20(*S*)-ginsenoside Rh2 inhibits the proliferation and induces the apoptosis of KG-1a cells through the Wnt/β-catenin signaling pathway

YI CHEN[1], ZE-HONG LIU[1], JING XIA[2], XIAO-PENG LI[1], KE-QIONG LI[1], WEI XIONG[1], JING LI[1] and DI-LONG CHEN[1]

[1]Laboratory of Stem Cell and Tissue Engineering, Department of Histology and Embryology, Chongqing Medical University, Chongqing 400016; [2]Department of Human Anatomy, Chongqing Medical and Health School, Chongqing 408000, P.R. China

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Abstract. Previous research has shown that total saponins of *Panax ginseng* (TSPG) and other ginsenoside monomers inhibit the proliferation of leukemia cells. However, the effect has not been compared among them. Cell viability was determined by Cell Counting Kit-8 assay, and ultra-structural characteristics were observed under transmission electron microscopy. Cell cycle distribution and apoptosis were determined by flow cytometry (FCM). Real-time fluorescence quantitative-PCR, western blotting and immunofluorescence were used to measure the expression of β-catenin, TCF4, cyclin D1 and NF-κBp65. β-catenin/TCF4 target gene transcription were observed by ChIP-PCR assay. We found that 20(*S*)-ginsenoside Rh2 [(*S*)Rh2] inhibited the proliferation of KG-1a cells more efficiently than the other monomers. Moreover, (*S*)Rh2 arrested KG-1a cells in the G0/G1 phase and induced apoptosis. In addition, the levels of β-catenin, TCF4, cyclin D1 mRNA and protein were decreased. The ChIP-PCR showed that (*S*)Rh2 downregulated the transcription of β-catenin/TCF4 target genes, such as cyclin D1 and c-myc. These results indicated that (*S*)Rh2 induced cell cycle arrest and apoptosis through the Wnt/β-catenin signaling pathway, demonstrating its potential as a chemotherapeutic agent for leukemia therapy.

Introduction

Leukemia is a type of hematological malignancy of the blood and the bone marrow characterized by an abnormal increase in immature hemamegba called 'blasts' (1). Chemotherapy drugs kill cancer cells as well as normal cells, leading to significant side effects (2). As a step in overcoming this limitation in chemotherapy, medicines prepared from natural traditional Chinese medicine (TCM) are currently being considered as anticancer agents (3,4). In the past decades, a series of studies have demonstrated that many extracted products from natural plants can kill cancer cells including leukemia cells *in vitro* (5).

Ginsenosides (total saponins of *Panax ginseng*, TSPG), are important components extracted from the root of *Panax ginseng*. TSPG as an extract of a medicinal plant is used in a wide range of ailments and has been reported to have various potent biological functions, including improvement of physical and mental capacity, reduction of fatigue, regulation of the central nervous system and antitumor effects (6-10). TSPG includes many monomers, such as ginsenoside Rb1 (Rb1), ginsenoside Re (Re), ginsenoside Rg1 (Rg1), ginsenoside Rg3 (Rg3), 20(*S*)-ginsenoside Rh2 [(*S*)Rh2] and compound K. TSPG shows enhancement of proliferation and differentiation of bone marrow cells (9-12). To date, TSPG and its monomers have not been compared in regard to their effects on cell viability, thus research on the effects of TSPG and other monomers on proliferation inhibition in KG-1a cells is warranted.

(*S*)Rh2 belongs to the protopanaxadiol family and has attracted attention in the research concerning chemoprevention and chemotherapy (13-15). Recently, researchers have found that (*S*)Rh2 exhibits anticancer functions and inhibits the growth and induces the apoptosis of various cancer cell lines, including leukemia cells (10,16-20). Evidence has proven that (*S*)Rh2 displays a marked inhibitory effect on leukemia cells, although its specific molecular mechanism is not well understood. Moreover, the Wnt pathway is apparently related to tumor progression. Therefore, we hypothesized that there may be some correlation between (*S*)Rh2 and the Wnt pathway.

The Wnt signaling pathway plays an important role in embryogenesis and shows little or no activity in fully differentiated cells (21). Self-renewal, proliferation and differentiation are tightly controlled during normal hematopoiesis to ensure lifelong hematopoietic stem cell homeostasis and blood production (22). Deregulation of these processes results in hematologic dysplasia, deficiency, myeloproliferation or leukemia. The unnatural activity of the Wnt pathway is

Correspondence to: Jing Li or Dr Di-Long Chen, Department of Histology and Embryology, Chongqing Medical University, Chongqing 400016, P.R. China
E-mail: xinmengyuandlc@163.com
E-mail: cybauw@hotmail.com

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involved in the development of several types of cancer (23). For acute myeloid leukemia (AML), it has been confirmed that Wnt genes are overexpressed and activated (24). The importance of the Wnt pathway for AML cell survival has been demonstrated by the fact that inhibition of the pathway results in the decline of cell growth (25). (*S*)Rh2 can obviously inhibit proliferation, differentiation and promote apoptosis of leukemia cells, yet its mechanisms are not fully clear. For the purpose of exploiting new strategies for tumor treatment, it is important to illuminate the concrete mechanism of (*S*)Rh2 in regards to its effect on leukemia, which is vital to clinical experimental research.

Materials and methods

Reagents and cell culture. TSPG, Rb1, Re, Rg1, Rg3 and (*S*)Rh2 were purchased from Nanjing Ze Lang Pharmaceutical Technology (Nanjing, China). Structures of Rb1, Re, Rg1, Rg3 and (*S*)Rh2 are shown (Fig. 1). These agents were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and stored at -20˚C. Human leukemia KG-1a cells were cryopreserved in our laboratory, and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (HyClone, Waltham, MA, USA) at 37˚C in air 5% CO_2 incubator at constant humidity, and maintained for logarithmic growth by passaging cells every 48 h.

CCK-8 assay. The CCK-8 assay was employed for determination of cell viability. Briefly, $1.0x10^4$ cells/well were planted in 96-well plates and cultured for different times. At the end of the time, 10 μl CCK-8 working solution was added to each well and then cultured at 37˚C for 2 h. Then plates were detected at 450 nm on a spectrophotometric plate reader. The drug concentration resulting in 50% inhibition of growth (IC_{50}) was determined.

