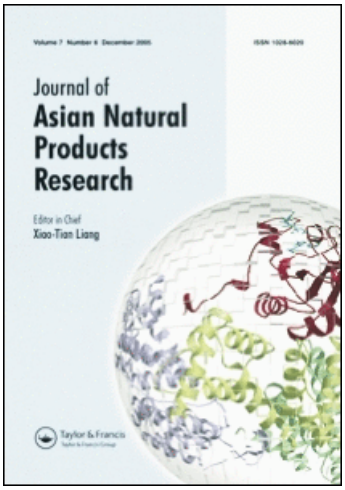

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Pseudolaric acid B-induced apoptosis through p53-dependent pathway in human gastric carcinoma cells

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Pseudolaric acid B (PLAB, **1**), a natural diterpenoid compound, was isolated from *Pseudolarix kaempferi* Gordon. It has shown antifungal, antifertility, and antiangiogenic properties in previous studies. Recently, increasing evidence has confirmed that **1** exhibits antitumor effects in several tumor cell lines, but the underlying mechanism has not been fully elucidated. The aim of this study was to investigate the mechanism of PLAB-induced cell apoptosis in MGC803 cells. The results showed that **1** significantly inhibited the proliferation of MGC803 cells at 0.01–10 μM and the IC_{50} value was 0.91 μM for 48 h. PLAB-induced apoptosis in MGC803 cells was confirmed by DNA fragmentation assay and Hoechst33342/PI staining. PLAB-treated MGC803 cells were arrested at G_2 phase, which was associated with a marked increment of the expression of cyclin-dependent kinase inhibitor p21. The induction of p21 appeared to be transcriptionally up-regulated and was p53-dependent. In addition, PLAB induced Fas/APO-1 and caspase-3 expressions that were also correlated with apoptosis. Meanwhile, **1** decreased the mRNA expression of bcl-2, which is an antiapoptosis factor. In conclusion, **1** induced apoptosis through p53-dependent pathway in human gastric carcinoma cells. These findings suggest that **1** may be a novel promising agent for treating human gastric carcinoma.

Keywords: pseudolaric acid B; p53; Fas; caspase-3; bcl-2; apoptosis

1. Introduction

Pseudolaric acid B (PLAB, **1**) is one of the most important natural diterpenoid compounds isolated from the root bark of *Pseudolarix kaempferi* Gordon [1] and displays potent antifungal, antifertile, and antiangiogenic properties [2–5]. In addition, **1** has cancer chemopreventive activity and inhibits *in vitro* growth of a number of human cancer cell lines, including KB, A-549, Hct-8, P-388, and L-1210 tumor cells [6]. Studies in several human carcinoma cells have shown that **1** induces growth inhibition, cell cycle arrest, and apoptosis [7–9]. However, the underlying mechanisms of action and cellular targets for these effects of **1** are not well characterized at the present time.

Apoptosis has been well characterized by a variety of hallmark events including rapid reduction in cellular volume, chromatin condensation, and internucleosomal DNA cleavage. Induction of apoptosis is suggested to be one of the major modes of action of chemotherapeutic anticancer drugs on malignant cells [10]. Activation of p53 in response to abnormal proliferative signals and stress including DNA damage leads to cell cycle arrest, and over-expression of p53 inhibits cellular proliferation and induces apoptosis [11]. The above effects probably indicate

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a role in the ability of p53 to act as a tumor suppressor and a number of chemopreventive agents have been shown to exert their antitumorigenic activity through p53-dependent mechanisms [12].

To search for a new candidate drug for the treatment of human gastric carcinoma, we evaluated the growth inhibition activity and cell cycle arrest effect of **1**. In addition, to elucidate the anticancer mechanism of **1**, we also assayed the protein levels of p53, Fas/APO-1, caspase-3, and the mRNA level of bcl-2, which are strongly associated with the pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents. A present study has reported that **1** can induce apoptosis in AGS human gastric cancer cells via the activation of caspase-3 and down-regulation of bcl-2 [13]. However, our results showed that the compound **1** exerted strong cytotoxic activity against human gastric carcinoma cells by a way of p53-mediated apoptosis and induced cell cycle arrest at G_2 phase.

