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Deguelin inhibits expression of IκBα protein in Raji and U937 cells[1]

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Key words

deguelin; IκBα; nuclear factor kappa B; tumor necrosis factor-α; apoptosis; Raji cells; U937 cells

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Abstract

Aim: To determine whether deguelin can regulate the expression of nuclear factor kappa B (NF-κB) binding protein (IκBα) in U937 human leukemia cells and Raji human B lymphoma cells. **Methods:** The localization of IκBα protein was investigated by using an immunofluorescence method. The expression of IκBα and NF-κB /p65 proteins in Raji and U937 cells were investigated by using Western blotting. Apoptosis was detected through annexin V/PI double-labeled cytometry. **Results:** IκBα localized in the cytoplasm in untreated and deguelin-treated cells. After treatment with tumor necrosis factor α (TNF-α) or deguelin plus TNF-α for 15 min, there was a substantial reduction in the amount of IκBα protein. The expression of IκBα was downregulated by deguelin in Raji and U937 cells. Deguelin induced apoptosis in U937 cells. **Conclusion:** Deguelin inhibited the expression of IκBα protein in U937 and Raji cells. The anti-proliferative activity of deguelin is related to the signal pathway of NF-κB.

Introduction

Several natural compounds, especially plant products and dietary constituents, have been found to have chemopreventive activities in both *in vitro* and *in vivo* model systems[1]. Their mechanisms of action vary widely, with many suppressing cell growth and modulating cell differentiation, and a few inducing apoptosis. Deguelin, which is isolated from several plant species, including *Mundulea serice* (Leguminosae), has been shown to have cancer-chemopreventive effects in models of both skin and mammary tumorigenesis[2,3].

The transcription factor nuclear factor kappa B (NF-κB) has been identified as a critical component of several signal transduction pathways. One important function of NF-κB is its ability to protect cells from apoptosis[4]. NF-κB is a heterodimer comprising p50 and p65 subunits. It is sequestered in the cytoplasm by association with a binding protein known as Iκ Bα (NF-κB cytoplasmic inhibitor), which masks the nuclear localization signal of NF-κB. A variety of external or internal signals modify IκBα/NF-κB complexes by phosphorylating the serine residues of IκBα and affect the subsequent degradation of IκBα.

Raji human B lymphoma cells are resistant to nuclear apoptosis induced by various stimuli[5]. Like other mature B cells, Raji cells contain constitutive NF-κB-binding activity in the nucleus[6]. The U937 human monocytic leukemia cell line was derived from a patient with generalized "histiocytic" lymphoma. This cell line is a well-established model for studying the induction of apoptosis and differentiation[7]. We demonstrated previously that deguelin inhibited the proliferation of human Burkitt's lymphoma cells, such as Daudi cells, by regulating the expression of cyclin D1 and pRb protein[8].

IκBα, similar to cyclin D1, has been shown to be regulated by NF-κB. In the present study, we examined the effect of deguelin on the expression of the IκBα gene product by immunoblotting and immunofluorescence assay. We focus on changes in the expression of IκBα protein in Raji cells and U937 cells after deguelin treatment, and further explore the anticancer molecular mechanisms of deguelin *in vitro*.

Materials and methods

Drugs and reagents Deguelin (Sigma, St Louis, MO, USA) was initially dissolved in dimethylsulfoxide (Me_2SO,

<1 %), stored at -20 ºC and thawed before use. Tumor necrosis factor α (TNF-α) was purchased from PeproTechEC (London, UK; 2×10^7 U/mg). The Annexin V-FITC Detection Kit II was purchased from BD Biosciences Pharmingen (San Jose, CA, USA), and the RPMI-1640 medium and Me_2SO were purchased from Sigma. Fetal calf serum (FCS), anti-IκBα (SC-371), anti-p65 (SC-372) and horseradish peroxidase (HRP)-conjugated secondary antibodies (goat IgG-HRP, SC-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Chemiluminescence (ECL) reagent kits were purchased from Pierce Biotechnology (Rockford, IL, USA). The Raji and U937 cell lines were obtained from the China Center for Typical Culture Collection (Wuhan, China). All cell groups were grown in an RPMI-1640 culture medium containing 10% FCS and *L*-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 U/mL) at 37 ºC in a humidified atmosphere containing 5% CO_2.