Transmission electron microscopic observation. For the ultra-structural characteristic observation assay, cells were harvested and fixed at $1.0x10^7$ with 2.5% glutaraldehyde for 6 h at 4˚C and then with 1% osmium tetroxide for 2 h prior to dehydration with ethyl alcohol. Ultra-thin sections (60 nm) were prepared and placed on grids, stained with 2% uranyl acetate solution and 0.2% lead citrate in 0.1 M NaOH. The cells were observed by a H-600 transmission electron microscope (Hitachi, Japan).

Cell cycle assay. The KG-1a cells were plated at a concentration of $2.0x10^5$ cells/ml and incubated with (*S*)Rh2 or TSPG for 48 h. The cells were collected by centrifugation at 1,000 x g for 3 min, added to 70% ethanol and then washed once with PBS. The cells were then resuspended in 1 ml of PBS containing 2.5 μg/ml ribonuclease and 50 μg/ml propidium iodide (Beyotime Institute of Biotechnology, Shanghai, China), incubated in the dark for 30 min at room temperature and analyzed using flow cytometry (FCM).

Cell apoptosis assay. Briefly, for the Annexin V assay, the cells were seeded at a concentration of $2.0x10^5$ cells/ml and incubated with (*S*)Rh2 or TSPG for 48 h. Samples were prepared based on the instructions provided together with the Annexin V apoptosis kit. Briefly, after treatment for the indicated times, the cells were collected and washed twice with binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$. Next, $1.0x10^5$ cells were resuspended in 100 μl of binding buffer, and then 5 μl of Annexin V-FITC and 10 μl of propidium iodide (50 μg/ml, stock concentration) were added to the cell suspension. After gently mixing, the cells were incubated for 15 min at room temperature, and then 400 μl of binding buffer was added to get the sample ready. Quantification of cell death was analyzed with a BD FACScan.

Table I. Primer pairs for qRT-PCR.

Gene name	Sequence (5'-3')
β-catenin	F: CAAAGCCTCAGGTCATAAACA
	R: GTGGGATGGTGGGTGTAAGA
TCF4	F: TGAGGTCCTGATGCGGTTGG
	R: TCGCCTTTGTTCTCCTTGATGC
Cyclin D1	F: AGGCTGGCTTCATCCACT
	R: CACCAAGGGTTAATTCTTCA
NF-κBp65	F: CCCCACGAGCTTGTAGGAAAG
	R: CCAGGTTCTGGAAACTGTGGAT
GAPDH	F: CATCAAGAAGGTGGTGAAGCA
	R: CGTCAAAGGTGGAGGAGTGG

F, forward; R, reverse.

Immunofluorescence staining. In brief, KG-1a cells cultured in 6-well plates were treated with DMSO or (*S*)Rh2 (60 μM) for 24 h. The cells were washed with ice-cold phosphate buffered saline (PBS) three times and fixed with 4% paraformaldehyde. The cell membrane was ruptured by 0.3% tristone, and closed with mountain goat serum (HyClone, Waltham, MA, USA). Antibodies against β-catenin (1:100), TCF4 (1:100), cyclin D1 (1:100), NF-κBp65 (1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, USA) were added respectively. Anti-rabbit secondary fluorescent antibody was added and incubated overnight for 1 h, and stained with PI (Beyotime Institute of Biotechnology, Shanghai, China). Then, 50% glycerol was used for mounting, and imaging was carried out under a fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time PCR (qRT-PCR). Following treatment, the cells were harvested and total RNA was immediately extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For expression analysis of β-catenin, TCF4, cyclin D1 and NF-κBp65 genes, 2 μg of total RNA was used to synthesize first-strand DNA with reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA). Quantitative RT-PCR (qRT-PCR) was performed with a Green PCR Master Mix kit (Shanghai, Shine Co, China). Briefly, one microliter of first-strand cDNA and gene-specific primers were used along with Hotstart Fluo-PCR Mix in a 20-μl reaction under the

