

Original Article

Effect of betulinic acid on the regulation of Hiwi and cyclin B1 in human gastric adenocarcinoma AGS cells

Li-jing YANG^{1, #}, Yan CHEN^{1, #}, Qi MA², Jun FANG¹, Jing HE¹, Yi-quan CHENG¹, Qiu-ling WU^{1, *}

¹Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China; ²Vascular Biology Institute, University of Miami, Miller School of Medicine, Miami, Florida 33136, USA

Aim: To investigate the effect of betulinic acid (BA) on the proliferation, apoptosis and cell cycle of gastric adenocarcinoma cell AGS *in vitro* and elucidate the underlying mechanisms.

Methods: The effect of BA on the proliferation of AGS cells was measured by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) assay. Apoptosis was analyzed by using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-labeled flow cytometry (FCM) and Hoechst 33258 staining. The influence of BA on cell cycle of AGS cells was tested by PI staining. Both FCM and reverse transcription-PCR (RT-PCR) technologies were applied to detect the expression of Hiwi and Cyclin B1.

Results: BA exhibited significant cell proliferation inhibition, as well as its potency of inducing apoptosis in AGS cells *in vitro* in a time- and dose-dependent manner. The IC₅₀ value for 24 h was 18.25 µg/mL (95% confidence interval: 15.16 to 27.31 µg/mL). Cells treated with BA showed increased cell population in G₂/M phase, with decreased S phase population. The expression of Hiwi and Cyclin B1 was down-regulated in BA-treated AGS cells in a dose-dependent manner.

Conclusion: BA exerted potent effect on growth inhibition, G₂/M cell cycle arrest and induction of apoptosis in AGS cells *in vitro*, possibly associated with the down-regulation of Hiwi and its downstream target Cyclin B1 expression. The potent antitumor capacity of BA suggested that it could be a promising new experimental anticancer agent in human gastric adenocarcinoma treatment.

Keywords: betulinic acid; gastric adenocarcinoma AGS cells; Hiwi; cyclin B1; cell cycle; apoptosis

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Introduction

Betulinic acid (BA), isolated from birch trees, is a natural pentacyclic triterpene which has demonstrated anti-HIV, anti-HBV and anti-malarial abilities^[1–3]. In addition, BA is believed to play an important role in delaying or preventing carcinogenesis. It was proved to be able to kill melanoma cells via induction of apoptosis^[4]. Many other cancer cell lines derived from a variety of different malignancies such as leukemia, prostate, ovarian, breast, lung, and colon cancers were also found to be sensitive to apoptosis induced by BA^[5–7]. BA did not induce apoptosis in normal cells. And in animal studies, no adverse effect was observed at concentrations up to 100 mg/kg body weight^[8]. However, whether BA can inhibit the proliferation of human gastric adenocarcinoma AGS cell remains unknown.

The cytotoxic mechanism of BA, which is linked to its alter-

ation in cell apoptosis and cell cycle, is controversial. Treatment with BA resulted in up-regulation of the pro-apoptotic Bax in neuroblastoma, glioblastoma and melanoma cells, whereas anti-apoptotic Bcl-XS was found at elevated levels in BA-treated neuroblastoma cells^[9–11]. An increase in Bcl-2 protein levels was reported in glioblastoma cells^[10]. BA also triggered up regulation of Mcl-1, another anti-apoptotic Bcl-2 family protein, in melanoma cells, whereas no changes in Mcl-1 levels were detected in squamous cell carcinoma cells^[11–13]. While BA was found to reduce the expression of p21 protein in melanoma cells, an increase of p21 protein was observed upon treatment with BA in glioblastoma cells^[10, 14]. Therefore how BA-mediated cell cycle changes are linked to its antitumor activity remains to be addressed. Since bcl-X_L, p21, Cyclin D1, and Cdc2-Cyclin B complex expression modulated by BA are also regulated by Hiwi^[15–17], it is very possible that BA exerts its antitumor effect through directly affecting a major target Hiwi and then indirectly impacting other factors.

The Piwi family comprises Hili, Hiwi1, Hiwi2 and Hiwi3 in humans, Mili, Miwi and Miwi2 in mice and Piwi, Aubergine

The two authors contributed equally to this work.

* To whom correspondence should be addressed.

E-mail wuqiling@medmail.com.cn

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(Aub) and Ago3 in flies. This family is united via a common domain structure – PAZ and PIWI – and a common molecular mechanism: short, guide-strand-mediated target recognition and, in the majority of cases, a capacity for target slicing. Genetic and biochemical studies first linked Piwi proteins to signaling processes that affect cellular differentiation and development^[18]. In addition, numerous groups have shown that Piwi proteins function as part of the RNAi machinery to mediate posttranscriptional gene, chromatin silencing and cell cycle regulation^[17, 19]. Moreover, Piwi proteins are proved to serve as effector molecules that provide specificity in gene-silencing pathways^[20]. Dicer processed micro-RNAs or small interfering RNAs (siRNA) bind Piwi proteins to form ribonucleoprotein complexes termed RNA induced silencing complexes (RISCs), which are thought to mediate mRNA degradation, translational repression, or chromatin silencing in a homology-dependent manner^[20]. It has been estimated that Piwi protein-dependent RNAi controls the expression of more than 30% of genes in the human genome^[20].