MTT assay The antiproliferative effect of deguelin against different group cells was determined by using the MTT dye uptake method. Briefly, the final concentrations of deguelin were 5, 10, 20, 40 and 80 nmol/L. Each concentration of deguelin was added to 6 wells, respectively. The plates were in the presence or absence of the indicated test samples for 24 h. Thereafter, 20 μL MTT solution [5 mg/mL in phosphate-buffered saline (PBS)]was added to each well. After incubation for 4 h at 37 ºC, the supernatant was removed and 150 μL Me_2SO was added. When the blue crystals were dissolved, the optical density (*OD*) was detected in a microplate reader at a wavelength of 570 nm using a 96-well multiscanner autoreader (Biotech Instruments μQuant, NY, USA). The following formula was used: cell proliferation inhibited (%)=[1-(*OD* of the experimental samples/*OD* of the control)]×100% (n=6. Mean±SD).

Western blot analysis

Preparation of nuclear extracts for NF-kB The nuclear extracts were prepared according to the method described by Schreiber *et al*[9]. Briefly, 2×10^6 cells were washed with cold PBS and suspended in 0.4 mL hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 12.5 μL 10% Nonidet P-40. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts was stored at -80 ºC . The nuclear pellet was resuspended in 25 μL ice-cold nuclear extraction buffer. After 30 min with intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were obtained. The protein content was measured by using the Bradford method. If the nuclear extracts were not used immediately, they were stored at -80 ºC.

Preparation of whole-cell lysates Cells were harvested and lysed in 100 μL of lysis buffer by incubation on ice for 30 min, and the extracts were centrifuged at 18 000×*g* for 15 min to remove cell debris. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad). After the addition of 5×loading buffer, the samples were incubated at 95 ºC for 5 min and then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membranes and probed with anti-IκBα and anti-p65 antibody (at a 1:1500 dilution). The antigen-antibody complexes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies at a final dilution of 1:1500 in Western washing solution. After the mixture was washed 3 times with Tris-buffered saline, antibody binding was visualized by using enhanced chemiluminescence and autoradiography. Quantification of the bands was carried out using the Quantity One densitometric analysis software (Bio-Rad).

Indirect immunofluorescence U937 cells were fixed with 4% paraformaldehyde for 5 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. To block nonspecific antibody binding, slides were incubated with 3% milk/PBS at room temperature for 30 min. Then slides were incubated with rabbit polyclonal antihuman IκBα antibody (dilution, 1:50 in 1% milk/PBS). After overnight incubation, the slides were washed and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibodies (1:50 in 1% milk/PBS) at 37 ºC for 1 h. Finally, slides were washed 3 times for 5 min each with PBS and covered with glycerol (1:9 in PBS). Slides were examined by using an fluorescence microscope (Olympus, Tokyo, Japan). Photographs were taken with a camera, and images were created using a software package.

Annexin V/PI double-labeled cytometry For detection of apoptotic cells, expression of Annexin-V-FITC and exclusion of PI were simultaneously detected by using two-color flow cytometry. The U937 cells treated or untreated with deguelin (40 or 80 nmol/L) for 24 h were washed with PBS and resuspended in binding buffer containing 5 μL of FITC-labeled anti-Annexin-V antibody and 10 μL of 20 μg/mL PI. After incubation for 10 min at room temperature in a light protected area, all specimens were quantified on the FACS can (Becton Dickinson, CA, USA)

Statistical analysis All data are expressed as mean±SD, and were analyzed using SPSS 10.0 for Windows 98. The linear *t*-test was used for statistical analysis, and $P<0.05$ was considered to be statistically significant.

Results

Effects of deguelin on proliferation of U937 and Raji

cells U937 and Raji cells were treated with different concentrations of deguelin (5, 10, 20, 40 and 80 nmol/L) for 24 h, resulted in the inhibition of cell proliferation in a dose- dependent manner. The *OD* value of the deguelin-treated group was significantly lower than that of the untreated group (Figure 1). The IC_{50} values after 24 h treatment for the U937 cells and Raji cells were 21.61 and 45.37 nmol/L, respectively.

