

Full-length article

Regulating expressions of cyclin D1, pRb, and anti-cancer effects of deguelin on human Burkitt's lymphoma Daudi cells *in vitro*¹

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Key words

deguelin; Daudi cells; cyclin D1; pRb; apoptosis

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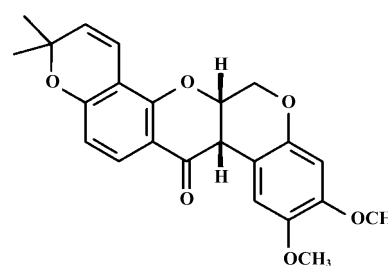
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Abstract**Aim:** To investigate anticancer effects and molecular mechanism of deguelin on human Burkitt's lymphoma Daudi cells *in vitro* and compare the cytotoxicities of deguelin on Daudi cells and human peripheral blood mononuclear cells (PBMC).**Methods:** The effects of deguelin on the growth of Daudi cells were studied by 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) assay. Apoptosis were detected through Hoechst 33258 staining and Annexin V/PI double-labeled cytometry. The effect of deguelin on the cell cycle of Daudi cells were studied by a propidium iodide method. The expressions of cyclin D1 and pRb were checked by Western blot. **Results:** The proliferation of Daudi cells were decreased in deguelin-treated group with a 24-h IC₅₀ value of 51.55 nmol/L. Deguelin induced Daudi cells apoptosis was in a time- and dose-dependent manner. G₀/G₁ phase increased and S phase decreased in Daudi cells treated with deguelin. With deguelin 0, 5, 10, 20, and 40 nmol/L treatment for 24 h, G₀/G₁ phase increased from 37.34% to 56.56%, whereas S phase decreased from 37.72% to 21.36%. PBMC was less sensitive to the cytotoxic effect of deguelin than Daudi cells. The expression of cyclin D1 and pRb protein were decreased sharply in Daudi cells treated with deguelin. **Conclusion:** Deguelin is able to inhibit the proliferation of Daudi cells by regulating the cell cycle that arrested cells at G₀/G₁ phase and inducing the cell apoptosis. Moreover, deguelin selectively induced apoptosis of Daudi cells with low toxicity in PBMC. The antitumor effects of deguelin were related to down-regulating the expression of cyclin D1 and pRb protein.**Introduction**

A large number of natural products have been evaluated as potential chemopreventive agents. Deguelin (Figure 1), a natural plant-derived rotenoid, most commonly used as an insecticide in Africa and South America, has been isolated from several plant species, including *Mundulea sericea* (*Leguminosae*). Its main active composition is rotenone, and it has very strong photodissociation^[1]. The recent research has indicated that deguelin usually has a very strong antitumor function, and can suppress many kinds of tumors cell at nmol levels^[1]. Notably, deguelin can suppress colon-cancer cell HT-29 growth^[2], inhibit the growth of precancerous and cancerous lung cells, and induce premalignant and malignant human bronchial epithelial (HBE) cells apoptosis

**Figure 1.** Structure of deguelin.

with no toxic effects on normal cells^[3]. These recent experiments verified that deguelin could lead the cell cycle to block and induce apoptosis, however, its mechanism is not yet completely clear.

Burkitt's lymphoma (also called small noncleaved cell lymphoma) is a type of on-Hodgkin's lymphoma, a cancer in the lymphatic system. Despite recent advances in radiotherapy, chemotherapy, and stem cell transplant, the severe morbidity from lymphoma has not been improved^[4]. Much effort has been focused on the discovery and development of new chemopreventive agents, especially agents targeted at mechanisms known to be involved in the process of carcinogenesis. Therefore, we have sought to identify novel agents that can prevent lymphoma carcinogenesis effectively but with minimal toxicity.

This study was designed to explore the mechanism of deguelin-induced apoptosis in Daudi cells. We studied deguelin on human Burkitt's lymphoma Daudi cells *in vitro* and compared the cytotoxicities of deguelin on Daudi cells with human peripheral blood mononuclear cell (PBMC), and focused on the changes in the expression of cyclin D1 and phosphor-Rb, and analyzed its underlying mechanism.

