Deguelin exerts anticancer activity of human gastric cancer MGC-803 and MKN-45 cells *in vitro*

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Abstract. During the pathogenesis of gastric cancer, Akt signaling is considered as a pivotal inducer of gastric cancer development. Here we report the identification of anticancer activities of deguelin, a natural agent that inhibits Akt signaling. When applied to MGC-803 and MKN-45 cells, deguelin suppressed the proliferation and arrested cell cycle by p21-mediated inhibition of cyclin E. We further present *in vitro* evidence that deguelin promoted apoptosis of cancer cells by decreasing the phospho-Akt signaling and affecting expression of the apoptosis-associated genes Bax and Bcl-2. Additionally, deguelin was found to suppress the migration and invasion of gastric cancer cells. Taken together, these results indicated that deguelin exerted anticancer activity of human gastric cancer MGC-803 and MKN-45 cells *in vitro*.

Introduction

Gastric cancer is biologically aggressive and one of the leading causes of cancer-related deaths (1). Although multiple therapeutic modalities exist, including surgical excision, radiation therapy and chemotherapy, the clinical results remain unsatisfactory due to gastric cancer often being diagnosed in an advanced stage or metastatic stage (2). Hence, great research efforts have been explored to exploit novel molecular markers and detailed molecular mechanisms contributing to improving diagnostic and therapeutic management of gastric cancer.

The molecular basis of gastric cancer is complicated and involves the interactions between genetic and environmental factors. Among these etiologies, phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) pathway is believed to be important in gastric cancer development (3-5). Tian *et al* demonstrated that the expression of Akt and PI3K were remarkably higher in tumor tissue than that in normal tissue (3). In addition, Ye *et al* found that PI3K/Akt signaling pathway plays a crucial role in the formation and progression of gastric cancer (4). Simultaneously, phosphorylated-Akt expression significantly correlated with a poor prognosis (5). Therefore, PI3K/Akt pathway may represent a considerable therapeutic target for gastric cancer.

Deguelin, a natural component derived from leguminous plants, has been reported to prevent breast cancer (6), tobacco carcinogen-induced lung carcinogenesis (7), prostate cancer (8) and squamous cancer (9) by blocking Akt activation. Many studies have demonstrated that deguelin exerts its anticancer effect by inhibiting cell viability, cell growth, migration and invasion, inducing apoptosis, targeting cell cycle arrest and anti-angiogenesis (7,10,11).

Therefore, deguelin may provide an alternative potential approach for gastric cancer treatment. Here, we investigated that deguelin not only inhibited the proliferation, invasion, migration but also induced apoptosis in gastric cancer MGC-803 and MKN-45 cells *in vitro*.

Materials and methods

Cell culture and reagents. Human gastric cancer cell lines MGC-803 and MKN-45 were purchased from Shanghai institute of Cell Biology, Chinese Academy of Sciences and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) under a humidified 5% CO_2 atmosphere at 37˚C. Deguelin (MedChem Express, Monmouth Junction, NJ, USA) was dissolved in dimethyl sulfoxide (DMSO) at concentration of 10 mM as stock solution and stored at -20˚C. Before using, deguelin was diluted directly to the desired dose with an identical final concentration of DMSO.

Cell counting kit-8 (CCK-8) cell proliferation assay. CCK-8 (Dojindo, Kumamoto, Japan) assay was used to determine

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the inhibitory effect of deguelin on the proliferation of two cancer cell types. The MGC-803 ($3x10^3$/well) and MKN-45 ($5x10^3$/well) cells were plated in 96-well plates with 200 μl of medium. Before the cells were treated with various concentrations of deguelin (0, 1, 5, 10, 25 and 50 μM), they were starved in serum-free medium for 24 h to allow for cell synchronization, and then tested at 72 h. At the testing time-point, 10 μl of sterile CCK-8 solution was added to each well and incubated for an additional 1.5 h at 37˚C. The optical density values at 450 nm were measured with a microplate reader (Thermo Scientific, Waltham, MA, USA). The inhibitory rate was calculated using the following formula: Inhibitory rate (%) = (A_{450} of control cells-A_{450} of treated cells)/(A_{450} of control cells) x100%. The assays were repeated using six cell samples. In addition, for the cell viability test after treatment of deguelin, the MGC-803 ($1.5x10^3$/well) and MKN-45 ($2.5x10^3$/well) cells were plated in 96-well plates with the medium of 200 μl and incubated with various concentrations of deguelin (0, 1, 10 and 25 μM), then tested using CCK-8 assay at days 1, 2, 3 and 4.

Morphology observation. After treated with deguelin (0, 1, 10 and 25 μmol/l) for 48 h, MGC-803 and MKN-45 cells were observed under an inverted phase contrast microscope to determine their morphology changes.

Cell cycle analysis. After deguelin treatment for 48 h, cells were collected, fixed in 70% ethanol and incubated at 4˚C overnight. Cells were then centrifuged and stained in ice-cold phosphate-buffered saline (PBS) solution containing 100 μg/ml propidium iodide (PI), 0.1% Triton X-100, and 1 mg/ml RNase for 0.5 h. After washing, the samples were analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA).

Annexin V/FITC-PI assay. The apoptotic assays were performed by an Annexin V-FITC/PI apoptosis detection kit and detected as the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the 10^6 cells were washed using 500 μl binding buffer, centrifuged at 300 x g and stained with 10 μl Annexin V-FITC solution in room temperature for 30 min. Before testing, 5 μl PI solution was added to each sample. The apoptosis rate was evaluated by flow cytometry. At least 10,000 cells for each sample was analyzed. The analyses were repeated in three cell samples.

Real-time quantitative PCR. Total RNAs from control and deguelin-treated cells were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse translated with AMV reverse transcriptase (Promega, Madison, WI, USA). To synthesize complementary DNA, 2 μg total RNA per sample was added to the reaction system of a final volume of 20 μl containing 4 μl 5X buffer, 2 μl dNTP, 1 μl oligo(dT), 0.5 μl RNase inhibitor, 0.5 μl AMV reverse transcriptase and ddH_2O to meet the final volume. After incubated at 30˚C for 10 min, 45˚C for 60 min, 98˚C for 5 min and 5˚C for 5 min, the amplified complementary DNA (cDNA) products were mixed with Power SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Quantitative polymerase chain reaction (qPCR) was conducted using a real-time thermal cycler (Stratagene, La Jolla, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA content was used to normalize with cDNA, and the comparative Ct method was used to analyze data. The primers for real-time qPCR analysis are shown in Table I. Each assay was performed in triplicate and repeated in three cell samples.