2. Results and discussion

2.1 1 induces cell growth inhibition of human cancer cells

The structure of **1** used in our experiments is shown in Figure 1. To study the effect of **1** on human cancer cells, the cells were treated with various concentrations of **1** for 48 h and the cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction methods. Results indicated that **1** possessed potent anticancer activity, the order of the concentration (μM) of 50% cytotoxicity (IC_{50}) against the various human cancer cell lines was MGC803 (0.91) < SMMC7721 (3.4) < LOVO (13.6). The MGC803 cells were chosen for further study and the results demonstrated that the treatment with **1** (0.01–10 μM) resulted in a dose- and time-dependent inhibition of proliferation of MGC803 cells. This effect was more pronounced at 24-, 48-, and 72-h post-treatment (Figure 2). The inhibition of cell growth might be a result of the induction of apoptosis and/or cell cycle arrest. Therefore, **1** mediating to perturb the cell cycle and to induce programed cell death was further evaluated.

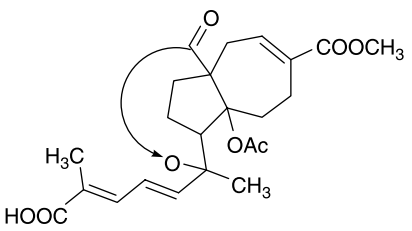

Figure 1. Structure of **1**.

2.2 1 induces cell cycle arrest at G_2 phase in MGC803 cells

Treatment of MGC803 cells with **1** resulted in a dose- and time-dependent inhibition of cell growth when compared with their untreated controls at all the time points observed. This may involve an arrest of cells at specific check points in the cell cycle. After the treatment with **1** (10 μM) for 24, 48, and 72 h, the results showed an appreciable arrest of cells in G_2/M, and the change was accompanied with a concomitant decrease in cell number in G_1 and S phases (Figure 3 and Table 1).

2.3 Effect of 1 on apoptosis markers of DNA fragmentation and apoptotic body formation in MGC803 cells

To characterize cell death induced by **1**, the integrity of genomic DNA and morphology of nuclear were examined on the analysis of DNA integrity by DNA ladder mode. The results showed that PLAB-treated MGC803 cells expressed apoptosis at 1.0 and 10 μM of **1** in a dose- and time-dependent manner (Figure 4). A quantitative evaluation of apoptosis was sought using a fluorescence microscope and MGC803 cells were observed by Hoechst33342/propidium (PI). The results showed that 10 μM of **1** induced 14.7, 32.8, and 49.1% of apoptotic cells in MGC803 cells at 24, 48, and 72 h (Figure 5), which is consistent with the above.

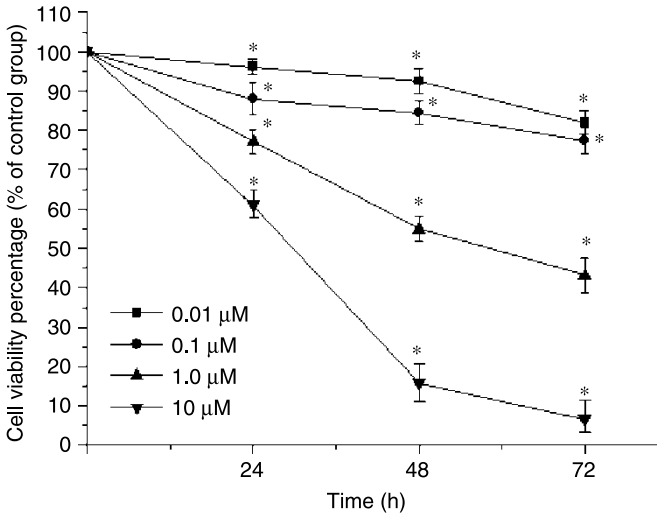

Figure 2. Effect of **1** on the viability of MGC803 cells. The cells were treated with 0.1% dimethylsulfoxide (DMSO) as a control or various concentrations of **1** for 24, 48, and 72 h, and then measured using MTT assay. The values represent percentage of viability of control culture from three independent experiments. Data represent the mean ± SD. *$P < 0.05$ compared with control group.

2.4 1 modulated p53 and p21/WAF1 expression

For investigating the mechanism of **1** against MGC803 cells, results showed that **1** (10 μM) exhibited a time-dependent p53 protein levels after 12 h of treatment (Figure 6(A)). The protein levels of p21, a downstream target of p53, were also found to increase after treatment with 10 μM **1** for 24 h (Figure 6(B)). Based on the above results, it suggested that PLAB-mediated apoptosis and cell cycle arrested at G_2 phase in MGC803 cells was p53/p21-dependent.

2.5 1 affected Fas/APO-1 expression

A recent study showed that over-expression of p53 could induce cell surface expression of Fas/APO-1. The results indicated that Fas level increased after treatment with 10 μM **1** for 24 h (Figure 6(C)). These data suggested that Fas/APO-1 was also associated with the PLAB-induced apoptosis in MGC803 cells.