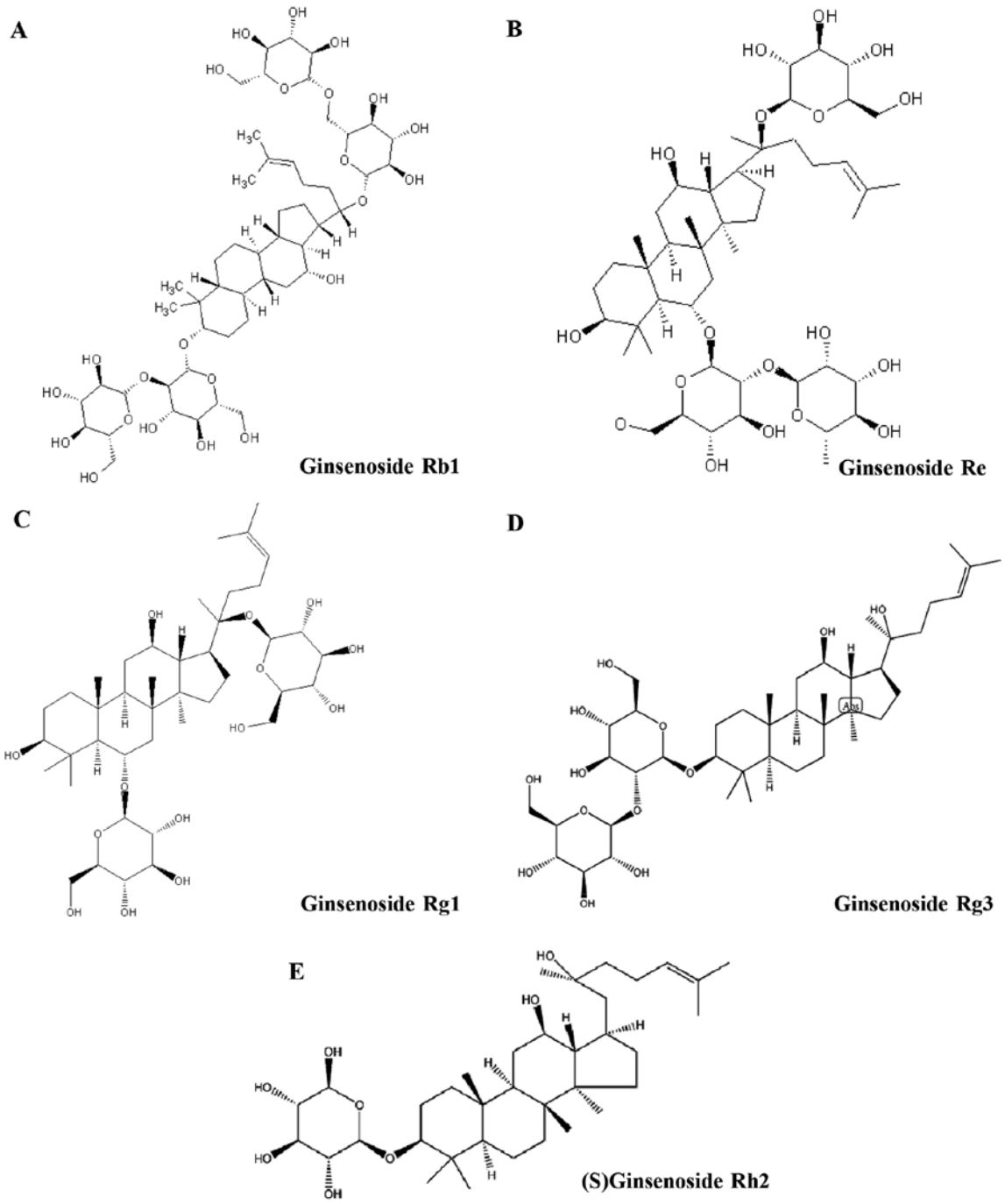

Figure 1. Chemical structure of ginsenoside Rb1 (A), Re (B), Rg1 (C), Rg3 (D) and (*S*)Rh2 (E).

following conditions: pre-denaturation at 95˚C for 5 min, 35 cycles of denaturation at 95˚C for 10 sec, annealing at 57˚C for 15 sec, and extension at 72˚C for 20 sec. Each sample was performed in triplicate and was quantified based on the formula $2^{-\Delta Ct}$. The primer pairs for qRT-PCR are listed in Table I.

Western blotting. Following treatment with (*S*)Rh2 for 48 h, the cells were lysed in ice-cold RIPA lysis buffer. The lysates were centrifuged at 15,000 x g for 10 min at 4˚C to obtain the proteins. The protein content of the cell extracts was determined by bicinchoninic acid (BCA). A total of 30-40 μg of protein was electrophoresed on 10-15% SDS-PAGE gels and transferred to PDVF membranes. The membranes were blocked, incubated with the primary Abs at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:2,000 dilutions). Labeled bands were detected by Immun-Star TMHRP Chemiluminescent kit, and images were captured and the intensity of the bands was quantified by the Bio-Rad VersaDoc™ image system (Bio-Rad, Regents Park, Australia).

Chromatin immunoprecipitation (ChIP) assays. For the ChIP assay, KG-1a cells were treated with DMSO (control), or 60 μM (*S*)Rh2 for 48 h. Treated cells were cross-linked with 1% formaldehyde for 15 min at room temperature. The cross-

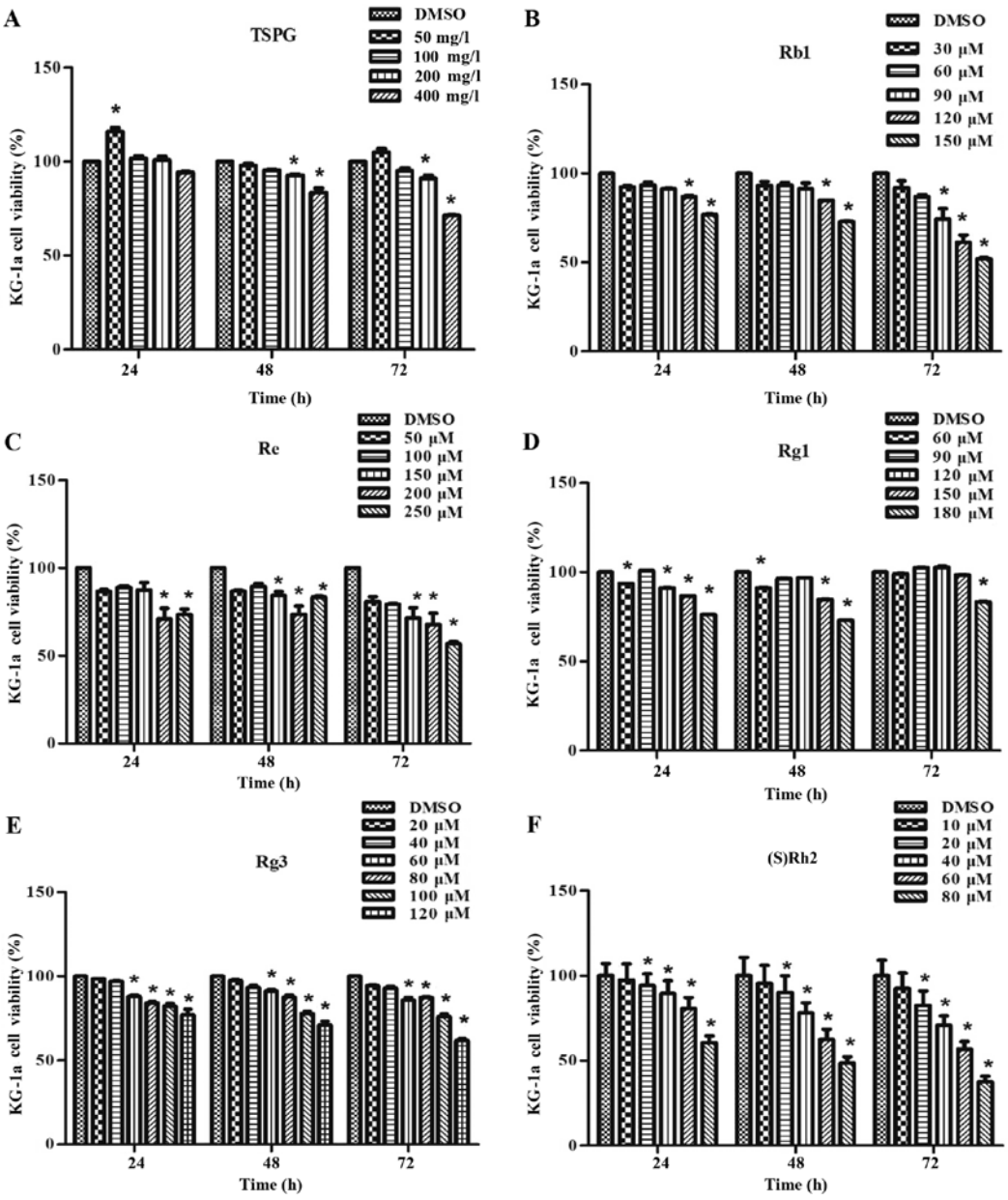

Figure 2. Effect of TSPG, Rb1, Re, Rg1, Rg3 and (*S*)Rh2 on the cell viability of KG-1a cells as determined by the CCK-8 assay. KG-1a cells were incubated with or without various concentrations of TSPG, Rb1, Re, Rg1, Rg3 and (*S*)Rh2 for 24, 48 and 72 h. Data are presented as mean ± SEM. (n=3) for each group. *p<0.05, a significant difference compared with the control by one-way ANOVA. Similar results were observed in replicate experiments.