It has recently been observed that seminoma tumors are often associated with increased levels of Hiwi mRNA^[21]. Coincidentally, Hiwi was first reported as a gene that is expressed in undifferentiated haematopoietic stem cells, but not in differentiated cells^[22]. Likewise, the expression of the *D melanogaster* orthologue of Hiwi, Piwi, had previously been reported to promote mitosis in stem cells^[18]. In addition, the chromosomal locus 12q24.33 that includes the Hiwi gene has been linked to testicular germ cell tumors^[23]. Conversely, deletion of this region is associated with hypogonadism^[24]. Together, these observations are consistent with a scenario in which overexpression of certain Piwi proteins is associated with increased mitosis in undifferentiated cells. So Hiwi plays a critical core role in the carcinogenesis and can be served as a new therapeutic target for human cancer. Mechanisms regulating Hiwi may provide novel opportunities for anti-tumour drug development.

In this study, we examined the efficiency of BA on gastric adenocarcinoma AGS cell proliferation and apoptosis, and explored its relationship with the regulation of Hiwi and Cyclin B1 gene expression. We demonstrated that BA down-regulated the expression of Hiwi and its downstream targets Cyclin B1 in AGS cells, accompanied by apoptosis induction, cell cycle arrest and proliferation inhibition.

Materials and methods

Reagents and cell cultures

BA (C₃₀H₄₈O₃, molecular weight 456.7), from the Alexis Co, (USA), was initially dissolved in 100% demethylsulfoxide (DMSO), stored at -20 °C, and thawed before use. Dulbecco's modified Eagle's medium (DMEM) was from Gibco Co, (USA). Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co, Ltd (China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT), DMSO, propidium iodide (PI), Hoechst 33258, RNase A, and Ficoll-Hypaque were from Sigma Co, (USA). Annexin V-fluorescein isothiocyanate (FITC)/PI reagent Kit was from Nanjing Key-

Gen Biotech Co, Ltd (China). Trizol reagent kit was from Invitrogen Co (USA). Reverse transcription-PCR (RT-PCR) reagent kit was from TOYOBO Co (Japan). Primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co, Ltd (China). Anti-Hiwi antibody was from Abcam Co (UK). Anti-Cyclin B1 antibody and FITC-labeled secondary antibody were from Santa Cruz Biotech Co (USA). Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation. AGS cells were a present from Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and placed in a humidified incubator with 95% air and 5% CO₂ at 37 °C.

MTT assay

The effect of BA on proliferation of AGS cells was examined using a MTT assay. AGS cells were maintained in DMEM medium until mid-log phase and plated at a density of 1×10⁴ cells per well in 96-well plate in triplicates. Cells were allowed to attach overnight, then incubated with various concentrations of BA for 24 h, 36 h and 48 h, respectively. PBMCs were plated at a density of 5000 cells per well in 96-well plate as control cells, followed by adding BA immediately. The final concentrations of BA were 9, 12, 15, 18 and 21 µg/mL, while the group dissolved with maximal DMSO concentration served as the control. After incubation, 20 µL MTT solution (5 mg/mL) was added and the cells were further incubated at 37 °C for 4 h. The supernatant was discarded and 150 µL DMSO was added. The plate was gently shaken until the blue crystals were dissolved. Absorbance (A) at 570 nm wavelength was read using a 96-well multiscanner autoreader (Biotech Instruments, NY, USA). Relative cell proliferation was calculated with the following formula: cell proliferation inhibition of = [1 - (A of experimental samples / A of the control)] × 100%.

Annexin V-FITC/PI double-labeled flow cytometry

For detection of apoptotic cells treated with BA, the expression of Annexin V-FITC and exclusion of PI were detected by two-color flow cytometry (FCM). Cells incubated with different concentrations of BA (9, 12, 15 and 18 µg/mL) for 24 h were collected and washed with PBS, then resuspended in 100 µL binding buffer. Samples were incubated with 5 µL Annexin V-FITC in dark for 10 min at 4 °C, then volume was adjusted to 500 µL with binding buffer. PI (5 µL) was added and samples were incubated for another 10 min at 4 °C. Fluorescence was measured with a flow cytometer (Becton Dickinson, USA).

Hoechst 33258 staining

Nuclear fragmentation of AGS cells treated with 15 µg/mL BA was visualized by Hoechst 33258 staining. AGS cells were plated in 6-well plates at the density of 1×10⁵ cells. After being allowed to attach overnight, cells were treated with 15 µg/mL BA for 24 h and collected by trypsin digestion method. Cells were fixed in 4% paraformaldehyde for 10 min at room tem-

perature and re-suspended in 50 μL PBS before deposition on poly lysine-coated cover slips. After 30 min, the adhered cell were permeabilized with 0.1% Triton X-100 for 5 min at 4 $^{\circ}\text{C}$ and incubated with Hoechst 33258 for 30 min at room temperature. Cover slips were rinsed with PBS, mounted on slides with glycerol, and imaged with an Olympus BH-2 fluorescence microscope (Tokyo, Japan).

Cell cycle analysis

AGS cells treated with BA at various concentrations were collected, washed with PBS, and then suspended in 70% ethanol at 4 $^{\circ}\text{C}$ overnight. Cells were incubated with 20 μL 0.1% RNase A for 15 min at room temperature, and incubated with 50 $\mu\text{g}/\text{mL}$ PI for 15 min. DNA content was examined by using FCM and the cell cycle analysis was performed using CellQuest software (Becton Dickinson, USA).