2.6 Effects of PLAB-induced caspase-3 activation

Caspases are believed to play central roles in mediating various apoptotic responses and are activated in a sequential cascade of cleavages from their inactive forms. In this study, there were significantly time-dependent increases in caspase-3 activity after **1** treatment for 24, 36, and 48 h (Figure 6(D)). These results suggested that caspase-3 appeared to participate in this apoptotic pathway.

2.7 Regulation of bcl-2 mRNA expression by 1

After exposure to 10 μM **1** for 12, 24, and 48 h, bcl-2 mRNA expression was down-regulated remarkably in MGC803 cells (Figure 7).

2.8 Discussion

In this study, we found that **1** displayed a significantly inhibitory effect on the proliferation of several human carcinoma cell lines and it is the most remarkable in PLAB-treated MGC803 cells. Furthermore, our results showed that **1** (1.0 and 10 μM) resulted in the induction of apoptosis in cells, as evidenced by DNA fragmentation and apoptotic body of apoptotic markers detection. More interestingly, the

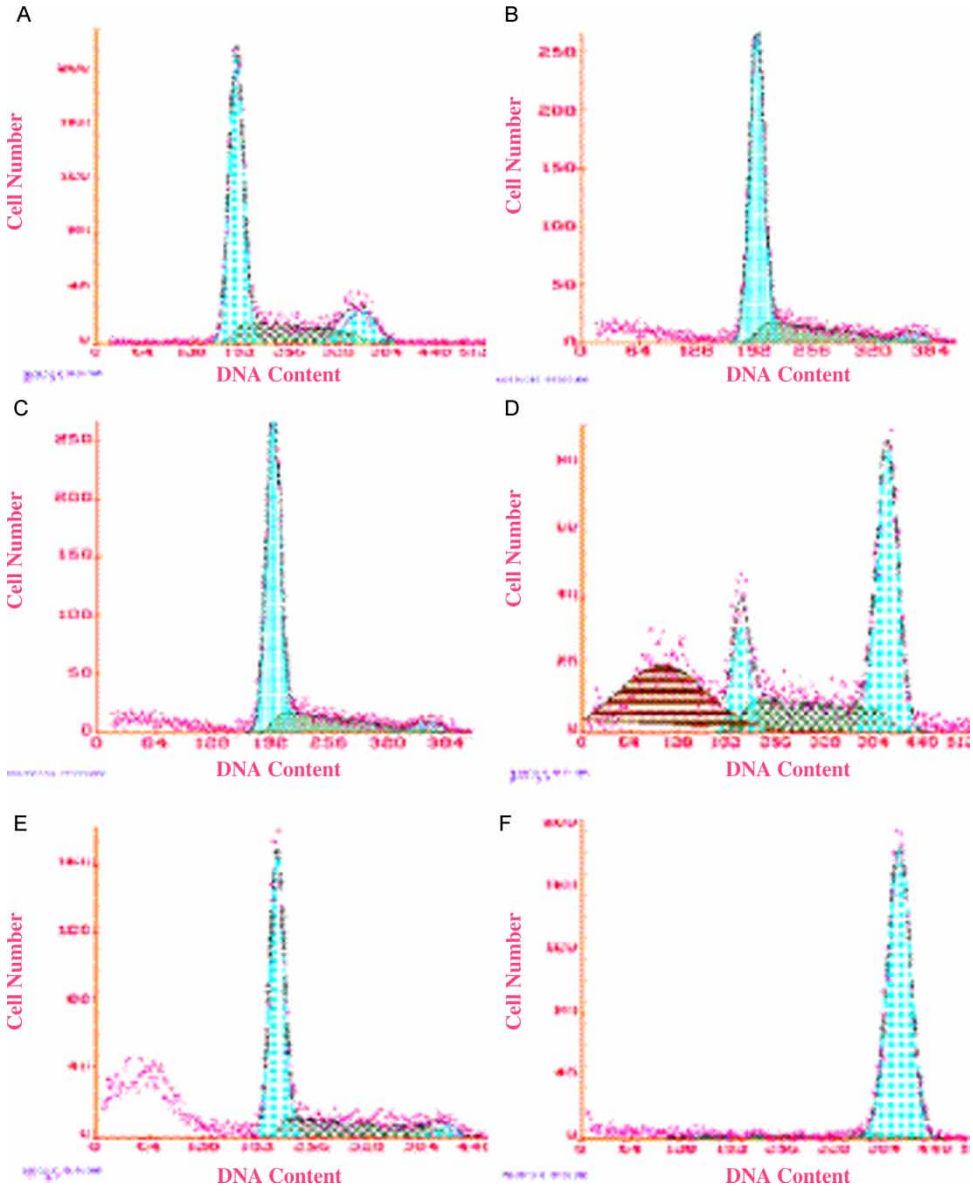

Figure 3. The cell cycle of MGC803 cells detected by flow cytometry (FCM). (A) Control for 24 h ($\%G_1 = 71.0$, $\%G_2 = 8.6$, $\%S = 20.3$); (B) **1** for 24 h ($\%G_1 = 60.3$, $\%G_2 = 15.0$, $\%S = 24.7$); (C) control for 48 h ($\%G_1 = 71.4$, $\%G_2 = 4.1$, $\%S = 24.1$); (D) **1** for 48 h ($\%G_1 = 14.9$, $\%G_2 = 55.0$, $\%S = 30.1$); (E) control for 72 h ($\%G_1 = 74.0$, $\%G_2 = 4.9$, $\%S = 21.1$); (F) **1** for 72 h ($\%G_1 = 1.5$, $\%G_2 = 92.1$, $\%S = 6.4$).

MGC83 cells treated with **1** (10 μM) for 48 h exhibited a dramatic accumulation of cells in G_2 phase of the cell cycle.

The tumor suppressor gene p53 plays an important role in tumor growth inhibition and induction of apoptosis [14]. Many studies demonstrated that the induction of p21 might be due to a p53-dependent as well as a p53-independent pathway [15]. The p21 could inhibit G_1–S-phase transition resulting in G_1-phase cell cycle arrest. However, many evidences have recently

Table 1. The effect of 10 μM **1** on cell cycle distribution of MGC803 cells (%, $\bar{x} \pm s$, $n = 3$).

