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# Effects of Geldanamycin on Expression of Bcl-2 in Human Cervical Cancer HeLa Cells

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**OBJECTIVE** Geldanamycin, a natural product of *Streptomyces geldanus*, binds the heat shock protein 90 (Hsp90), a cell chaperone protein that interacts with Bcl-2. In this study, we investigated whether geldanamycin (GA) inhibits proliferation of HeLa cells through induction of apoptosis by decreasing the level of Bcl-2 expression.
**METHODS** HeLa cells, a human cervical cell line, were cultured in vitro and treated with different concentrations of GA (0, 0.02, 0.2, 2, 10 μmol/L) for 24 h. or were treated for different lengths of time at a GA concentration of 10 μmol/L. Proliferation of the cells was analyzed by an MTT assay, and cell apoptosis was determined by staining the cells with annexin V. In addition, cellular mRNA levels for Bcl-2 and Hsp90 were determined by the semi-quantitative polymerase chain reaction (PCR), and the levels of Bcl-2 and Hsp90 protein expression were determined by Western blots.
**RESULTS** Treatment of cells with GA was found to inhibit HeLa cell proliferation in a concentration and time-dependent manner. The inhibition was a result of increased cellular apoptotic levels. Further analyses showed that while the mRNA and protein expression levels of Hsp90 were not affected, GA treatment significantly reduced the level of Bcl-2 mRNA and protein expression in a concentration-dependent manner that correlated with the observed inhibition of cell proliferation.
**CONCLUSION** GA can inhibit proliferation and increase apoptosis of HeLa cells by decreasing the transcription and expression of an anti-apoptotic gene bcl-2, probably through interaction and functional inhibition of Hsp90.

**KEY WORDS: cervical carcinoma, geldanamycin, prolification, apoptosis, Hsp90, Bcl-2.**



## Introduction

Globally, cervical cancer (CC) is the second most common cause of cancer-related mortality among women, causing approximately 40,000 and 234,000 deaths annually in developed nations and developing countries respectively. Most of these deaths occur in women with large or locally advanced cervical cancer. The conventional medical treatment of CC is combined therapy including surgery, radiotherapy, and chemotherapy. An early manifestation of CC is vaginal hemorrhage, that is often ignored by patients. CC is therefore often diagnosed at an advanced stage, when potentially curative surgical or combined therapy becomes less feasible[1,2].

New tests and treatment methods for CC are under development with the aim to reduce the incidence rate and death from this disease. As our understanding of the genetics and molecular biology of cancer has increased, there has been a shift over the last decade in

the approaches used in the discovery of novel cancer therapeutics[3]. In contrast to development of cytotoxic agents in early studies, focus has moved to the development of treatments that target the molecular pathways responsible for cell malignancy. The majority of cancers involve multiple molecular abnormalities that are likely to be involved in malignant cellular progression. These new findings have reinforced the idea that inhibition of multiple targets will be required to cure most human cancers[4]. It is this consideration that drives the increasing amount of interest in targeting the heat shock protein 90 (Hsp90) molecular chaperone[5] for cancer treatment.

Hsp90 is over-expressed in many cancers and exerts its chaperone function to ensure the correct conformation, activity, intracellular localization, and proteolytic turnover of a range of proteins that are involved in cell growth, differentiation and survival[6]. Of particular importance, Hsp90 is essential for the stability and the function of many oncogenic-client proteins, which contribute to the hallmark traits of cancer. Inhibition of Hsp90 function has been shown to cause degradation of client proteins via the ubiquitin-proteasome pathway[5-7], which results in the simultaneous depletion of multiple oncoproteins, the combined down-regulation of signals propagated through numerous oncogenic-signaling pathways, and modulation of all aspects of the malignant phenotype[5,8]. The ability to deliver a combined effect through a single drug target may have promise in treating cancers driven by multiple molecular abnormalities and could also reduce the opportunity for resistance development following treatment[3,5].

Geldanamycin (GA) is a natural benzoquinone ansamycin product of *Streptomyces geldanus* that binds the protein chaperone, Hsp90[9]. Several studies have suggested that Hsp90 is connected with cell apoptosis. However, whether there is a direct interaction between Hsp90 and Bcl-2 in regulation of cell apoptosis is still not established. As GA binds to Hsp90, interfering with its function, we investigated our hypothesis that treatment with GA may inhibit the function of Hsp90 and interfere with the expression of Bcl-2, resulting in apoptosis.

