Chin J Integr Med 2024 Jul;30(7):588-599

Chinese Journal of Integrative Medicine

Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
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Original Article

Ginsenoside Rd Induces Differentiation of Myeloid Leukemia Cells via Regulating ERK/GSK-3β Signaling Pathway*

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ABSTRACT **Objective**: To investigate the role of ginsenoside Rd (GRd) in acute myeloid leukemia (AML) cell differentiation. **Methods**: AML cells were treated with GRd (25, 50, 100 and 200 μg/mL), retinoic acid (RA, 0.1g/L) and PD98059 (20 mg/mL) for 72 h, cell survival was detected by methylthiazolyldiphenyl-tetrazolium bromide and colony formation assays, and cell cycle was detected by flow cytometry. Cell morphology and differentiation were observed by Wright-Giemsa staining, peroxidase chemical staining and cellular immunochemistry assay, respectively. The protein expression levels of GATA binding protein 1 (GATA-1), purine rich Box-1 (PU.1), phosphorylated-extracellular signal-related kinase (p-ERK), ERK, phosphorylated-glycogen synthase kinase-3β (p-GSK3β), GSK3β and signal transducer and activator of transcription 1 (STAT1) were detected by Western blot. Thirty-six mice were randomly divided into 3 groups using a random number table: model control group (non-treated), GRd group [treated with 200 mg/(kg·d) GRd] and homoharringtonine (HTT) group [treated with 1 mg/(kg·d) HTT]. A tumor-bearing nude mouse model was established, and tumor weight and volume were recorded. Changes of subcutaneous tumor tissue were observed after hematoxylin and eosin staining. WT1 and GATA-1 expressions were detected by immunohistochemical staining. **Results**: The cell survival was inhibited by GRd in a dose-dependent manner and GRd caused G_0/G_1 cell arrest ($P<0.05$). GRd treatment induced leukemia cell differentiation, showing increased expressions of peroxidase and specific proteins concerning erythrogenic or granulocytic differentiation ($P<0.05$). GRd treatment elicited upregulation of p-ERK, p-GSK-3β and STAT1 expressions in cells, and reversed the effects of PD98059 on inhibiting the expressions of peroxidase, GATA-1 and PU.1 ($P<0.05$). After GRd treatment, tumor weight and volume of mice were decreased, and tumor cells underwent massive apoptosis and necrosis ($P<0.05$). WT1 level was decreased, and GATA-1 level was significantly increased in subcutaneous tumor tissues ($P<0.05$ or $P<0.01$). **Conclusion**: GRd might induce the differentiation of AML cells via regulating the ERK/GSK-3β signaling pathway.

KEYWORDS ginsenoside Rd, myeloid leukemia, survival, differentiation, extracellular signal-related kinase signaling pathway

Acute myeloid leukemia (AML) is a malignant tumor caused by abnormal clonal proliferation of hematopoietic stem cells, where bone marrow hematopoietic cells lose their ability to further differentiate and mature, leading to termination of cell growth in the immature stage and the production of massive non-nucleated cells. These cloned non-nuclear cells proliferate and aggregate in bone marrow, peripheral blood and other hematopoietic tissues, as well as invade other organs and tissues, thereby inhibiting the normal hematopoietic system.[1,2] The main clinical manifestations of AML are fever, infection, anemia, and extramedullary infiltration.[3,4] The incidence of AML is about 1.6 among 100,000 patients and continues to increase, while elderly people are main victims of all leukemias (70%).[5,6]

Currently, the main treatment options for leukemia are combined chemotherapy, differentiation induction therapy and stem cell transplantation therapy,[7-11] among which differentiation induction therapy has

*Supported by the General Program of National Natural Science Foundation of China under Grant (No. 81873113), the Natural Science Foundation of Zhejiang Province (No. LY18H290004)

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DOI: https://doi.org/10.1007/s11655-023-3561-z

become the mainstay for the treatment of AML due to its low toxicity and high safety. Differentiation induction therapy can induce tumor cells to differentiate into normal or nearly normal cells by chemical drugs, and reverse malignant phenotypes such as abnormal proliferation, invasion, and metastasis of tumor cells, thereby achieving the goal of tumor treatment.[12,13] Activation of extracellular signal-related kinase (ERK) is a key step in the cascade mediating cell proliferation in response to a variety of extracellular signals.[14] ERK2 and c-Jun N-terminal kinase (JNK) were reported to be involved in the generation of retinoid responses in the HL-60 AML cell line.[15] ERK inhibitor could offset the suppressive effect of L-ascorbic acid on the leukemic progenitor cell growth.[14] It has been reported that VPS9D1 antisense RNA 1 (VPS9D1-AS1) knockdown inhibits the MEK/ERK signaling pathway, and enhances the inhibitory effect of chidamide on the proliferation of AML cells.[16] These studies suggest that ERK pathway plays a regulatory role in the development of AML.

At present, many Chinese herbs and their monomer preparations have been proved to be highly effective in inducing the differentiation of leukemia cells.[17,18] For instance, ginsenoside Rd (GRd) is an active ingredient of *Radix Ginseng*, which can promote the proliferation and differentiation of neural stem cells and bone marrow stromal cells *in vitro*.[19,20] However, the function of GRd on treating leukemia is rarely reported in existing studies, and the effect of GRd on the differentiation of leukemia cells has not been reported as well. The present study strives to investigate GRd-induced differentiation in AML cells, with the purpose of discovering an effective differentiation inducer for AML treatment.

METHODS

Cell Culture and Treatment

AML cell lines (KG-1, K562, Hel and NB4) purchased from American Type Culture Collection (Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ℃ under the atmosphere of 5% CO_2. The cells in logarithmic growth phase were treated with GRd (CAS: 52705-93-8, purity: over 98%, HY-N0043, MedChemExpress, China) at different concentrations (25, 50, 100 and 200 μg/mL) for 72 h to detect cell viability.[21,22] Likewise, KG-1, K562 and NB4 cells were treated with GRd at different concentrations (25, 50, 100 and 200 μg/mL) for 14 days to detect cell proliferation.

Cell Viability Detection

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) kit (C0009, Beyotime Biotechnology, China) was used to detect cell viability according to the instructions. Briefly, cells were treated with GRd at different concentrations (25, 50, 100 and 200 μg/mL) for 72 h. After treatment, 25 μL MTT solution was added into KG-1, K562, Hel and NB4 cells and kept at 37 ℃ for 3–5 h. Subsequently, the supernatant was removed, and 200 μL dimethyl sulfoxide (DMSO) was added into the cells. Eventually, the absorbance was measured at 490 nm using a microplate reader (Molecular Devices, SpectraMax iD3, USA).

