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Herbal diterpenoids induce growth arrest and apoptosis in colon cancer cells with increased expression of the nonsteroidal anti-inflammatory drug-activated gene

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Abstract

Novel chemotherapeutic agents derived from active phytochemicals could be used as adjuvants and improve the anti-carcinogenicity of standard drug treatments. However, their precise mechanisms of action are sometimes unclear. In this study, the anti-carcinogenic effect of the herbal diterpenoid pseudolaric acid B (PAB) on the growth and apoptosis of colon cancer cells was investigated, and to compare that with the more toxic compound triptolide. PAB induced growth inhibition and apoptosis in HT-29 cells, which were associated with cell cycle arrest at the G_2/M phase, modulation of cyclin expression and downregulation of the protooncogene c-*myc*. In addition, PAB also inhibited bcl-x_L expression, induced cleavage of procaspase-3 and its substrate poly(ADP-ribose) polymerase (PARP), which together caused DNA fragmentation and nuclear chromatin condensation. Concomitantly, the modulation of the growth-related and apoptotic factors by PAB was accompanied by the increased protein and gene expression of the nonsteroidal anti-inflammatory drug-activated gene (NAG-1), which occurred along with cyclooxygenase-2 inhibition. The effects of PAB on PARP cleavage and NAG-1 overexpression were not reversible upon removal of the drug from the culture medium. Similar cytotoxic and pro-apoptotic effects were also attained by treating the HT-29 cells with another diterpenoid triptolide, but its actions on cell cycle progression and on the upstream transcriptional regulation of NAG-1 both took place in a less coherent manner. These findings exemplify the potential of herbal terpenoids, particularly PAB, in modulating colon cancer carcinogenesis through known molecular targets and precise mechanism of action.

Keywords: Pseudolaric acid B; Triptolide; Apoptosis; Growth arrest; Colon cancer; NAG-1

1. Introduction

In animal and cell culture studies, the growth and metastasis of cancer can be delayed by many phytochemicals and herbal extracts. Novel chemotherapeutic agents derived from active phytochemicals could thus be used as adjuvants to improve the anti-cancer rates achievable with standard treatments (McCarty, 2001). Terpenoids from plant origin have demonstrated excellent anti-neoplastic functions by suppression of tumor proliferation and induction of apoptosis (Takahashi et al., 2002). The diterpene triepoxide triptolide (Fig. 1A) isolated from the herb *Tripterygium wilfordii* hook f. has received supporting evidences for being a potential anti-neoplastic agent (Shamon et al., 1997). It was found that triptolide could inhibit the growth and metastasis of various solid tumors and is capable of acting synergistically with conventional chemotherapeutic drugs (Chang et al., 2001; Yang et al., 2003). The proposed modes of anti-neoplastic action of triptolide include the activation of the death receptor-mediated apoptotic pathway by inhibition of nuclear factor-κB (NF-κB) or members of the inhibitor of apoptosis (IAP) family (Lee et al., 1999, 2002), as well as the prevention of cell proliferation (Kiviharju et al., 2002). PG490-88, a derivative of triptolide, had also been reported to induce

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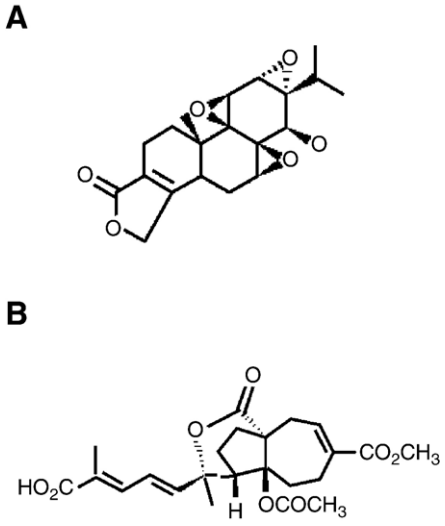


Fig. 1. Chemical structures of (A) triptolide and (B) PAB.

colon tumor regression in nude mice xenograft (Fidler et al., 2003). However, triptolide manifested a potent but non-specific cytotoxicity in a series of mammalian cells (Shamon et al., 1997), which has precluded its further development into potential anti-neoplastic drug.