Western blot analysis. Two different gastric cell lines were isolated after 6 h of incubation in the absence or different concentration of deguelin. Cells were scraped in RIPA lysis buffer and the protein content of cellular extracts were quantified by BCA assay. For western blot analysis, the prepared protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes and incubated with the appropriate antibodies. For estimation of p-Akt/total-Akt protein, we used two different specific antibodies: monoclonal anti-p-Akt and monoclonal anti-total-Akt (both from Cell Signaling Technology, Beverly, MA, USA). The blots were probed with β-actin antibody (Cell Signaling Technology) for equal loading control. The secondary antibody was goat anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive proteins were detected by the enhanced chemiluminescent (ECL) protocol (Amersham Biosciences, Piscataway, NJ, USA).

4',6-Diamidino-2-phenylindole (DAPI) staining. MGC-803 and MKN-45 cells were cultured as described, and then treated with various concentrations of deguelin for 48 h. Apoptosis was further determined by nucleus morphology using DAPI staining (Sigma-Aldrich, St. Louis, MO, USA). After being fixed and stained with DAPI, the cells were examined under a fluorescent microscope (Olympus, Tokyo, Japan).

Cell migration assay. The MGC-803 and MKN-45 cells at a density of $2x10^5$ cells/well were cultured in 6-well plates. When reaching 90% confluence, the cells were scratched by a sterile yellow 200 μl pipette tip and then washed three times with PBS. The cells were then placed in fresh RPMI-1640 medium containing 1% FBS with treatment of deguelin or DMSO for 24 h. Five random fields were selected and photographed with an inverted microscope, respectively.

Cell invasion assay. The ability of deguelin to inhibit cell invasion was tested using Transwell assay. The Transwell is in 24-well plate with an insert of 8-μm pore size polyethylene terephthalate membrane (Corning Life Sciences, Tewksbury, MA, USA). The cells were starved in serum-free medium overnight, then trypsinized and resuspended in serum-free medium. The cells at a density of $6x10^4$ cells/well were seeded in the upper Transwell chamber which was coated with Matrigel (BD Biosciences, Bedford, MA USA), then incubated with DMSO or deguelin (1 and 10 μM) for 24 h, and 500 μl of complete growth medium was added to the bottom. Cells on the upper membrane were wiped off with a cotton swab. Invaded cells on the lower membrane were fixed with 4% paraformaldehyde, stained with DAPI and three random fields were counted under a fluorescent inverted phase contrast microscope at x200 magnification.

Statistical analysis. All data were expressed as mean ± standard deviation (SD). Student's t test was used to analysis the

Table I. Primers used in quantitative PCR analysis.

Gene	Primer sequence (5'-3')	Annealing temperature (˚C)	Product size (bp)
Bax	Sense: TGCTTCAGGGTTTCATCCAG Antisense: GGCGCCAATCATCCTCTG	58	170
Bcl-2	Sense: AATCAAACAGAGGTCGCATGCTGG Antisense: TTGTGGCCTTCTTTGAGTTCGGTG	58	192
Cyclin E1	Sense: AGTGGCGTTTAAGTCCCCTG Antisense: CAGTTTTGAGCTCCCCGTCT	58	326
p21	Sense: TCCAGCGACCTTCCTCATCCAC Antisense: TCCATAGCCTCTACTGCCACCATC	58	108
GAPDH	Sense: TCACCATCTTCCAGGAGCG Antisense: CTGCTTCACCACCTTCTTGA	58	572

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

difference between the deguelin-treated and control groups. The analysis was conducted with the statistical software SPSS 22 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

