22	
ROCK2	F: 5'-CGCTGATCCGAGACCCT-3'	60
	R: 5'-TTGTTTTTCCTCAAAGCAGGA-3'22	

AT – annealing temperature, qRT-PCR – quantitative real-time polymerase chain reaction.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells in the experimental (5-Aza-Dc and TSA) group and the control group were cultured for 48 hours. After centrifugation, RPMI-8226 and U266 cells were harvested. Total RNA in cells was extracted with TRIzol reagent according to manufacturer's instructions. The purity and concentration of extracted RNA were detected by ultraviolet spectrometry photometer. Total RNA (1 μg) was transcribed into cDNA by High Capacity cDNA Reverse Transcription Kit with a reaction volume of 20 μL. Synthesized cDNA was used for subsequent experiments or stored at –20°C. Real-time fluorescence quantitative PCR amplification of target gene was carried out using the ABI 7500 real-time PCR instrument and SYBR Premix Ex Taq™ II kit. GAPDH was used as internal control. Primer sequences used in this study were shown in Table 1 [21,22]. The reaction system included: 10 μL SYBR Premix Ex Taq, 2 μL cDNA, 0.8 μL upstream primers, 0.8 μL downstream primers, 0.4 μL ROX Reference Dye, and RNase-free ddH2O to be filled up to 20 μL. The mRNA expressions of RhoC, ROCK1, and ROCK2 in cells of the experimental and control groups were detected using relative quantitative method of cycle threshold (Ct) value comparison ($2^{-\Delta\Delta Ct}$). Relative gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. Δ Ct=Ct (target gene)–Ct (GAPDH), ΔΔCt=Δ Ct (experimental group)–ΔCt (control group). Additionally, the expression level of genes in the control group was considered as "1".

Western blot

After 48 hours of incubation, cells in the experimental group (5-Aza-Dc and TSA) and control group were collected by centrifugation. Meanwhile, the concentration of cells was adjusted to 10^8 cells/mL. Total protein was extracted according to the instructions of radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was used to detect the concentration of extracted proteins. According to the quantitative results of protein, an appropriate volume of total protein samples was added to the protein gel electrophoresis sample buffer and gently mixed. The sample volume of per well was 4 μg. Subsequently, extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking, the membranes were incubated with primary antibodies of RhoC, ROCK1, and ROCK2 (1: 1000, 1: 400 and 1: 5000 diluted in 5% skim milk in Tris buffered saline with Tween 20 (TBST), respectively) overnight. After washing with 1xTBST for 3 times (slowly shaking at room temperature) with 10 minutes for each time, the membranes were incubated with secondary antibody (1: 3000 dilution) at 37°C for 1 hour. Then the membranes were washed with 1xTBST for 3 times, with 10 minutes for each time. Anti-rabbit IgG was conjugated to horseradish peroxidase for 15 minutes, and HRP-ECL reagent smear was developed. Finally, immuno-reactive bands of western blotting for RhoC (band detected at molecular weight of approximately 23 kDa), ROCK1, and ROCK2 (both at around 160 kDa), β-actin (at approximately 42–43 kDa) were visualized by United States UVP analytical instrument.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as a mean ± standard deviation. The *t*-test was used to compare the difference between 2 groups. One-way analysis of variance was for comparison among different groups. $P<0.05$ was considered statistically significant.

Results

Effects of Rho and ROCK inhibitors on myeloma cell proliferation

RPMI8226 and U266 cells were treated with RhoA inhibitor CCG-1423, Rac1 inhibitor NSC23766 and ROCK inhibitor fasudil for 12, 24, and 48 hours, respectively. CCK-8 assay results demonstrated that cell viability rates were significantly decreased in a time- and dose-dependent manner at higher drug concentrations ($P<0.05$), indicating that Rho and ROCK inhibitors could inhibit cell proliferation (Figures 1, 2). Compared with the control group, no significant difference was observed in cell viability rates of the lowest drug concentration ($P>0.05$), indicating that the lowest dose of drug concentration had no significant

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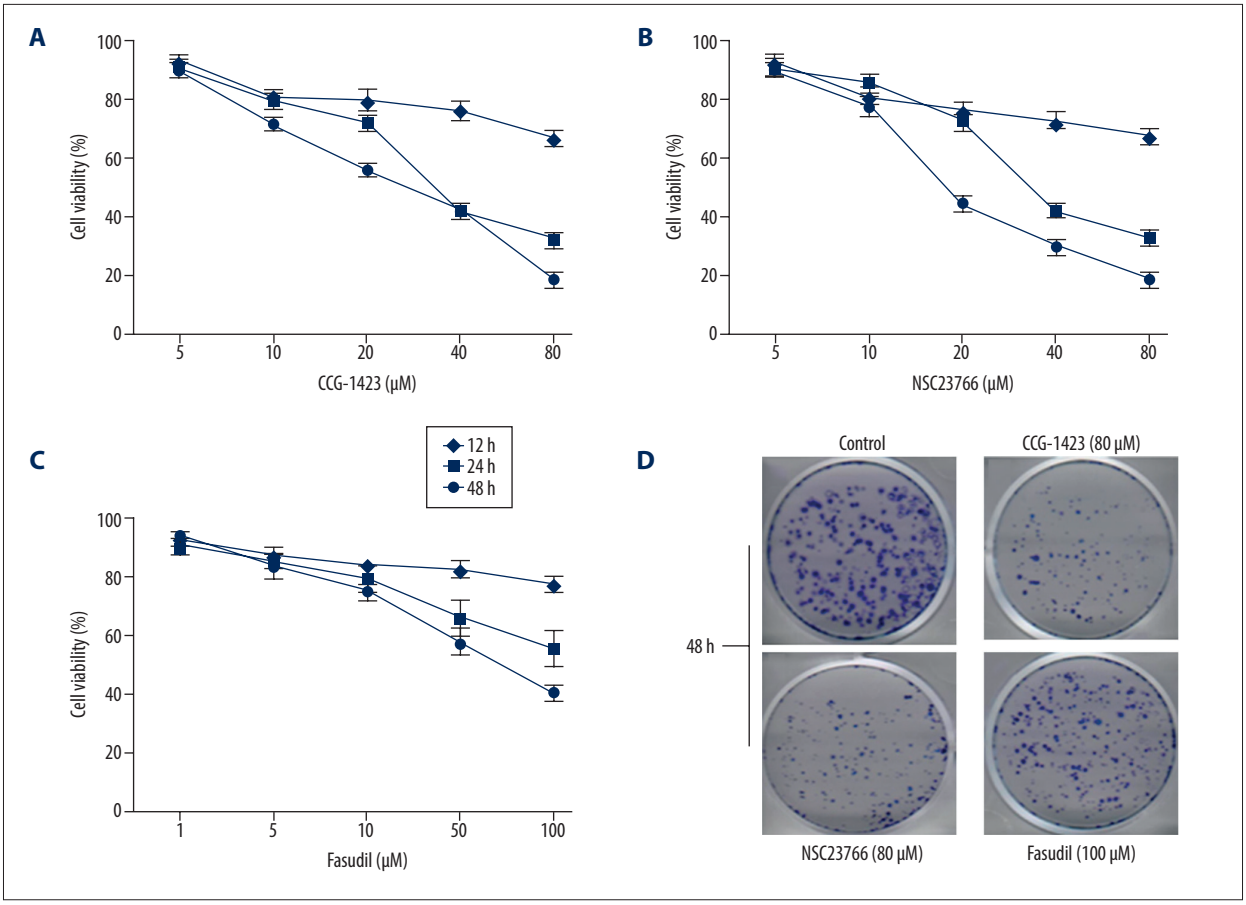