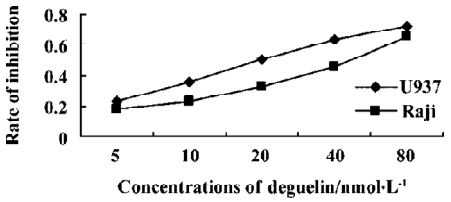

Figure 1. Antiproliferation effect of deguelin on U937 and Raji cells. U937 and Raji cells were treated with different concentrations of deguelin (5, 10, 20, 40, and 80 nmol/L) for 24 h. Growth inhibition was determined using an MTT assay and shown as an inhibitory rate. n=6. Mean±SD.

Effect of deguelin on IκB-α degradation in Raji cells IκBα protein level in Raji cells was reduced after 24 h treatment with deguelin at concentrations of 5, 10, 20, 40 and 80 nmol/L or after treatment with deguelin at a concentration of 40 nmol/L for 0, 2, 8, 12, 24, and 48 h. This indicates that deguelin promotes IκBα protein degradation in Raji cells in a concentration- and time-dependent manner (Figure 2A, 2B, Figure 4).

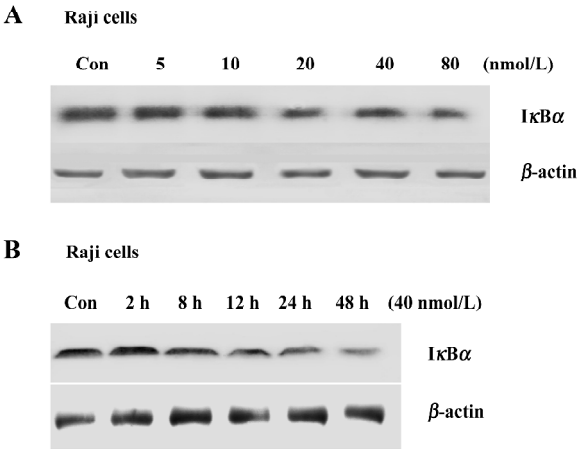

Figure 2. Effect of deguelin on IκBα degradation in Raji cells. Cells were treated with deguelin at concentrations of 5, 10, 20, 40 and 80 nmol/L for 24 h (A) or treated with deguelin at a concentration of 40 nmol/L for 0 (control), 2, 8, 12, 24, or 48 h (B). IκBα protein in whole cell lysates was prepared and 30–50 μg was subjected to 10% SDS-PAGE. Immunoblotting revealed IκBα to be a 37 kDa protein, and representative blots are shown here.

Effect of deguelin and TNF-α on IκBα degradation in U937 cells IκBα protein level in U937 cells was reduced after 24 h treatment with deguelin at concentrations of 5, 10, 20, 40 and 80 nmol/L or after treatment with deguelin at a concentration of 20 nmol/L for 0, 2, 8, 12, 24 and 48 h. These findings indicate that deguelin promotes IκBα protein degradation in U937 cells in a concentration- and time-dependent manner (Figure 3A, 3B, Figure 4). IκBα protein showed abrupt and complete depletion within 48 h after treatment with deguelin. On the other hand, IκBα protein showed only a partial decline in a dose-dependent manner compared with the control cells.