Materials and methods

Drugs and reagents Deguelin was purchased from the Sigma Chemical Company (St Louis, MO, USA) and initially dissolved in dimethylsulfoxide (Me₂SO), and stored at -20 °C, and thawed before use. MTT was purchased from Janssen Chimica Company (New Brunswick, NJ, USA). RPMI-1640 medium, Hoechst 33258, and Me₂SO were purchased from Sigma Co. Anti-cyclin D1 and anti-phosphor Rb antibodies were purchased from Santa Cruz (California, USA). Chemiluminescence (ECL) reagent kits were purchased from Pierce Biotechnology, Inc (Rockford, IL, USA). Daudi cells line was obtained from China Center for Typical Culture Collection (Wuhan, China). PBMC were obtained by Ficoll-Hypaque density gradient centrifugation. All kinds of cells were grown in RPMI-1640 culture medium containing 10% fetal calf serum (FCS) and 2 mmol/L L-glutamine at 37 °C in a 5% CO₂ incubator.

MTT assay The antiproliferative effects of deguelin against different group cells were determined by the MTT dye uptake method. Briefly, the Daudi cells and PBMC (40 000 per well) were incubated in triplicate in a 96-well plate. Different concentrations of deguelin were added, and the final concentrations were 0, 5, 10, 20, 40, 80, and 160 nmol/L. The plates were in the presence or absence of the indicated test samples for 0, 24, 36, 48, 60, and 72 h. The group in maximal Me₂SO dissolution concentration acted as the control group. Thereafter, 20 μL MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) was added to each well. After 4 h at 37 °C, the supernatant was removed and 150 μL

Me₂SO was added. When the blue crystal was dissolved, the optical density (OD) was detected at 570 nm wavelength using a 96-well multiscanner autoreader (Biotech Instruments μQuant, New York, USA). The following formula was used: Cell proliferation(%)=[1-(OD of the experimental samples/OD of the control)]×100%.

Hoechst 33258 staining Deguelin-induced apoptosis was monitored by the extent of nuclear fragmentation. Nuclear fragmentation was visualized by Hoechst 33258 staining of apoptotic nuclei. The apoptotic cells were collected by centrifugation, washed with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Subsequently, the cells were washed and resuspended in 20 μL PBS before deposition on poly lysine-coated coverslips and were left to adhere on cover slips for 30 min at room temperature and then the coverslips were washed twice with PBS. The adhered cells were then incubated with 0.1% Triton X-100 for 5 min at room temperature and rinsed with PBS three times. The coverslips were treated with Hoechst 33258 for 30 min at 37 °C, rinsed with PBS and mounted on slides with glycerol-PBS. The cells were viewed with an Olympus BH-2 fluorescence microscope (Tokyo, Japan).

Annexin V-PI assay The cells were collected and washed with PBS, followed by being resuspended in binding buffer (HEPES-NaOH 10 mmol/L, pH 7.4, NaCl 140 mmol/L, CaCl₂ 2.5 mmol/L). The samples were incubated with 5 μL Annexin-V in dark for 15 min, washed with binding buffer and resuspended in 1% formaldehyde in the binding buffer at 4 °C for 30 min. After being washed with binding buffer again, the cells were stained with 500 μL PI (1 μg/ml, Sigma) for 15 min then measured on flow cytometry (Stanford, Becton Dickinson, USA).

DNA content and cell cycle analysis Untreated and treated cells were collected, after cultured in the presence or absence of deguelin for the indicated time, rinsed with PBS, and suspended in 75 % ethanol at -20 °C overnight. Fixed cells were centrifuged at 1200×g and washed with PBS twice. To detect DNA content, cells were contained in the dark with PI 50 mg/L and 0.1 % RNase A in 400 μL PBS at 25 °C for 30 min. Stained cells were analyzed on FACSsort (Becton Dickinson). The percentage of apoptotic cells was determined using the CellQuest software program.

Western blot analysis Lysates were prepared from 1×10⁷ cells by dissolving cell pellets in 100 μL of lysis buffer Na₂HPO₄ pH 7.4 20 mmol/L, 150 mmol/L NaCl, 1% Triton X-100, 1% aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 g/L leupeptin, 100 mmol/L NaF, and 2 mmol/L Na₃VO₄. Lysates were centrifuged at 18 000×g for 15 min and the supernatant was collected. Protein content was determined

using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2 mol/L DTT) was added to the lysates. Lysates were heated to 100 °C for 5 min, and 80 µg of protein was loaded into each well of a 10% SDS-PAGE gel. Resolved proteins were electrophoretically transferred to nitrocellulose and blocked with 5% non-fat milk, and the primary antibodies cyclin D1 and phosphor Rb (dilution 1:200), respectively. After overnight incubation at 4 °C the blots were washed, exposed to HRP-conjugated corresponding secondary antibodies for 1 h, and finally detected by ECL. Quantification of the bands was carried out using densitometric analysis software, Quantity One (Bio-Rad), and processed as described previously.