Group (h)	G_1	G_2	S
24			
Control	72.1 ± 1.2	6.7 ± 0.5	21.2 ± 0.8
1	60.8 ± 1.1*	13.6 ± 0.7*	25.6 ± 0.6*
48			
Control	71.8 ± 1.3	4.9 ± 0.5	23.3 ± 1.0
1	15.3 ± 0.4*	60.8 ± 1.4*	23.9 ± 1.2
72			
Control	72.6 ± 1.5	5.2 ± 0.4	22.2 ± 0.9
1	1.8 ± 0.3*	91.6 ± 0.3*	6.8 ± 0.2*

Data represent the mean ± SD. *$P < 0.05$ compared with control group.

been accumulated to suggest that p21 is involved in the suppression of G_2/M-phase progression [16–18]. Our data showed that **1** treatment resulted in a time-dependent increment of p53 and p21 proteins in MGC803 cells and demonstrated a significant up-regulation of the p21/WAF at 24 h after over-expression of p53 protein at 12 h and it may be an important molecular mechanism through which **1** inhibited the growth of cancer cells. Thus, we suggested that the induction of p21 was a p53-dependent event in PLAB-treated MGC803 cells and it played some roles in G_2 arrest induced by **1** in MGC803 cells.

The Fas/APO-1 is an apoptosis-signaling receptor molecule on the surface of a number of cell types. A recent study showed that the Fas/APO1-mediated apoptosis is subjected to the regulation by cell cycle-dependent mechanisms, one of which is probably the function of the p53 antigen [19] and over-expression of p53 can induce cell surface expression of Fas/APO-1 [20]. We demonstrated that **1** treatment resulted in the up-regulation of Fas expression. This result revealed that Fas might participate in PLAB-induced apoptosis through p53-dependent mechanisms in MGC803 cells.

Bcl-2 is an upstream effect molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis. Bcl-2 is found at inappropriately high levels in more than half of all human tumors. In this study, a decrease in bcl-2 mRNA expression was observed in MGC803 cells. This result suggested that down-modulation of bcl-2 might be molecular mechanism through

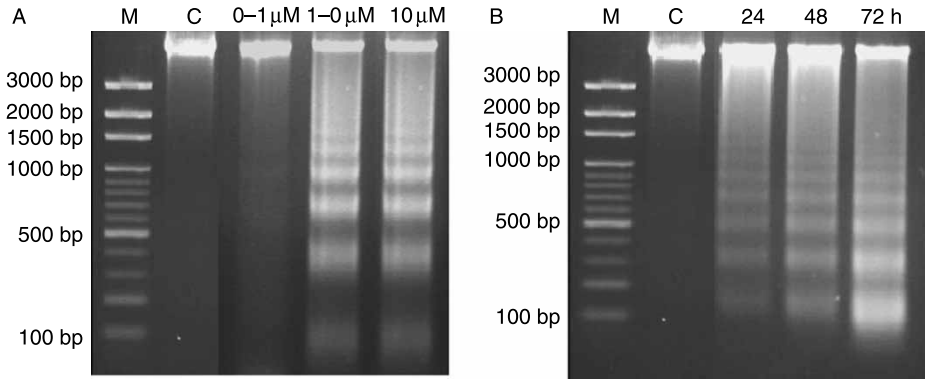

Figure 4. DNA fragmentation induced by **1** in MGC803 cells. (A) The cells were treated with 0.1, 1.0, and 10 μM of **1** for 48 h. (B) The cells were treated with 10 μM of **1** for 24, 48, and 72 h. Lane M was DNA marker. Lane C was treated with 0.1% DMSO.