After treatment with TNF-α (10 ng/mL) or TNF-α (10 ng/

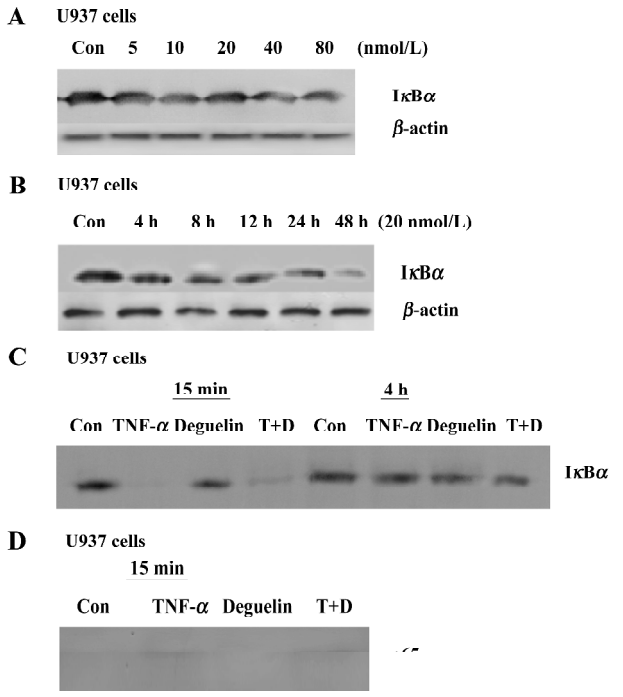

Figure 3. Effect of deguelin and TNF-α on IκBα degradation in U937 cells. (A) IκBα degradation after treatment with deguelin at concentrations of 5, 10, 20, 40 and 80 nmol/L for 24 h. (B) IκBα degradation after treatment with deguelin at a concentration of 20 nmol/L for 0, 2, 8, 12, 24, or 48 h. IκBα protein in whole cell lysates was prepared and 30–50 μg protein was subjected to 10% SDS-PAGE. (C) IκBα degradation after treatment with normal saline (NS), 20 nmol/L deguelin, and/or 10 ng/mL TNF-α for 15 min or 4 h. Cytoplasmic proteins were prepared, and 30 μg was subjected to 10% SDS-PAGE. IκBα was identified as a 37 kDa protein. (D) p65 level after NS, 20 nmol/L deguelin, and/or 10 ng/mL TNF-α treatment for 15 min. Nuclear extracts for NF-κB/p65 were prepared and 30–50 μg was subjected to 10% SDS-PAGE. Immunoblotting revealed p65 to be a 65 kDa protein, and representative blots are shown here. T, TNF-α; D, deguelin.

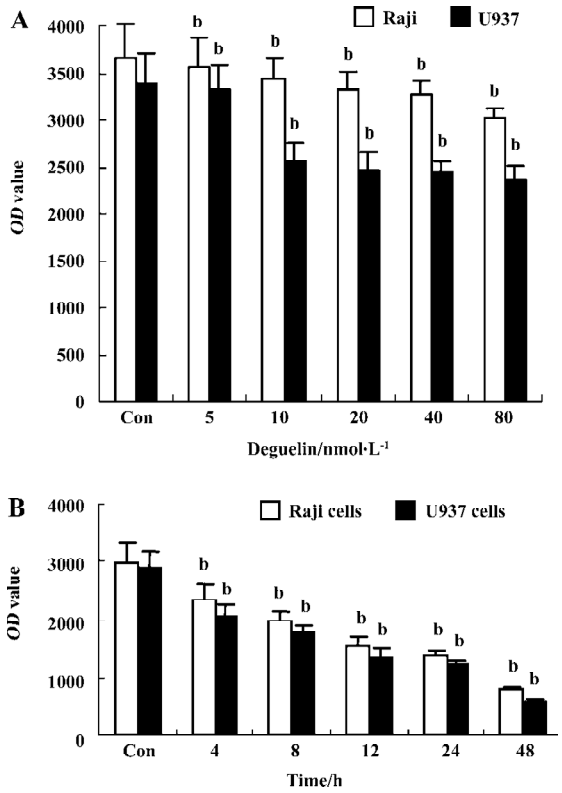

Figure 4. Concentration (A)- and time (B)-dependent effects of deguelin on expression of IκBα in Raji and U937 cells. n=3. Mean±SD. $^bP<0.05$ *vs* control.

mL) plus deguelin (20 nmol/L) for 15 min, the IκBα protein level was decreased. Nuclear extracts for NF-κB/p65 were prepared and imunoblotting revealed that there was p65 protein expression in the nucleus after treatment with TNF-α or TNF-α plus deguelin for 15 min, but no p65 protein was found in control and deguelin-treated groups (Figure 3C, 3D).

Effect of deguelin on the subcellular localization of IκBα in U937 cells After treatment with TNF-α (10 ng/mL) or deguelin (20 nmol/L) plus TNF-α (10 ng/mL) for 15 min, there was a substantial reduction in the amount of IκBα protein in the cytoplasm compared with controls. After treatment with deguelin 20 nmol/L for 4, 24, or 48 h, IκBα protein expression was gradually reduced in a time-dependent manner compared with control cells (Figure 5).