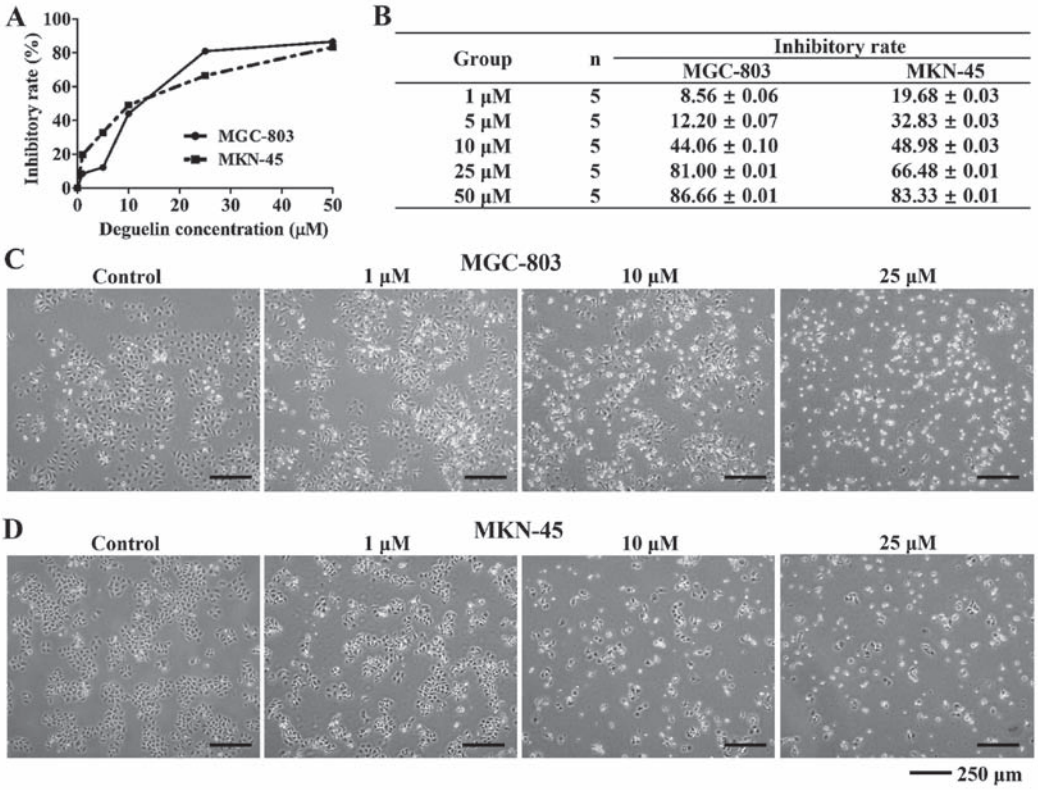
Results

Inhibitory effect of deguelin on the growth of MGC-803 and MKN-45 cells. CCK-8 assay showed inhibitory effect of deguelin on the growth of MGC-803 and MKN-45 cells. As shown in Fig. 1A and B, after various doses (1, 5, 10, 25 and 50 μM) of deguelin treatment on MGC-803 and MKN-45 cells for 72 h, the IR (inhibitory rate) of MGC-803 cells was 8.56±0.06, 12.20±0.07, 44.06±0.10, 81.00±0.01 and 86.66±0.01%, respectively and the IR of MKN-45 cells was 19.68±0.03, 32.83±0.03, 48.98±0.03, 66.48±0.01 and 83.33±0.01%, respectively. The IC_{50} (72 h) value for MGC-803 and MKN-45 was 11.83 and 9.33 μM, respectively. As the dose of deguelin increased, the IR of MGC-803 and MKN-45 cells improved gradually.

The effect of deguelin on the cell morphology. As the IC_{50} described above, doses of 1, 10 and 25 μM deguelin were chosen to perform the rest of the assays. As shown in Fig. 1C and D, after incubated with different concentrations of deguelin (1, 10 and 25 μM) for 48 h, the number of deguelin-treated cells was less than that of blank control cells. In contrast to the cells of control group which exhibited normal features with typical adherent, membrane intact morphology, deguelin treatment groups showed obvious apoptotic morphology with membrane distorted morphology with dose-dependent severity.

Deguelin inhibited cell proliferation and arrested the cell cycle of MGC-803 and MKN-45 cells. Deguelin treatment resulted in a dose- and time-dependent decrease in cell viability (Fig. 2A and B). To address whether the inhibitory effect of deguelin on the growth of cancer cells was accompanied by blocking the cell cycle, FCM assay was performed. Previous experiments suggested that a very high death rate occurred in 25 μM deguelin-treated cells and therefore we chose 1 and 10 μM deguelin treatment group to conduct the cell cycle assay. The assay indicated that deguelin altered the cell cycle distribution after 48 h treatment in both tested cell lines. For MGC-803 cells, deguelin induced cells cycle arrest at G_0/G_1 phase (Fig. 2C and D). Compared with control group (57.27±1.48%), the percentage of G_0/G_1 phase cells of deguelin treatment groups (67.53±1.72% at 1 μM, 72.1±4.10% at 10 μM) increased (P<0.05), and S and G_2/M phases decreased accordingly. For MKN-45 cells, low dose of deguelin induced cell cycle arrest at S phase, while high dose of deguelin induced cell cycle arrest at G_2/M phase (Fig. 2E and F). Compared with control group (22.53±1.11% at S phase, 11.60±0.90% at G_2/M phase), the percentage of S phase cells of low dose-treated group (34.27±1.31%) increased (P<0.05), and the percentage of G_2/M phase cells of high dose-treated group (32.93±2.44%) increased (P<0.01).

Deguelin promotes apoptotic effects on cultured MGC-803 and MKN-45 cells. Apoptosis was assessed in MGC-803 and MKN-45 cells after treatment with 1, 10 and 25 μM deguelin for 48 h. The early (10.87±0.46% at 1 μM, 20.20±3.21% at 10 μM and 52.70±4.88% at 25 μM), late (5.17±1.28 at 1 μM, 17.60±4.25% at 10 μM and 22.90±4.58% at 25 μM) and total (16.03±1.60% at 1 μM, 37.80±7.02% at 10 μM and 75.60±7.08% at 25 μM) apoptosis rates were increased significantly in MGC-803 cells compared with control (8.03±1.31% of early, 4.60±0.50% of late and 12.63±1.76% of total apoptosis rate) (Fig. 3A and B). Similarly, the early (19.20±0.79 at 1 μM, 39.13±4.01% at 10 μM and 62.63±2.00% at 25 μM), late (3.73±0.66 at 1 μM, 18.57±2.36% at 10 μM and 29.23±1.70% at 25 μM) and total (22.93±0.90 at 1 μM, 57.70±6.33% at 10 μM and 91.87±3.37% at 25 μM) apoptosis rates were also increased in MKN-45 cells compared with control (2.00±0.28% of early, 1.07±0.33% of late and 3.07±0.46% of total apoptosis rate) (Fig. 4A and B). This suggested that deguelin prominently induced apoptosis in gastric cancer cells. DAPI staining showed that many apoptotic bodies with condensed chromatin and apoptotic nucleus fragmentations were observed in



Group	n	Inhibitory rate	
		MGC-803	MKN-45
1 μM	5	8.56 ± 0.06	19.68 ± 0.03
5 μM	5	12.20 ± 0.07	32.83 ± 0.03
10 μM	5	44.06 ± 0.10	48.98 ± 0.03
25 μM	5	81.00 ± 0.01	66.48 ± 0.01
50 μM	5	86.66 ± 0.01	83.33 ± 0.01

Figure 1. Inhibitory rates of deguelin on the growth of MGC-803 and MKN-45. Human gastric cancer MGC-803 and MKN-45 cells were incubated with 0, 1, 5, 10, 25 and 50 μM of deguelin for 72 h, and then examined by cell counting kit-8 (CCK-8). (A and B) Effect of different concentration of deguelin on the inhibitory rate of MGC-803 and MKN-45. Deguelin induced cell morphological change and photographed by a phase-contrast microscope and decreased the total human gastric cancer (C) MGC-803 and (D) MKN-45 cells for 48 h. Data represent mean ± SD. Original magnification, x100. The bar represents 250 μm.

deguelin-treated cells (1 and 10 μM for 48 h), but almost none in untreated cells (Fig. 3C and 4C). Notably also, not only did deguelin reduce the proliferation of MGC-803 and MKN-45 cells, but also visibly induced apoptosis.