linked cells were then resuspended in 0.3 ml of lysis buffer (50 mM Tris-HCl, pH 8.1/1% SDS/10 mM EDTA protease inhibitors) and subjected three times for 10 sec followed by centrifugation for 10 min. The average size of the sheared fragment was expected to be 300-1,000 bp. Immunoprecipitates were collected three times with 1% SDS/0.1 M $NaHCO_3$. Eluates were pooled and heated at 65˚C for 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick spin kit (Qiagen, Chatsworth, CA, USA). For PCR, 1 μl from a 50 μl DNA extraction and 38 cycles of amplification were used with the following promoter-specific primers: c-myc forward, 5-GCTTGGCGGGAAAAAGAAGGG-3 and reverse, 5-AGAGCTGCCTTCTTAGGTCG-3; cyclin D1 forward, 5-GTTCCTGGAAGGGCGACTAA-3 and reverse, 5-GGGGTGGGATCTGAGATTTG-3.

Statistical analysis. The intensity of the immunoreactive band was determined by a densitometer (Bio-Rad, Hercules, CA, USA). Data are expressed as the mean ± SEM of three independent experiments, and one-way ANOVA analysis was conducted using the statistical software SPSS 22.0. Each treatment group was compared with the control group with Dunnett's t-test, and P-value <0.05 was considered to indicate a statistically significant result.

Results

TSPG, Rb1, Re, Rg1, Rg3 and (*S*)Rh2 *inhibit the growth of KG-1a cells*. We assessed the effects of TSPG, Rb1, Re, Rg1, Rg3 and (*S*)Rh2 on the cell viability of KG-1a cells using Cell Counting Kit-8 assays. (*S*)Rh2 was found to have a marked

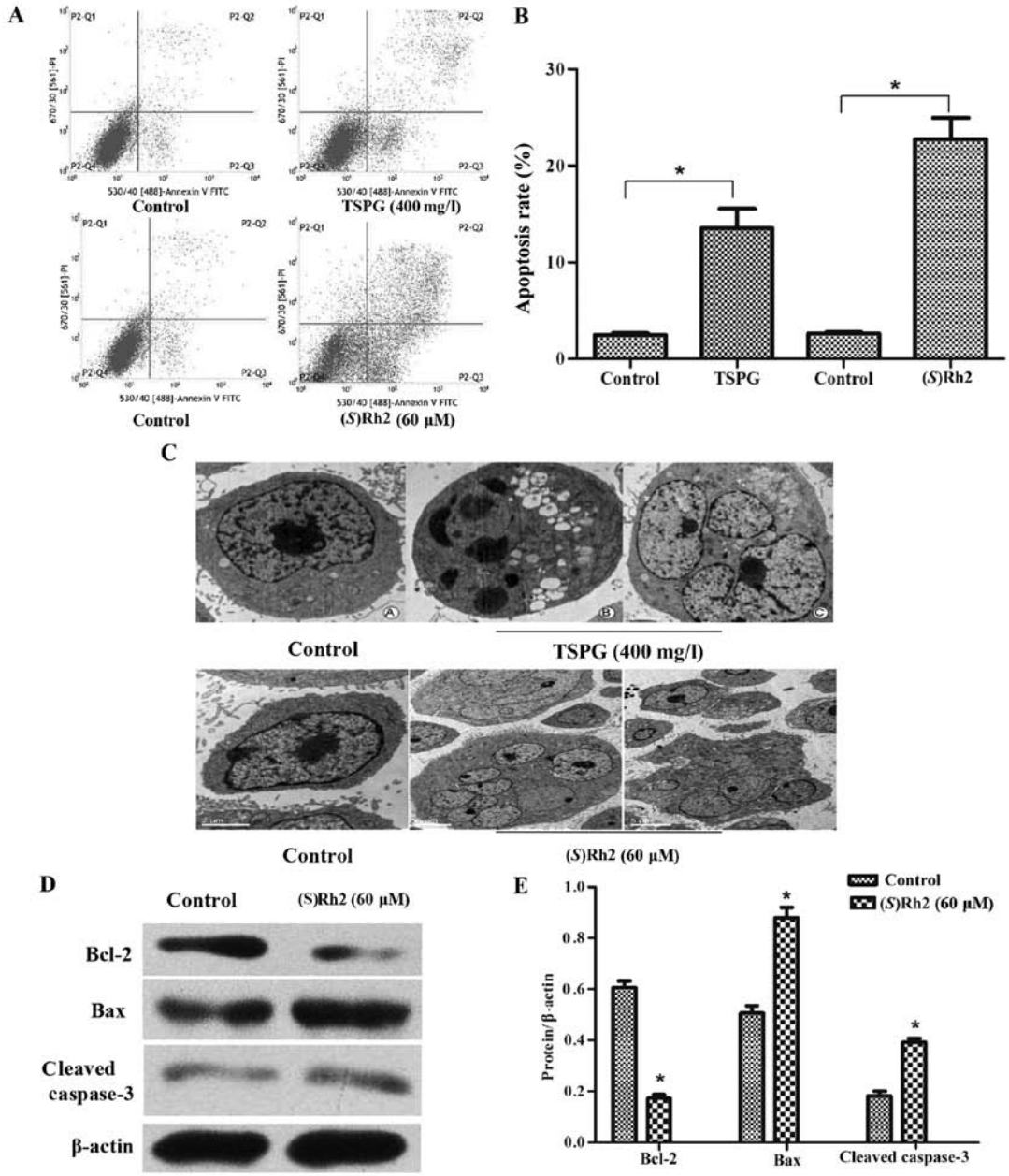

Figure 3. TSPG and (*S*)Rh2 induce KG-1a cell apoptosis. (A and B) KG-1a cells were treated with control, 400 mg/l TSPG and 60 μM (*S*)Rh2 for 48 h followed by assessment of apoptosis. Data are presented as mean ± SEM. (n=3) for each group. $^{*}p<0.05$, a significant difference compared with the control by one-way ANOVA. (C) KG-1a cells were treated with control, 400 mg/l TSPG and 60 μM (*S*)Rh2 for 48 h and analyzed by transmission electron microscope (TEM x 8,000). (D and E) KG-1a cells were treated with the control and 60 μM (*S*)Rh2 for 48 h, effects of (*S*)Rh2 on the expression of cell apoptosis-related proteins (Bax, Bcl-2 and cleaved caspase-3). Experiments were repeated three times and similar results were achieved.