Pseudolaric acid B (PAB; Fig. 1B) is another natural diterpenoid that can be extracted from the root bark of the tree *Pseudolarix kaempferi* Gordon, which has been used in Chinese folk medicine for the treatment of fungal infections (Li et al., 1995). Crude extract of the herb as well as purified PAB had both demonstrated potent cytotoxicity against several tumor cells, including those of the colon (Pan et al., 1990). Nevertheless, unlike triptolide that have been studied for years, little was known about the anti-neoplastic mechanism of this novel herbal compound. It was proposed that subtypes of the nuclear peroxisome proliferator-activated receptor (PPAR) are cellular targets of PAB in its action (Jardat et al., 2002). According to the limited data on its mode of action, PAB could induce apoptosis through activation of the mitogen-activated protein kinase (MAPK) pathway (Gong et al., 2004). Furthermore, a latest study on the anti-cancer activity of PAB in tumor cell lines had identified microtubules as its molecular target (Wong et al., 2005). It is therefore interesting to explore the precise mechanism of action of PAB in colon cancer cells.

Among novel molecular targets of anti-carcinogenic agents, the nonsteroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1) has been actively investigated in recent years. It is a divergent member of the transforming growth factor-β (TGF-β) superfamily. TGF-β is recognized as an important negative regulator of colonic epithelial cell growth. It is generally suggested that the TGF-β pathway is a potent tumor suppressor of human colorectal cancer. NAG-1, like other TGF-β proteins, also possesses anti-tumorigenic activity. NAG-1 induction in colon cancer cells by NSAID treatment would induce apoptosis and lead to a cyclooxygenase (COX)-independent anti-tumorigenic effect (Baek et al., 2001b). Beside NSAID, other novel pro-apoptotic and anti-tumorigenic agents that also cause NAG-1 overexpression include PPARγ ligands (Baek et al., 2004b). Since PAB is also a PPAR agonist (Jardat et al., 2002), we want to reveal whether NAG-1 is also a target for its actions.

In the present study, we attempted to investigate the actions of PAB in modulating the apoptotic and growth-inhibitory factors in human colon cancer cells, and to correlate with its effect on NAG-1 expression. Besides, its mode of action in colon cancer cells would be compared with that of another diterpenoid triptolide to see if they share common molecular target(s) and act via similar pathway.

2. Materials and methods

2.1. Herbal drugs

Triptolide and most chemicals used in this study (unless specified) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PAB was provided by Dr Pauline Chiu (Department