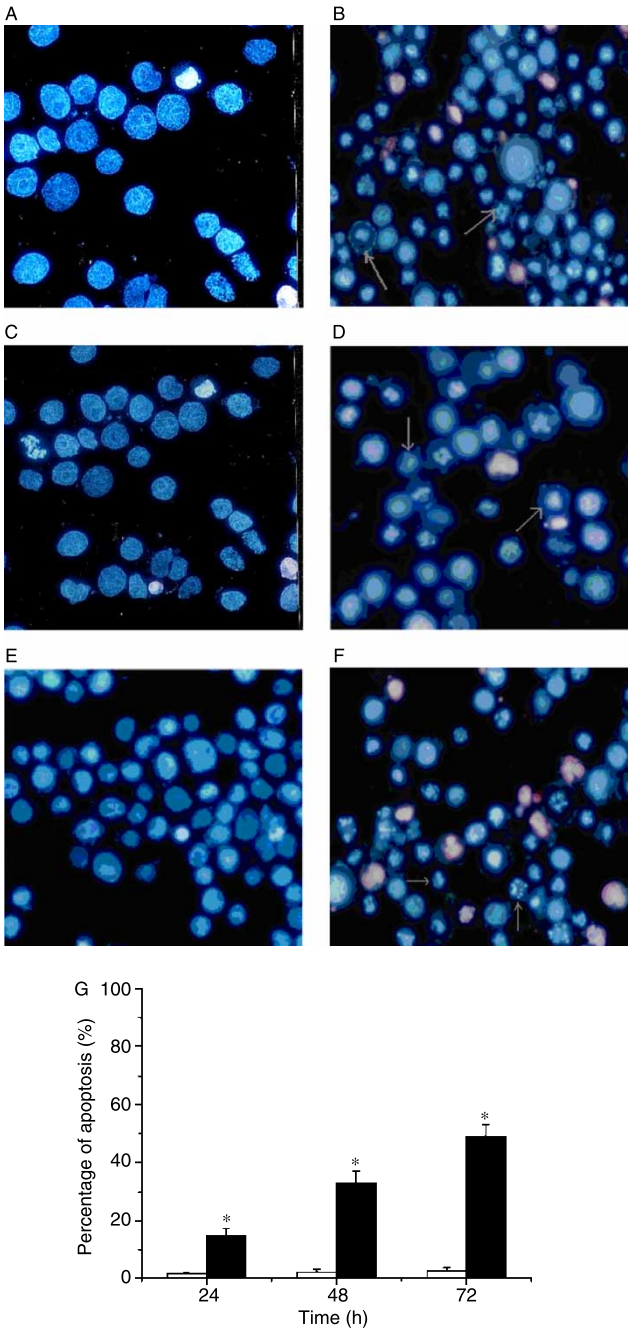

Figure 5. Apoptosis was observed using a fluorescence microscope (20 × 20). MGC803 cells were treated with 10 μM **1** for 24, 48, and 72 h. The harvested cells were stained with 40 μl Hoechst33342/PI. The stained cells were then examined under fluorescence microscope. (A) Control group for 24 h. (B) Apoptosis was clearly induced with 10 μM **1** for 24 h. (C) Control group for 48 h. (D) Apoptosis was clearly induced with 10 μM **1** for 48 h. (E) Control group for 72 h. (F) Apoptosis was clearly induced with 10 μM **1** for 72 h. (G) Percentage of apoptosis was calculated from the ratio of apoptotic cells to total cells. Results were expressed as the mean ± SD of three independent experiments. $^*P < 0.05$ compared with control group.