Relative gene and protein expression under the treatment of deguelin. Deguelin exerted its antitumor activity by inducing cell cycle arrest, apoptosis and inhibiting cell proliferation. Further experiments revealed the effect of deguelin on the apoptotic and proliferative gene expression. After treated *in vitro* with 1 and 10 μM deguelin for 48 h. Deguelin upregulated the gene expression of *Bax* (Fig. 5A and E) and downregulated that of *Bcl-2* (Fig. 5B and F) of MGC-803 and MKN-45 cells. The expression of *Bax* and *Bcl-2* in MGC-803 and MKN-45 cells showed significant difference from the control cells (P<0.05 for all) (Fig. 5A, B, E and F). The gene expression of *cyclin E1* was downregulated and that of *p21* was upregulated dramatically in a dose-dependent manner. The expression of *cyclin E1* and *p21* of MGC-803 and MKN-45 cells showed significant difference from the control cells (P<0.05 for all) (Fig. 5C, D, G and H).

Demonstration of deguelin-induced apoptosis of cancer cells by mediating Akt signal pathway. To test Akt inhibition by deguelin, MGC-803 and MKN-45 cells were treated *in vitro* with 1 and 10 μM deguelin for 6 h. Consistent with previous studies in other tumor cells (6,12,13), deguelin downregulated the expression of p-Akt (Fig. 5I). This result implied that deguelin induced apoptosis in gastric cells by downregulating Akt activity.

Deguelin inhibits the migration of MGC-803 and MKN-45 cells in vitro. To evaluate the effect of deguelin on a migration ability of MGC-803 and MKN-45 cells, wounded scratch assay was performed on both cell lines. The result showed that 24 h after scratching the cell migration of both cell lines was significantly inhibited by deguelin treatment (Fig. 6). MGC-803 and MKN-45 cells in the control group efficiently migrated into the scratched area (Fig. 6A and C). MGC-803 cells of the control group migrated 67.07±4.67% of the scratched area, whereas the 1 μM deguelin-treated MGC-803 cells migrated 37.12±1.53% of the area (P<0.05) and the 10 μM deguelin-treated MGC-803 cells migrated 31.69±0.81% of the area (P<0.05) (Fig. 6A and B). Blank control MKN-45 cells migrated 29.78±3.67% of the area, whereas the MKN-45 cells of the 1 μM and the 10 μM deguelin-treated group, respectively, migrated 11.14±1.09 and 3.96±1.03% of the area (P<0.05), and the migration rate was significantly lower in deguelin-treated group than that in blank control groups (P<0.05) (Fig. 6C and D).

Deguelin inhibits the invasion of MGC-803 and MKN-45 cells in vitro. Because the enhanced invasion properties of gastric cancer cells are the critical parameters in the development of gastric cancer, we wondered if deguelin would affect the cell behavior of MGC-803 and MKN-45 cells *in vitro*. Transwell chamber invasion assay results showed that, when compared with the control group (129.1±32.38), the invasion of the 1 μM deguelin-treated MGC-803 cells (36.89±9.67) and the 10 μM deguelin-treated MGC-803 cells (12.67±2.62) was significantly

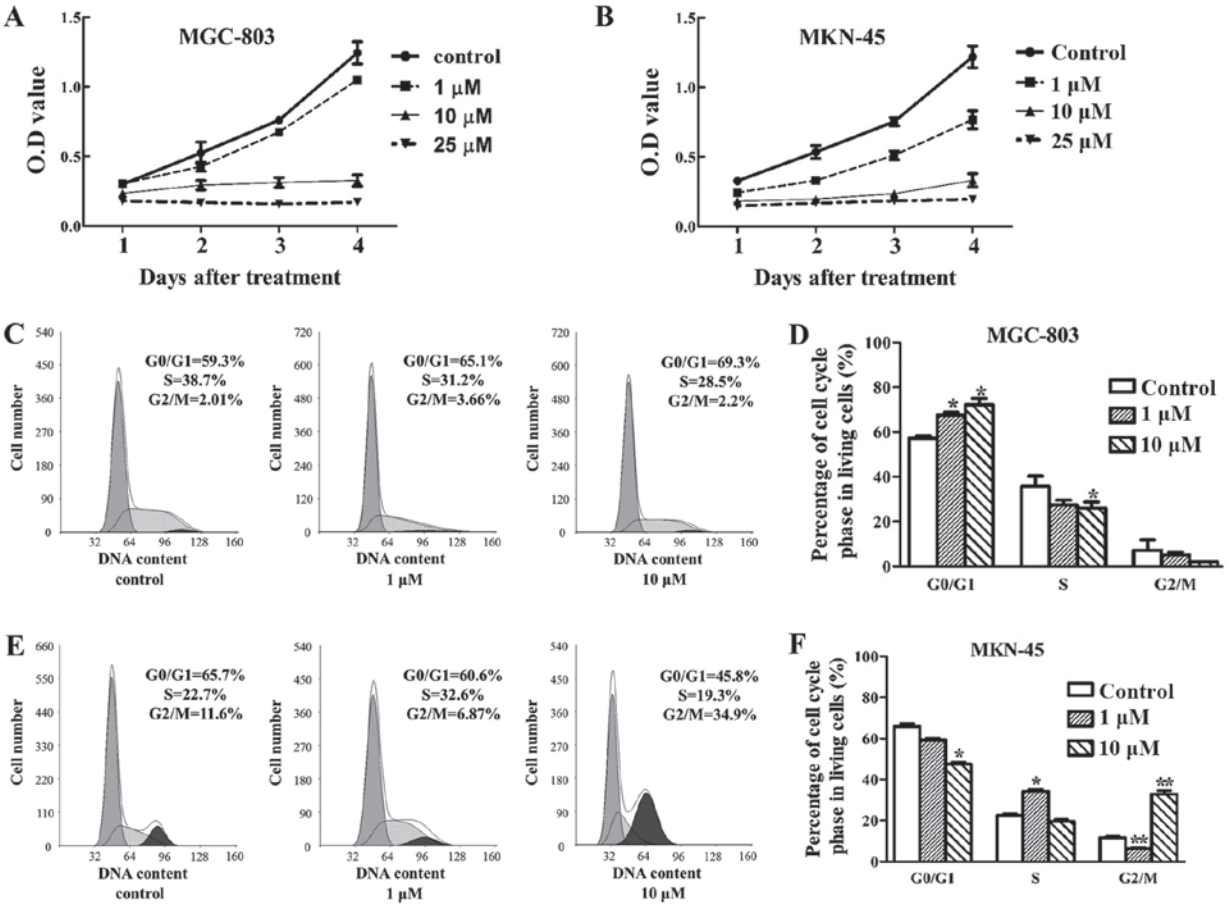


Figure 2. Treatment with deguelin inhibits gastric cancer cell proliferation. (A) The cell viabilities of (A) MGC-803 and (B) MKN-45 treated with or without deguelin (1, 10 and 25 μM) were measured with a cell counting kit-8 (CCK-8) assay at days 1, 2, 3 and 4. After treatment with deguelin for 48 h, gastric cell lines were subjected to flow cytometry analysis. (C and D) The effect of deguelin (1 and 10 μM) on the cell cycle profiles of MGC-803 cell lines was further evaluated. (E and F) The effect of deguelin (1 and 10 μM) on the cell cycle profiles of MKN-45 cell lines was further evaluated. All the experiments were repeated in three independent experiments. G_0/G_1, gap between end of M phase and start of S phase; S, DNA duplication phase; G_2, gap between end of S phase and start of M phase; M, mitosis. *P<0.05, significant difference between control group and the experiment group; **P<0.01, significant difference between control group and the experiment group.