## Materials and Methods

### *Cell culture and reagents*

The HeLa cell line was kindly provided by National Key Laboratory of Experimental Hematology, Institute of Hematology, CAMS and PUMC (Tianjin, China). The HeLa cells were cultured in RPMI 1640 (Gibco BRL, Rockville, MD) with 10% fetal calf serum (FCS) plus penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were cultured in an incubator with a humidified atmosphere containing 5% $CO_2$ at 37℃ and divided upon reaching confluence. GA was obtained from Alexis (Lausen, CH, Switzerland) and diluted in dimethyl sulfoxide (DMSO) for all experiments. The MTT assay kit was purchased from Sigma (St. Louis, MO, USA).

### *Inhibition of cell growth*

The growth inhibition rate of cells following GA treatment was determined by the tetrazolium dye assay (MTT). After trypsinization, the HeLa cells were counted and seeded into 96-well flat-bottomed plates (Costar, Charlotte, NC) at $1 \times 10^5$/well in serum-free RPMI 1640 and incubated at 37℃ for 24 h. Serial dilutions of GA dissolved in 20 μl DMSO (prepared as described above) were added to the wells in triplicate giving a final volume of 200 μl. Cells in control wells received 20 μl DMSO with no GA. The cells were then incubated for an additional 24 h.

In addition, another series of HeLa cells were treated with 10 μmol/L GA for different lengths of time, ie., 0, 3, 12, 24, 48, 72 h. GA was added to the wells every 24 h. At the end of incubation, 20 μl MTT (5 mg/ml in PBS) was added to each well. After another 4 h of incubation, the plates were centrifuged (400 ×*g* for 10 min) to pellet the cells, the supernatant removed, and 100 μl DMSO added to the wells. After stirring for 5 min the optical density was monitored in an ELISA reader at 546 nm (Model A-5082, SLT Lab Instruments. Grodig, Austria). Cell growth inhibition rate was calculated as-follows: (OD in untreated well – OD in treated well)/OD in untreated well × 100%.

### *Detection of cell apoptosis*

Cell apoptosis was detected by an Annexin V–fluorescein-isothiocyanate/propidiumiodide (FITC/PI) double-stain assay employing an Annexin V-FITC Apoptosis Detection Kit I (BD, San Diego, USA) flowing the manufacturer's protocol. Briefly untreated cells or cells treated with different concentrations of GA were washed with cold PBS and re-suspended in 1× binding buffer (0.01 M HEPES, pH 7.4, 0. 14 M NaCl, 2.5 mM $CaCl_2$) at a concentration of $1 \times 10^6$ cells/ml. The cells were then transferred (100 μl, $1 \times 10^5$ cells) to a 5 ml culture tube followed by addition of FITC-conjugated Annexin V (5 μl) and PI (2 μl). After incubation for 15 min at room temperature in the dark, additional 400 μl of 1× binding buffer was added to each tube and the cells were immediately analyzed on a flow cytometer. Apoptotic cells were defined as $FITC^+/PI^-$ cells. Gated cells were then plotted for annexin V-FITC and PI in a 2-way dot plot to assess the percentage of apoptotic cells.

### *RT-PCR analysis of Bcl-2 and Hsp90 mRNA*

Cultured HeLa cells were treated with different concentrations of GA for 24 h, and the total cellular RNA was extracted. Two μg of total RNA from each sample was used for cDNA synthesis using the SuperScriptTM III First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The synthesized cDNAs were subjected to PCR

analysis using 30 cycles. Each cycle consisted of 60 s at 94℃ for denaturation, 60 s at 55℃ (Bcl-2), 53℃ (Hsp90), 55℃ (GAPDH) for annealing, and 60 s at 72 ℃ for extension. The PCR products were stained with ethidium bromide after agarose ge1 electrophoresis and photographed with GAPDH serving as the control. Integrated density values (IDV of these mRNA levels were normalized with respect to GAPDH gene expression with Tanon Image Note software ( Scion, Frederick, MD). The actual sequences of specific primers are summarized as follow[10,11]. Bcl-2 (F: 5'-TGC ACC TGA CGC CCT TCA C; R: 5'-AGA CAG CCA GGA GAA ATC AAA CAG); Hsp90 (F: 5'-TGG CAG TCA AGC ACT TTTC TGT; R:5'-TGA TGA ACA CAC GGC GGA); GAPDH (F: 5'-TGA AGG TCG GAG TCA ACG GAT TTG G; R: 5'-CAT GTG GGC CAT GAG GTC CAC CAC).