Detection of the expression of Hiwi and Cyclin B1 protein

AGS cells at 1×10^6 treated respectively with 0, 9, 12, 15 and 18 $\mu\text{g}/\text{mL}$ BA for 24 h were collected, washed with PBS, and then fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells were resuspended in PBS containing 1% heat-inactivated FBS and incubated for 1 h, then incubated with anti-Hiwi or anti-Cyclin B1 antibody (1:100) at 4 $^{\circ}\text{C}$ overnight. Cells were washed with PBS containing 1% heat-inactivated FBS and incubated with FITC-labeled secondary antibody (1:100) at 37 $^{\circ}\text{C}$ for 30 min. Samples were analyzed with a FACSsort (Becton Dickinson, USA). In each test, 1×10^4 cells were collected and the mean fluorescence intensity represented the expression level of protein in the BA-treated AGS cells.

Reverse transcriptional RCR

2×10^5 AGS cells per well in 6-well plates were incubated with 0, 9, 12, 15, 18 and 21 $\mu\text{g}/\text{mL}$ BA for 24 h, respectively. Cells were lysed with Trizol reagent and total RNA was extracted. cDNA was synthesized according to the manufacturer's instruction of TOYOBO kit. The primers were as follows: Hiwi (Genebank accession N_Q AF104260.2), 5'-TCTGTT-GTCAAGTAATCGGAAGG-3', 5'-AGACTTTGAGCCCA-TCTACCAG-3', the PCR products were 359 bp; Cyclin B1 (Genebank accession N_Q NM_031966.2), 5'-GCCTATTTGGT-TGATACTGC-3', 5'-ATCTGTCTGATTGGTGCTTAGT-3', the PCR products were 501 bp; β -actin (Genebank accession N_Q NM_001101.3), 5'-CIGTCCCTGTATGCCTCTG-3', 5'-ATGTCACGCACGATTTC-3', the PCR products were 218 bp. The following PCR conditions were used: 94 $^{\circ}\text{C}$ for 30 s; 94 $^{\circ}\text{C}$ for 30 s, 57 $^{\circ}\text{C}$ (Hiwi) or 53 $^{\circ}\text{C}$ (β -actin and Cyclin B1) for 30 s, 72 $^{\circ}\text{C}$ for 1 min, 30 cycles; 74 $^{\circ}\text{C}$ for 10 min; 4 $^{\circ}\text{C}$ for 30 min. After amplification, 5 μL aliquots of products were electrophoresed on 1.7% agarose gel. DNA bands were quantified using Smart View Bio-electrophoresis Image Analysis System. The ratio between Hiwi (or Cyclin B1) and β -actin band density represented the relative expression level of the target gene.

Statistical analysis

All experiments were repeated three times. All data were expressed as mean \pm SD and processed by SPSS 13.0 statistical software for Window. One-way ANOVA and Student-Newman-Keuls (SNK) test were applied for comparison between each group and $P < 0.05$ was considered to be statistically significant.

Results

Effect of BA on proliferation of AGS cells

The cytotoxicity of BA to AGS cells at 0, 9, 12, 15, 18 and 21 $\mu\text{g}/\text{mL}$ for various time was measured using MTT assay. AGS cells were treated with various concentrations BA for 0, 24, 36 and 48 h, respectively, which resulted in a significant decrease of cell viability of AGS cells in a dose- and time-dependent manner (Figure 1). The IC₅₀ of BA to AGS cells for 24, 36 and 48 h were 18.25 $\mu\text{g}/\text{mL}$ (95% confidence interval: 15.16 to 27.31 $\mu\text{g}/\text{mL}$), 15.86 $\mu\text{g}/\text{mL}$ (95% confidence interval: 12.18 to 21.17 $\mu\text{g}/\text{mL}$) and 12.99 $\mu\text{g}/\text{mL}$ (95% confidence interval: 8.64 to 16.04 $\mu\text{g}/\text{mL}$), respectively. After being treated with 21 $\mu\text{g}/\text{mL}$ BA for 24 h, AGS cells inhibition ratio was 63.2% \pm 3.4%, while PBMCs inhibition ratio was 10.1% \pm 2.8% ($P < 0.05$).

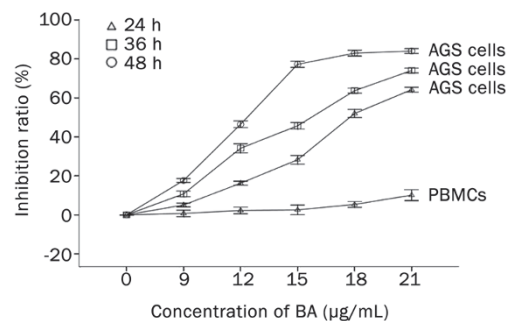


Figure 1. Inhibition of proliferation by BA in AGS cells. Cells were treated *in vitro* with various concentrations of BA (9, 12, 15, 18 and 21 $\mu\text{g}/\text{mL}$) for 24, 36 and 48 h, respectively, while PBMCs treated with BA for 24 h were served as control. Growth inhibition was detected by MTT assay and shown as an inhibition ratio. Data were mean \pm SD of three independent experiments.