inhibited in a dose dependent manner (P<0.05) (Fig. 7A and B). Deguelin (1 and 10 μM)-treated MKN-45 cells exhibited much lower invasion ability compared to the blank control as evidenced by decreasing the number of cells migrated through the Matrigel (Fig. 7C and D). The invasion ability of MKN-45 cells treated with deguelin was dose-dependently decreased compared to that of DMSO treated MKN-45 cells (13.90±6.76 at 1 μM and 5.60±1.85 at 10 μM). These results indicated that deguelin was able to inhibit invasive ability of gastric cancer cells.

Discussion

Gastric cancer is a highly mortal malignancy with few effective therapies. Resulted from both genetic and environmental risk factors, such as gene mutations, cigarette smoking, *helicobacter pylori* and intake of salty and smoked food (14), gastric cancer is a heterogeneous and multifactorial disease. Most patients with aggressive gastric cancer fail to respond to surgery and radiotherapy, but they are sensitive to systemic chemotherapy as palliative care (15,16). Therefore, the exploitation of potential alternative chemotherapy drug for gastric cancer is highly encouraging. Deguelin, a natural component of the flavonoid family products, has been used as a promising chemopreventive and therapeutic agent against various cancer cells (13,17,18).

Deguelin has been reported to inhibit the proliferation of different cancer cells, including breast cancer cells, prostate cancer cells and lung squamous cell cancer cells (6,8,9). This study revealed that proliferation of two different gastric cancer MGC-803 and MKN-45 cell lines were inhibited in a time- and dose-dependent manner by deguelin treatment (Fig. 1A and B). Some previous studies demonstrated the anti-proliferative effect of deguelin in different cancer cells was related to G_0/G_1 phase, S phase or G_2/M phase arrest (10,19,20). Murillo *et al* found that deguelin promoted cell cycle arrest at G_0/G_1 phase in colon cancer cells (10). Our observations were in accord with an overall efficacy of deguelin in inducing a G_0/G_1 arrest in MGC-803 cells (Fig. 2C and D). In another study, premalignant and malignant human HBE cells treated with deguelin were observed to arrest at G_2/M phase (7). Deguelin treatment of MKN-45 cells resulted in S phase arrest at lower dose but G_2/M phase arrest at higher dose (Fig. 2E and F). Indeed, more future studies are needed to identify the underlying mechanisms responsible for the action of deguelin to fully understand the seemingly puzzling role of this compound.

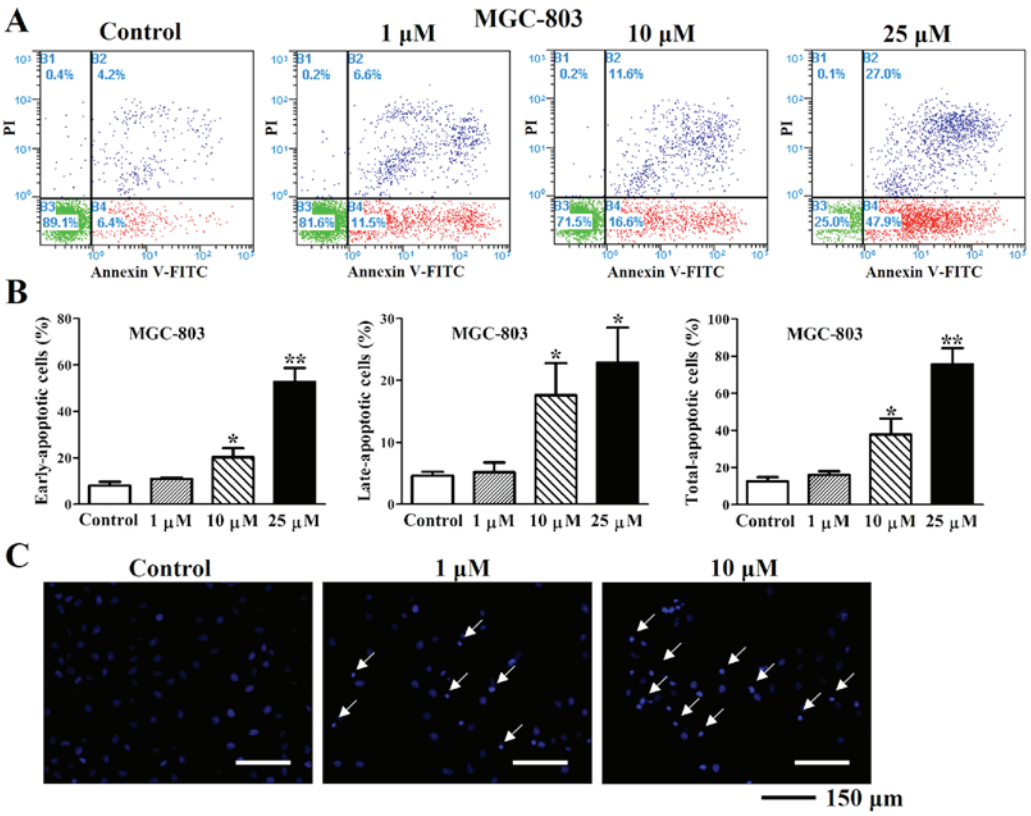


Figure 3. Deguelin triggered MGC-803 cell apoptosis. MGC-803 cells were exposed to complete medium containing indicated concentrations of deguelin (1, 10 and 25 μM) or control dimethyl sulfoxide (DMSO) for 48 h. (A) Histograms show the percentages of viable, early and late apoptotic cells. The lower left quadrant represents the viable cells, the lower right quadrant represents the early apoptotic cells and the upper right quadrant represents the late apoptotic cells. (B) Quantification of early, late and total apoptotic cells induced by deguelin. (C) The impact of deguelin on cell apoptosis was further determined by 4',6-diamidino-2-phenylindole (DAPI) staining. DAPI nuclear staining indicated chromatin condensation. Arrows represent cells exhibiting apoptosis. The bar represents 150 μm. *P<0.05 vs. the control cells. **P<0.01 vs. the control cells.