Figure 1. RhoA, Rac1 and ROCK inhibitors could inhibit the proliferation of RPMI-8226 cells in a dose- and time-dependent manner. (**A**) After treatment of RhoA inhibitor CCG-1423 for 12, 24, 48 hours, cell viability was determined by CCK-8. (**B**) Rac1 inhibitors NSC23766 could inhibit proliferation of RPMI-8226 cells. (**C**) Compared with CCG-1423 and NSC23766 groups, the viability of cells was higher in cells treated with ROCK inhibitor fasudil for 48 hours. (**D**) Plate cloning experiments demonstrated that RhoA, Rac1 and ROCK inhibitors could significantly inhibited proliferation of RPMI-8226 cells.

toxicity on RPMI-8226 and U266 cells. Furthermore, cell proliferation was not significantly inhibited in a time-dependent manner in the lowest drug concentration group.

Effects of Rho and ROCK inhibitors on myeloma cell apoptosis

RPMI8226 and U266 cells were treated with CCG-1423, NSC23766, and fasudil for 24 hours. Subsequently, cell apoptosis rates were detected by flow cytometry. The apoptosis rates of RPMI8226 cells in CCG-1423 group were significantly increased in a dose-dependent manner ($P<0.01$). With the increase of drug concentration, the apoptosis rates of RPMI8226 cells were also increased in NSC23766 group. No statistical difference was observed in the apoptosis rates of RPMI8226 cells between 20 mol/L NSC23766 group and control group ($P=0.378$), indicating that the concentration of 20 mol/L NSC23766 had no significant apoptosis-inducing effects. No statistical significance was found in apoptosis rate of RPMI8226 cells between 20 mol/L and 40 mol/L fasudil groups ($P=0.566$). However, compared with control group and 100 mol/L fasudil group, statistically significant difference was found in the apoptosis rates of RPMI8226 cells in 20 mol/L or 40 mol/L fasudil groups ($P<0.05$). Similarly, compared with the control group, the apoptosis rate of U266 cells was significantly increased in a dose-dependent manner. The apoptosis rate of U266 cells increased in a dose-dependent manner from 20.82±1.475 (%) to 60.72±1.205 (%) in CCG-1423 group ($P<0.05$). NSC2376 and fasudil could obviously induce cell apoptosis in a dose-dependent manner at higher drug concentration. Among the 3 drugs, the apoptosis rate in CCG-1423 group were the highest, which was relatively lower in fasudil group (Figures 3, 4). These results indicated that the growth inhibition of fasudil was partly achieved by apoptosis induction. CCG-1423, NSC23766, and fasudil could remarkably inhibit the human multiple myeloma cell lines RPMI-8226 and U266. There were statistically significant differences among groups ($P<0.05$), except for the difference between NSC23766 20 μmol/L group and NSC23766 40 μmol/L group in U266 cells ($P>0.05$).