Colony Formation Assay

KG-1, K562 and NB4 cells were respectively treated with GRd at different concentrations (25, 50, 100 and 200 μg/mL) for 14 days. The treated cells were then centrifuged at 1,000 × *g* for 5 min at 4 ℃, and were rinsed twice in Dulbecco's phosphate buffered saline (DPBS, D8662, Sigma-Aldrich, Germany). The above mentioned cells were cultured in Iscove's modified Dulbecco medium (IMDM, PM150510, Procell, China) consisting of 30% new bovine serum, 1% L-glutamine, penicillin-streptomycin, and 0.3% agar as viscous support. The experiments were performed in triplicate with 10^3 cells per well in a 24-well plate, and incubated at 37 ℃ in a humidified atmosphere supplemented with 5% CO_2 for 7 days. The colony number (≥40 cells) was counted under the microscope.

Cell Cycle

KG-1, K562 and NB4 cells were treated with GRd at different concentrations (25, 50 and 100 μg/mL) for 14 days. Thereafter, all cell suspensions were transferred to a centrifugal tube and centrifuged at 1,000 × *g* for 5 min at 4 ℃. After the supernatant was discarded, cells were fixed by 3 mL 70% ice ethanol for 48 h and then stained by 400 μL 50 μg/mL propidium iodide (PI, 85-BMS500PI, Multi Sciences, China) solution. Finally, the stained cells were analyzed by a flow cytometer (version 10.0, FlowJo, FACS Calibur™, BD, USA).

Wright-Giemsa Staining

Wright-Giemsa Stain kit (9990710, Thermo Fisher Scientific, USA) was used to observe cell morphology.

Chin J Integr Med 2024 Jul;30(7):588-599

In brief, K562 cells were treated with GRd (25 and 50 μg/mL) or the positive control retinoic acid (RA, 0.1 g/L, U1674004, Sinopharm Chemical Reagent Beijing Co., Ltd., China). Afterwards, the cells were centrifuged at 1,000×*g* for 5 min at 4 ℃, the culture medium was discarded and the cell smear was prepared. After natural drying, the cells were immersed in Wright-Giemsa staining solution for 5 min. Finally, the cell morphology was observed under an optical microscope (BX-51, Olympus, Japan).

Peroxidase Chemical Staining

For the preparation of compound benzidine dye solution, 0.3 g 4,4'-DiaMinobiphenyl (CAS:92-87-5, purity: higher than 99%, Beijing Ouhe Technology Co., Ltd., China), 1 mL sodium nitroferricyanide (Ⅲ) dihydrate (CAS: 13755-38-9, Beijing Ouhe Technology Co., Ltd., China), and 99 mL ethanol were thoroughly mixed.

K562 cells were treated with the positive control RA (0.1 g/L), or GRd (25 and 50 μg/mL). In addition, K562 cells received treatments of GRd (25 μg/mL) and PD98059 (a non-ATP competitive ERK inhibitor; 20 mg/mL, S1805, Beyotime Biotechnology, China). The treated cells were then centrifuged at 1,000×*g* for 5 min at 4 ℃, the culture medium was discarded and the cell smear was prepared. Next, 3–8 drops of compound benzidine dye were added into the cell smear and held for 2 min. Next, 3–8 drops of hydrogen peroxide solution (SW010764, Beijing Ouhe Technology Co., Ltd., China) were added into the smear and held for another 10 min. Finally, the cell smear was washed by water, naturally dried, and observed under an optical microscope.

Cellular Immunochemistry Assay

The cells were inoculated into the culture flask at a concentration of 1×10^5 cells/mL. K562 cells were treated with GRd (25 and 50 μg/mL) or the positive control RA, and centrifuged at 1,000×*g* for 5 min at 4 ℃. Five days later, immunochemistry chemical staining was performed for identifying the cells. The cell smear was washed by phosphate buffer saline (PBS) 3 times and fixed on ice acetone (01000356-25 g, Beijing Ouhe Technology Co., Ltd., China) for 15 min. Next, the dried cells were washed by PBS 3 times. After that, the cells were incubated first with 0.5% Triton X-100 (ST795, Beyotime Biotechnology) for 20 min and then with 3% hydrogen peroxide solution (H_2O_2, S0051, Beyotime Biotechnology) for 15 min, followed by being washed 3 times using DPBS (14190250, Thermo Fisher Scientific). Thereafter, the serum was used to incubate the cells for 20 min. Next, the cells were incubated with primary antibodies [anti-purine rich Box-1 (PU.1), anti-GATA binding protein 1 (GATA-1), anti-γ-globulin and anti-cEPB antibodies] at 4 ℃ overnight and washed by PBS for 3 times, followed by being further incubated with a secondary antibody at 37 ℃ for 30 min and washed with PBS 3 times. Subsequently, cells were stained with DAB developing kit (P0202, Beyotime Biotechnology) for 10 min, and washed by distilled water for 1 min. Afterwards, the cells were stained by hematoxylin (Shanghai Ruji Biotechnology Development Co., Ltd., China) for 1 min, washed by tap water for 30 min and then sealed by gum. Ultimately, the erythroid and granulated cells were observed under an optical microscope.

Western Blot

After the cells were washed twice by cold PBS, the total proteins were lysed in RIPA lysis buffer (Tianjin Yitailong Technology Co., Ltd., China) and then boiled at 100 ℃ for 5 min of denaturation. Next, protein sample (20 μg) was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose blotting membranes (A10464264, GE Healthcare Life Science, USA), subsequent to which the membranes were sealed by 5% skim milk at room temperature for 1 h. Afterwards, the membranes were incubated with primary antibodies (obtained from Cell Signaling Technology, CST) against GATA-1 (rabbit, 1:1,000, 4589), PU.1 (rabbit, 1:1,000, 2258), phosphorylated-extracellular signal-related kinase (p-ERK, rabbit, 1:500, 4370S), ERK (rabbit, 1:500, 4695S), phosphorylated-glycogen synthase kinase-3 beta (p-GSK3β) (rabbit, 1:1,000, 5558), GSK3β (rabbit, 1:1,000, 12456), signal transducer and activator of transcription 1 (STAT1; rabbit, 1:1,000, 14995, CST), GAPDH (rabbit, 1:1,000, 5174), and β-actin (rabbit, 1:1,000, 4970) at 4 ℃ overnight. Following these, the membranes were further incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-mouse or goat anti-rabbit IgG (H+L) (Protein Tech, USA) for 2 h and washed by PBS 3 times. Finally, the protein bands were detected by electrochemiluminescence (ECL) Western blot kit (MAB5350, Sigma-Aldrich, USA) and scanned by a super sensitive multifunctional imager (Image J, version 4.7, National Institutes of Health, USA).