Effect of deguelin on apoptosis of U937 cells There was little binding of annexin V-FITC to untreated U937 cells (0.07%). Binding of annexin V-FITC was increased by 14.4% and 40.3% following treatment with deguelin at concentrations of 40 and 80 nmol/L for 24 h, respectively. The P value for the test for interaction was 0.003 (Figure 6).

Discussion

Activation of the NF-κB signaling pathway has been linked to resistance to chemotherapeutic drugs, and its downregulation, by means of NF-κB inhibitors, lowers re-

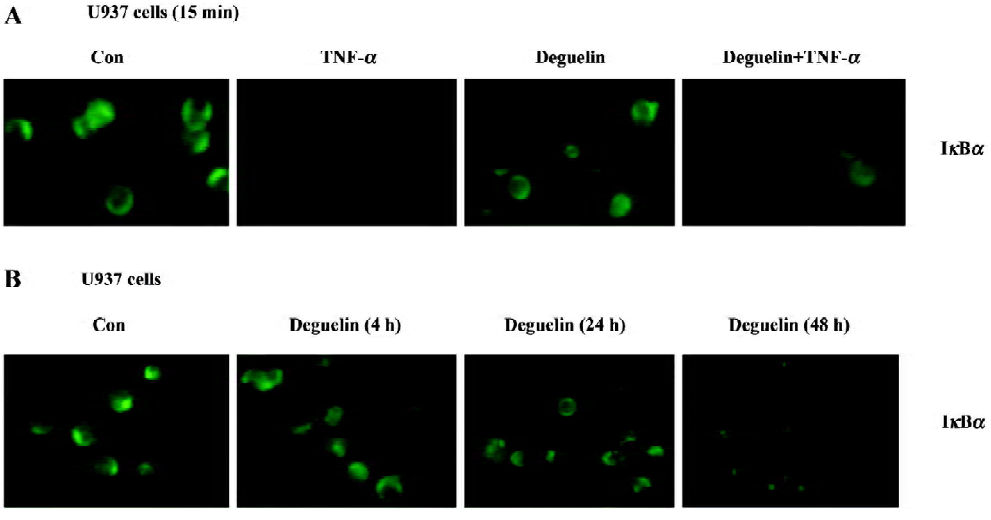

Figure5. Effect of deguelin and TNF-α on the subcellular localization of IκBα in U937 cells. (A) IκBα localizes in the cytoplasm in untreated and deguelin-treated cells. After treatment with TNF-α or deguelin plus TNF-α for 15 min, there were substantial reductions in the amount of IκBα protein. (B) After treatment with deguelin 20 nmol/L for 4, 24 or 48 h, IκBα protein expression was gradually reduced in a time-dependent manner compared with control cells. ×400.

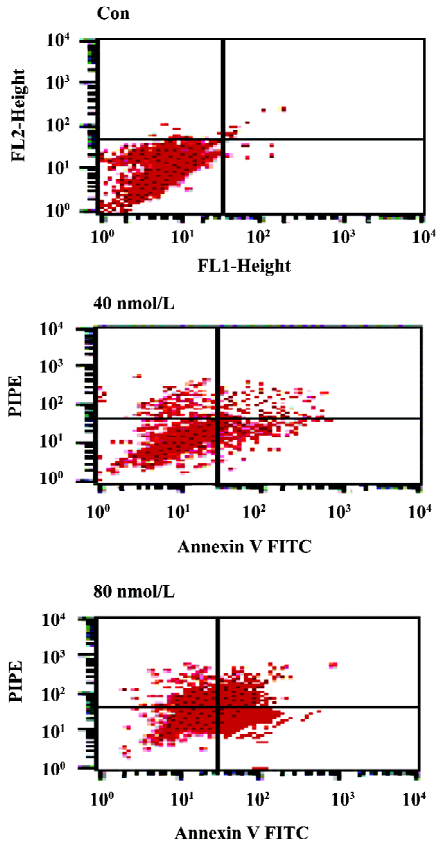

Figure 6. Deguelin-induced apoptosis in U937 cells. Cells were untreated or treated with deguelin (40 or 80 nmol/L) for 24 h.