inhibitory effect on KG-1a cells at a lower concentration, compared with Rb1, Re, Rg1 and Rg3. (*S*)Rh2 had the lowest IC_{50} values (in the mid-μM range) compared with the other ginsenosides tested. Rb1, Re, Rg1, Rg3 did not significantly decrease cell viability even at the high-μM range. These results showed that (*S*)Rh2 decreased the viability of the KG-1a cells. TSPG had a lesser effect on viability than the other members of the ginsenosides in the KG-1a cells (Fig. 2).

TSPG and (*S*)Rh2 *induce the apoptosis of KG-1a cells.* Further experiments were performed using KG-1a cells to evaluate the effect of TSPG and (*S*)Rh2 on apoptosis and the mechanism involved. To determine whether the cell growth inhibitory effect of TSPG and (*S*)Rh2 is associated with the induction of cell apoptosis, we used Annexin V/7-AAD double staining. Annexin V^+ and 7-AAD^- cells were designated as early apoptotic and Annexin V^+ and 7-AAD^+ cells were designated as necrotic. A higher number of apoptotic cells was observed in the TSPG (400 mg/l) and (*S*)Rh2 (60 μM) treatment groups than that in the control group (Fig. 3A). Analysis of the cell population revealed distinct sets of populations. Following treatment with TSPG (400 mg/l) and (*S*)Rh2 (60 μM) for 24 h, the percentage of Annexin V^+ and 7-AAD^- apoptotic cells increased gradually from 3.10 to 8.63 and 19.53%, while the percentage of $FITC^+$/7-AAD^+ necrotic cells increased from 2.17 to 6.68 and 16.52% (Fig. 3B). These results revealed

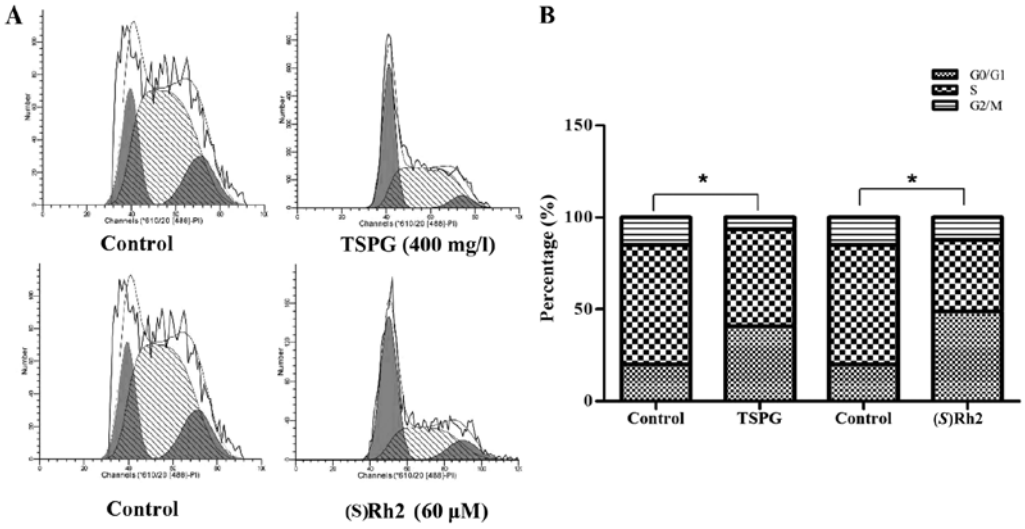

Figure 4. TSPG and (*S*)Rh2 induce KG-1a cell cycle arrest. KG-1a cells were treated with control, 400 mg/l TSPG or 60 μM (*S*)Rh2 for 48 h. (*S*)Rh2 (60 μM) induced KG-1a cell cycle arrest at the G0/G1 phase, but TSPG (400 mg/l) also had an effect on cycle arrest. Data are presented as mean ± SEM. (n=3) for each group. $^{*}p<0.05$, a significant difference compared with the control by one-way ANOVA. Experiments were repeated three times, and similar results were achieved.

that (*S*)Rh2 was significantly more potent at inducing apoptosis than TSPG. To further characterize the TSPG- and (*S*)Rh2-induced apoptosis, we observed ultra-microstructures in the KG-1a cells following treatment with TSPG (400 mg/l) or (*S*)Rh2 (60 μM) for 48 h. These cells had different levels of apoptosis, chromatin occured margination, nucleoli decreased or disappeared, and apoptotic bodies increased (Fig. 3C). In addition to its anti-proliferative effects, we also noted that (*S*)Rh2 caused an increase in apoptosis in the KG-1a cells. Thus, we assessed the expression of several apoptosis-related proteins, including Bcl-2, Bax and cleaved caspase-3. The effect on apoptosis was consistent with the cell apoptosis assay. (*S*)Rh2 increased the expression of Bcl-2 and cleaved caspase-3 (Fig. 3D and E). Cleaved caspase-3 was activated. We demonstrated that (*S*)Rh2 induced apoptosis via both the intrinsic and extrinsic pathways.

(*S*)Rh2 *induces cell cycle arrest at the G0/G1 phase in the KG-1a cells*. CCK-8 results showed that TSPG and (*S*)Rh2 inhibited the proliferation of the KG-1a cells. To determine whether the cell growth inhibitory effect of (*S*)Rh2 is associated with cell cycle arrest, changes in cell cycle distribution were detected by flow cytometry. Compared with the percentage in the control group, the KG-1a cells after treatment with (*S*)Rh2 (60 μM) showed an increased percentage of cells in the G0/G1 phase from 20.08±3.12 to 48.76±4.22%; the percentage in the S phase decreased from 64.87±0.52 to 39.22±0.86%; the percentage in the G2/M phase decreased from 15.05±3.92 to 12.02±2.81% (Fig. 4A and B), and the difference was statistically significant ($p<0.05$). (*S*)Rh2 induced KG-1a cell cycle arrest at the G0/G1 phase. TSPG also had an effect on cell cycle arrest (data not shown). To further explore the underlying mechanisms, we studied the expression of cell cycle-associated proteins. We found that (*S*)Rh2 decreased the level of cyclin D1. The decreased expression of cyclin D1 may be associated with the G0/G1 cell cycle arrest induced by (*S*)Rh2. The results shown that (*S*)Rh2 induced KG-1a cell cycle arrest at the G0/G1 phase.