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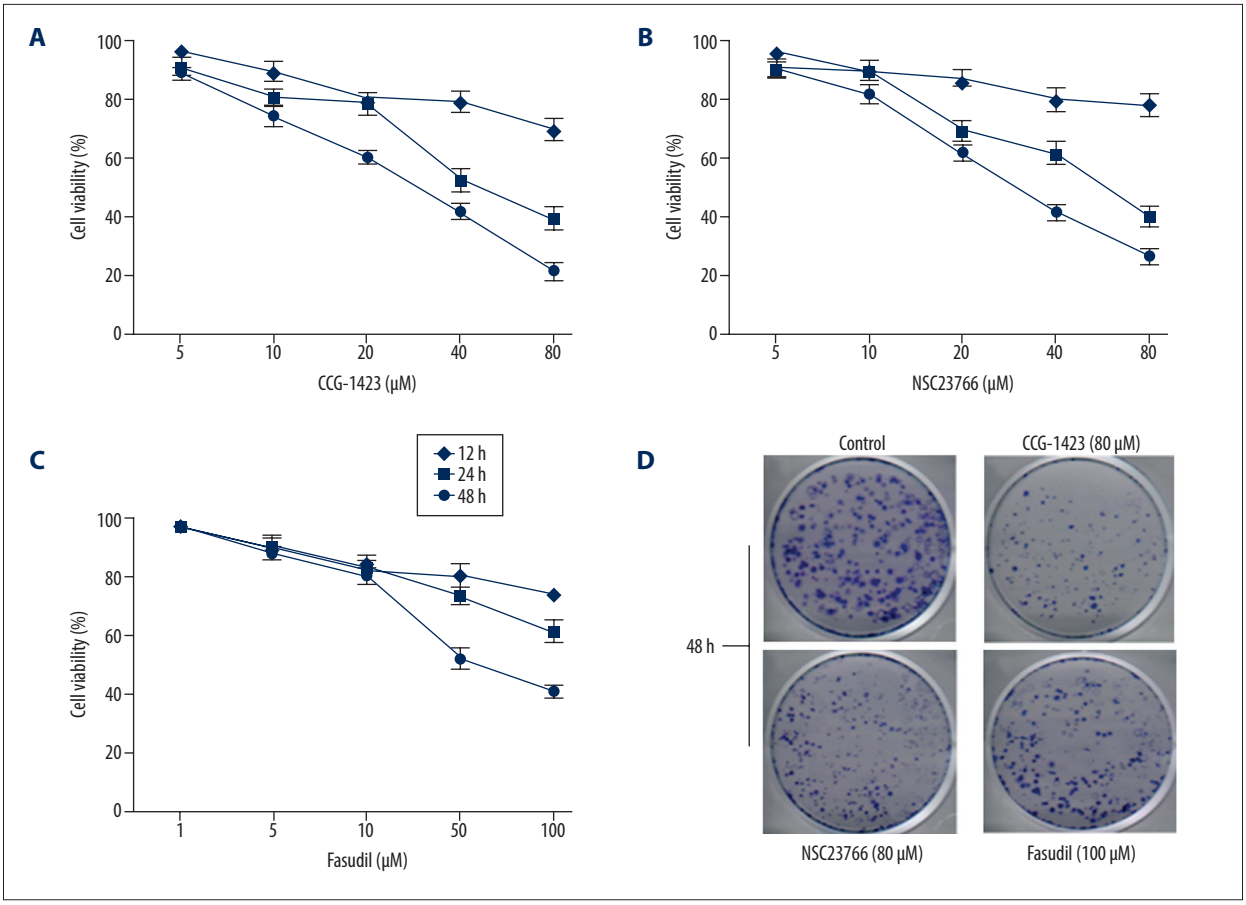

Figure 2. Rac1 and ROCK inhibitors could inhibit the proliferation of U266 cells in a dose- and time-dependent manner. (**A**) After CCG-1423 treatment for 12, 24, 48 hours, cell viability rates were significantly decreased. (**B**) Rac1 inhibitors NSC23766 could inhibit proliferation of U266 cells. (**C**) Similarly, fasudil could obviously inhibit cell proliferation. (**D**) Plate cloning experiments demonstrated that RhoA, Rac1, and ROCK inhibitors could significantly inhibited proliferation of U266 cells.

Effects of 5-Aza-Dc and TSA on RhoC, ROCK1, and ROCK2 mRNA expression in myeloma cells

The SYBR Green I non-probe method and the relative quantitative method of cycle threshold (Ct) value comparison ($2^{-\Delta\Delta Ct}$) were used to detect the expression of target genes. Results were analyzed in combination with dissolution curve following PCR. RPMI8226 cells were first treated with 0.5 μmol/L 5-Aza-Dc for 48 hours. The mRNA expression of RhoC, ROCK1, and ROCK2 was 3.23±0.099, 2.875±0.202, and 2.0355±0.0898, respectively. The mRNA expression of RhoC, ROCK1, and ROCK2 was 1.24±0.057, 1.3355±0.0219, and 1.2075±0.0318 in RPMI8226 cells treated with 10 μmol/L 5-Aza-Dc for 48 hours, respectively. Meanwhile, after RPMI8226 cells were also treated with 0.2 μmol/L TSA for 48 hours, the mRNA expression of RhoC, ROCK1, and ROCK2 was 1.928±0.003, 1.5605±0.086, and 1.619±0.0297, respectively. The mRNA expression of RhoC, ROCK2, and ROCK1 was 1.1435±0.04, 1.096±0.0028, and 0.3945±0.078 in RPMI8226 cells treated with 2.0 μmo/L TSA for 48 hours, respectively. Furthermore, after RPMI8226 cells were treated with 5.0 μmol/L 5-Aza-Dc plus 0.2 μmol/L TSA for 48 hours, the mRNA expression of RhoC, ROCK1, and ROCK2 was 1.9105±0.0134, 1.29±0.042, and 1.3535±0.0347, respectively. The mRNA expression of RhoC, ROCK1, and ROCK2 was 1.051±0.0184, 0.875±0.0212, and 0.113±0.0099 in RPMI8226 cells treated with 5.0 μmol/L of 5-Aza-Dc plus 2.0 μmol/L of TSA for 48 hours, respectively. Similarly, qRT-PCR results showed that after treatment of 5-Aza-Dc and TSA in U266 cells for 48 hours, compared with the control group, the relative mRNA expression level of RhoC, ROCK2, and ROCK1 was significantly declined, and the difference among groups was statistically significant ($P<0.05$). These results suggested that 5-Aza-Dc and TSA could obviously reduce the mRNA expression of RhoC, ROCK2, and ROCK1 in RPMI-8226 and U266 cells and were dose-dependent ($P<0.05$). The mRNA expression of RhoC, ROCK1, and ROCK2 was lowest in mixed group, indicating that 5-Aza-Dc and TSA could effectively inhibit mRNA expression of RhoC, ROCK1/ROCK2. In addition, a synergistic effect was observed (Figures 5, 6).