Abnormalities of cell cycle checkpoint regulators have been recognized as critical factors in the development of human cancers. Cyclin-dependent kinase (CDK) inhibitor p21 is a significant element in this regulatory cascade (21), and is shown to be associated with the prognosis of gastric cancer (22). p21 is a negative regulator of cell cycle progression (21). Overexpression of p21 has been identified as a crucial element resulting in cell cycle arrest at G_0/G_1, S and G_2/M phase (23). Radhakrishnan *et al* found that p21 was specifically associated with cyclin E, rather than cyclin D1, cyclin A, CDK4 or PCNA (23). Their finding are consistent with our results of increased expression of *p21* and decreased that of *cyclin E1* after deguelin treatment in gastric cancer cells (Fig. 5C, D, G and H). These results suggested that p21-mediated inhibition of cyclin E could be one of the factors that is responsible for the proliferation inhibition and cell cycle arrest of gastric cancer with deguelin treatment.

Deguelin induced apoptosis in a wide array of cancer cell types *in vitro*, including lung squamous cell carcinoma cells, colon cancer cells and head and neck squamous cell cancer cell lines (9,10,12). In the present study, we found that deguelin could induce apoptosis of gastric cancer MGC-803 and MKN-45 cell lines *in vitro* in a dose-dependent manner (Figs. 3 and 4), which is consistent with the effect of deguelin performed in other cancer cells (9,11,12). The induction effect of apoptosis could be mediated by the suppression of various molecular pathways, such as PI3K-Akt, IKK-IκBα-NF-κB, EMT, HSP90 and AMPK-mTOR-survivin pathways (11,24,25). Among all the target pathways suggested for deguelin, the PI3K/Akt pathways have the strongest support for inducing cell apoptosis (7,26-29). As shown in Fig. 5, compared to the control group, phosphorylation of Akt was dramatically decreased by treatment with deguelin in a dose-dependent manner in both cell lines, suggesting that Akt pathway was responsible for deguelin-induced cell apoptosis inhibition. To further corroborate the induced-apoptosis effects of deguelin, investigation on Akt and other pathways is still necessary.

In addition, based on the references of related literatures (38,39), we assumed that another mechanism of deguelin induced-apoptosis in gastric cancer cells may be related to the mitochondria-mediated (also called the intrinsic) apoptosis pathways. Mitochondria-dependent apoptosis is mediated by the proteins of Bcl-2 family (30,31), including antiapoptotic and proapoptotic proteins such as Bcl-2/xL and Bax/Bak (32-37). Bcl-2 family proteins change the mitochondrial membrane permeability required for the release of cytochrome *c* (38) (39,40). Bcl-2 blocks the release of cytochrome *c*, whereas Bax localizes to mitochondria and enhances the release of cytochrome *c*, promotes nuclear fragmentation and induces cell death (41). Therefore, we measured the gene expression of *Bcl-2* and *Bax* to clarify the probable mechanism. Gene expression study by RT-qPCR has shown that deguelin persuaded apoptosis through intrinsic pathway

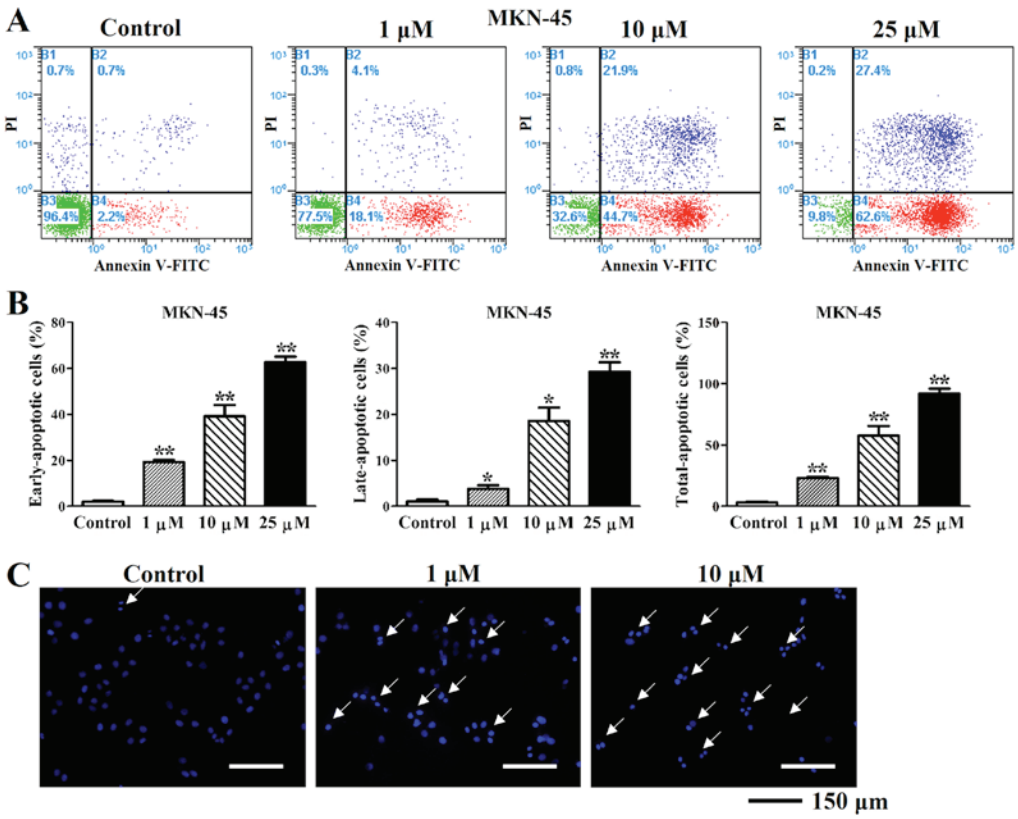


Figure 4. Deguelin triggers MKN-45 cells apoptosis. MKN-45 cells were exposed to complete medium containing indicated concentrations of deguelin (1, 10 and 25 μM) or control dimethyl sulfoxide (DMSO) for 48 h. (A) Histograms show the percentages of viable, early and late apoptotic cells. The lower left quadrant represents the viable cells, the lower right quadrant represents the early apoptotic cells and the upper right quadrant represents the late apoptotic cells. (B) Quantification of early, late and total apoptotic cells induced by deguelin. (C) The impact of deguelin on cell apoptosis was further determined by 4',6-diamidino-2-phenylindole (DAPI) staining. DAPI nuclear staining indicated chromatin condensation. Arrows represent cells exhibiting apoptosis. The bar represents 150 μm. *P<0.05 vs. the control cells; **P<0.01 vs. the control cells.