Effect of BA on apoptosis of AGS cells

Apoptosis rate of AGS cells treated with various concentrations of BA (0, 9, 12, 15 and 18 $\mu\text{g}/\text{mL}$) for 24 h was analyzed by Annexin V-FITC/PI double-labeled flow cytometry. The degree of early apoptosis was quantitatively expressed as a percentage of the Annexin V-FITC-positive but PI-negative cells, while late apoptosis was quantitatively expressed as a percentage of the Annexin V-FITC-positive and PI-positive cells. The apoptosis rate was the sum of early apoptosis and late apoptosis. There was little binding of Annexin V-FITC in untreated and 9 $\mu\text{g}/\text{mL}$ BA treated AGS cells (Figure 2). However, after treatment of cells with BA at 12, 15 and 18 $\mu\text{g}/\text{mL}$ for 24 h, the apoptosis rate was 9.90% \pm 0.44%, 29.13% \pm 1.57%

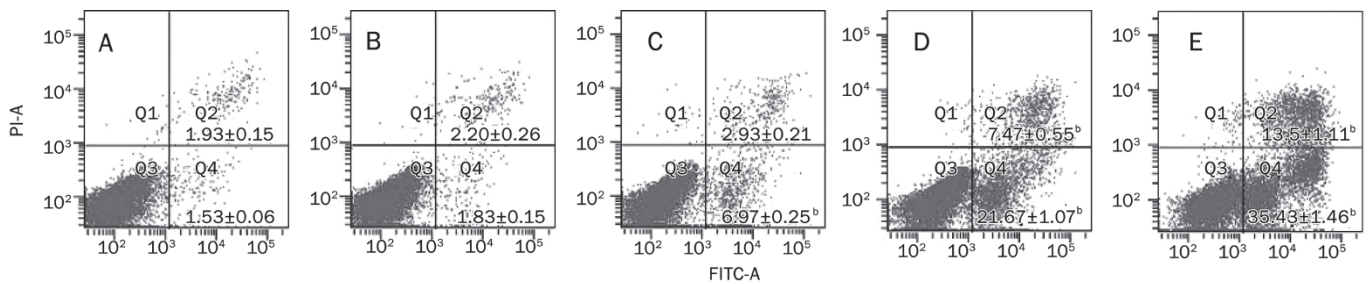


Figure 2. Effect of BA on apoptosis rate of AGS cells. Apoptosis ratio was detected with FCM using Annexin V-FITC/PI double staining. Q2 quadrant represented late apoptosis, while Q4 quadrant represented early apoptosis. (A) 0 µg/mL; (B) 9 µg/mL; (C) 12 µg/mL; (D) 15 µg/mL; (E) 18 µg/mL. The figures were representative of three separate experiments. ^b*P*<0.05 vs control group.

and 48.96%±2.55%, respectively, which were statistically higher than the control 3.47%±0.21% (Figure 2). The nuclear fragmentation of AGS cells treated with 15 µg/mL BA for 24 h was stained by Hoechst 33258 (Figure 3). Apoptotic body containing nuclear fragments was found in BA-treated cells, the nuclear envelope appeared lytic and the cytoplasm shrunk. In contrast, the cells in the culture without BA showed normal cell nuclei morphology.

Cell cycle analysis

After incubation with BA, AGS cells were analyzed using FCM to analyze alterations in cell cycle distribution. DNA content distribution in AGS cells was changed after 24 h treatment with various concentrations of BA (0, 9, 12, 15, and 18 µg/mL) (Figure 4). As the dose of BA increased, the percentage of cells in G₂/M phase was increased with a concomitant sub-G₁ apoptotic peak, and that in S phase decreased in a dose-dependent manner. There were minimal changes in G₀/G₁ phase

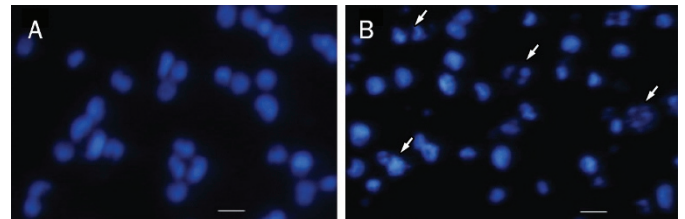


Figure 3. Effect of BA on apoptosis of AGS cells. Morphological changes of AGS cells treated with 15 µg/mL BA for 24 h which were stained with Hoechst33258 and detected using a fluorescence microscope. Fragmented or condensed nuclei indicative of apoptosis could be observed in the BA-treated groups as the arrows indicated (B), while the untreated cells showed normal cell nuclei morphology (A). White bars: 25 µm (Magnification×400).

cell population. As compared with control sample, the ratio of apoptotic cells in BA-treated samples was higher. This sug-