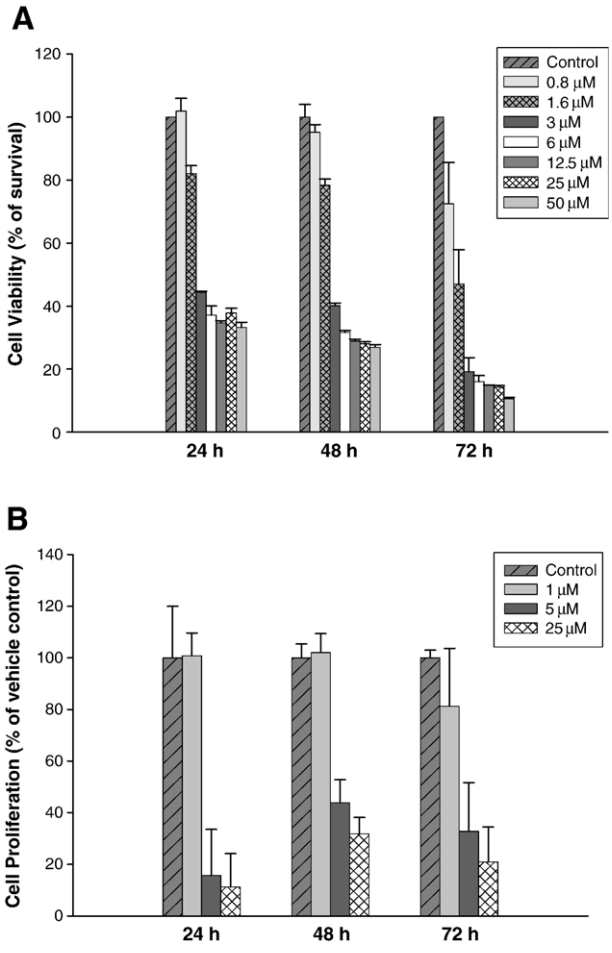


Fig. 2. Effects of PAB on (A) cell growth inhibition and (B) cell proliferation in HT-29 cells. Cells cultured in D-MEM supplemented with 10% FBS were incubated with various concentrations of PAB for 24, 48 or 72 h. The cytotoxic effect and number of proliferating cells were determined by MTT assay and BrdU ELISA, respectively. Data represent the percent reduction in the number of viable/proliferating cells after drug treatment relative to that of vehicle-treated control cells (100%). Data were obtained from 3–5 independent experiments.

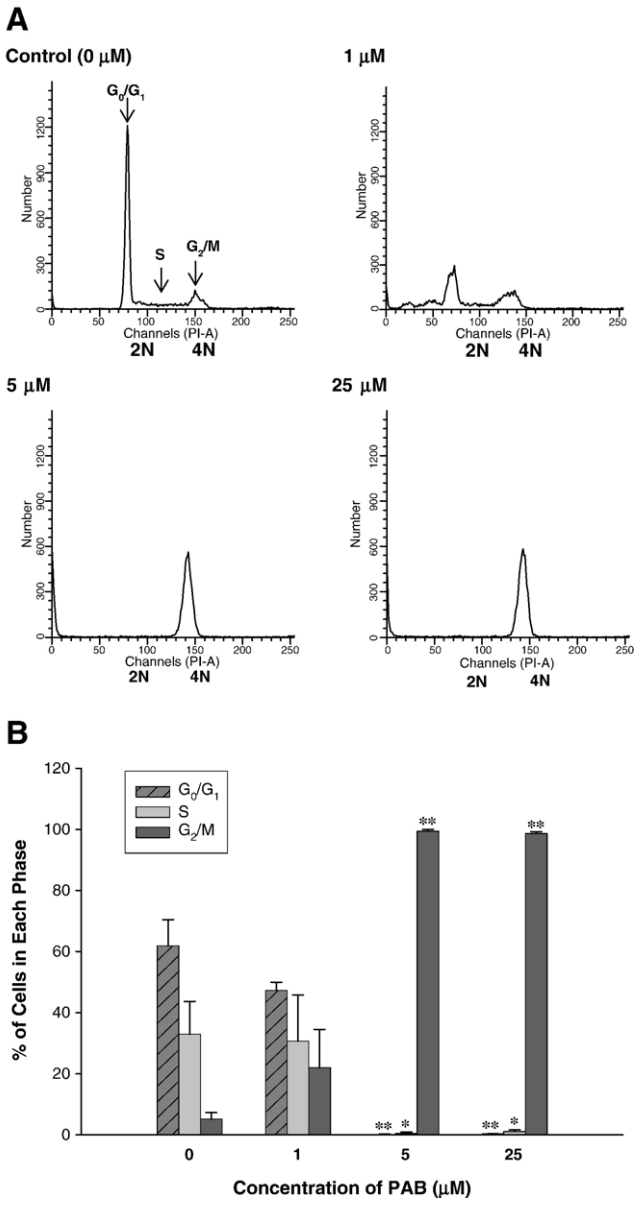


Fig. 3. Cell cycle analysis of HT-29 cells treated with PAB. Cells were stained with PI after 48 h exposure to PAB (1, 5, 25 μM) and analyzed by flow cytometry. (A) DNA histograms of vehicle-(Control) or drug-treated cells are shown. Axes *X* and *Y* illustrate the DNA content and number of cells, respectively. (B) Percentages of cell population in different phases of the cell cycle were analyzed using the ModFit LT software version 3.0 (Beckton Dickinson). Data were obtained from 3 independent experiments and analyzed by one-way ANOVA with Turkey's post-hoc test. $P<0.01$ (*) and $P<0.001$ (**) indicate significant difference between control and PAB-treated cells.