***Protein extraction and Western blotting***

HeLa cells were stimulated with different concentrations of GA for 24 h. After stimulation, the cells were lysed in cold RIPA buffer (50 mM Tris, with 150 mM sodium chloride, 1.0% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) plus a cocktail of protease inhibitors (1 mM phenylmethyl sulfonylfluoride (PMSF), 5 mM dithiothreitol (DTT), 1 μg/ml aprotinin, and 1 μg/ml leupeptin). After centrifugation to remove cell debris, the protein level in each sample was determined using a BCATM Protein Assay Kit (Pierce, Rock ford, USA) with bovine serum albumin (BSA) standards. Supernatants (a total protein amount of 100 ng) were mixed with protein gel loading buffer and were separated by 12% SDS-PAGE (polyacrylamide gel electrophoresis). The separated proteins were subsequently blotted onto a nitrocellulose membrane with a BioRad 200/2.0 semi-dry blotter. The membranes were blocked in 5% defatted milk powder in PBST for 1 h at room temperature and then incubated with the indicated primary antibodies (rabbit polyclonal anti-human Hsp90 (Cellsignal), mouse monoclonal anti-human Bcl-2 (Santa Cruz, California, USA), or mouse monoclonal anti-human GAPDH (Santa Cruz, California, USA) respectively) prepared in PBST buffer (at a concentration of 0.5 μg/ml) at 4℃ overnight. Blots were developed by incubation with secondary antibodies (anti-rabbit IgG-HRP for Hsp90 and anti-mouse IgG-HRP for Bcl-2 and GAPDH respectively, 1:5000 dilution) for 1 h at room temperature. The enhanced chemiluminescence (ECL) detection system(Pierce, Rockford, USA) and ECL film were used to visualize the presence of proteins on the nitrocellulose membrane, and the intensity of protein bands was quantified by densitometry using the Tanon Image note software.

***Statistical analysis***

To detect differences between data sets, the *t*-test (2-tailed) was applied. $P < 0.05$ was considered statistically significant.

To detect differences between data sets, specifically for cell survival (MTT and annexin V) data, a Student *t*-test (2-tailed) was applied in which $P < 0.05$ was considered statistically significant. RT-PCR and Western blot results are shown as the ratio between densitometry units (band intensity) in experimental conditions versus controls. These values were calculated from 3 independent experiments. A ratio of 1 (along the dotted line) indicates the experimental groups had results identical to the controls.

## Results

***Inhibition of HeLa cell proliferation by GA***

As shown in Fig.1A, GA inhibited growth of the HeLa cells in a concentration-dependent manner, even at the lowest level tested. Fig.1B shows that the inhibitiory effect of of 10 μmol/L GA increased with time, and reached a plateau at 24 h. As determined by the MTT assay, most of the Hela cells died in the presence of 10 μmol/L GA, over a 72-h period.

***Effects of GA treatment on HeLa cell apoptosis***

The Annexin V-(FITC/PI) double-stain assay revealed different levels of apoptosis in HeLa cells after 24 h of treatment by increasing concentrations of GA. The results showed that GA treatment induced HeLa cell apoptosis in a concentration-dependent manner. As seen in Fig.2, when cultured in the presence of 10 μmol/L of GA for 24 h, there was nearly a 9-fold increase in apoptosis compared to the controls (5.83 *vs.* 0.66).

***RT-PCR analysis of Bcl-2/Hsp90 mRNA***

RT-PCR was used to examine if changes in mRNA were related to GA-induced HeLa cell apoptosis. The mRNA expression level of Bcl-2 was markedly down-regulated by GA treatment in a concentration-dependent manner (Fig.3). On the other hand, treatment with GA had no effect on the level of Hsp90 mRNA. The results shown are representative from 3 independent experiments.

***Western blotting analysis of Bcl-2 /Hsp90 proteins***

Western blots was used to determine the expression level of related proteins in cells after treatment by GA. The results showed that the inhibitory effects on Bcl-2 expression in HeLa cells were dependent on the concentration of GA. Furthermore, it was also observed that GA had no effect on Hsp90 expression (Fig.4). Similar results were obtained in 3 independent experiments.