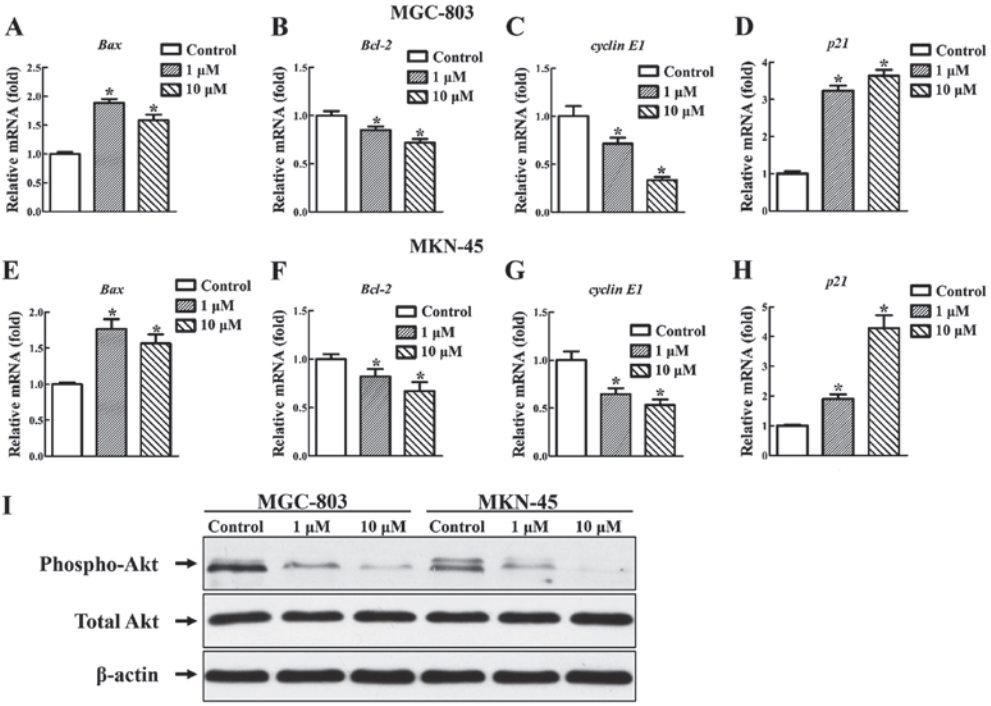


Figure 5. Relative gene and protein expression under the treatment of deguelin. After treatment with deguelin (1 and 10 μM) for 48 h, MGC-803 cells were subjected to real-time qPCR to detect the effects of deguelin on the gene expression levels of (A) *Bax*, (B) *Bcl-2*, (C) *cyclin E1* and (D) *p21*, and MKN-45 cells were subjected to real-time qPCR to detect the effects of deguelin on the gene expression levels of (E) *Bax*, (F) *Bcl-2*, (G) *cyclin E1* and (H) *p21*. (I) MGC-803 and MKN-45 cells were treated with increasing doses of deguelin (0, 1 and 10 μM) for 6 h and harvested for western blot (WB) analysis to assess the p-Akt signaling as indicated. *P<0.05 compared to dimethyl sulfoxide (DMSO) control of each cell line.