of Chemistry, The University of Hong Kong), which had been isolated from the root bark of *P. kaempferi* as reported previously (Zhou et al., 1983). In brief, a benzene extract was treated with 5% $NaHCO_3$. The acidic component was chromatographed on a silica gel column, eluted with C_6H_6 and C_6H_6-EtOAc mixture to give PAB. PAB was purified by repeated flash column chromatography on silica gel using hexane-ethyl acetate and dichloromethane–acetone mixtures. The purity of PAB purity was confirmed by high-performance liquid chromatography (HPLC) using an Alltima C18 column and a mobile phase of methanol/water on an Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) (Wong et al., 2005). Both PAB and triptolide were checked to have the purity of >98%. Their stock solutions were prepared by dissolving the compounds in <0.1% dimethylsulfoxide (DMSO) and stored at −20 °C for in vitro experiments. Cells in the control groups were treated with the culture medium containing an equal volume of DMSO. No significant difference was found between the groups treated with DMSO and with culture medium alone in all experiments.

2.2. Cell culture

The human colon adenocarcinoma HT-29 cells (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. HT-29 cells were seeded on 96-well plates at a concentration of 2×10^3 cells per well with complete culture medium. After overnight incubation, the cells were exposed to various concentrations of PAB or triptolide for 24–72 h.

2.3. Assessment of cell viability by MTT assay

The growth-inhibitory activity of PAB and triptolide was determined by measuring cell viability using the MTT assay. Following treatment of the test compounds, 30 μl of methylthiazolyldiphenyl-tetrazolium bromide (MTT) stock solution was added to each well and incubated at 37 °C for 3 h. The supernatant was removed and DMSO (100 μl) was added to solubilize the formazan crystals, with the absorbance being measured spectrophotometrically at 540 nm. Three to five independent experiments had been conducted for each treatment or control group.

2.4. Cell proliferation assay

Colon cancer cell proliferation was determined by using a 5′-bromo-2′-deoxyuridine (BrdU) colorimetric assay kit according to the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). It is a non-radioactive colorimetric ELISA that measures the BrdU incorporated into newly synthesized cellular DNA. In brief, 5×10^3 HT-29 cells were cultured in D-MEM supplemented with 10% FBS in 96-well plates, and exposed to PAB or triptolide. The cells were then fixed, incubated with anti-BrdU antibody and substrate for reactions. Color development correlates to the number of proliferating cells and was measured spectrophotometrically at 450 nm and using 690 nm as reference wavelength.

2.5. Assessment of cell cycle distribution by flow cytometry

Subconfluent HT-29 cells (3.3×10^5) were cultured in 60 mm dishes with PAB or triptolide. The cells were then harvested by

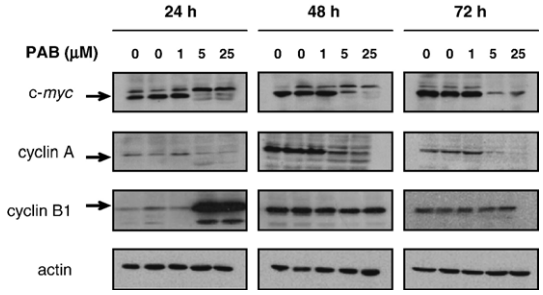


Fig. 4. Expression of c-*myc*, cyclin A and cyclin B1 proteins in HT-29 cells treated with PAB (1, 5, 25 μM) or vehicle control (0 μM) for 24–72 h was detected by Western blotting. β-actin was used as internal control. Data shown are representative immunoblots from at least 3 independent experiments with similar findings. Arrows indicate the target bands in the immunoblots.

trypsinization and fixed overnight with ice-cold 75% ethanol. After being washed with PBS, cells were stained with PBS containing 0.1% Triton X-100, 100 mg/ml of RNase A and 50 μg/ml of propidium iodide (PI) on ice for 1 h. The analysis of samples was performed by using flow cytometry (FACS Canto™, Beckton Dickinson Biosciences, San Jose, CA, USA). Cell cycle phase distributions were calculated from the resultant DNA histogram using the ModFit LT version 3.0 software, and expressed as a percentage of cells in the respective phases. In addition, the percentage of apoptotic cells was defined as those found in the sub-G1 region.