(*S*)Rh2 *affects the localization and expression of β-catenin, NF-κBp65, TCF-4, and cyclin D1 protein in the KG-1a cells*. Since nuclear β-catenin is a hallmark of the activated Wnt/β-catenin signaling, we performed immunofluorescence to determine the localization of β-catenin to further validate the activation of Wnt/β-catenin signaling. As shown in Fig. 5A, treated cells exhibited strong green fluorescence in the cytoplasm, and a small amount of green fluorescence could be visible in the nucleus. Compared with the control group, β-catenin was obvious altered in the cytoplasm. TCF4 was expressed mainly in the nucleus and the expression of TCF4 in the nucleus, was significantly decreased after (*S*)Rh2 treatment for 48 h (Fig. 5B). Cyclin D1 was expressed in the cytoplasm or nucleus. The expression level was significantly reduced after (*S*)Rh2 treatment for 48 h (Fig. 5C). NF-κBp65 is mainly in the nucleus or cytoplasm, and its expression in the nucleus in the cells following treatment with (*S*)Rh2 for 48 h was significantly reduced (Fig. 5D). Taken together, these data suggest that (*S*)Rh2 induced KG-1a cell cycle arrest at the G0/G1 phase, and may be responsible for the downregulation of the Wnt/β-catenin signaling pathway enhancing the cell cycle arrest of KG-1a cells.

(*S*)Rh2 *affects the mRNA level and protein expression of β-catenin, TCF-4, cyclin D1 and NF-κBp65 in the KG-1a cells*. KG-1a cells were treated with (*S*)Rh2 (60 μM) for 48 h. We assessed the mRNA level and protein expression of several Wnt/β-catenin pathway-related and cell cycle-related proteins, including β-catenin, TCF-4, cyclin D1 and NF-κBp65 (Fig. 6). Treatment with (*S*)Rh2 (60 μM) for 48 h caused significant downregulation of the mRNA expression of β-catenin, TCF-4, NF-κBp65 and cyclin D1 in the KG-1a cells, compared with the control group (Fig. 6A). We also found that in the (*S*)Rh2 treatment group reduced protein expression of β-catenin, TCF4, NF-κBp65 and cyclin D1 was noted in the KG-1a cells (Fig. 6C and D). (*S*)Rh2 affected the gene transcription of c-myc and cyclin D1 in the KG-1a cells. The ChIP-PCR assay

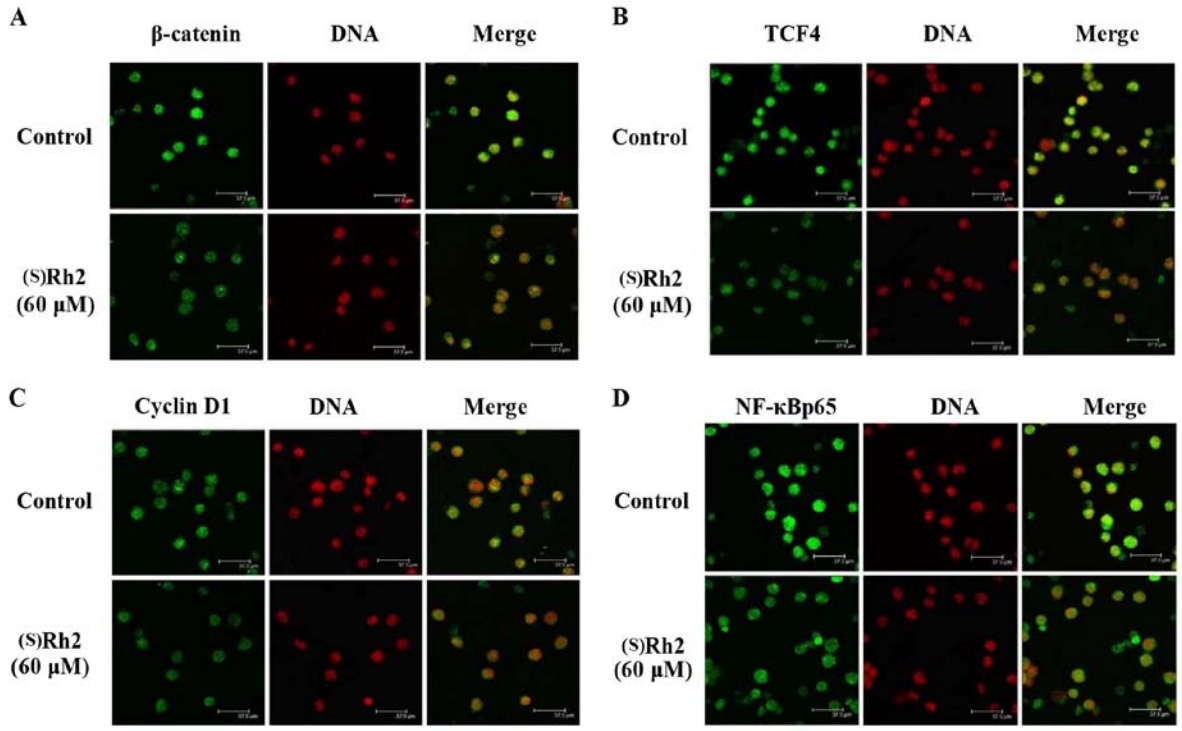

Figure 5. (*S*)Rh2 affects β-catenin, TCF-4, cyclin D1 and NF-κBp65 protein localization in the KG-1a cells. (A) Immunostaining of β-catenin transfer from the nucleus to the plasma membrane. β-catenin, PI, and the merging of PI and β-catenin are shown. (B) Immunostaining of decreased TCF4 in the nucleus, TCF4, PI and the merging of PI are shown. (C) Immunostaining of decreased cyclin D1 in the nucleus and plasma membrane. Cyclin D1, PI and the merging of PI are shown. (D) Immunostaining of significantly reduced NF-κBp65 in the nucleus or cytoplasm. NF-κBp65, PI and the merging of PI are indicated. Experiments were repeated three times and similar results were achieved.