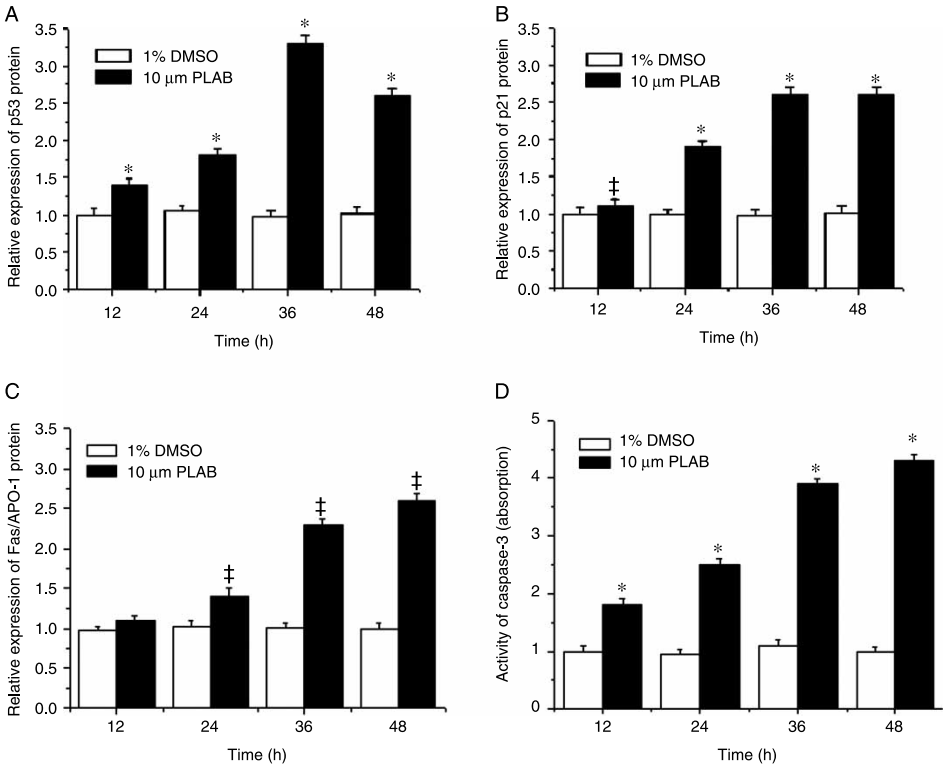

Figure 6. (A) Effect of **1** on the expression of p53 protein in MGC803 cells by p53 pan ELISA kit. (B) Effect of **1** on the expression of p21 protein in MGC803 cells by p21 ELISA kit. (C) Effect of **1** on the expression of Fas protein in MGC803 cells by Fas/APO-1 ELISA kit. (D) Effect of **1** on the activity of caspase-3 in MGC803 cells by caspase-3 activity assay kit. The cells were treated with 0.1% DMSO as a control or 10 μM **1** for 12, 24, 36, and 48 h. Results were expressed as the mean ± SD of three independent experiments, which were calculated from the ratio of absorbance reading in PLAB-treated well to that of control well. $*P < 0.05$ compared with control group.

which **1** induced apoptosis and is consistent with Prof. Li's observation [13].

Caspases play a critical role in the apoptosis. Fas binds the adapter protein, with death domain, that recruits caspase-8. Caspase-8, as an initiator, directly activates caspase-3, which is one of the principal caspases found in apoptotic cells [21]. In our study, results showed that the activity of caspase-3 was significant increase in a time-dependent manner in MGC803 cells. These findings suggested that Fas might be an upstream activator of caspase-3 of PLAB-induced apoptosis in MGC803 cells.

Based on the outcome of this study and the available literature knowledge, as shown in the scheme in Figure 8, we suggest multiple pathways by which **1** results in apoptotic cell death. This may be mediated via cell cycle arrest, modulation in p53 pathway, and down-regulation of bcl-2 expression. In summary, however, based on the present findings, it is suggested that **1** induced apoptosis through p53-dependent pathway in human gastric carcinoma cells and has strong potential development for as a chemopreventive and possibly as a therapeutic agent against gastric cancer.

3. Experimental

3.1 Chemicals

DMSO, ribonuclease (RNase), Hoechst33342, PI, MTT, other chemicals and cell culture