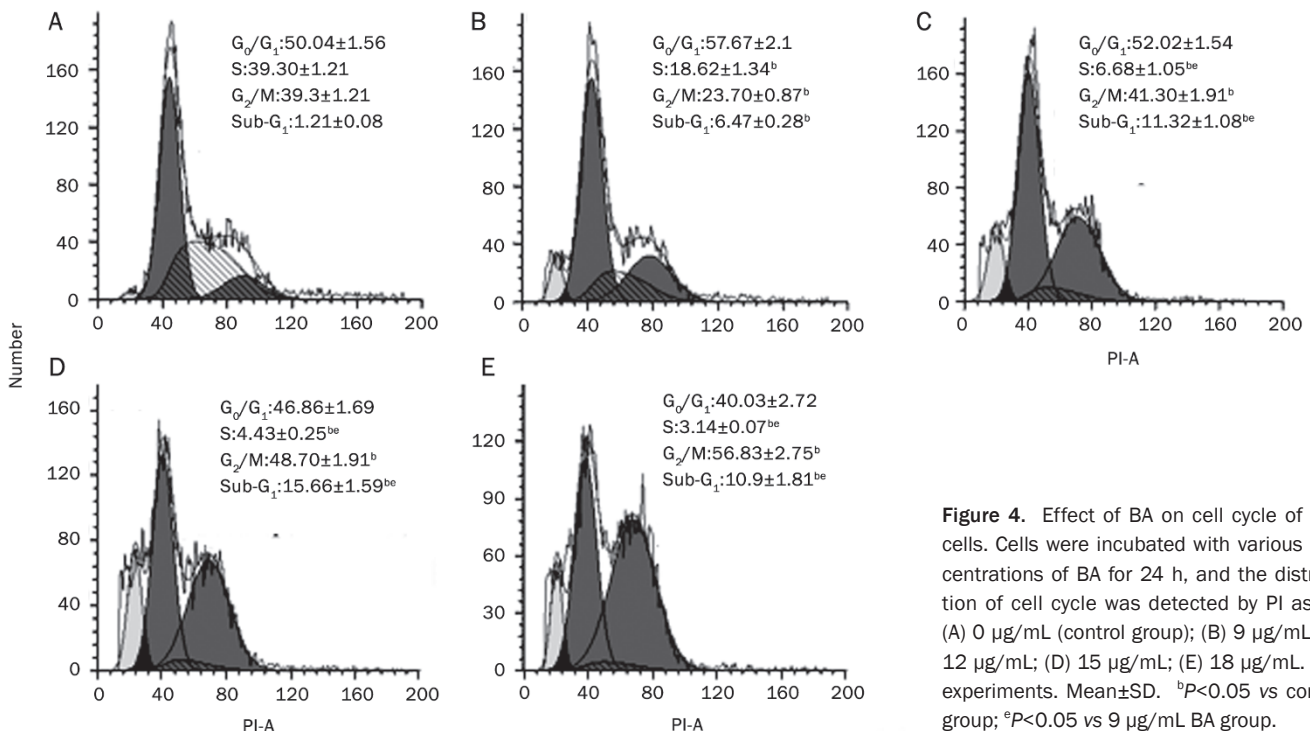


Figure 4. Effect of BA on cell cycle of AGS cells. Cells were incubated with various concentrations of BA for 24 h, and the distribution of cell cycle was detected by PI assay. (A) 0 µg/mL (control group); (B) 9 µg/mL; (C) 12 µg/mL; (D) 15 µg/mL; (E) 18 µg/mL. *n*=3 experiments. Mean±SD. ^b*P*<0.05 vs control group; ^e*P*<0.05 vs 9 µg/mL BA group.

gested that BA might inhibit cell proliferation by inducing cell G₂/M arrest, followed by apoptosis in AGS cells.

Expression of Hiwi and Cyclin B1 protein in AGS cells

Extensive investigations have implicated that the overexpression of Hiwi is known for tumor cell growth and survival response, which performs multiple functions ranging from posttranscriptional gene, chromatin silencing and cell cycle regulation. The present study and other findings have demonstrated that BA potently inhibited cell proliferation and induced apoptosis of AGS cells and many other cell lines. We therefore hypothesized that BA might be disrupting these cellular processes by primarily inhibiting the Hiwi gene. To test this hypothesis, we first examined whether constitutive deregulation of Hiwi existed in AGS cells. Since CyclinB1, which is under the control of Piwi protein, also plays a key role in tumorigenesis including gastric cancer, we further investigated whether BA could also inhibit Cyclin B1 activity in AGS cells. FCM was used to detect the expression of Hiwi and Cyclin B1 protein in AGS cells, and the mean fluorescence intensity represented the expression levels of Hiwi and Cyclin B1 protein. The wave crest representing the fluorescence intensity shifted left after AGS cells were treated with BA for 24 h (Figure 5A). In this experiment, the mean fluorescence intensity value of Hiwi protein in untreated group was 1405±21, while that in the groups treated with 9, 12, 15 and 18 µg/mL BA was down-regulated to 1159±17, 681±13, 645±10 and 621±8, respectively (Figure 5B). The Cyclin B1 protein expression in AGS cells was also down-regulated by BA. The mean fluorescence intensity of Cyclin B1 in the control group was 512±14, while that in the groups treated with 9, 12, 15 and 18 µg/mL BA was 447±13, 415±11, 384±8 and 351±9, respectively (Figure 5C). The results indicated that BA down-regulated the expression of Hiwi and Cyclin-B1 protein in a dose-dependent manner.

Detection of Hiwi and Cyclin B1 mRNA by RT-PCR

In order to identify whether the decrease of Hiwi and Cyclin B1 proteins was mediated at the transcriptional level by a decrease of Hiwi and Cyclin B1 mRNA expression, or at the post-translation level, we performed reversed transcriptase PCR (RT-PCR). Highest expression of Hiwi mRNA was detected in control group, with a mean value of 1.319±0.045 as compared with β-actin. After incubation with 9, 12, 15, 18 and 21 µg/mL BA for 24 h, Hiwi/β-actin ratio in AGS cells was decreased to 0.822±0.094, 0.674±0.065, 0.506±0.041, 0.272±0.023 and 0.209±0.027, respectively (Figure 6A, Figure 6C). The Cyclin B1 mRNA expression was also down-regulated. The expression level of Cyclin B1 mRNA in 9 µg/mL BA-treated group was not significantly different from that in the control (1.273±0.017 vs 1.304±0.021, *P*>0.05), but the differences in AGS cells treated with 12, 15, 18 and 21 µg/mL were statistically significant, with mean values of the Cyclin B1 mRNA expression being 1.124±0.022, 0.945±0.018, 0.875±0.028 and 0.501±0.027, respectively (Figure 6B, Figure 6D).