Animal Ethics Statement

On the premise of obtaining the approval from Ethics Committee of Zhejiang Tongde Hospital (Approval No. ZJTDH2019120121), and animal experiments were conducted in strict accordance with the guidelines of the Animal Welfare Act.

Establishment of Tumor-Bearing Model in Nude Mice

Totally 36 male athymic nude mice (aged 6 weeks, weighing approximately 25 g) were purchased from Shanghai Lab. Animal Research Center [License No. SCK (Shanghai) 2008-0016]. During the experiment, all the mice were kept in a controlled specific pathogen-free (SPF) environment with a temperature of 22±2 ℃ and a humidity of 40%–70% under a 12 h light/dark cycle and given free access to food and water. Later, 1×PBS (100 μL) was used to resuspend the K562 cells (1×10^7/mL), and the cell suspension was injected into the mice via the right flank to establish a tumor-bearing model.

The model mice were randomly divided into 3 groups (12 mice in each group) using a random number table: model control group (non-treatment), GRd group [treated with 200 mg/(kg•d) GRd, which is equivalent to 9.1 times clinical administration] and homoharringtonine (HTT) group [treated with 1 mg/(kg•d) HTT, which is equivalent to 9.1 times clinical administration].[23,24] After a week, the short and long diameters of mice were measured by vernier calipers every 3 days. All the mice were fed for 4 weeks and then the experiment was terminated. The mice were sacrificed when obvious tumor burden hampered their movement or feeding behavior on daily inspection or at the end of 4 weeks, whichever came first. Mice were sacrificed by intraperitoneal injection of pentobarbital sodium (100 mg/kg). Tumor weight and volumes were noted at the time of sacrifice. Tumor volume was calculated by the formula: tumor volume = $(L\times W^2)/2$. This study was approved by the Committee of Experimental Animals of Zhejiang Chinese Medical University (approval No. ZSLL-2013-105). Every effort was made to minimize the pain and discomfort to the animals.

Hematoxylin and Eosin Staining

The sections containing subcutaneous tumor tissues were stained by hematoxylin and eosin (HE). Concretely, the subcutaneous tumor tissues derived from the nude mice were fixed by formaldehyde (SF877503, Sinopharm Chemical Reagent Beijing Co., Ltd., China), dehydrated by gradient alcohol (80%, 90%, 95% and 100%), and treated with xylene (10023418, Sinopharm Chemical Reagent Beijing Co., Ltd., China). Next, the tissues were immersed in wax, embedded, and cut into sections. Subsequently, the sections were dewaxed by xylene, rehydrated by ethanol at the concentrations of 100%, 95%, 80% and 70% for 2 min, and rinsed in distilled water twice. Then, the sections were stained with hematoxylin for 20 min, and washed under running water. After being washed by tap water for 10 min, the sections were further stained by eosin (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., China) for 15 min. Later, the sections were dehydrated in 100% ethanol for 15 min and then treated with xylene for 15 min. Finally, the sections were sealed by the neutral gum, whose pathological changes were observed under an optical microscope (CKX31, Olympus, Japan).

Immunohistochemical Staining

The subcutaneous tumor tissues of the mice were fixed with formaldehyde, dehydrated in gradient alcohol, and transparentized by xylene, followed by being immersed in wax, embedded, and sectioned into tissue slices. Next, the slices were dewaxed by xylene, rehydrated by ethanol at the concentrations of 100%, 95%, 80% and 70% for 2 min, and washed by PBS twice. Afterwards, 30 mL EDTA antigen repair buffer (P0086, Beyotime Biotechnology) was diluted by 1,500 mL distilled water, and then the diluted buffer was boiled in a microwave box. Subsequently, the tissue slices were fully immersed in the buffer above to repair the antigen, heated for 10 min, naturally cooled, and rinsed in PBS 3 times. Following these, each tissue slice was added with 3 drops of 3% H_2O_2, incubated at room temperature for 15 min under the light to eliminate the activity of endogenous peroxidase, and then rinsed in PBS 3 times. Later, the slices were incubated with primary antibodies against WT1 (83535, CST) and GATA-1 (3535, CST) at 4 ℃ overnight and washed by PBS 3 times, followed by being incubated with the secondary antibody at 37 ℃ for 30 min and washed by PBS 3 times. Then, the slices were stained with DAB developing kit (P0202, Beyotime Biotechnology) for 10 min and washed by distilled water for 1 min. After these, the slices were stained by hematoxylin for 1 min, washed by tap water for 30 min and then sealed by gum. Finally, the slices were observed under an optical microscope.

Chin J Integr Med 2024 Jul;30(7):588-599

Flow Cytometry for Detecting Cell Apoptosis

K562, KG-01 and NB4 cells were washed with pre-cooled PBS twice and then diluted in 200 μL binding buffer to a concentration of 1×10^6 cells/mL. In accordance with the guidance of Annexin V-FITC kit (556547, Shanghai Weijin Biotechnology Co., Ltd., China), 5 μL Annexin V and 5 μL PI were added to 100 μL cell suspension. Subsequently, cells were incubated at room temperature for 15 min in the dark, and then resuspended with 400 μL binding buffer. Finally, cells were loaded in a flow cytometer (BD FACSVerse™, Becton Dickinson, USA) for the determination of their apoptosis rates.

Statistical Analysis

Graphpad Prism software (version 6.01; GraphPad Software, Inc., USA) was introduced for statistical analysis. All the experiments were independently repeated 3 times. The data were shown as mean ± standard deviation ($\bar{x} \pm s$) of at least 3 independent experiments. The differences among multiple groups were analyzed by One-Way Analysis of Variance (ANOVA), followed by Tukey's *post hoc* test. $P<0.05$ was considered to be statistical significance.

RESULTS

GRd Inhibited Survival of AML Cells

The result revealed that GRd inhibited the viability of 4 AML cells in a dose-dependent manner ($P<0.05$ or $P<0.01$, Figure 1A). As Figure 1B denoted, GRd inhibited KG-1, K562 and NB4 cell proliferation in a dose-dependent manner, and GRd at 200 μg/mL induced the notably suppression ($P<0.01$). Details of KG-1, K562 and NB4 cell colonies are presented in Figure 1C. Moreover, flow cytometry results showed that GRd could cause cell arrest in G_0/G_1 phase ($P<0.01$, Figure 2).