2.6. DAPI staining for nuclear damage

HT-29 cells (1×10^5 per well) were cultured for 48 h with varying concentrations of PAB or 0.1% DMSO (control). The cells were washed with PBS three times and then fixed with 4% paraformaldehyde for 10 min. After washing with PBS for three more times, cells were then incubated with ice cold methanol at −20 °C for another 10 min. Finally, the cells were stained with PBS containing 1 μg/ml of 4′,6-diamidino-2-

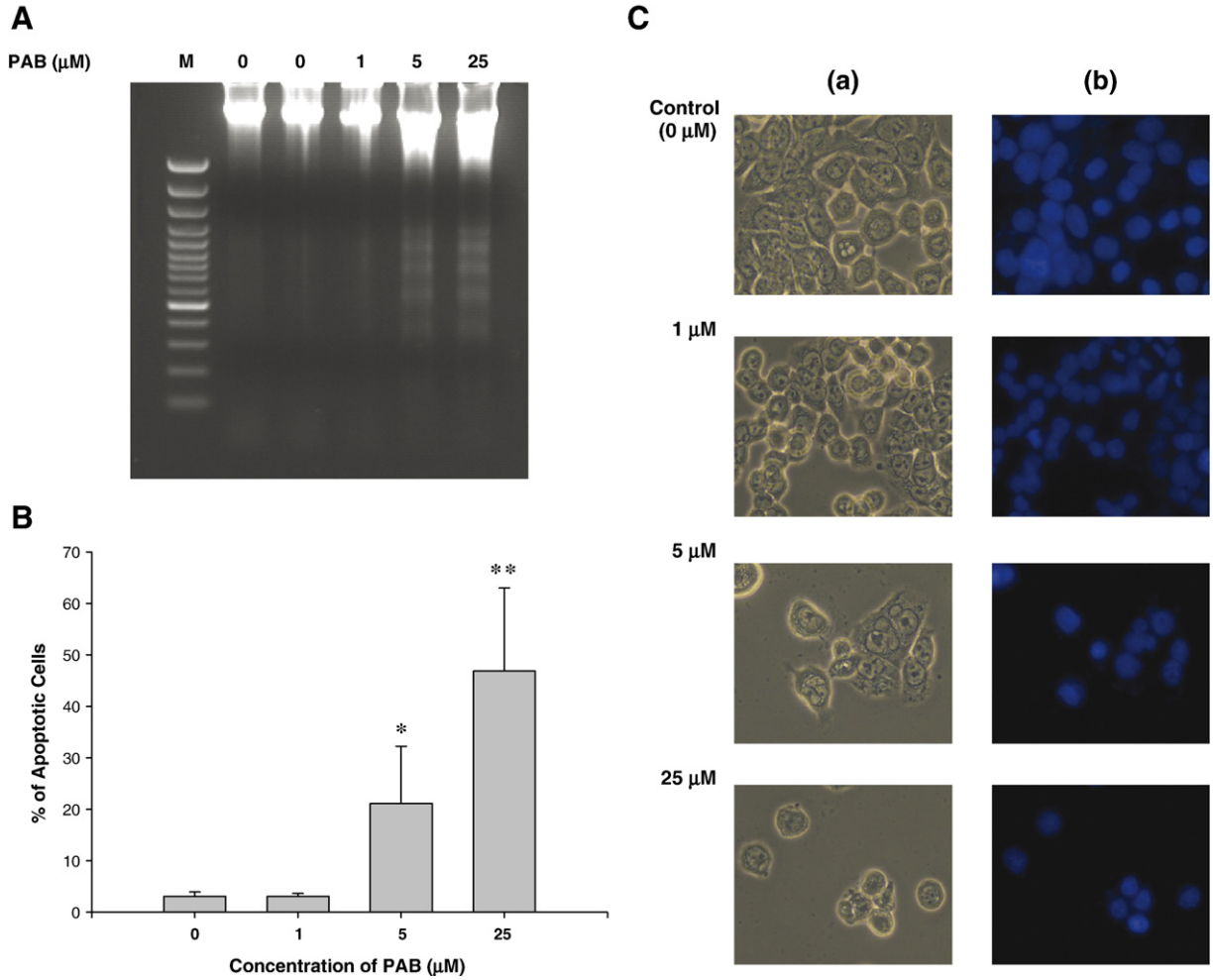


Fig. 5. Induction of apoptosis in HT-29 cells after 48 h exposure to PAB (1, 5, 25 μM). (A) DNA laddering pattern formation in 1.8% agarose gel indicates that the two higher PAB concentrations had caused DNA fragmentation [*M*=DNA marker; vehicle-treated control: 0 μM PAB]. (B) The amount of apoptotic cells was quantified by PI staining and analyzed by flow cytometry using the ModFit LT software version 3.0 (Beckton Dickinson). Apoptotic cells were defined as those distributed in the sub-G_1 region of the DNA histograms. Results are expressed as means±S.E.M. of 3 independent experiments and analyzed by one-way ANOVA with Turkey's