Discussion

BA is a natural pentacyclic triterpene isolated from birth trees. It is a very potent anticancer compound that is capable of killing a plethora of tumor cells. However, antitumor activity and action mechanism of BA still remains elusive. Our results indicated that BA possessed significant cytotoxicity against AGS cells in a dose- and time-dependent manner, and IC₅₀ for 24 h was 18.25 µg/mL (95% confidence interval: 15.16 to 27.31 µg/mL), well below the established tolerance level. BA also induced distinct G₂/M phase arrest and apoptosis in AGS cells, accompanied with typical apoptotic morphological changes. It have been revealed numerous pro-apoptosis and cell cycle proteins (eg, Cyclin B1, CyclinD1 and Bcl-x_i) were targeted by BA^[15-17]. Because Bcl-x_i, Cyclin D1 and Cyclin B1

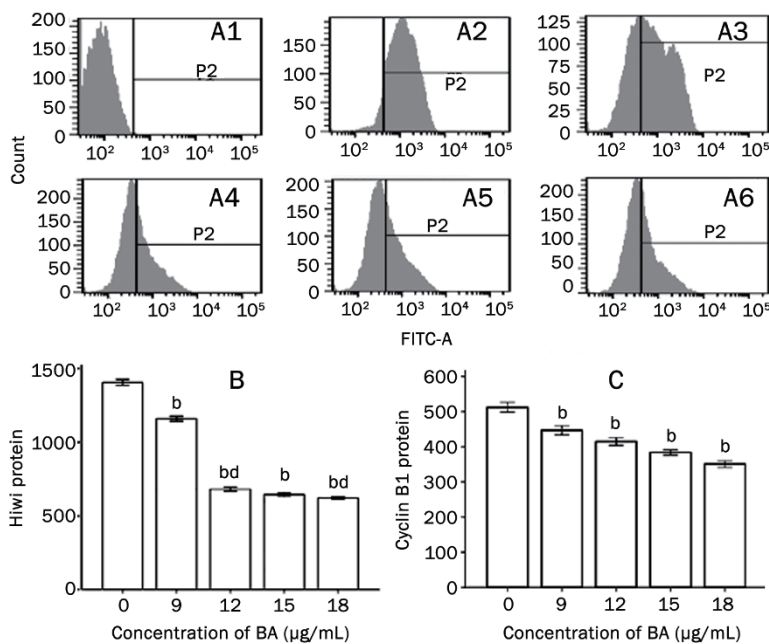


Figure 5. The expression of Hiwi and Cyclin B1 protein in the AGS cells treated with various concentrations of BA. Untreated cells served as controls. Mean fluorescence intensity represented the expression levels of Hiwi and Cyclin B1. Panel A: The graphs of flow cytometry of Hiwi protein in AGS cells treated with various concentration of BA. (A1) negative control; (A2) 0 µg/mL (control); (A3) 9 µg/mL; (A4) 12 µg/mL; (A5) 15 µg/mL; (A6) 18 µg/mL. Panel B: Mean fluorescence intensity of Hiwi protein in AGS cells. Panel C: Mean fluorescence intensity of Cyclin B1 protein. *n*=3 experiments. Mean±SD. ^b*P*<0.05 vs control group; ^{bd}*P*>0.05 vs 15 µg/mL BA-treated group.

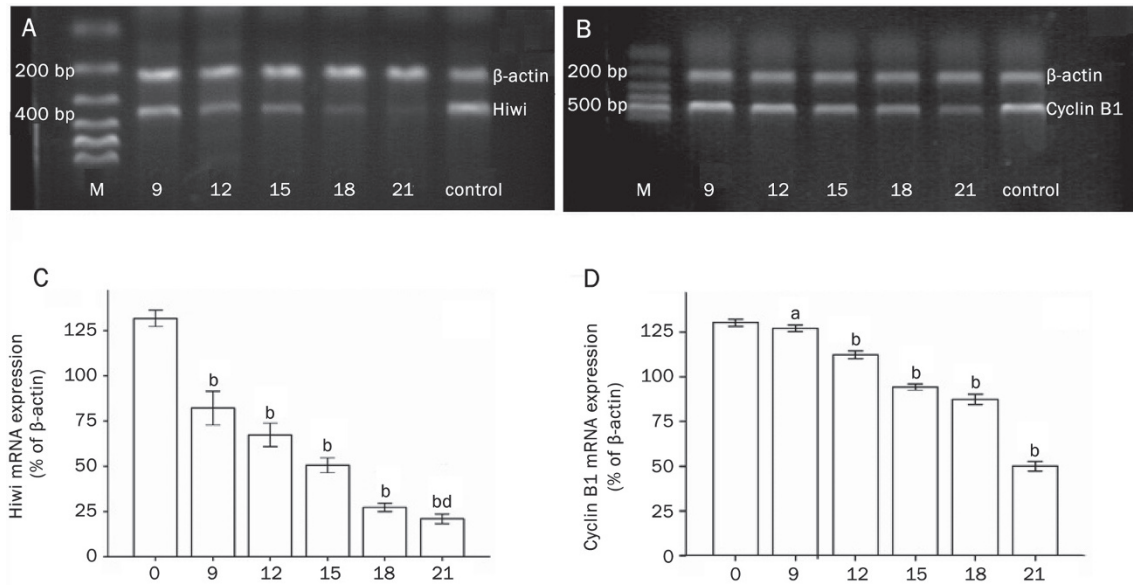


Figure 6. Effect of BA on mRNA expression of Hiwi (panel A) and Cyclin B1 (panel B) in AGS cells. M: marker, 9: 9 μ g/mL, 12: 12 μ g/mL, 15: 15 μ g/mL, 18: 18 μ g/mL, 21: 21 μ g/mL, control: 0 μ g/mL (control group). $n=3$ experiments. Mean \pm SD. ^a $P>0.05$, ^b $P<0.05$ vs control group; ^d $P>0.05$ vs 18 μ g/mL BA-treated group.