Statistical analysis All data were expressed as mean±SD using SPSS 10.0 for windows 98. Using linear *t*-tests for statistics analyses, *P* values of less than 0.01 or 0.05 were considered to be statistically significant.

Results

Effects of deguelin on proliferation of Daudi cells and PBMC Daudi cells and PBMC treated with deguelin for 0 h, 24 h, 36 h, 48 h, 60 h, and 72 h, respectively, resulted in the inhibition of cell proliferation in a dose- and time-dependent manner. The *OD* value of the deguelin-treated group decreased significantly compared with the untreated group and PBMC group. There are significant differences between the deguelin-treated and untreated group (Figure 2). It has also shown great difference between deguelin-treated and PBMC group (Figure 3). The *IC*₅₀ value of 24 h on Daudi cell was 51.55 nmol/L, whereas the *IC*₅₀ value of 24 h on PBMC was 1.63 µmol/L. The *IC*₅₀ value of 24 h on PBMC was higher than Daudi cell group with great significance (*P*<0.01).

Hoechst 33258 staining We used Hoechst 33258 staining to investigate the changes in the nucleus of cells, and many apoptotic bodies containing nuclear fragments were found in deguelin-treated (40 nmol/L for 24 h) cells, but none in untreated cells. Arrows indicate apoptotic nuclear fragmentation (Figure 4). At the same time, cytoplasmic shrinkage was observed in cells cultured with deguelin.

Annexin V-PI assay All untreated cells were Annexin-V negative, which meant no apoptosis occurred. In contrast, when Daudi was treated with deguelin, apoptotic cells became Annexin-V positive and PI negative almost all the apoptosis occurred in cells undergoing the G1-phase. With the increasing dose of deguelin, early apoptotic cells increased from (15.46%±0.62%) to (18.48%±2.98%) with 20

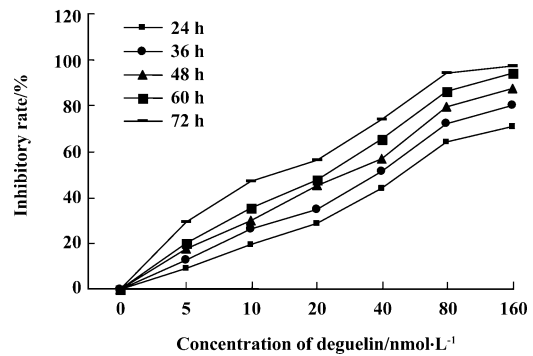


Figure 2. Antiproliferation effect of deguelin on Daudi cells. Daudi cells were treated with various concentrations of deguelin as indicated for 0, 24, 36, 48, 60, and 72 h. Growth inhibition was determined using a MTT assay and shown as an inhibitory rate. *n*=3.

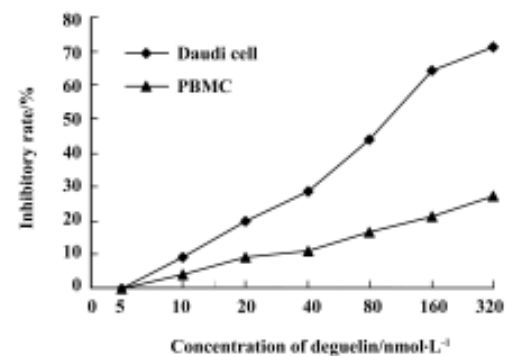


Figure 3. Comparison of the cytotoxicities of deguelin on Daudi cells and human peripheral blood mononuclear cell (PBMC). *n*=3.

nmol/L and 40 nmol/L deguelin, respectively (Figure 5). It meant that deguelin could induce G1-phase specific apoptosis.

Cell cycle analysis Figure 6 illustrates changes in DNA content distribution treated with deguelin 0, 5, 10, 20, 40, and 80 nmol/L for 24 h. As the treatment dose increased, the percentage of cells in G₁/G₀ phase increased and S phase decreased accordingly. Treated with 0, 5, 10, 20, 40, and 80 nmol/L deguelin for 24 h, the rate of G₁/G₀ phase cells were increased by 37.34%, 40.91%, 44.69%, 52.45%, and 56.56% in a dose-dependent manner. Meanwhile, the rate of S phase cells were decreased by 37.72%, 35.97%, 34.91, 24.11, and 21.36% in a dose-dependent manner. It showed few changes in G₂/M phase. These results illustrated that deguelin arrested the Daudi cells at G₁/G₀ phase *in vitro*.