sistance to various types of therapy in tumor cell lines. The IκBα protein is ubiquitously expressed as an NF-κB cytoplasmic inhibitor. In response to various stimuli, IκBα is rapidly degraded to allow NF-κB nuclear translocation and NF-κB target gene transcription. Because IκBα itself is the product of an NF-κB-regulated gene, it is rapidly resynthesized and can then migrate to the nucleus to terminate the first phase of NF-κB activation. Because the IκBα gene is an NF-κB target gene, it has been shown to be regulated by NF-κB. The expression of IκBα was downregulated in Raji and U937 cells treated with deguelin. Our results indicate that deguelin induced apoptosis in U937 cells and inhibited the expression of IκBα protein in U937 and Raji cells. The anti-proliferative activity of deguelin is related to the signaling pathway of NF-κB.

It has been reported that TNF-α induces IκBα degradation[10,11]. Our data also indicate that IκBα degradation is induced by treatment with TNF-α for 15 min in U937 cells. Recently, it has been reported that deguelin is a powerful inhibitor of PI3K[12]. Chun *et al* investigated whether deguelin could enhance sensitivity to chemotherapeutic drugs of human U937 leukemia cells and acute myeloid leukemia (AML) blasts with an activated PI3K/Akt network[12]. They found that deguelin might be useful in the future for increasing sensitivity to therapeutic treatments of leukemia cells with an active PI3K/Akt signaling network. Crowell *et al* found that deguelin decreased cancer-related increased AKT activity via the PI3K pathway in lung carcinogenesis[13]. Deguelin effectively inhibited AKT in premalignant cells. Because activated AKT can affect numerous cellular functions via intermediary molecules, including mTOR, NF-κB, and p53, which control cell survival, growth and proliferation, effective inhibition of PI3K/AKT by deguelin means it can affect the activation of NF-κB.

Although deguelin decreases the expression of IκBα, p65 is released for translocation from the cytoplasm into the nucleus, where it interacts with p50 and binds specifically to the NF-κB target DNA sequence. Following specific binding to DNA, transcriptional activation of NF-κB is regulated through specific phosphorylation of p65 at several distinct sites. At present, we do not know the precise mechanisms of transcriptional inhibition by deguelin in the nucleus. We hypothesize that deguelin inhibits IκBα protein expression through transcriptional inhibition of the IκBα gene, probably via NF-κB targets such as triptolide. Triptolide potently inhibits the expression of IκBα mRNA and protein, so more p65 is released for translocation into the nucleus and specific binding to DNA. Triptolide almost completely blocked TNF-α-induced NF-κB activation by inhibiting p65 transactivation, but not DNA binding. Curcumin is an upstream inhibitor of NF-κB and prevents the activation of inhibitory B (IκB) kinase (IKK), an enzyme required for the activation of NF-κB. Triptolide inhibits NF-κB activity further downstream by interfering with the NF-κB-mediated transcription process. We hypothesize that deguelin inhibits NF-κB activity also further downstream. So NF-κB would not be activated and thereby an increase in apoptosis was caused by deguelin.

Numerous studies in animals have demonstrated that deguelin has potent chemopreventive activity against a wide variety of different tumors. Deguelin has cancer chemopreventive effects in skin and mammary tumorigenesis models, and additional studies are warranted to characterize the cancer chemopreventive or chemotherapeutic potential of this substance more fully[14]. Deguelin inhibits the growth of colon cancer cells through the induction of apoptosis and cell cycle arrest. Deguelin inhibits the proliferation of human Burkitt's lymphoma cells by regulating the expression of cyclin D1 and pRb protein[8]. IκBα, Bcl-2, Bcl-x_L, interleukin-6, and cyclin D1 are all NF-κB target genes, and

are regulated by NF-κB[15,16].

Overall, our results indicate that deguelin can inhibit the expression of IκBα protein in U937 and Raji cells in a dose- and time-dependent manner. Deguelin might inhibit IκBα protein expression through transcriptional inhibition of the IκBα gene, but the precise mechanisms of transcriptional inhibition by deguelin are unknown. Our findings suggest that the anti-proliferative activity of deguelin is related to the signal pathway of NF-κB.

Acknowledgement

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