GRd Induced Differentiation of AML Cells

Western blot result revealed that the expressions of GATA-1 and PU.1 were increased in K562 and KG-1 cells treated with GRd ($P<0.05$ or $P<0.01$, Figures 3A and 3B). In addition, the expression of PU.1 was increased in NB4 cells treated with GRd ($P<0.01$, Figure 3C). After GRd and RA treatments, some cells showed pseudopodia and increased cell vacuoles under the optical microscope. Moreover, Wright-Giemsa staining results uncovered that the cytoplasm of cells became lighter and the ratio of nuclear plasma was decreased (Figure 3D).

The POX chemical staining assay results demonstrated that the expression of POX was increased in ALM cells after GRd and RA treatments (Figure 4). Furthermore, the results of cellular immunochemical experiments revealed that the specific protein expressions of PU.1 and γ-globulin were upregulated in KG-1 cells treated with GRd and RA ($P<0.01$), and the expressions of GATA-1 and PU.1 were also elevated in K562 cells after GRd and RA treatments ($P<0.05$ or $P<0.01$, Figure 5).

GRd Induces AML Cell Differentiation via ERK Pathway

Western Blot was performed to detect the expressions of p-ERK and ERK in AML cells, and the result uncovered that the ratio of p-ERK/ERK was overtly increased in KG-1, K562 and NB4 cells treated with 25, 50 and 100 μg/mL GRd ($P<0.01$, Figure 6).

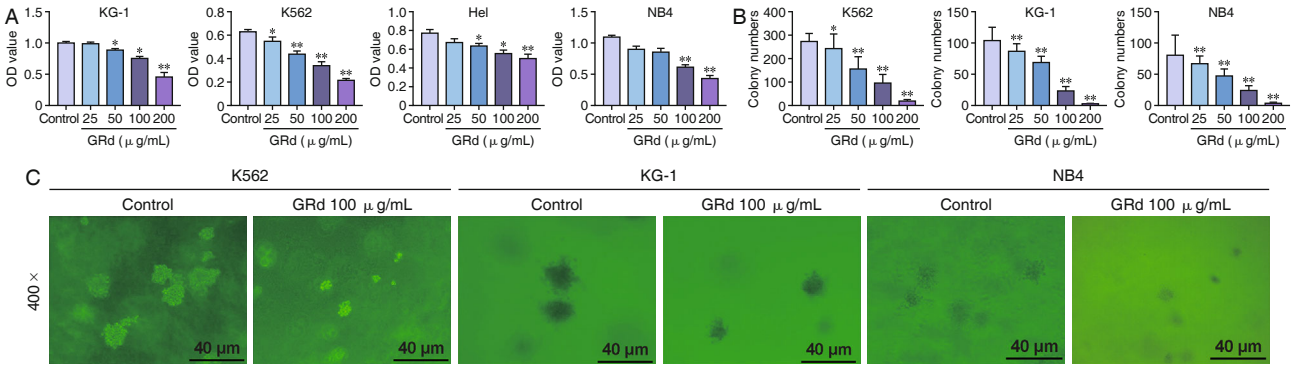

Figure 1. GRd Inhibited Proliferation of AML Cells

Notes: A: Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to detect the viability of AML cells (KG-1, K562, Hel and NB4) after treatment with different concentrations of GRd (0, 25, 50, 100 and 200 μg/mL). B: Colony formation assay was performed to determine the cell proliferation after treatment with different concentrations of GRd (0, 25, 50, 100 and 200 μg/mL). $\bar{x} \pm s$, $n=4$. C: KG-1, K562 and NB4 cell colonies (magnification: 400×, scale bar: 40 μm). $^*P<0.05$; $^{**}P<0.01$ *vs.* control. GRd: ginsenoside Rd; AML: acute myeloid leukemia. The same below

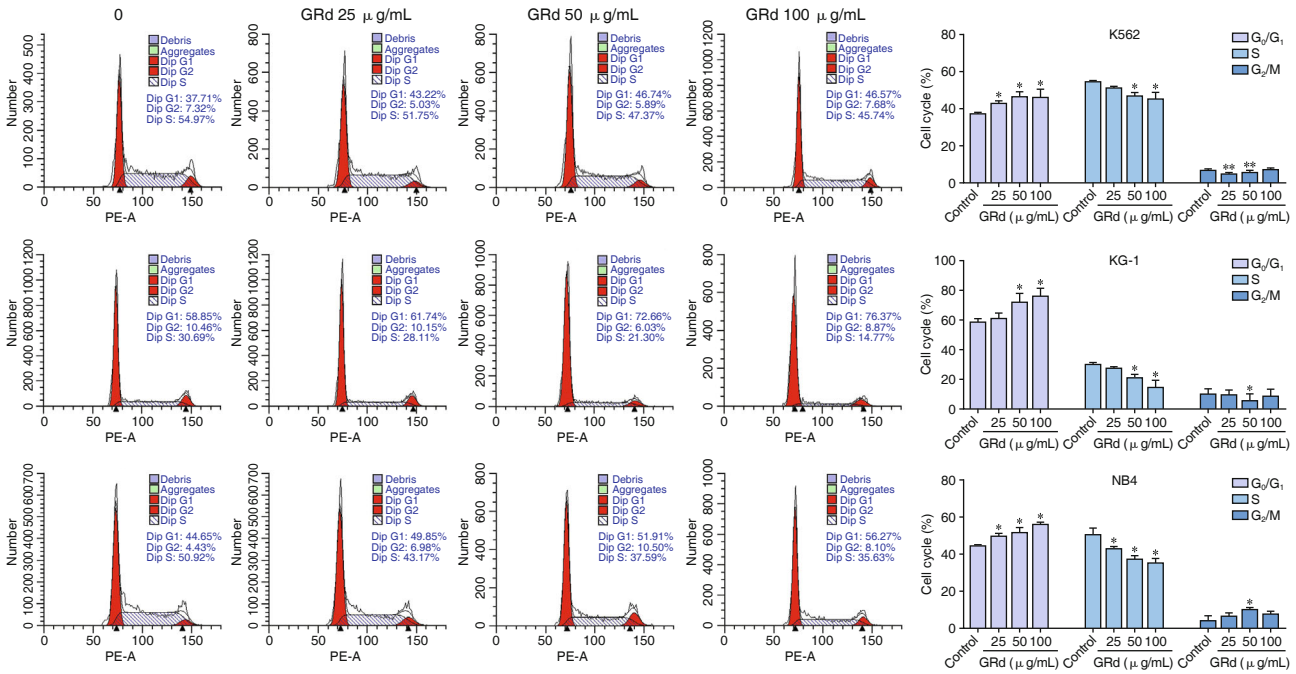

Figure 2. GRd Inhibited AML Cell Cycle ($\bar{x} \pm s$, n=3)