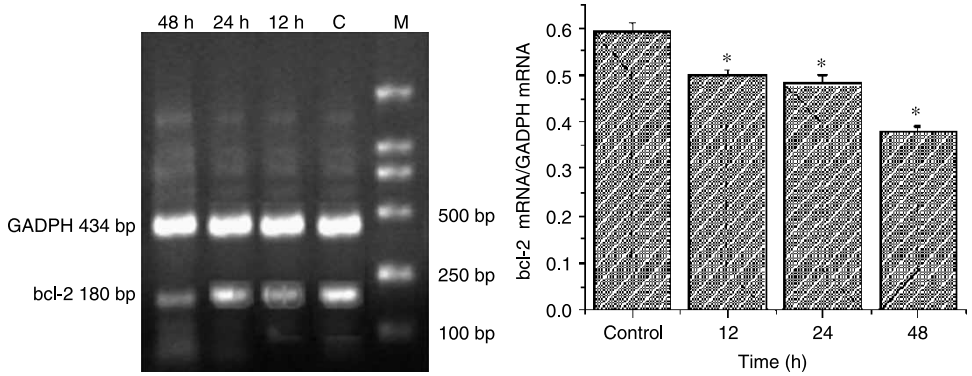

Figure 7. Effect of 10 μM **1** on mRNA expression of bcl-2 in MGC803 cells. The cells were treated with 10 μM **1** for 12, 24, and 48 h. Lane M was DNA marker. Lane C was treated with 0.1% DMSO. $^*P < 0.05$ compared with control group.

medium RPMI-1640 were purchased from Sigma Chemical Co. (St Louis, MO, USA). P53 pan ELISA kit, p21 ELISA kit, and Fas/APO-1 ELISA kit were obtained from Roche Applied Science. Capase-3 cellular activity assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai City, China). **1** ($C_{23}H_{28}O_8$, FW432) was kindly provided by Chinese Academy of Medical Science. All were dissolved in DMSO at 10 mM as a stock solution. The final concentration of DMSO in the culture medium did not exceed 0.1%. All stock solutions were stored at −70°C and were further diluted to appropriate concentrations with medium before use.

The human gastric carcinoma cell line MGC803, human hepatoma cell line SMMC7721, and human colon carcinoma cell line LOVO were established by Cancer Research Center, Peking University. Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum 100 U/ml penicillin G and 100 μg/ml streptomycin (North China Pharmaceutical Group Corporation, Shijiazhuang City, China).

3.2 *Assessment of cell growth*

To evaluate the effect of **1** on cell growth, cells were seeded on a 96-well cell culture plate (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5×10^4 cells/well in a volume of 100 μl. Twenty-four hours later, each well was incubated with **1** at various concentrations (0.01, 0.1, 1.0, and 10 μM). After 48 h, MTT solution was added to each well and cells were incubated for 4 h. The water-insoluble formazon was formed during agents incubated in each well. DMSO was added, and the soluble formazon dye was quantified using a microplate reader (Bio-Rad model 550, Bio-Rad, Hercules, CA, USA).

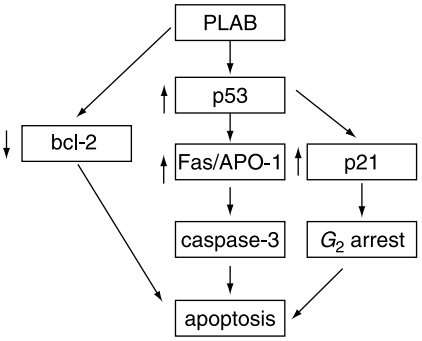

Figure 8. Proposed model for PLAB-mediated cell cycle dysregulation and apoptosis of human gastric carcinoma cells. The G_2 arrest in PLAB-treated MGC803 cells appeared to be p53-dependent. In addition, **1** induced Fas/APO-1 and caspase-3 expression that was also correlated with apoptosis. **1** decreased in the mRNA expression of bcl-2 and this antiapoptotic factor might be also participated in the apoptotic process.

3.3 Apoptosis analysis by Hoechst33342/PI

After being treated with 10 μM **1** for 24, 48, and 72 h, MGC803 cells were trypsinized, collected by centrifugation at 1000 rpm for 5 min, and washed twice with PBS. The cells were fixed with 3.7% paraformaldehyde at room temperature for 2 h, centrifuged, and washed with PBS, stained with 40 μl of Hoechst33342/PI at 1:1 ratio for 30 min at 37°C. At the end of incubation, the cells were washed and resuspended in PBS for the observation of nuclear morphology under a fluorescence microscope (Nikon E800, Nikon, Tokyo, Japan). The percentage of apoptosis was calculated from the ratio of apoptotic cells to total cells in the PLAB-treated MGC803 cells.

3.4 Detection of DNA fragmentation

MGC803 cells (4.0×10^5/ml) were treated with 0.1% DMSO or **1** (1.0 and 10 μM) for 24, 48, or 72 h, washed in cold PBS, and then gently homogenized in extraction buffer (10 mM Tris–HCl, 10 mM EDTA, and 0.2% Triton X-100 at pH 8.0). The samples were incubated for 10 min on ice and then centrifuged at 12,000*g* for 10 min. The supernatant containing fragmented DNA, but not intact chromatin, was extracted with phenol and chloroform precipitated with ethanol. The pellet was rinsed with 70% ethanol, air-dried, and dissolved in 1× TE (10 mM Tris–HCl and 10 mM EDTA at pH 8.0). After digestion with 0.1 mg/ml RNaseA at 37°C for 1 h, the DNA was fractionated in a 2% agarose gel and stained with 10 μg/ml ethidium bromide.