## Discussion

Hsp90, as a molecular chaperone, plays an important role in cell viability, and is synthesized in response to various stresses, including carcinogenesis. The chap-

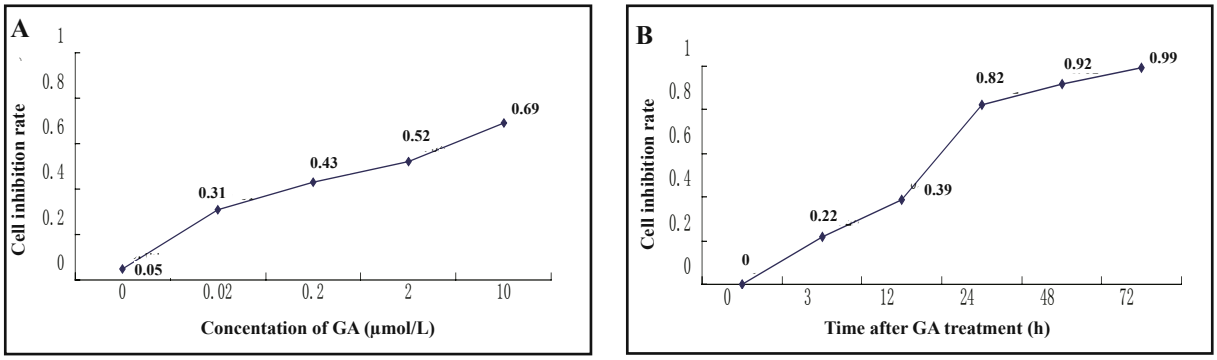


**Fig.1. Inhibitory effects of GA on HeLa cells.** A, GA inhibited cellular proliferation in a concentration-dependent manner (lowest level 0.02 μmol/L). B, The inhibition of proliferation by GA increases with time. After 3 h of GA treatment inhibitory effects were obvious, reaching a plateau at 24 h. By 72 h nearly all cells had died.

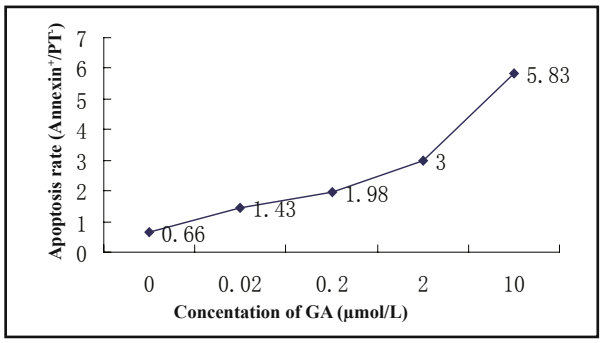


**Fig.2. Apoptosis induced by GA in HeLa cells at 24 h of treatment.** GA promoted the apoptosis in a concentration-dependent manner, reaching a peak at 10 μmol/L.

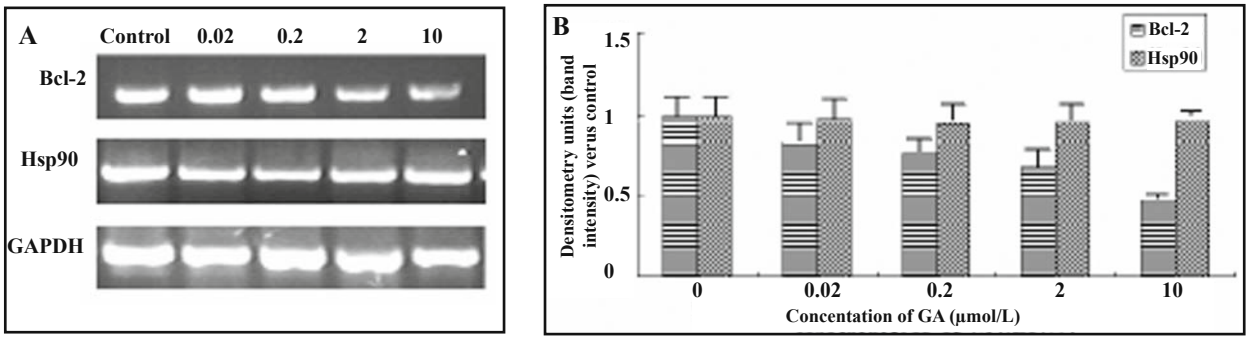


**Fig.3. GA inhibition of Bcl-2 mRNA expression in HeLa cells.** A, GA inhibited Bcl-2 mRNA expression, but had no effect on Hsp90 mRNA expression. Total RNA extracts were obtained from HeLa cells, which were cultured with μmol/L levels of GA in serum-free RPMI for 24 h. B, RT-PCR results were quantified by densitometry and are shown as the ratio between densitometry values in treated versus control cells. Each experiment was repeated 3 times.