Notes: Cell cycle was measured by flow cytometry after cells were treated with different concentrations of GRd (0, 25, 50, 100 and 200 μg/mL). *P<0.05, **P<0.01 *vs.* control

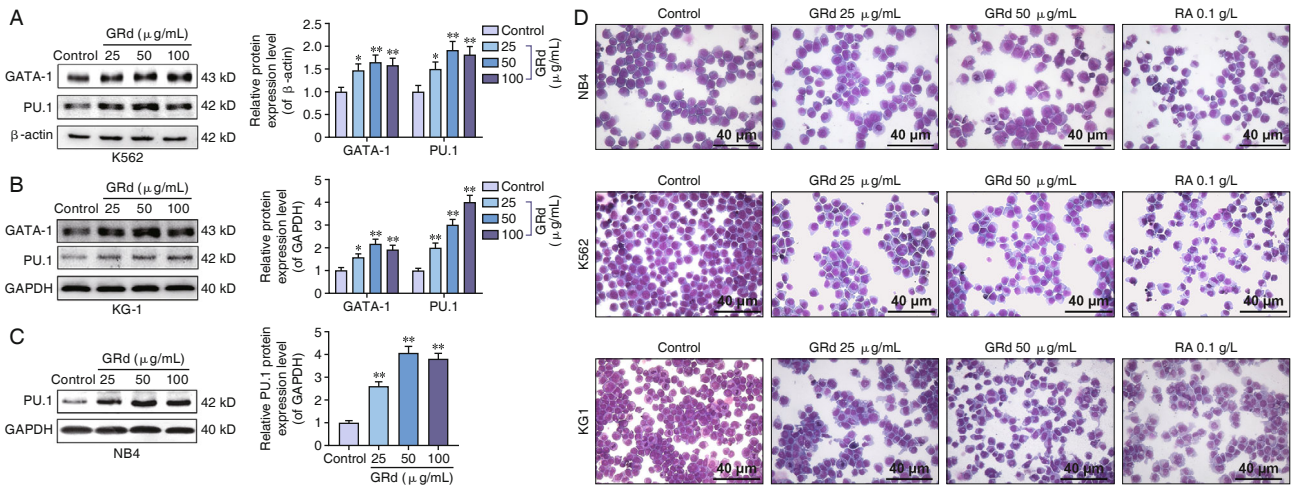

Figure 3. GRd Induced Differentiation of AML Cells

Notes: A: The expressions of GATA-1 and PU.1 in K562 cells treated with GRd (0, 25, 50, 100 μg/mL) detected by Western blot. β-actin was the housekeeping control. B: Western blot was employed to quantify the expressions of GATA-1 and PU.1 in KG-1 cells after treatment with GRd (0, 25, 50, 100 μg/mL). GAPDH was the housekeeping control. C: The expression of PU.1 in NB4 cells treated with GRd (0, 25, 50, 100 μg/mL), GAPDH acted as the housekeeping control. $\bar{x} \pm s$ (n=3). D: The morphology of NB4, K562 and KG-1 cells observed by Wright-Giemsa staining after cells were treated with GRd (0, 25, 50 μg/mL) and RA (0.1 g/L). Magnification: 400×, scale bar: 40 μm. *P<0.05, **P<0.01 *vs.* control. GATA-1: GATA binding protein 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; RA: retinoic acid

In addition, the levels of p-GSK-3β/GSK-3β and STAT1 expressions were increased in K562 and NB4 cells treated with 25, 50 and 100 μg/mL GRd (P<0.05 or P<0.01, Figures 7A and 7B).

The ERK pathway inhibitor PD98059 was used to culture AML cells, and POX chemical staining assay was performed to observe NB4 cell differentiation. The results demonstrated that the expression of POX was obviously inhibited in NB4 cells treated with PD98059, whereas GRd promoted the expression of POX and reversed the effect of PD on inhibiting the POX expression (Figure 8A). Meanwhile, the result from Western blot revealed that the expressions of GATA-1 and PU.1 were conspicuously promoted in GRd-treated cells, and the alteration was reversed by PD treatment (P<0.01, Figure 8B).

Chin J Integr Med 2024 Jul;30(7):588-599

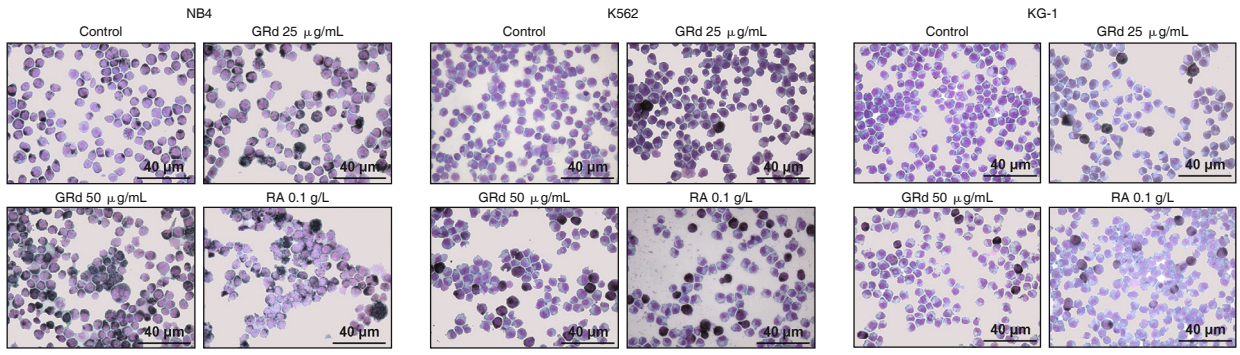

Figure 4. GRd Induced POX Expression in ALM Cells

Notes: Effect of GRd on inducing NB4, K562, and KG-1 cell differentiation evaluated by chemical staining of POX after cells were treated with GRd (0, 25, 50 μg/mL) and RA (0.1 g/L). Magnification: 400×, scale bar: 40 μm. POX: peroxidase