Cyclin D and pRb analysis by Western blot After treated with 0, 50, 100, 200, and 400 nmol/L deguelin for 24 h, both the expression of cyclin D1 and pRb were down-regulated dramatically in a dose-dependent manner. The *OD* values of

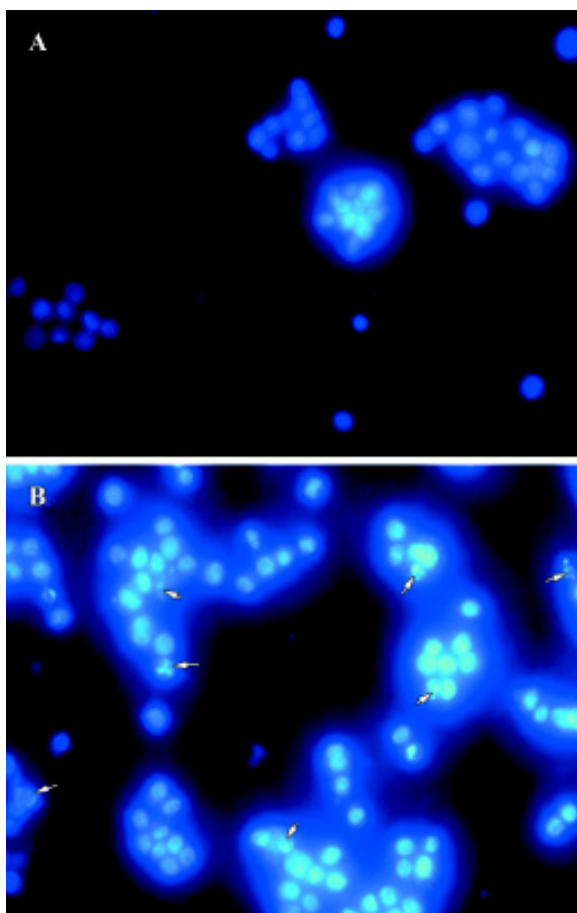


Figure 4. (A) Untreated Daudi cells; (B) Cells treated with 50 nmol/L deguelin for 24 h. Photographs were taken under an Olympus BH-2 fluorescence microscope ($\times 40$). Arrows indicate apoptotic nuclear fragmentation.

cyclin D1 and pRb on Daudi cells were greatly significant compared with the control cells, and *P* values were less than 0.05 or 0.01, respectively (Figure 7).

Discussion

Several natural compounds, especially plant products and dietary constituents, have been found to exhibit chemopreventive activities both *in vitro* and *in vivo* in model systems. Their mechanisms of action vary widely, with many suppressing cell growth or modulating cell differentiation and a few also inducing apoptosis. Rotenoids, which constitute a class of compounds from the flavonoid family, have chemopreventive activity, act by inhibiting NADH: ubiquinone oxidoreductase activity, and by suppressing steady-state mRNA levels and enzymatic activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity^[5]. One rotenoid, deguelin, has been