post-hoc test. $P<0.05$ (*) and $P<0.01$ (**) indicate significant difference between control (0 μM) and PAB-treated cells. (C) Study of morphologic alterations in HT-29 cells following PAB treatment for 48 h using DAPI staining (63× amplification): a. phase-contrast microscopy; b. fluorescence microscopy. Nuclear chromatin condensation and presence of granular apoptotic bodies were observed in the cells treated with 5 and 25 μM of PAB.

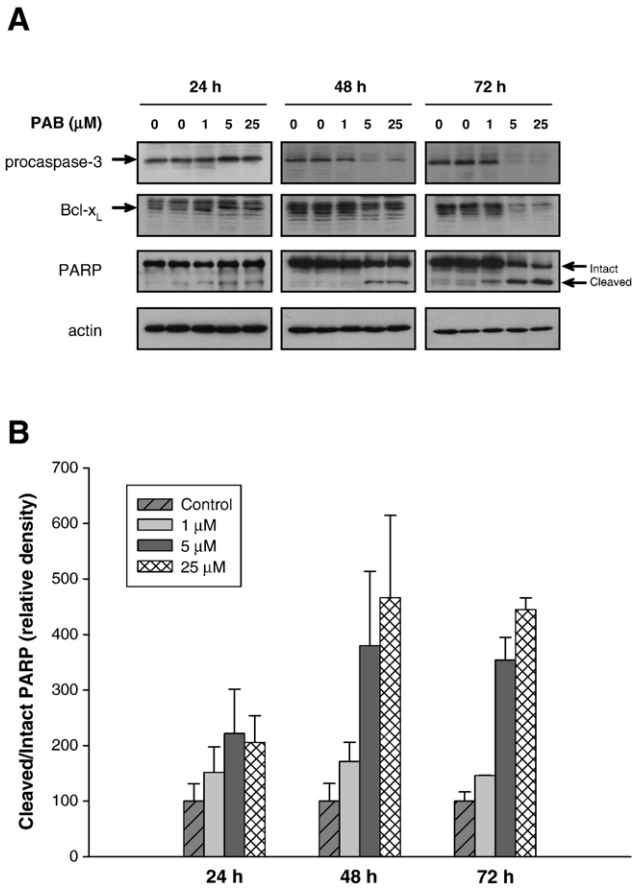


Fig. 6. (A) Expression of procaspase-3, Bcl-x_L proteins and PARP cleavage in HT-29 cells treated with PAB (1, 5, 25 μM) or vehicle control (0 μM) for 24–72 h was detected by Western blotting. β-Actin was used as internal control. Data shown are representative immunoblots from at least 3 independent experiments with similar findings. Arrows indicate the target bands in the immunoblots. (B) Increased cleavage of PARP was induced by PAB treatment in a concentration-dependent manner.

phenylindole (DAPI) for 10 min. The cells were observed for nuclear morphology under a fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) with a 63× magnification.