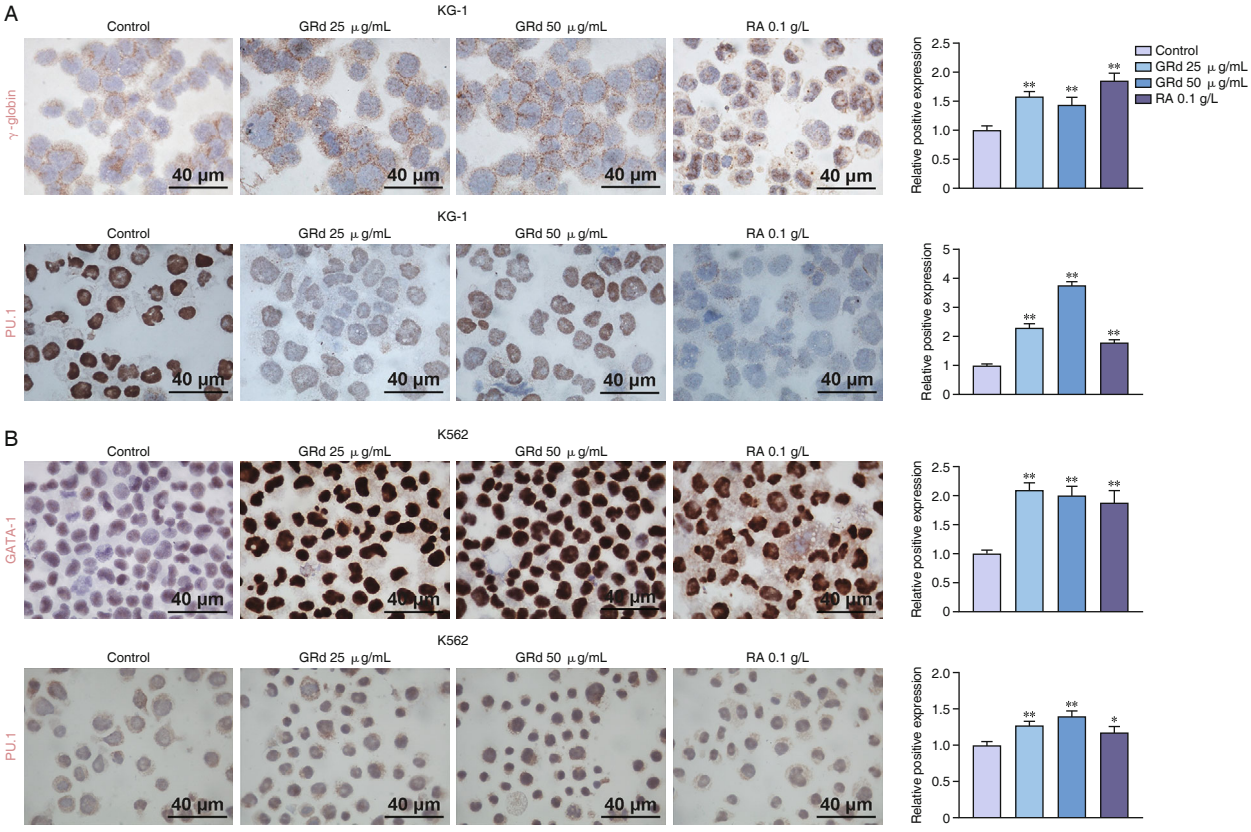

Figure 5. GRd Induced Expressions of Specific Proteins Concerning Erythroid or Granulocytic Differentiation in AML Cells

Notes: A: The expression of erythroid differentiation protein (γ-globulin) and granulocyte-differentiated protein (PU.1) in KG-1 cells detected by cytoimmunochemistry after cells were treated with GRd (0, 25, 50 μg/mL) and RA (0.1 g/L). B: The expression of erythroid differentiation protein (GATA-1) and PU.1 in K562 cells detected by cytoimmunochemistry after cells were treated with GRd (0, 25, 50 μg/mL) and RA (0.1 g/L). Magnification: 400×, scale bar: 40 μm. $\bar{x} \pm s$ (n=3); $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control

In addition, the effect of GRd on apoptosis of K562 cells was also detected. The result showed that the apoptosis rate of K562 cells in GRd group was increased compared with that in control group, and the apoptosis rate of K562 cells in GRd+PD980509 group was increased compared with those in control and PD980509 groups (P<0.01), while no significant change was observed between GRd and GRd+PD980509 groups (P>0.05, Figure 8C).

GRd Inhibited AML Tumor Development

To further analyze the role of GRd, the tumor model of nude mice was established and mice were treated with GRd and HTT. It was found that the tumor volume and weight were decreased significantly in the mice treated with GRd (P<0.05) or in the mice treated

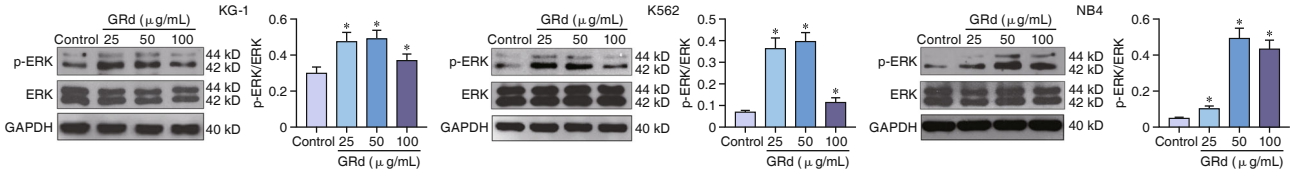

Figure 6. GRd Regulated ERK Pathway in AML Cells ($\bar{x} \pm s$, n=3)

Notes: A: The protein expressions of p-ERK and ERK in KG-1, K562 and NB4 cells after cells were treated with different concentrations of GRd (0, 25, 50, 100 μg/mL). *P<0.01 *vs.* control. GAPDH was used as the loading control. ERK: extracellular signal-related kinase; p-ERK: phosphorylated-extracellular signal-related kinase

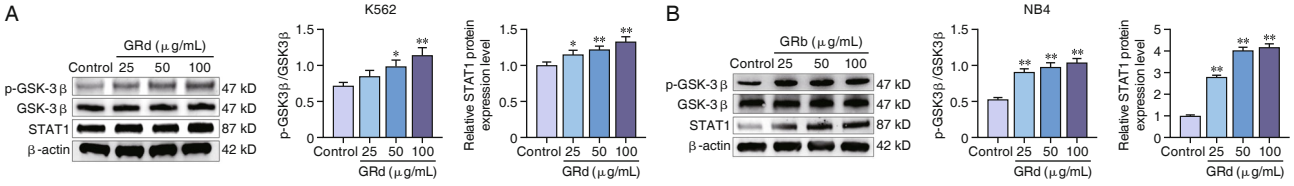

Figure 7. GRd Regulated p-GSK-3β and STAT1 Expressions in AML Cells ($\bar{x} \pm s$, n=3)