gene expression modulated by BA is regulated by Piwi protein, we investigated whether the anticarcinogenic effect of BA was mediated through modulation of Hiwi and Hiwi-regulated gene products. The significant decrease of the levels of both Hiwi mRNA and protein in AGS cells upon BA treatment strongly supported this hypothesis. BA also modulated Piwi-dependent gene products Cyclin B1 which are believed to be important for apoptosis and G_2/M arrest and serve as a common target for cancer drug development. It suggests that BA is an effective anti-gastric cancer reagent through the ability of inducing growth-arrest and apoptosis and cell cycle arrest via the downregulation of Hiwi.

Hiwi is human subfamily of Piwi family. PPD (PAZ Piwi domain) proteins form the core of RNA-induced silencing complexes (RISCs), which is required for gene silencing. Previous studies have shown that Piwi performs multiple functions ranging from epigenetic programming and repression of transposition to post transcriptional regulation^[25, 26]. Moreover, it has recently become evident that PPD proteins and Dicer also function in siRNA-independent and dependant pathways that regulate cell cycle event^[27]. High-level expression of Hiwi mRNA is related with high risk of tumor-related death in soft-tissue sarcomas patients^[28]. It had been reported that Hiwi expression was higher in gastric cancers than in normal mucosa or in mucosa with atrophic gastritis or intestinal metaplasia, and the suppression of Hiwi by antisense or RNAi inhibited the growth of gastric cancer cells and induced cell cycle arrest in G_2/M phase^[29]. These studies suggest that Hiwi might be an attractive target for future gastric cancer therapeutic drugs. Consistent with the above observation, we also found Hiwi was overexpressed in AGS cells. Moreover, this is the first report to suggest that BA could downregulate the lev-

els of both Hiwi mRNA and protein, which inhibited growth, induced apoptosis and cell cycle arrest of AGS cells.

Overexpression of the Piwi in yeast resulted in cell cycle delay at the G_2/M boundary^[17]. For example in *Drosophila*, altering the expression level of Piwi leads to changes in the proliferation rate of germline stem cells^[18]. Furthermore, the Piwi signaling network exhibits cross talk with the Hedgehog signaling machinery^[30], a developmental patterning system that influences the cell cycle through the actions of Patched1 on cyclin B1^[31]. Piwi is required for regulated hyperphosphorylation of the cyclin-dependent kinase Cdc2^[32], which controls the transition from the G_2 phase of the cell cycle to mitosis through binding with Cyclin B. Inhibitory phosphorylation of Cdc2 is required to prevent the onset of mitosis in situations where damaged or unreplicated DNA is present^[33]. In our study, BA induced cell cycle arrest at G_2/M and simultaneously decreased the expression of Hiwi in AGS cells in a dose-dependent manner. We could infer that BA down-regulated Hiwi through inhibiting Cyclin B1. In agreement with this hypothesis, we found that BA treatment could strongly inhibit CyclinB1 concurrently with Hiwi.

Taken together, our results demonstrated that BA presented potent effect on growth inhibition, G_2/M cell cycle arrest and induction of apoptosis in AGS cells *in vitro* via down-regulating Hiwi and Cyclin B. Based on the outcome of this study, it is suggested that Hiwi might be a novel target for gastric cancer treatment and BA could be used as a potent agent for the management of gastric cancer. However, further studies are needed to establish a cause-and-effect relationship between Hiwi/Cyclin B pathway and BA effect. Furthermore, it will also be important to validate these findings in the *in vivo* animal models, which could better relate to human situations.

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Author contribution

Yan CHEN and Qiu-ling WU designed research; Li-jing YANG performed research; Jun FANG and Jing HE managed analytical tools and reagents; Li-jing YANG and Yi-quan CHENG analyzed data; Li-jing YANG and Qi MA wrote the paper.