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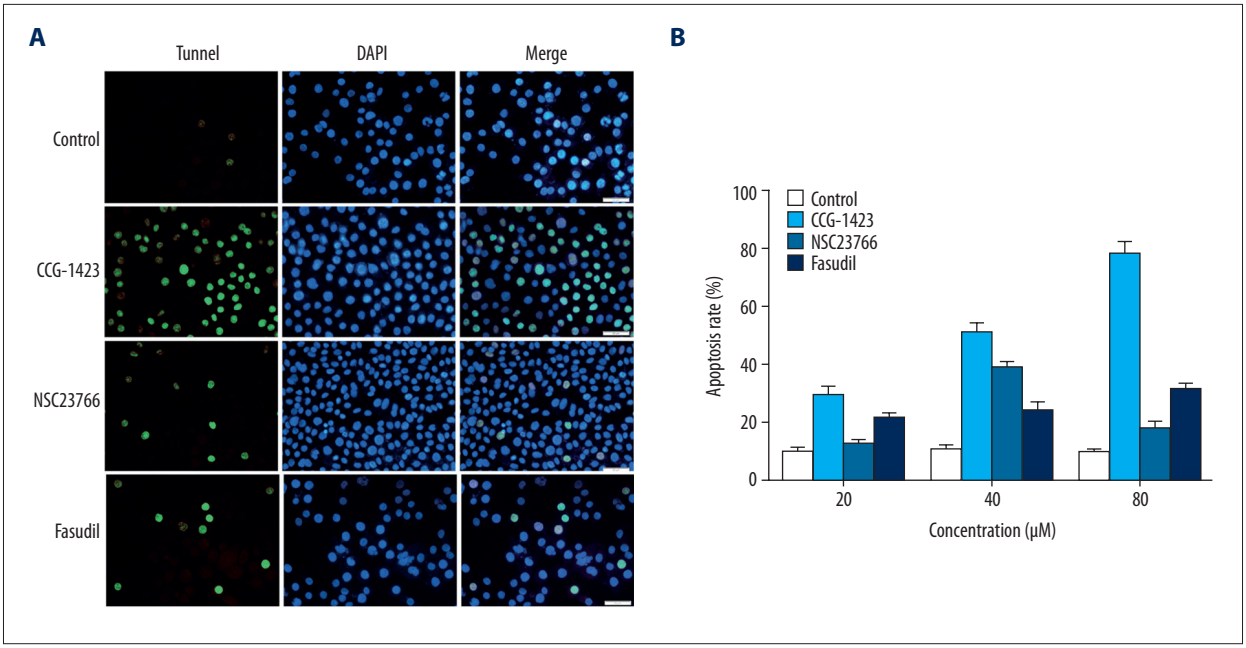

Figure 3. Effect of CCG-1423, NSC23766, and fasudil on rates of apoptosis in RPMI-8226 cells. (**A**) TUNEL assay indicated that CCG-1423, NSC23766, and fasudil promoted apoptosis of RPMI8226 cells. (**B**) After treatment with CCG-1423, NSC23766, and fasudil for 24 hours, the apoptosis rates of RPMI8226 cells were significantly higher than those of the control group, which were dose-dependent ($P<0.05$).

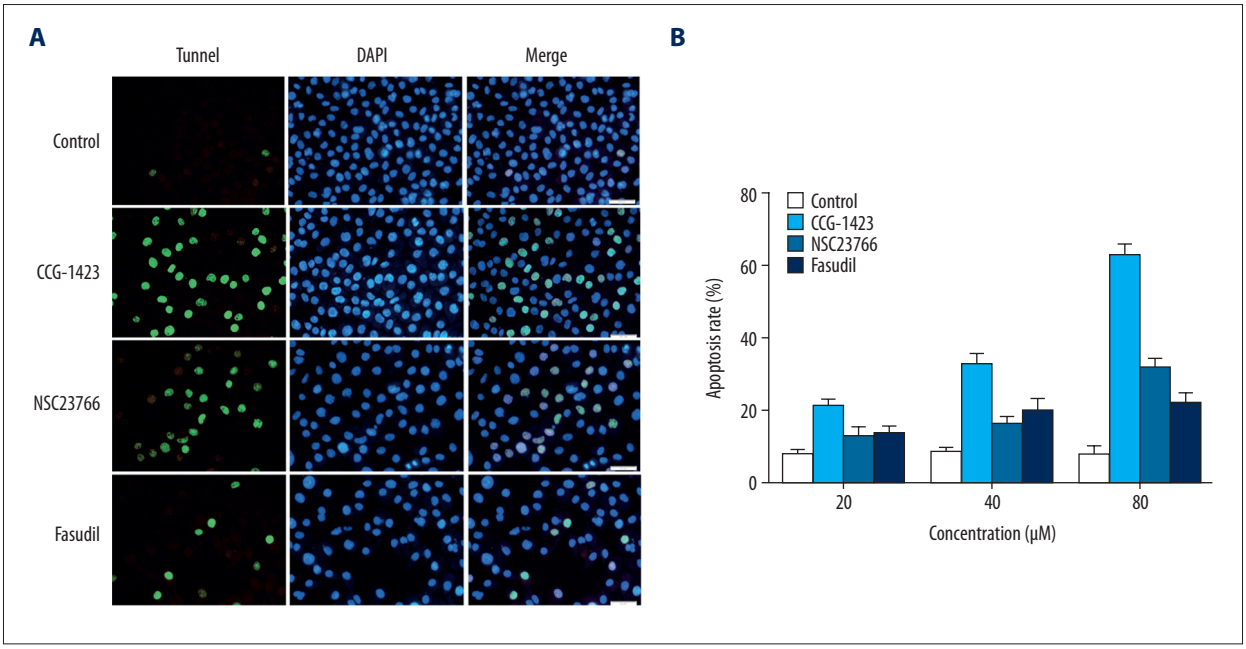

Figure 4. Effect of CCG-1423, NSC23766, and fasudil on rates of apoptosis in U266 cells. (**A**) TUNEL assay indicated that CCG-1423, NSC23766, and fasudil promoted apoptosis of RPMI8226 cells. (**B**) After treatment with CCG-1423, NSC23766, and fasudil for 24 hours, the apoptosis rates of U266 cells were significantly higher than those of the control group, which were dose-dependent ($P<0.05$).