3.5 FCM analysis

Cells were seeded in six-well plates and cultured with 0.1% DMSO or **1** (10.0 μM) for 24, 48, or 72 h. At the end of incubation, the cells were harvested by trypsinization (0.5% trypsin/2.6 mmol/l EDTA), washed twice with ice-cold PBS, and fixed in 70% ethanol at −20°C. These samples were then washed twice with ice-cold PBS, treated with RNase (10 μg/ml), stained with PI, and analyzed with the FACSC alibur flow cytometer (Becton Dickinson, Heidelberg, Germany). All of the results were obtained from three independent experiments.

3.6 Measurement of p53, p21, and Fas protein levels

For detecting the expression of p53, p21 proteins, and Fas/Apo-1 in the MGC803 cells, p53 pan ELISA kit, p21 ELISA kit, and Fas/Apo-1 ELISA kit were used. Briefly, MGC803 cells were treated with 10 μM **1**, and 0.1% DMSO as a control for 12, 24, 36, and 48 h, the samples to be assayed (the lysate collected from approximately 10^6 cells) were placed (triplicated) in a streptavidin-coated 96-well microtiter plate precoated with biotinylated mouse monoclonal antibody specific for p53, p21, and Fas/Apo-1 proteins. After incubation with horseradish peroxidase-conjugated sheep polyclonal detector antibody for 2 h at room temperature, the unbound materials were removed by several washings. Finally, a chromogenic substrate (tetramethylbenzidine) was added and enzymatically converted to a colored solution with intensity proportional to the amount of p53, p21, and Fas/Apo-1 proteins in the sample. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad model 550, Bio-Rad); p53, p21, and Fas/Apo-1 concentrations were determined by interpolating from the standard curve obtained with the known concentrations of p53, p21, and Fas/Apo-1 proteins. Relative expression of p53, p21, and Fas/Apo-1 proteins was calculated from the ratio of absorbance of test sample to that of control.

3.7 Reverse transcription-PCR

The cells were treated with 10 μM of **1** for 12, 24, and 48 h. Qualitative reverse transcription-PCR (RT-PCR) was done by generating sense strand RNA for bcl-2 and human glyceraldehydes-3-phosphate dehydrogenase (GADPH). Oligonucleotides to the

corresponding human ligand-binding domain were synthesized by TaKaRa Biotechnology (Dalian City, China): for bcl-2 sense 5′-TGC AACCTG AGCGC CTTCAC-3′ and antisense 5′-TGACT GAT TCG ACCATT TGAG CCC-3′; for GADPH sense 5′-CTT TGGT ATCGTG GAAG GAC-3′ and antisense 5′-GAAATGA-GCTTGACAAAGTG-3′. Total RNA was obtained from our experimental cell lines (Trizol, Invitrogen Life Technology, Paisley, UK), and 1μg and reverse transcribe using random hexamers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). The RT procedure involved incubation time and temperature as follows: 10 min at room temperature, 45 min at 42°C, and 5 min at 90°C. The RT product was divided into two PCRs (bcl-2 and GADPH). The PCR used 94°C for 5-min denaturation temperature and 60°C for 1-min annealing temperature, and 72°C for 1-min elongation temperature. The reaction was carried out 35 cycles. The inductions of PCR were separated on a 1.5% agarose gel and then analyzed by Image Master VDS (Pharmacia Biotech, Uppsala, Sweden) using Total Lab software.

3.8 Measurement of caspase-3 activity

The activity of caspase-3 activity was determined using the caspase-3 activity kit. To evaluate the activity of caspase-3, cell lysates were prepared after their respective treatment with various designated treatments. Assays were performed on 96-well microtiter plates by incubating 10 μl protein of cell lysate per sample in 80 μl reaction buffer (0.1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM Nad, and 10% glycerol) containing 10 μl caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37°C for 4 h. Samples were measured with an ELISA reader at an absorbance of 405 nm. The detailed analysis procedure was described in the manufacturer's protocol.

3.9 Statistical analysis

The results were expressed as the mean ± SD and statistically compared with the control group or compared between different drugs by using the one-way AVOVA and multiple comparison of SAS Base 6.12 software (SAS Inc., Cary, NC, USA). $P < 0.05$ was statistically significant difference.

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