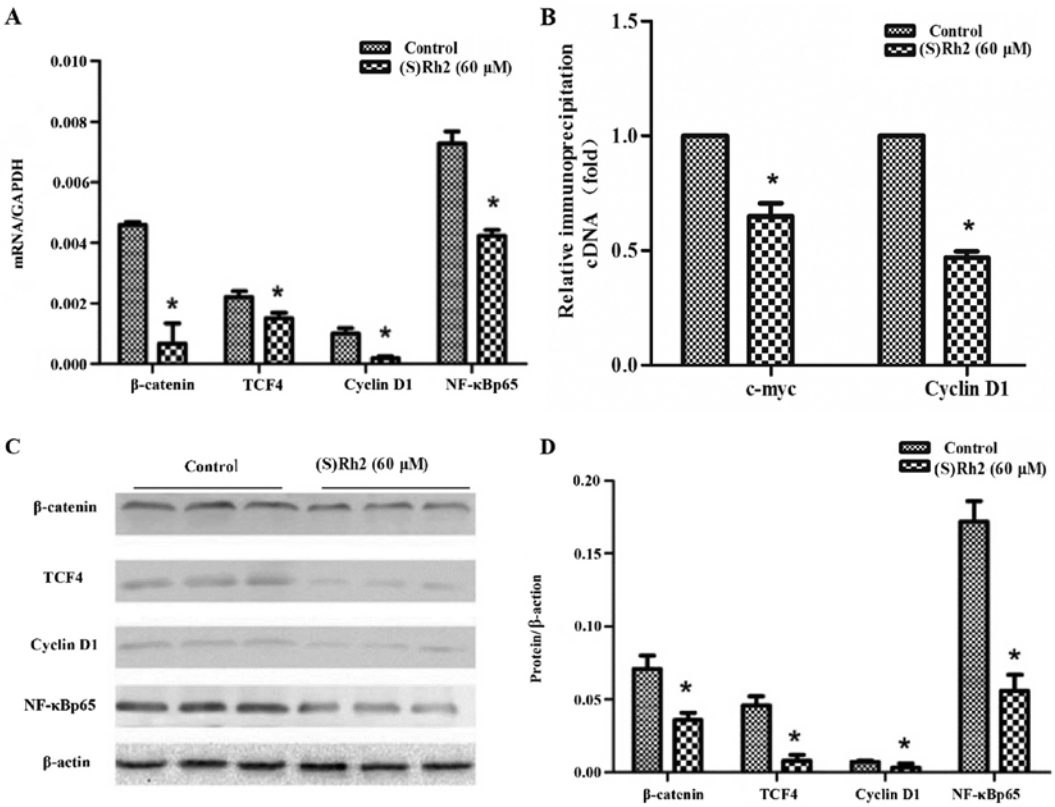

Figure 6. (*S*)Rh2 affects the Wnt/β-catenin/TCF4/cyclin D1 signaling pathway. (A) KG-1a cells were treated with (*S*)Rh2 (60 μM) for 48 h. Expression of the mRNA of genes in the Wnt/β-catenin/TCF4/cyclin D1 signaling pathway was determined by RT-PCR. (B) KG-1a cells were treated with (*S*)Rh2 (60 μM) for 48 h. Expression of the β-catenin/TCF4 target genes, such as c-myc, cyclin D1, involved in apoptosis and proliferation, was also diminished as determined by ChIP-PCR. ChIP assay was performed using TCF4 IgG. (C and D) KG-1a cells were treated with (*S*)Rh2 (60 μM) for 48 h. Expression of the affected Wnt/β-catenin/TCF4/cyclin D1 signaling pathway proteins was determined by western blotting, β-actin immunoblots indicate loading of equal amounts of protein. Experiments were repeated three times, and similar results were achieved.

shown that (*S*)Rh2 decreased the β-catenin/TCF4 target gene transcription, such as c-myc and cyclin D1. These genes are involved in apoptosis and proliferation, The ChIP-PCR assay was performed using TCF4 IgG (Fig. 6B). The downstream genes of the Wnt/β-catenin pathway including β-catenin, NF-κB and cyclin D1 were also downregulated, further suggesting that (*S*)Rh2 induced KG-1a cell cycle arrest at the G0/G1 phase and apoptosis by the Wnt/β-catenin pathway.

Discussion

While cytotoxic agents are often used as traditional chemotherapy drug, there is an ongoing search for more effective specific agents that spare normal host tissues. Numerous natural TCM products have anticancer properties. These compounds generally have low toxicity and have protective effects against a variety of cancers (26). On the basis of research *in vitro*, we and other researchers have demonstrated that a structure-activity relationship exists among the ginsenosides, with the panaxadiols generally being more active than the panaxatriols in the killing of cancer cells, and the glycerogelatin compounds being less active as the number of sugar moieties increases (Fig. 1). To our knowledge, (*S*)Rh2 is one of the most active ginsenosides to be evaluated. (*S*)Rh2 demonstrated strong effects, and was more effective at lower doses than TSPG, Rb1, Re, Rg1, Rg3 in KG-1a cells. The obvious cancer-specific effects of these compounds can be distinguished from the chemical structure. These may be ascribed to their differential chemical structure. Anticancer activities increase with a decrease in the number of sugar moieties in a ginsenoside molecule. Ginsenosides with four or more sugar molecules, for example Rb1, showed no significant anti-proliferative effects; while Re with three sugar residues weakly inhibited the growth of KG-1a cells; Rg3 and Rg1 (with two sugar residues), and (*S*)Rh2 (with one sugar residue) have been found to inhibit various types of cancer cells and also enhance the efficacy of chemotherapy agents when treated in combination (27). (*S*)Rh2 showed a 2- to 14-fold relatively stronger anti-proliferative effect than TSPG, Rb1, Re, Rg1, Rg3. (*S*)Rh2 inhibited the proliferation and induced the apoptosis of KG-1a cells although the possible molecular mechanisms require further study.