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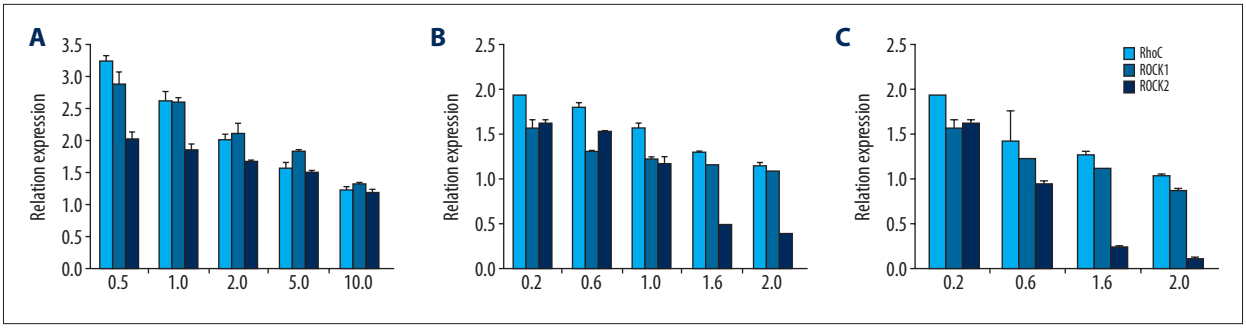

Figure 5. The mRNA expression of RhoC, ROCK1, and ROCK2 in RPMI8226 cells after treated with 5-Aza-Dc, TSA, and combined 5-Aza-Dc and TSA. (**A**) The mRNA expression of RhoC, ROCK1, and ROCK in RPMI8226 cells with different concentrations of 5-Aza-Dc. 5-Aza-Dc could effectively reduce the expression of RhoC, ROCK1, and ROCK ($P<0.05$). (**B**) The mRNA expression of RhoC, ROCK1, and ROCK with different concentrations of TSA. Slimily, TSA could reduce the expression of RhoC, ROCK1, and ROCK ($P<0.05$). (**C**) The mRNA expression of RhoC, ROCK1, and ROCK with 5 μmol/L 5-Aza-Dc combined TSA group. When the 2 drugs were combined, the mRNA expression of RhoC, ROCK1, and ROCK were significantly decreased. 5-Aza-Dc – 5-aza-2-deoxycytidine; TSA – trichostatin A.

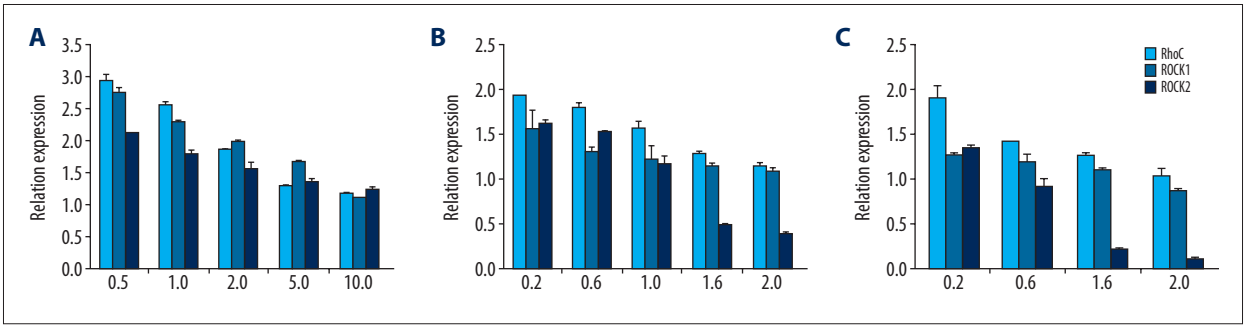

Figure 6. The mRNA expression of RhoC, ROCK1, and ROCK2 in U266 cells after treated with 5-Aza-Dc, TSA, and combined 5-Aza-Dc and TSA. (**A**) 5-Aza-Dc could effectively reduce the expression of RhoC, ROCK1, and ROCK in U266 cells ($P<0.05$). (**B**) Slimily, TSA could reduce the expression of RhoC, ROCK1, and ROCK in U266 cells ($P<0.05$). (**C**) When the 2 drugs were combined, the mRNA expression of RhoC, ROCK1, and ROCK were significantly decreased in U266 cells ($P<0.01$). 5-Aza-Dc – 5-aza-2-deoxycytidine; TSA – trichostatin A.

Effects of 5-Aza-Dc and TSA on RhoC, ROCK1, and ROCK2 proteins expression in myeloma cells