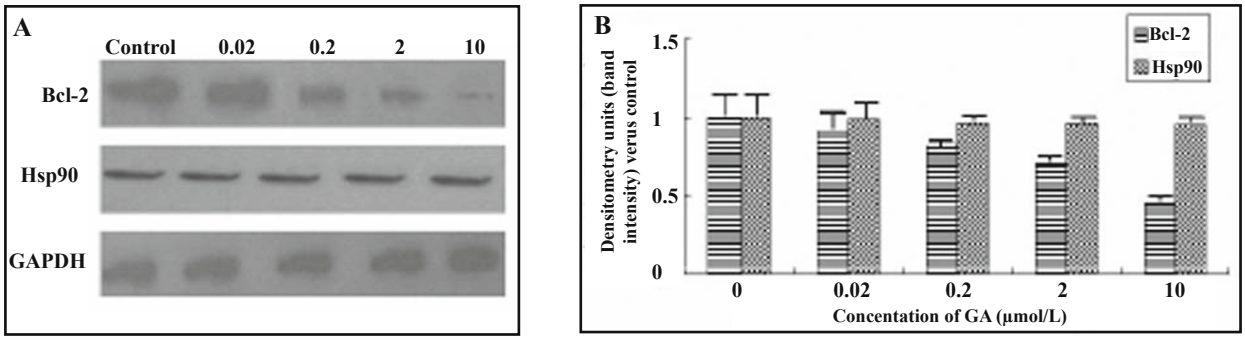


**Fig.4. GA inhibited Bcl-2 protein expression in HeLa cells.** A, GA treatment inhibited Bcl-2 protein expression in HeLa cells but had no effect on Hsp90 protein expression. B, Western blot analysis results were quantified by densitometry and are shown as the ratio between densitometry values in treated versus control cells. Each experiment was repeated 3 times.

erone activity of Hsp90 depends on its transient N-terminal dimerization, which activates its intrinsic and essential ATPase activity[12]. It is known that the natural product, GA, exerts its anti-tumor effect by binding to the N-terminal ATPase domain of Hsp90 to inhibit its chaperone function[13-15]. Therefore, GA has become an important tool to study the function of Hsp90. It has been shown that Hsp90 can stabilize several cellular proteins thereby preventing their degradation. Hsp90 may prevent cell apoptosis by stabilizing the RIP-1 anti-apoptotic protein 14 and binding to APAF-1, preventing its oligomerization[11].

Bcl-2 is an anti-apoptotic protein that is involved in multiple apoptotic pathways and acts some what downstream in the apoptotic cascade. In previous studies, Hsp90 has been reported to associate with Bcl-2 proteins. Therefore, in the present study, to find the molecular pathways by which Hsp90 promotes cervical carcinoma cell survival and apoptosis, we used HeLa cells to investigate the effect of GA, as GA is a known Hsp90 inhibitor. Our results showed that GA inhibited growth of HeLa cells. Furthermore, we performed early apoptotic analysis, and carried out analysis of Bcl-2 and Hsp90 expression in GA treated and untreated HeLa cells.

The Hela cells were treated with GA over a range of concentrations for different lengths of time, and survival of cells was analyzed by an MTT assay. It was found that inhibition of cell growth by GA treatment was concentration and time-dependent. We thus chose 24 h treatment for further analysis. Previous studies had shown that GA plays an important role in regulating Hsp90-induced cell apoptosis in vitro[16]. In our study, we found that GA treatment promotes apoptosis of HeLa cells, in agreement with results from previous studies.

By employing the RT-PCR and Western blot analyses, we assessed the level of expression of Bcl-2 after treatment of the cells with GA. As shown above, Bcl-2 expression in cells was down-regulated by GA treatment. Here, we showed that the expression of Bcl-2 can be decreased by treating cells with the Hsp90-specific antibiotic, GA. However, GA had no effect on Hsp90 expression. These results suggest that the effect of GA on the HeLa cell growth was due to inhibition of the molecular chaperone function of Hsp90, but not the reduction of Hsp90 expression. GA may competitively bind to the Hsp90 N-terminus and block its interaction with other client proteins, including Bcl-2. Because Hsp90 stabilizes different proteins and prevents their cellular degradation, our results indicated that Hsp90 may fail to bind Bcl-2 in the presence of GA, and as a result, Bcl-2 is rapidly degraded causing cell apoptosis.

The results from this study further suggest that Hsp90 is involved in the process of tumor formation, and the potential anti-tumor effects of GA may lead to its use in clininal trials for treatment of a variety of malignancies. Furthermore, these results suggest that tumors may be suppressed through multiple approaches involving tumor-gene therapy.

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