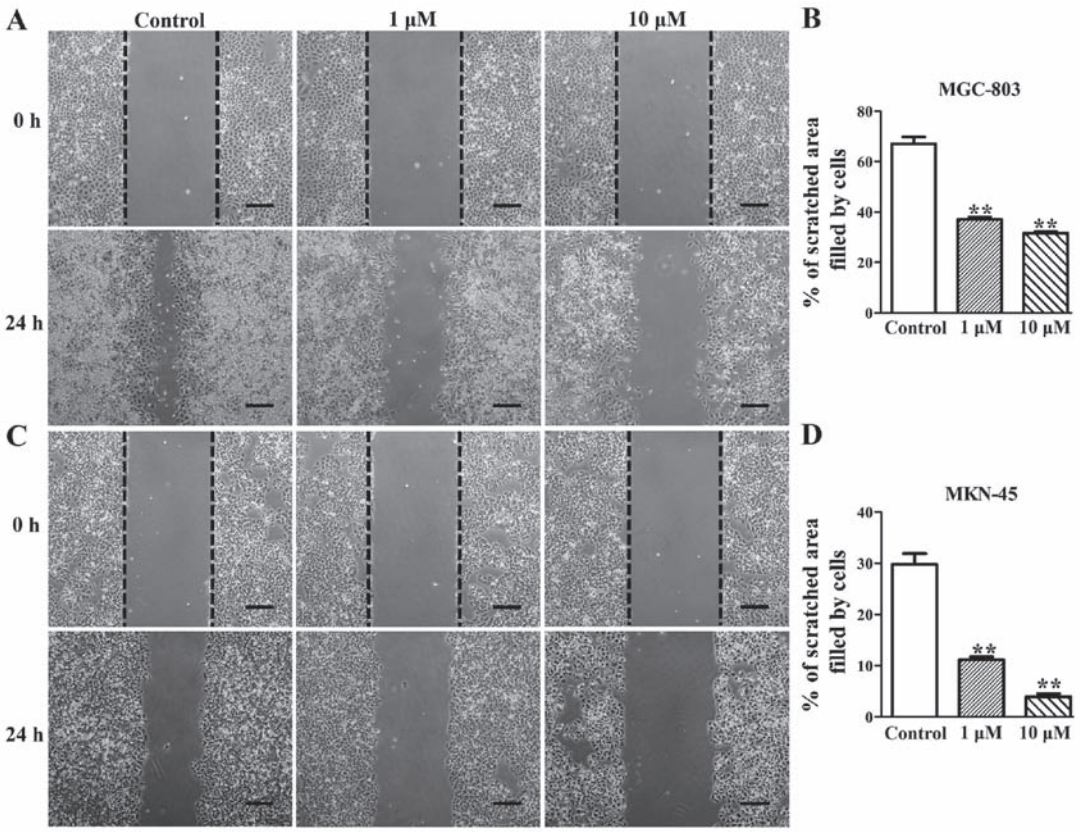


Figure 6. Deguelin inhibits the migration of MGC-803 and MKN-45 cells *in vitro*. Cells were manually scratched with a 200 μl pipette tip (black dash lines) and then incubated with or without deguelin for 24 h. (A and B) The scratched areas filled by migrated MGC-803 cells were observed at 24 h post-scratching and was quantified using IPP software. (C and D) The scratched areas filled by migrated MKN-45 cells were observed at 24 h post-scratching and was quantified using IPP software. The bar represents 250 μm. **P<0.01 compared to DMSO control of each cell line.

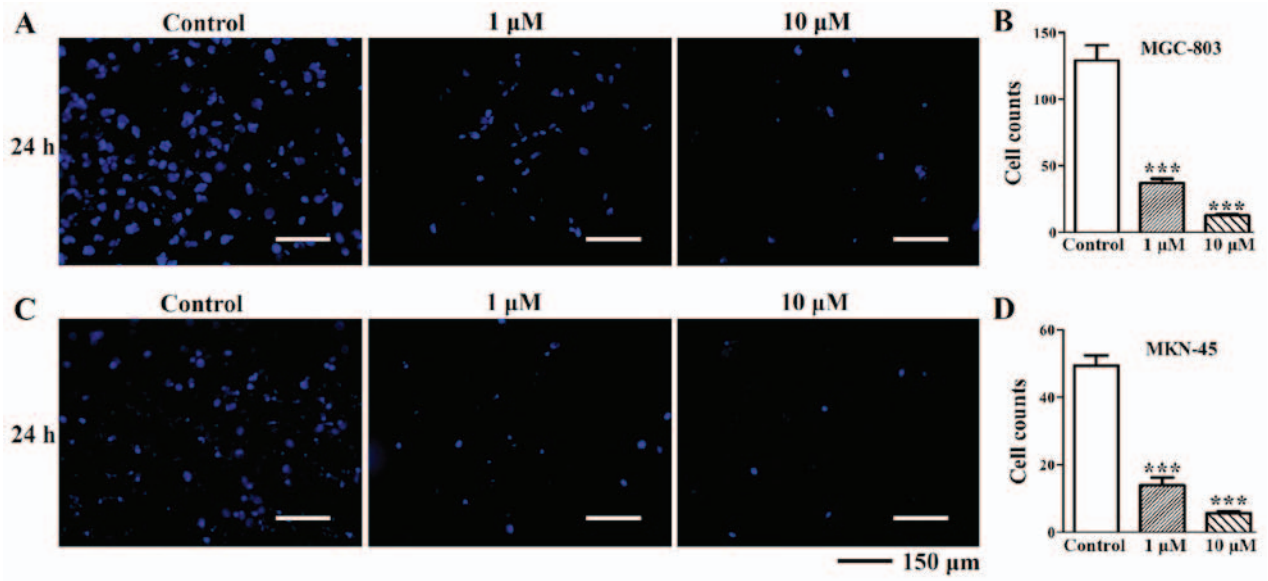


Figure 7. Deguelin inhibits the invasion of MGC-803 and MKN-45 cells *in vitro*. The MGC-803 and MKN-45 cells were treated with deguelin, and the inhibitory effect of deguelin on their invasion ability was evaluated by Transwell assay. (A and C) Representative images show the invasive cells after a 24-h deguelin treatment. The invasive cells were visualized by imaging the DAPI-labeled nuclei. (B and D) The invasive cell number of each cell line was quantified and counted in three random fields. All the experiments were repeated in three independent experiment. The bar represents 150 μm. ***P<0.001 compared to the dimethyl sulfoxide (DMSO) control.

by upregulating the pro-apoptotic gene *Bax* and down-regulating the anti-apoptotic gene *Bcl-2* in a dose-dependent manner (Fig. 5A, B, E and F). In conclusion, deguelin induced gastric cancer cell lines apoptosis via two pathways including the regulation of Bax/Bcl-2 ratio and inhibition of Akt activation.

The motility of gastric cancer cells plays a critical role in development of metastasis. A number of reported studies have demonstrated the effect of deguelin on the migration and invasion of different cancer types (25,42-44). Hu *et al* found that the treatment of deguelin inhibited lung cancer migration via downregulating Akt and the MAPK pathway (25). In another study, deguelin inhibited the migration and invasion of human osteosarcoma cells via the inhibition of MMP-2/9 *in vitro* by inhibiting the expression of GRB2, FAK and RhoA (44). However, the effects of deguelin on motility of gastric cancer MGC-803 and MKN-45 cell lines have not been reported. As shown in Figs. 6 and 7, the results showed that deguelin reduced cell migration and invasion of gastric cancer MGC-803 and MKN-45 cell lines *in vitro*. Therefore, deguelin would be a useful candidate to suppress cancer progression and metastasis. Based on the literature, deguelin suppressed the migration and invasion of cancer cells by complicated mechanisms such as reducing the expression and inactivation of FAK, RhoA-ROCK, MAPK and Akt. The mechanism that occurred in the effect of deguelin on the migration and invasion of gastric cancer cells need further investigation.

In conclusion, this study demonstrated that deguelin exhibited anticancer effect by inhibiting cell proliferation, causing cell cycle arrest, inducing apoptosis and suppressing cell migration and invasion of human gastric cancer *in vitro*. To provide a guide for further clinical trials, more studies on the precise mechanism of deguelin treatment of gastric cancer, however, are still needed. Since a number of reported animal studies have proved the antitumor activity of deguelin at doses of 3-5 mg/kg using xenograft models of different cancer types *in vivo* (24,43,45). These bionic models as well as our study warrant further *in vivo* investigations of deguelin efficacy. Collectively, deguelin may represent a promising anticancer agent capable of combating the progression and metastasis of gastric cancer.

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Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WK and XZ performed the experiments and wrote the manuscript. SG and PW designed the experimental plan and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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