In this study, the protein expression of RhoC, ROCK1/ROCK2 in RPMI8226 and U266 cells were determined by western blot. The protein expression of RhoC, ROCK1, and ROCK2 in RPMI8226 cells of the control group was 0.814±0.031, 0.777±0.015, and 0.723±0.025, respectively. After treatment of 10 mol/L 5-Aza-Dc in RPMI8226 cells for 48 hours, the protein expression of RhoC, ROCK2, and ROCK1 was 0.093±0.004, 0.104±0.017, and 0.060±0.005, respectively. The protein expression of RhoC, ROCK1, and ROCK2 was 0.077±0.007, 0.064±0.01, and 0.069±0.014 in cells treated with 2.0 mol/L TSA for 48 hours, respectively. After RPMI8226 cells were treated with 5.0 mol/L 5-Aza-Dc plus 2.0 mol/L TSA for 48 hours, the protein expression of RhoC, ROCK1, and ROCK2 was 0.07±0.017, 0.052±0.021, and 0.061±0.013, respectively. In U266 cells, the protein expression of RhoC, ROCK1, and ROCK2 in the control group was 0.932±0.045, 0.878±0.019, and 0.801±0.036, respectively. After U266 cells were treated with 10 mol/L 5-Aza-Dc for 48 hours, the protein expression of RhoC, ROCK2, and ROCK1 was 0.115±0.017, 0.102±0.009, and 0.061±0.021, respectively. The protein expression of RhoC, ROCK1, and ROCK2 protein was 0.217±0.025, 0.154±0.014, and 0.127±0.012 in cells treated with 2.0 mol/L TSA for 48 hours, respectively. After U266 cells were treated with 5.0 mol/L 5-Aza-Dc plus 2.0 mol/L TSA for 48 hours, the protein expression of RhoC, ROCK1, and ROCK2 was 0.094±0.018, 0.076±0.038, and 0.042±0.013, respectively. Compared with the control group, 5-Aza-CdR or TSA treatment could significantly decrease the protein expression of RhoC, ROCK1, and ROCK2 in RPMI-8226 and U266 cells. However, the effects were much stronger after combined treatment of 5-Aza-CdR and TSA in a dose-dependent manner ($P<0.05$). The results showed that the 2 drugs could remarkably reduce the protein expression of RhoC, ROCK1, and ROCK2 in RPMI8226 and U266 cells, which had a synergistic effect (Figures 7, 8).

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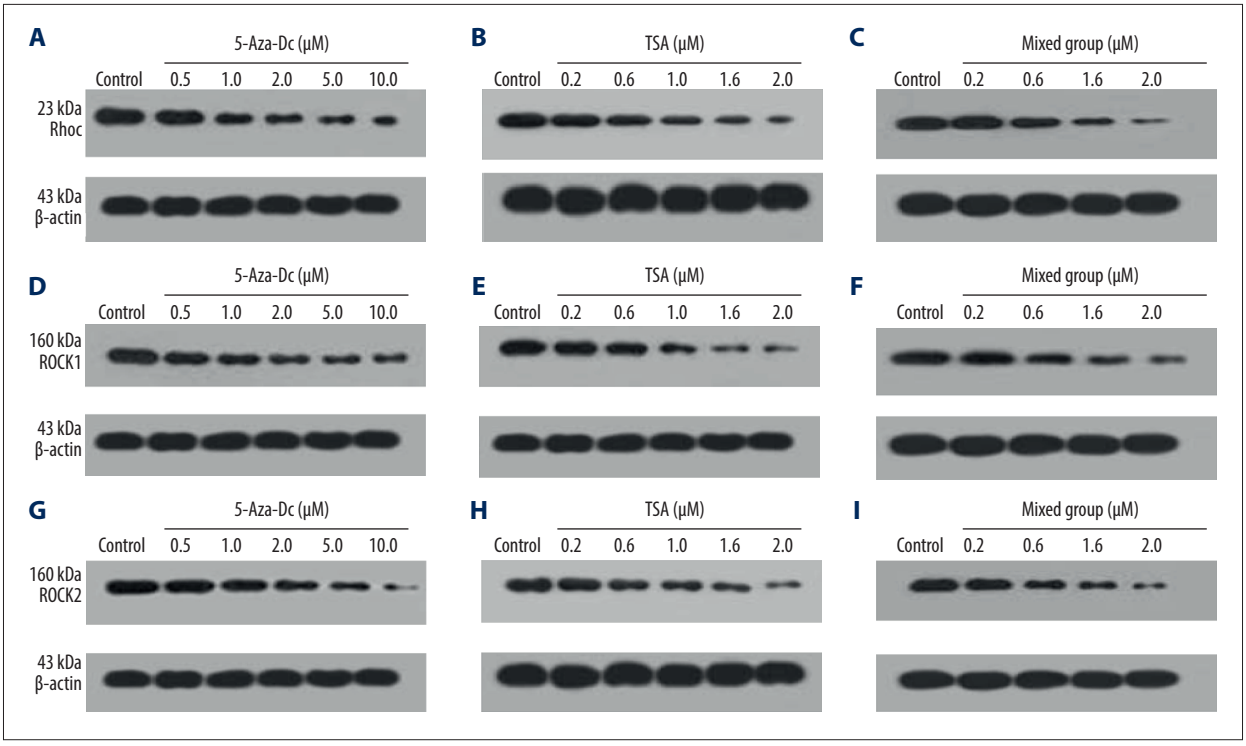

Figure 7. The protein expression of RhoC, ROCK1, and ROCK2 in RPMI8226 cells after treated with 5-Aza-Dc, TSA, and combined 5-Aza-Dc and TSA. (**A–C**) The protein expression of RhoC in control and experimental groups. (**D–F**) The protein expression of ROCK1 in control and experimental groups. (**G–I**) The protein expression of ROCK2 in control and experimental groups. The results showed that the protein expressions of RhoC, ROCK1, and ROCK2 in RPMI8226 cells were gradually decreased with different concentrations of 5-Aza-Dc and/or TSA treatment ($P<0.05$). 5-Aza-Dc – 5-aza-2-deoxycytidine; TSA – trichostatin A.