Notes: Western blot was performed to detect the expressions of p-GSK-3β, GSK-3β and STAT1 in K562 (A) and NB4 (B) cells after cells were treated with different concentrations of GRd (0, 25, 50, 100 μg/mL). GAPDH was used as a loading control. *P<0.05, **P<0.01, *vs.* control. p-GSK-3β: phosphorylated-glycogen synthase kinase-3 beta. STAT1: signal transducer and activator of transcription 1

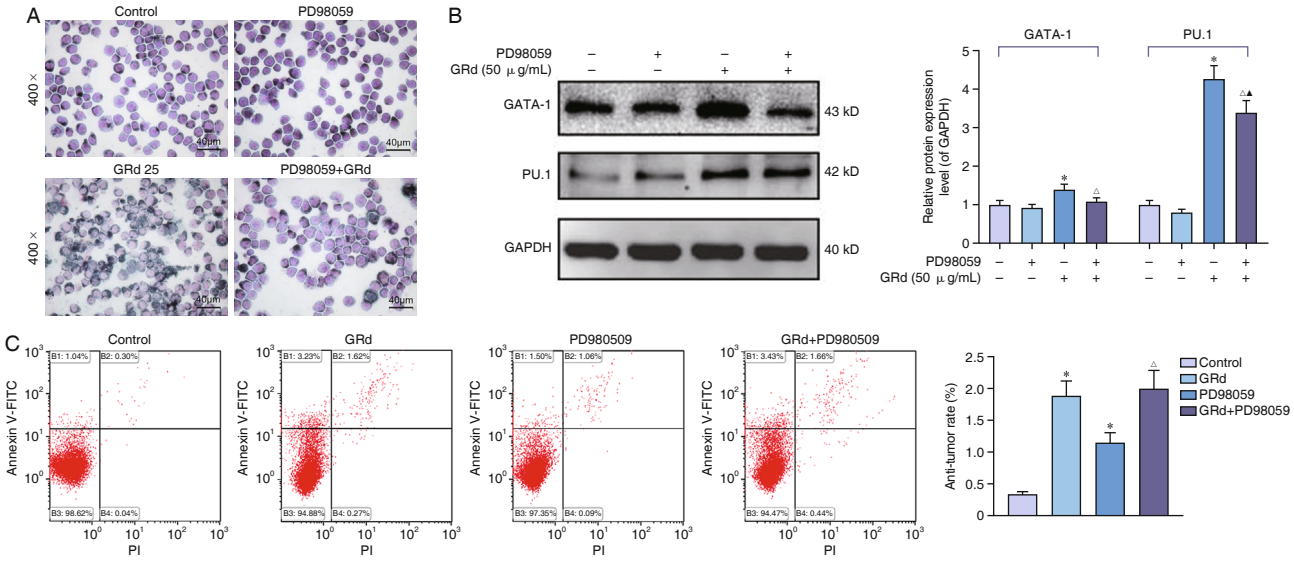

Figure 8. GRd Induced AML Cell Differentiation via ERK Pathway

Notes: A: Chemical stainning of POX was applied to observe NB4 cell differentiation (magnification: 400×, scale bar: 40 μm). B: The expressions of GATA-1 and PU.1 after cells were treated with GRd and PD98059 measured by Western blot. GAPDH was used as the loading control. C: The apoptosis of K562 cells detected by flow cytometry. $\bar{x} \pm s$ (n=3). *P<0.01 *vs.* control; △P<0.01 *vs.* GRd group; ▲P<0.01 *vs.* PD98059 group

with HTT (P<0.01, Figures 9A and 9B). Furthermore, the subcutaneous tumor tissues were observed after HE staining. It could be observed that the tumor tissue cells in the control group showed clear morphology, tight cell growth, and fewer apoptotic cells. The morphology of tumor tissue cells in the GRd treatment group and HTT group were unclear, with a significant increase in apoptotic and necrotic cells (Figure 9C). Immunohistochemical staining assay was performed to determine the expressions of WT1 and GATA-1 in subcutaneous tumor tissues. The results showed that WT1 expression was significantly reduced, however, GATA-1 expression was greatly increased in subcutaneous tumor tissues treated with GRd and HTT (P<0.05 or P<0.01, Figure 9D).

DISCUSSION

AML cells proliferate and accumulate in bone marrow and other hematopoietic tissues due to uncontrolled proliferation and differentiation dysfunction, and also invade other non-hematopoietic tissues and organs, thereby affecting the normal hematopoietic function.[1] In recent years, many Chinese herbal monomers such as vincristine, paclitaxel, and cytidine have been used in the

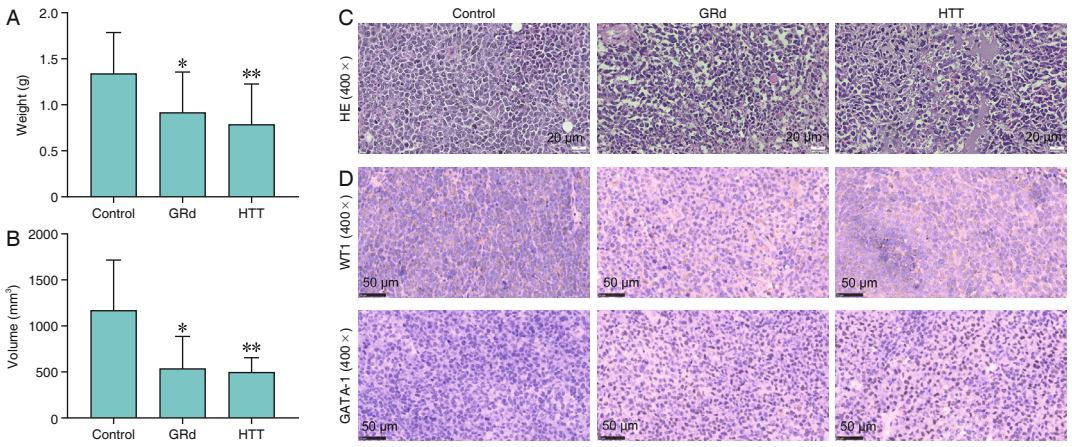

Figure 9. GRd Inhibited AML Tumor Development

Notes: A: Mouse tumor-bearing model was established and mice were treated with GRd [200 mg/(kg•d)] and HTT (homoharringtonine, 1 mg (kg•d), positive control), and the tumor weight was measured. B: The tumor volume of mice was calculated after the establishment of the tumor-bearing mouse model. $\bar{x} \pm s$ (n=12); C: HE staining was used to analyze the effects of GRd and HTT on subcutaneous tumor tissue of the mice. D: The protein expressions of WT1 and GATA-1 in subcutaneous tumor tissues of nude mice determined by immunohistochemical staining (magnification: 400 ×, scale bar: 50 μm). $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control.