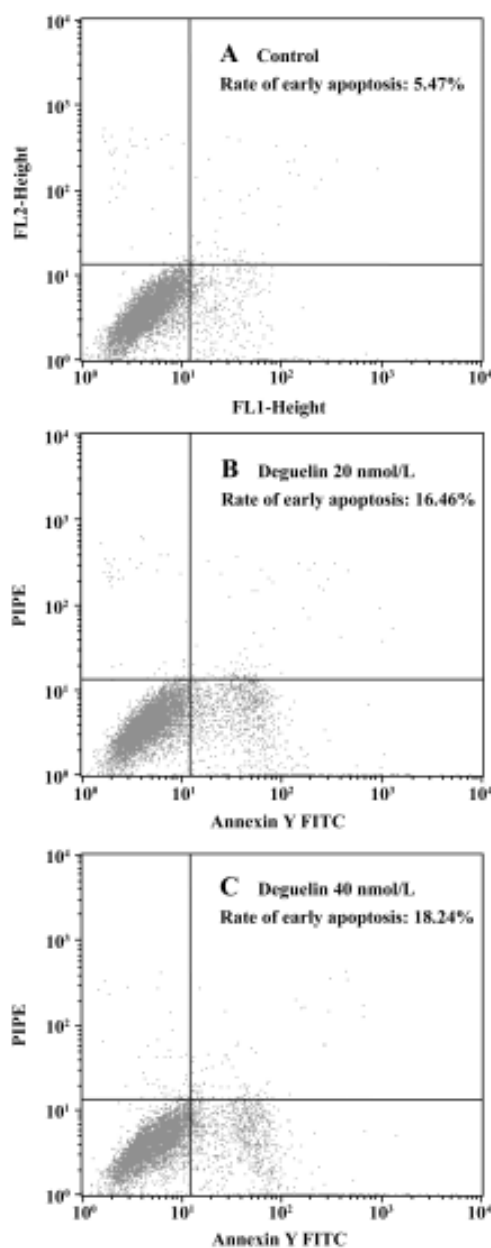
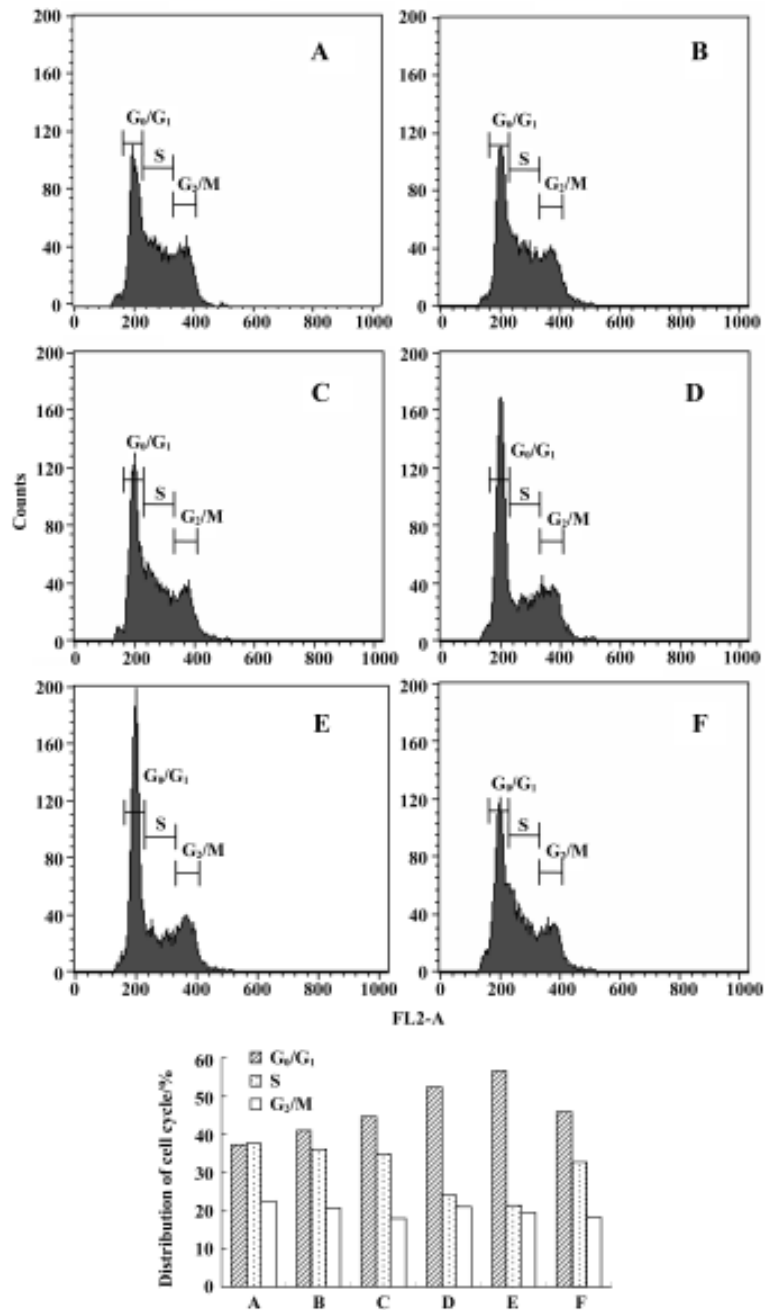


Figure 5. The rate of early apoptosis induced by deguelin with Annexin V-PI assay. A, B, and C show treatment with 0, 20, and 40 nmol/L deguelin for 24 h, respectively.

isolated from several plant species, including *Mundulea sericea* (Leguminosae)^[6]. Deguelin has been shown to have cancer-chemopreventive effects in models of skin, mammary, and lung tumorigenesis. Deguelin has been widely used as an insecticide in Africa, South Africa, and China^[7].