(*S*)Rh2 demonstrated anti-proliferative, and proapoptotic effects and regulation of cell cycle progression. Although TSPG may be marketed as an anticancer agent, it did not exert any appreciable effect on KG-1a cells. (*S*)Rh2 demonstrated a more potent effect and was more effective at lower doses than the other compounds. (*S*)Rh2 exhibited decreases in survival and proliferation, increases in apoptosis and cell cycle arrest at the G1 phase in KG-1a cells (Fig. 4). Apoptosis is an evolutionarily conserved form of cell suicide and is characterized by distinctive morphological changes. We observed that TSPG (400 mg/l) and (*S*)Rh2 (60 μM) increased condensed apoptotic nuclei (Fig. 3C), as evidenced by chromosomal condensation and formation of apoptotic bodies. TSPG and (*S*)Rh2 increased the percentage of Annexin V+/7-AAD$^-$ KG-1a cells as evidence of apoptosis (Fig. 3A and B). Our findings also revealed that (*S*)Rh2 can induce apoptosis in KG-1a cells more efficiently than TSPG.

The Wnt/β-catenin signaling pathway is an importantly drug discovery target (28). β-catenin is an important factor involved in the regulation of apoptosis and proliferation in the Wnt signaling pathway. The Wnt/β-catenin signaling pathway may have abnormal activation in acute leukemia cells (29). One study has demonstrated that the accumulation of β-catenin is prompted by increased β-catenin/TCF4 transcriptional activity in the cytoplasm and nucleus, and nuclear β-catenin accumulation implicates Wnt signaling pathway activation (30). Therefore, Wnt signaling pathway inhibitors can induce apoptosis and inhibit proliferation in acute leukemia (31). The immunofluorescence experiments confirmed that (*S*)Rh2 reduced the β-catenin protein transfer from the cytoplasm to the nucleus in KG-1a cells. Real-time PCR, immunofluorescence and western blotting confirmed that (*S*)Rh2 significantly reduced β-catenin mRNA and protein expression compared with the control group in the KG-1a cells. Thus, the Wnt/β-catenin pathway is likely to be an (*S*)Rh2 target for the treatment of acute leukemia.

TCF4 is a nuclear transcription factor and has a molecule 'switch' dual function (32). The Wnt/β-catenin pathway depends on the presence of different Wnt molecules that bind to the frizzled receptor. When Wnt/β-catenin pathway proteins are activated, several proteins that are important for the phosphorylation of β-catenin require the receptor. Binding of these proteins enables β-catenin to accumulate in the cytoplasm and translocate into the nucleus, where it interacts with members of the TCF4 transcription factor family to induce target gene expression (33,34). When Wnt/β-catenin pathway proteins are inactivated, several proteins are released from the frizzled receptor and TCF4 family to restraint target gene expression (35,36). Our experiment demonstrated that (*S*)Rh2 decreased the protein and mRNA expression of TCF4 in KG-1a cells. Immunofluorescence results showed that TCF4 is mainly located in the nucleus, and (*S*)Rh2 significantly reduced TCF4 expression. ChIP-PCR assays also showed that (*S*)Rh2 downregulated the transcription of β-catenin/TCF4 target genes, such as cyclin D1 and c-myc. β-catenin/TCF4 associated cell proliferation is provided by cyclin D1 and c-myc which have been reported to be regulated in the wnt pathway (37-39). Therefore, we speculate that (*S*)Rh2 can reduce nuclear β-catenin expression and restrain activation of β-catenin downstream genes in KG-1a cells.

Cyclin D1 is a Wnt/β-catenin downstream target gene (40,41). Wnt/β-catenin pathway activated cyclin D1, disrupting the cell cycle leading to cell abnormal proliferation in AML (42). TCF4/β-catenin complex can directly stimulate the activity of CDKs promoting cell cycle by upregulating cyclin D1. Cyclin D1 is a cell cycle-related oncogene, which significantly reduces the cells in the G1 phase by inactivation of (*S*)Rh2, suppressing malignant cell proliferation. The experiment showed that (*S*)Rh2 arrested KG-1a cells in the G0/G1 phase. Immunofluorescence and western blotting showed that cyclin D1 protein was significantly reduced. It can be seen that (*S*)Rh2 reduced cyclin D1 gene transcription and protein expression levels. ChIP-PCR also showed that (*S*)Rh2 downregulated β-catenin/TCF4 target gene transcription, such as cyclin D1 which is a downstream gene of the Wnt/β-catenin signaling pathways. Thus, these results indicate that (*S*)Rh2 induced cycle arrest at the G0/G1 phase, inhibited proliferation and promoted apoptosis in the KG-1a cells.

NF-κB is an important member of the Rel gene family that causes many hematologic malignancies and interacts with members of the TCF transcription factor family to induce target gene expression-induced apoptosis and cell cycle arrest (43,44). This study showed that the expression of NF-κBp65 was reduced and shifted to the cell cytoplasm from the nucleus following gradual treatment with (*S*)Rh2 in the KG-1a cells. The NF-κB and Wnt signaling pathways can regulate the same target gene transcription which is mainly associated with cell proliferation, cell cycle regulation and apoptosis (45). The irregular activation of gene promoter through NF-κB and TCF targeted activation of cyclin D1 and c-myc resulting in abnormal proliferation of tumor cells (46). (*S*)Rh2 inhibited proliferation and induced apoptosis possibly through inhibition of the Wnt and NF-κB pathways in the KG-1a cells.

In the present study, we found that (*S*)Rh2 played an antitumor role accompanied by β-catenin protein expression. When chromatin is in a state of relaxation, β-catenin can combine with TCF4 in the nucleus. Here, TCF4 competes with β-catenin to form a complex and is recruited to target promoters to suppress their expression, such as c-myc, Bcl-2, cyclin D1, thus inhibiting proliferation and promoting apoptosis in human leukemic cells (47). We detected that Bax, Bcl-2, cyclin D1 and cleaved caspase-3 protein was increased in the (*S*)Rh2-treated group. At the same time, (*S*)Rh2 inhibited proliferation and apoptosis through the Wnt/β-catenin signaling pathway in human leukemic KG-1a cells.

In conclusion, we initially considered that (*S*)Rh2 more efficiently inhibits the proliferation and induces the apoptosis than other monomers in KG-1a cells. The mechanism may be through the abnormal Wnt pathway by inhibiting the activation of β-catenin protein, thereby inhibiting its downstream protein TCF4 and cyclin D1 expression. Meanwhile, inhibition of the Wnt pathway in crosstalk also inhibits the NF-κB pathway. Therefore, we believe that (*S*)Rh2 induced proliferation inhibition and apoptosis through the Wnt/β-catenin signaling pathway in human leukemic KG-1a cells. Therefore, (*S*)Rh2 could be a potential and powerful chemopreventive agent to treat human leukemia, although further research must be carried out to fully investigate the mechanisms of action.

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