treatment of AML,[25-27] and anti-AML functions of Chinese herbal monomers such as triptolide, baicalin, and icariin have been recently discovered.[28-30] In addition, the functional mechanism of Chinese herbal monomers on inhibiting the proliferation and promoting the differentiation of AML cells has been investigated as well. At present, Chinese medicine and its monomer preparations have become important aspects in anti-leukemia pharmacology research.

GRd, an extract of *Panax notoginseng* and *Radix Ginseng*, is one of the main metabolites of diol ginsenoside in human intestinal tract, and has many biological activities.[31,32] Though GRd has been proved to have unique functions in the treatment of cardiovascular, cerebrovascular, and neurological diseases and exert anti-tumor effects,[33-35] its therapeutic effects on AML have not been reported yet. GRd has also been found to significantly inhibit the proliferation of human glioma U251 cells[36] and human cervical cancer cells.[37] Thanks to its effect in inhibiting phenylephrine-induced cardiomyocyte hypertrophy, GRd has been applied in the treatment of cardiomyocytes.[38] Ginsenoside Re and GRd have been manifested to enhance the expressions of cholinergic markers and neuronal differentiation in Neuro-2a cells.[19] GRd extracted from *Panax notoginseng* has been found to enhance the astrocyte differentiation from neural stem cells.[14] GRd can also induce the differentiation of MC3T3-E1 cells by activating AMPK/BMP-2/Smad signaling pathways.[20] In the current study, GRd at different concentrations was used to treat AML cells. It turned out that GRd inhibited the proliferation of AML cells in a dose-dependent manner, and the cells were arrested at G_0/G_1 phase, proving that GRd yielded an inhibitory effect on the development of AML cells.

Cell differentiation is an important biological process in the development and maturation of advanced organisms.[39] Abnormal cell differentiation can lead to the occurrence of diseases such as malignant tumors,[40] and cell differentiation disorder is a pivotal cause to AML. Cell differentiation is regulated by various proteins, and GATA-1 is only expressed in hematopoietic cell lines and has the function of regulating the proliferation and differentiation of erythroid cell lines.[41] PU.1 is mainly expressed in hematopoietic system. Besides, cells such as myeloid cells and B lymphocytes can regulate the transcription of myeloid genes, thereby regulating the differentiation of hematopoietic system. Several studies revealed that the absence of PU.1 could induce AML.[42,43] The results of the present study displayed that the expressions of GATA-1 and PU.1 were increased in AML cells after GRd treatment. The activation of STAT1 has a vital role in the terminal differentiation of immature leukemia cells.[44,45] In this research, RA treatment served as the positive control, which induced cell differentiation. Similar to the results after RA treatment, pseudopodia appeared in cells treated with GRd, and the ratio of nucleoplasm was decreased, whereas the expressions of POX and specific proteins concerning erythroid or granulocytic differentiation were

increased. These results indicated that GRd could promote the differentiation of AML cells.

ERK signaling pathway is closely related to cell proliferation and differentiation, and it acts on a variety of transcription factors through phosphorylation of downstream molecules, and promotes the transcription and expressions of target genes.[46] Currently, the activation of ERK/GSK-3β signaling pathway has been used to regulate cell differentiation.[47,48] It was found that LukS-PV could induce the differentiation of AML cells via activating ERK signaling pathway.[49] Several studies have shown that GRd promotes or inhibits cellular function by regulating ERK signaling pathway.[50-52] For example, GRd promotes neurite outgrowth of PC12 cells by upregulating GAP-43 expression via MAPK/ERK-dependent pathways.[53] At present, it is not clear whether GRd can promote the differentiation of AML cells through regulating ERK/GSK-3β signaling pathway. The results of this study exhibited that p-ERK and p-GSK-3β levels were increased in GRd-treated AML cells, and GRd reversed the effects of PD98059 on inhibiting the expressions of POX, PU.1 and GATA-1, suggesting that GRd could induce the differentiation of AML cells through upregulating ERK/GSK-3β signaling pathway-related proteins. Specifically, GRd promoted the differentiation of AML cells by upregulating POX, PU.1 and GATA-1 levels via activating ERK/GSK-3β signaling pathway.

GRd was evidenced to significantly reduce the volume and weight of breast tumor in a dose-dependent manner.[35] A previous study also discovered that GRd treatment obviously reduces the number of tumor lesions in mice inoculated with breast cancer 4T1 cells.[54] In this research, K562 cells were used to induce the formation of tumors in nude mice to further explore the inhibitory effect of GRd on solid AML tumors. Notably, the tumor weight and volume in GRd-treated mice were lower than those in the model control mice. In addition, the histological and pathological changes observed after HE staining showed that tumor cells underwent substantial apoptosis and necrosis. Moreover, WT1 level was decreased, however, GATA-1 level was visibly increased in the cells after GRd treatment. These results indicate that GRd can inhibit the development of tumors *in vivo*. However, there are limitations in this study. In *in vitro* experiment, nude mice were also treated with PD98059 to further analyze the effect of GRd-mediated ERK signaling pathway on tumors. In addition, the markers indicating the specific differentiation directions need to be further analyzed as well.

Collectively, this study attests through *in vivo* experiments that GRd can inhibit the proliferation and induce the differentiation of AML cells to hinder the development of AML tumor, hinting its potent potential as a beneficial anti-leukemic agent for differentiation therapy. The current findings further improve the understanding on GRd's role in AML at a molecular level.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Yin LM made substantial contributions to conception and design. Jiang YX and Yin LM drafted the article and critically revised it for important intellectual content. Jiang YX, Zhao YN, Yu XL and Yin LM were responsible for data acquisition, data analysis and interpretation. All authors read and approved the final version for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

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(Accepted July 17, 2023; First Online December 12, 2023)
Edited by TIAN Lin