This experiment also founded that deguelin could suppress Daudi cell selectively, and it had low toxicity on human peripheral blood monocular cells (PBMC). Deguelin-



	Control	5 nmol/L	10 nmol/L	20 nmol/L	40 nmol/L	80 nmol/L
G ₀ /G ₁	37.34	40.91	44.69	52.45	56.51	45.77
S	37.72	35.97	34.90	24.10	21.36	32.78
G ₂ /M	22.69	20.82	18.21	21.09	19.72	18.25

Figure 6. The distribution of cell cycle by deguelin with FACS_{Sort} (Becton Dickinson, USA). A, B, C, D, E, and F are the control, and deguelin 5, 10, 20, 40, and 80 nmol/L for 24 h, respectively.

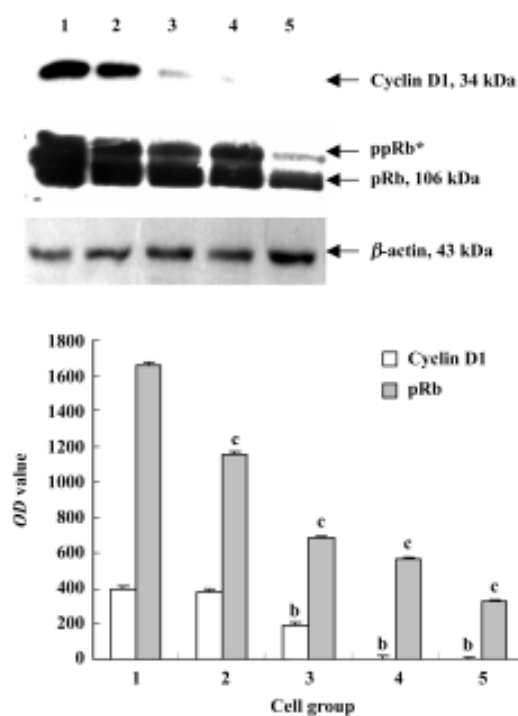


Figure 7. Effects of deguelin on the expression of cyclin D1 and pRb in Daudi cells. 1, 2, 3, 4, and 5 are control, and deguelin 50, 100, 200, and 400 nmol/L, respectively. $n=3$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$, compared with control. ppRb* was inactive form. pRb was active form.

induced antiproliferative effects on Daudi cells, indicating that deguelin may have potential as they accumulated in the G_0/G_1 phase of the cell cycle and underwent apoptosis in a dose- and time-dependent manner. We found that deguelin decreased the expression of cyclin D1 and pRb in Daudi cells, suggesting that changes in the ratio of cyclin D1 and pRb might contribute to the apoptosis-promoting activity of deguelin in these cells. Cyclin D1 and pRb are important proteins for regulating cell cycle. Chun *et al*^[4] found that deguelin could inhibit both premalignant and malignant HBE cells and these were more sensitive to deguelin than normal HBE cells. They also found deguelin increased the expression of Bax and decreased the expression of Bcl-2 in premalignant and malignant HBE cells, suggesting that changes in the ratio of Bax to Bcl-2 might contribute to the apoptosis-promoting activity of deguelin in these cells. This suggests that mechanisms of regulating cell cycle may contribute to the sensitivity of tumor cells to deguelin.

The results of Hoechst 33258 staining and Annexin V-PI assay showed that deguelin could induce Daudi cells apoptosis in a dose-dependent way. Arrows indicate apoptotic nuclear fragmentation. Apoptotic cells presented typi-

cal characteristics including the cell nuclear concentration, nucleus gathered by the core. With the increasing dose of deguelin, early apoptotic cells increased from 15.46% \pm 0.62% to 18.48% \pm 2.98% with 20 nmol/L and 40 nmol/L deguelin, respectively. The latest research materials indicate that deguelin can inhibit other tumor cell proliferation such as breast cancer, lung cancer, colon-cancer, and so on. Its underlying mechanism relates to inducing inhibition of cyclooxygenase-2 and extracellular signal-regulated kinase expression, participating in the phosphatidylinositol 3-kinase/Akt pathway^[7-9].