Discussion

MM is a common hematologic malignant tumor, accounting for 12% to 15% of hematologic malignancies and 1% of all malignant tumors [23,24]. In China, the incidence of MM is 1/100 000, which is lower than Western countries (4.3/100 000 in the United States). In recent years, new drugs such as immunomodulatory agents (IMiD) thalidomide, lenalidomide, and the proteasome inhibitor bortezomib have shown significant effects. Meanwhile, the disease-free survival of patients with MM is significantly prolonged. However, the majority of patients with short-term relief eventually die from MM recurrence and progression. Current studies have found that the occurrence and development of MM is related to a variety of cell factors, adhesion molecules, signal transduction pathways, and cytogenetic abnormalities. Our previous study has found that DLC-1 gene displays aberrant DNA methylation in multiple myeloma RPMI8226 cells, which was consistent with the results of Heller et al. [25] and Walker et al. [26]. Epigenetic modification is an important form of gene expression regulation, including methylation and histone acetylation [27]. This aberrant modification can inhibit gene transcription, sequentially resulting in the loss of tumor suppressor gene function. DNA methyltransferase inhibitors and histone deacetylase inhibitors can remove abnormal methylation, activate tumor suppressor genes, and play an anti-tumor role [28]. 5-Aza-Dc [29], a demethylation drug, can covalently bind to DNA methyltransferase and cause DNA methyltransferase inactivation, eventually achieving a demethylation effect. As a HDAC inhibitor, TSA can inhibit mammalian histone deacetylase activity, induce tumor cell cycle arrest, differentiation and apoptosis, and inhibit the growth of tumor cells. Researchers have shown that histone acetylation and DNA demethylation are able to achieve a synergistic effect by methyl-CpG binding proteins (MBDs) [30]. Our preliminary study has shown that 5-Aza-CdR and TSA can inhibit the methylation of tumor suppressor gene DLC-1 in RPMI8226 cells, effectively reverse DLC-1 gene expression. Furthermore, there is a synergistic effect between the 2 drugs [20].

In 2000, Clark et al. [31] found that the invasiveness and metastasis of melanoma was related to RhoC. RhoC is highly expressed in metastatic melanoma cells, and increased level of RhoC expression can induce the metastasis of melanoma *in vivo*. It has been reported that RhoC is high expressed in gastric cancer, breast cancer, lung cancer, liver cancer, ovarian cancer, and bladder cancer. Meanwhile, it affects the migration, invasion, and metastasis of tumor cells [32–34]. These studies have shown that the effect of RhoC is mainly achieved by

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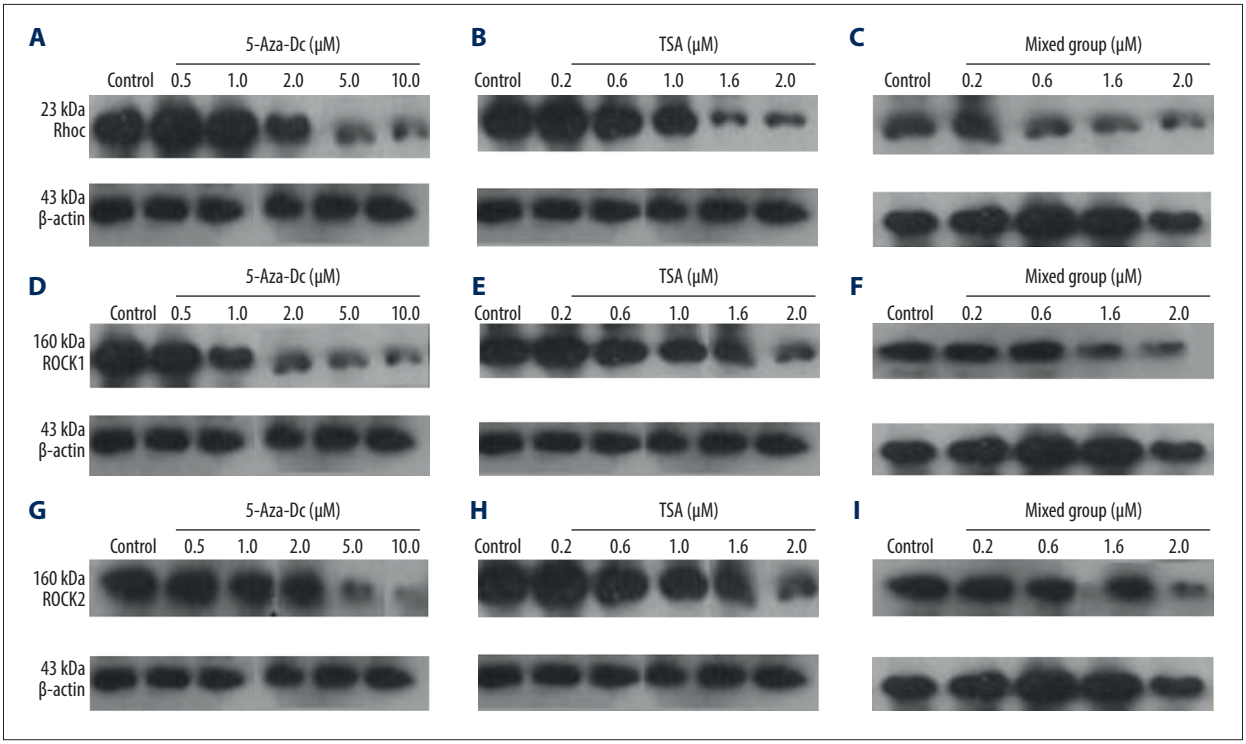

Figure 8. The protein expression of RhoC, ROCK1, and ROCK2 in U266 cells after treated with 5-Aza-Dc, TSA, and combined 5-Aza-Dc and TSA. (**A–C**) The protein expression of RhoC in control and experimental groups. (**D–F**) The protein expression of ROCK1 in control and experimental groups. (**G–I**) The protein expression of ROCK2 in control and experimental groups. The results showed that the protein expressions of RhoC, ROCK1, and ROCK2 in U266 cells were gradually decreased with different concentrations of 5-Aza-Dc and/or TSA treatment ($P<0.05$). 5-Aza-Dc – 5-aza-2-deoxycytidine; TSA – trichostatin A.