References

- Theo A, Masebe T, Suzuki Y, Kikuchi H, Wada S, Obi CL, et al. *Peltophorum africanum*, a traditional South African medicinal plant, contains an anti HIV-1 constituent, betulinic acid. *Tohoku J Exp Med* 2009; 217: 93–9.
- Yao D, Li H, Gou Y, Zhang H, Zhou H, Liu Z, et al. Betulinic acid-mediated inhibitory effect on hepatitis B virus by suppression of manganese superoxide dismutase expression. *FEBS J* 2009; 279: 2599–614.
- Ziegler HL, Franzyk H, Sairafianpour M, Tabatabai M, Tehrani MD, Bagherzadeh K, et al. Erythrocyte membrane modifying agents and the inhibition of *Plasmodium falciparum* growth: structure-activity relationships for betulinic acid analogues. *Bioorg Med Chem* 2004; 12: 119–27.
- Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat Med* 1995; 1: 1046–51.
- Ehrhardt H, Fulda S, Fuhrer M, Debatin KM, Jeremias I. Betulinic acid-induced apoptosis in leukemia cells. *Leukemia* 2004; 18: 1406–12.
- Kessler JH, Mullauer FB, de Roo GM, Medema JP. Broad *in vitro* efficacy of plant-derived betulinic acid against cell lines derived from the most prevalent human cancer types. *Cancer Lett* 2007; 251: 132–45.
- Jung GR, Kim KJ, Choi CH, Lee TB, Han SI, Han HK, et al. Effect of betulinic acid on anticancer drug-resistant colon cancer cells. *Basic Clin Pharmacol Toxicol* 2007; 101: 277–85.
- Zuco V, Supino R, Righetti SC, Cleris L, Marchesi E, Formelli F, et al. Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Lett* 2002; 175: 17–25.
- Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, et al. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. *Cancer Res* 1997; 57: 4956–64.
- Wick W, Grimmel C, Wagenknecht B, Dichgans J, Weller M. Betulinic acid-induced apoptosis in glioma cells: a sequential requirement for new protein synthesis, formation of reactive oxygen species, and caspase processing. *J Pharmacol Exp Ther* 1999; 289: 1306–12.
- Selzer E, Pimentel E, Wacheck V, Schlegel W, Pehamberger H, Jansen B, et al. Effects of betulinic acid alone and in combination with irradiation in human melanoma cells. *J Invest Dermatol* 2000; 114: 935–40.
- Selzer E, Thallinger C, Hoeller C, Oberkleiner P, Wacheck V, Pehamberger H, et al. Betulinic acid-induced Mcl-1 expression in human melanoma—mode of action and functional significance. *Mol Med* 2002; 8: 877–84.
- Thurnher D, Turhani D, Pelzmann M, Wannemacher B, Knerer B, Formanek M, et al. Betulinic acid: a new cytotoxic compound against malignant head and neck cancer cells. *Head Neck* 2003; 25: 732–40.
- Rieber M, Strassberg Rieber M. Induction of p53 without increase in p21^{WAF1} in betulinic acid-mediated cell death is preferential for human metastatic melanoma. *DNA Cell Biol* 1998; 17: 399–406.
- Lee JH, Schütte D, Wulf G, Füzesi L, Radzun HJ, Schweyer S, et al. Stem-cell protein Piwi2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-xl pathway. *Hum Mol Genet* 2006; 15: 201–11.
- Morris KV, Santoso S, Turner AM, Pastori C, Hawkins PG. Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. *PLoS Genet* 2008; 4: e1000258.
- Stoica C, Carmichael JB, Parker H, Pare J, Hobman TC. Interactions between the RNA interference effector protein Ago1 and 14-3-3 proteins. *J Biol Chem*. 2006; 281: 37646–51.
- Cox DN, Chao A, Lin H. Piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* 2000; 127: 503–14.
- Pal-Bhadra M, Bhadra U, Birchler JA. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol Cell* 2002; 9: 315–27.
- Hannon GJ. RNA interference. *Nature* 2002; 418: 244–51.
- Qiao D, Zeeman AM, Deng W, Looijenga LH, Lin H. Molecular characterization of Hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas. *Oncogene* 2002; 21: 3988–99.
- Sharma AK, Nelson MC, Brandt JE, Wessman M, Mahmud N, Weller KP, et al. Human CD34 (+) stem cells express the hiwi gene, a human homologue of the *Drosophila* gene piwi. *Blood* 2001; 97: 426–34.
- Summersgill B, Osin P, Lu YJ, Huddart R, Shipley J. Chromosomal imbalances associated with carcinoma in situ and associated testicular germ cell tumours of adolescents and adults. *Br J Cancer* 2001; 85: 213–20.
- Sathya P, Tomkins DJ, Freeman V, Paes B, Nowaczyk MJ. De novo deletion 12q: report of a patient with 12q24.31q24.33 deletion. *Am J Med Genet* 1999; 84: 116–9.
- Deng W, Lin H. Miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Dev Cell* 2002; 2: 819–30.
- Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, Elst H, et al. A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* 2007; 129: 69–82.
- Carmichael JB, Provost P, Ekwali K, Hobman TC. Ago1 and dcr1, two core components of the RNA interference pathway, functionally diverge from rdp1 in regulating cell cycle events in *Schizosaccharomyces pombe*. *Mol Biol Cell* 2004; 15: 1425–35.
- Taubert H, Greither T, Kaushal D, Bache M, Barter F, Kehlen A, et al. Expression of the stem cell self-renewal gene Hiwi and risk of tumour-related death in patients with soft-tissue sarcoma. *Oncogene* 2007; 26: 1098–100.
- Liu X, Sun Y, Guo J, Ma H, Li J, Dong B, et al. Expression of Hiwi gene in human gastric cancer was associated with proliferation of cancer cells. *Int J Cancer* 2006; 118: 1922–9.
- King FJ, Szakmary A, Cox DN, Lin H. Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the *Drosophila* ovary. *Mol Cell* 2001; 7: 497–508.
- Roy S, Ingham PW. Hedgehogs tryst with the cell cycle. *J Cell Sci* 2002; 115: 4393–7.
- Carmichael JB, Provost P, Ekwali K, Hobman TC. Ago1 and Dcr1, two core components of the RNA interference pathway, functionally diverge from rdp1 in regulating cell cycle events in *Schizosaccharomyces pombe*. *Mol Biol Cell* 2004; 15: 1425–35.
- Rhind N, Russell P. Tyrosine phosphorylation of cdc2 is required for the replication checkpoint in *Schizosaccharomyces pombe*. *Mol Cell Biol* 1998; 18: 3782–7.