The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. Non-dividing cells are not considered to be in the cell cycle. Severe defects in chromosomes block progression through the cell cycle, and can lead to cell suicide or apoptosis. In addition, cells have a finite lifespan, and at some point are no longer able to divide. Deguelin usually acts on different stages of the cell cycle of tumor cells and plays an antitumor role, and this may be one of its pharmacological mechanisms. In this report, Daudi cells were arrested in the G_0/G_1 phase mainly by deguelin, meanwhile the proportion of S phase was gradually reduced. After a different dose of deguelin for 24 h, cells in G_0/G_1 phase were most early influenced and increased gradually in a dose-dependent way with 40 nmol/L, and reached peak value. At the same time, cells in S phase were decreased gradually with 40 nmol/L resulting in the lowest value. The above data shows that deguelin usually regulates the G_1/S checkpoint. Deguelin regulating G_1/S check point was verified in other tumor cells. Chun *et al*^[4] found that after treatment with 1×10^{-9} mol/L deguelin on premalignant and malignant HBE cell, the rate of G_0/G_1 cells increased from 76.1% to 91.2%. At the same time, deguelin arrested premalignant and malignant HBE cells in G_2/M phase that increased from 9.6% to 40.2%.

Rapidly developing technology in the field of molecular biology enables estimation of control mechanisms of cancer cells at the molecular level. Much interest has focused on the proteins participating in cell cycle control. The sequential transcriptional activation of cyclins, the regulatory subunits of cell-cycle-specific kinases, are thought to regulate progress through the cell cycle^[10]. The orderly progression of dividing mammalian cells through the G_1 , S, G_2 , and M phases of the cell cycle is governed by a series of proteins called cyclins, which exert their effects by binding to and activating a series of specific cyclin-dependent kinases (CDK). Cyclins are therefore potential oncogenes. Cyclin D1 overexpression and/or amplification at its genomic locus, 11q13, are common features of several human cancers. The

cyclin D1 gene, which acts at the mid-portion of the G₁-S transition, is often overexpressed in human breast, colon, and squamous carcinomas, and several other types of cancer, and the cyclin E gene, which acts in late G₁ is also overexpressed and dysregulated in a variety of human cancers^[11]. In mechanistic studies we demonstrated that overexpression of cyclin D1 played a critical role in Burkitt lymphoma carcinogenesis. Therefore, cyclin D1 may be a useful biomarker in molecular epidemiology studies, and inhibitors of its function may be useful in both cancer chemo-prevention and therapy.

Retinoblastoma (Rb) is a rare tumor of the retina, associated with mutations of chromosome 13q14. The protein encoded by the Rb gene, pRb, normally plays a key role as a negative regulator of the G₁/S transition in the cell cycle by binding the transcription factor E₂F and preventing it from activating the transcription of genes required for the S phase^[12]. pRB gene product nuclear phosphoprotein undergoes differential phosphorylation during the cell cycle^[13]. A number of cellular proteins interact with hypophosphorylated pRb including: E₂F transcription factor, several cyclins, RBP-1, RBP-2, c-myc, N-myc, and p46. During G₁-phase, pRb is predominantly in a hypophosphorylated state and it becomes increasingly phosphorylated through the cell cycle until the end of mitosis when substantial dephosphorylation^[14-16]. To further investigate the role of cyclin genes in mammary tumorigenesis, we analyzed the expression of cyclins D1 and pRb in Daudi cells by Western blot. In this study, deguelin was shown to down-regulate the expression of cyclin D1 and pRb on Daudi cells in a dose-dependent way. This incident was closely related to arrest in G₀/G₁. This shows that deguelin can regulate the function of molecule cyclin D1 and control cell cycle, reduce the expression of pRb, and realize its regulation and control function of G₁/S checkpoints.

B-NHL plays an important role in blood system tumor. Though traditional chemotherapy and radiotherapy can achieve progress, the long-term result of treatment is not obvious^[17]. We chose Burkitt's lymphoma cell line Daudi cells as a research target. We found that deguelin could arrest Daudi cells in G₀/G₁ phase and induce apoptosis, and did not have any obvious poisonous function on the normal cell. As the source of deguelin is abundant and cheap, hopefully it will become a new-type of powerful antineoplastic medicine in treatment with B cells lymphoma.

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