regulation of ROCK. ROCK is known as one of the effector proteins of RhoC. Rho activation signal induces ROCK activation by phosphorylation of multi-amino acid sites, which gives rise to a series of phosphorylation/de-phosphorylation reaction in downstream signal path [35]. Mertsch et al. [22] have indicated that ROCK1 and ROCK2 play an important role in glioblastoma proliferation and metastasis. Wong et al. [36] have found that endogenous DLC-1 expression can attenuate cytoskeleton construction mediated by Rho/Rho kinase, and inhibit the formation of stress fibers and focal adhesion. Moreover, this inhibitory function is Rho GAP-dependent. However, changes in the expression of Rho/ROCK signaling pathway in multiple myeloma have seldom been reported so far. Our preliminary study has shown that the protein expression of Rac1 and RhoA is enhanced in RPMI8226 cells. After treatment with 5-Aza-Dc and TSA, the protein expression of Rac1 and RhoA is significantly decreased [20]. Based on those findings, the human multiple myeloma cell lines RPMI-8226 and U266 were treated with different concentrations of single or combined 5-Aza-CdR and TSA in this study. Meanwhile, the mRNA and protein expression levels of RhoC, ROCK1, and ROCK2 were detected. The results showed that the mRNA and protein expressions of RhoC, ROCK1, and ROCK2 were significantly increased in RPMI-8226 and U266 cells. However, 5-Aza-Dc and TSA could reduce their expression, the effect of which was more significant in the combined-drug group. These results again confirmed that there was a synergistic effect between the 2 drugs. This effect might be related to the inhibition of DLC-1 gene methylation status of 5-Aza-CdR and TSA. In addition, this resulted in the re-expression of DLC-1 gene, thereby inhibiting the expression of downstream signaling molecules.

NSC23766 is an inhibitor of Rac GTPase. It was identified as a surface groove suitable for Rac1, which is known to be critical for GEF specification. NSC23766 can effectively inhibit Rac1 binding and activation by Rac-specific GEF Trio or Tiam1 in a dose-dependent manner without interfering with the closely related Cdc42 or RhoA binding or activation by their respective GEFs or with Rac1 interaction with BcrGAP or effector PAK1 [37]. Studies have shown that blockade of Rac1 activity (NSC23766) induces G1 cell cycle arrest or apoptosis in breast cancer cells through downregulating cyclinD1, surviving, and X-linked inhibitor of apoptosis protein [38]. Fasudil is a class of calcium antagonists, which is also a specific Rho/Rho-kinase inhibitor. It can selectively bind to ATP-binding site of Rho/Rho-kinase catalytic domain, thereby blocking the activation of Rho/Rho kinase and its downstream signaling pathways [39]. Some researchers have reported that ROCK inhibitors effectively inhibit

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the proliferation of corneal endothelial cells, vascular smooth muscle cells, and non-small cell lung cancer cells [40–42]. The Rho family of small GTPases plays an important role in transduction of cell signaling events associated with several human cancers. CCG-1423 is a specific inhibitor of Rho signal transduction pathway and activation of serum response factor (SRP) transcription [43]. It can selectively inhibit DNA synthesis, proliferation and invasion, and induce apoptosis of RhoA-overexpressing cell lines at nanomolar to low micromolar concentrations [43]. Whether these 3 inhibitors can also inhibit cell proliferation and induce cell apoptosis in the human multiple myeloma cell lines RPMI-8226 and U266 remains unclear. Therefore, we treated RPMI-8226 and U266 cells with different concentrations of CCG-1423, NSC23766, and fasudil in this experiment. After RPMI-8226 cells were treated with the highest concentration of CCG-1423, NSC23766, and fasudil for 48 hours, cell viability was significantly decreased (13.9±1.626%, 15.405±2.835%, and 30.415±1.93%, respectively). Cell apoptosis rates were significantly increased by the highest concentration of CCG- 1423, NSC23766, and fasudil treatment for 24 hours (68.53±1.089%, 39.57±1.96%, and 32.38–7.606%, respectively). Similarly, the viability rate of U266 cells after treatment with CCG-1423, NSC23766, and fasudil in 48 hours decreased from 87.696±1.99 (%) to 19.331±2.326 (%), 89.58±4.017(%) to 23.28±2.808 (%), and 94.129±3.087 (%) to 41.041±1.711 (%), respectively. In the CCG-1423, NSC23766, and fasudil groups, the apoptosis rate of U266 cells in 24 hours increased from 20.82±1.475 (%) to 60.72±1.205 (%), 13.74±2.301 (%) to 33.29±1.474 (%), and 14.26±2.68 (%) to 26.79±5.747 (%), respectively. These results showed that CCG-1423, NSC23766, and fasudil could significantly inhibit cell proliferation and induce cell apoptosis in myeloma RPMI-8226 and U266 cells.

In this study, we found that single or combined 5-Aza-CdR and TSA effectively inhibited the mRNA and protein expressions of RhoC, ROCK1, and ROCK2 in RPMI-8226 and U266 cells. This experiment indicated that the inhibitors of Rho pathway-mediated signaling, including CCG-1423, NSC23766, and fasudil, could remarkably inhibit cell proliferation and induce cell apoptosis in MM cells. These results provided experimental evidence for the role of Rho/ROCK signaling pathway in multiple myeloma pathogenesis. Meanwhile, our findings suggested that we might downregulate the expression of RhoC, ROCK1, and ROCK2 to treat MM. Therefore, further research of Rho/ROCK signaling pathway and its medicine intervention in MM would reveal potential value for clinical application and provide important therapeutic targets. The molecular mechanism of signaling pathway needs further study, and the effect of MM therapy should also be combined with clinical features.

Conclusions

In conclusion, 5-Aza-Dc and TSA can effectively decrease the mRNA and protein expressions of RhoC, ROCK1, and ROCK2. Meanwhile, Rho and ROCK inhibitors can significantly inhibit cell growth and induce cell apoptosis in the human MM cell lines RPMI-8226 and U266. Our findings provide a potential strategy for the treatment of MM.

Conflict of interest

None.

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