2.7. DNA fragmentation assay

DNA fragmentation was studied as described previously (Grant et al., 1995). In brief, cells were lyzed in 10 mM Tris–HCl (pH 7.4) buffer with 0.5% SDS, 25 mM EDTA and 0.1 mg/ml proteinase K, and incubated at 50 °C for 12–18 h. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with two volumes of ice-cold absolute ethanol and 1/10 volume of 3M sodium acetate. Equal amounts of DNA were electrophoresed in 1.8% agarose gels impregnated with 0.1 μg/ml of ethidium bromide for 1 h at 70 V. DNA fragments in the form of a laddering pattern were visualized by ultraviolet translumination and recorded by a gel documentation system (AlphaImager 2200, Alpha Innotech Corp., San Leandro, CA, USA).

2.8. Immunoblotting

Cells were lyzed by incubation in lysis buffer with 0.1% SDS, 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 2 mM EDTA and 10% glycerol supplemented with the following proteinase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 5 μg/ml pepstatin A on ice for 30 min. Cell lysates (50 μg) were denatured in SDS and separated on 10 to 15% SDS-polyacrylamide gel by electrophoresis and blotted onto nitrocellulose membrane (Bio Rad, Hercules, CA, USA). After blocking for 1 h in room temperature, the membranes were immunoblotted with the respective primary antibodies (1:500 except β-actin) overnight at 4 °C, as indicated: mouse monoclonal anti-cyclin A (Upstate, VA, USA), mouse monoclonal anti-cyclin B1 (Upstate), mouse monoclonal anti-c-*myc* (Calbiochem, San Diego, CA, USA), mouse monoclonal anti-procaspase-3 (Zymed Laboratories Inc, CA, USA), mouse monoclonal anti-Bcl-x_L (Zymed), mouse monoclonal anti-PARP (Beckton Dickinson), mouse monoclonal PPAR-γ (Santa Cruz, CA, USA), rabbit monoclonal anti-COX-2 (Lab Vision Corp, CA, USA), rabbit polyclonal anti-NAG-1 (Upstate), and mouse monoclonal anti-β-actin (internal control; 1:10000; Sigma). Following incubation with the appropriate secondary antibodies (1:5000) conjugated with horseradish peroxidase for 1 h in room temperature, the protein expression signals were detected by the enhanced chemiluminescence kit (Amersham Biosciences, IL, USA).

2.9. Real-time polymerase chain reaction (PCR)

HT-29 cells were harvested by trypsinization and RNA was extracted by using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA). Single-stranded cDNA was synthesized from 5 μg of total RNA using SuperScript™ Reverse Transcriptase (Invitrogen). Real-time fluorescence detection was carried out with Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). For each PCR, assay mix containing unlabeled PCR primers, TaqMan® MGB probes (FAM™ dye-labeled) and TaqMan® Universal PCR Master Mix (1x; Applied Biosystems) was mixed with each

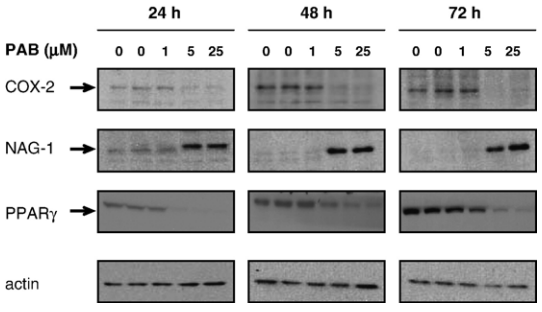
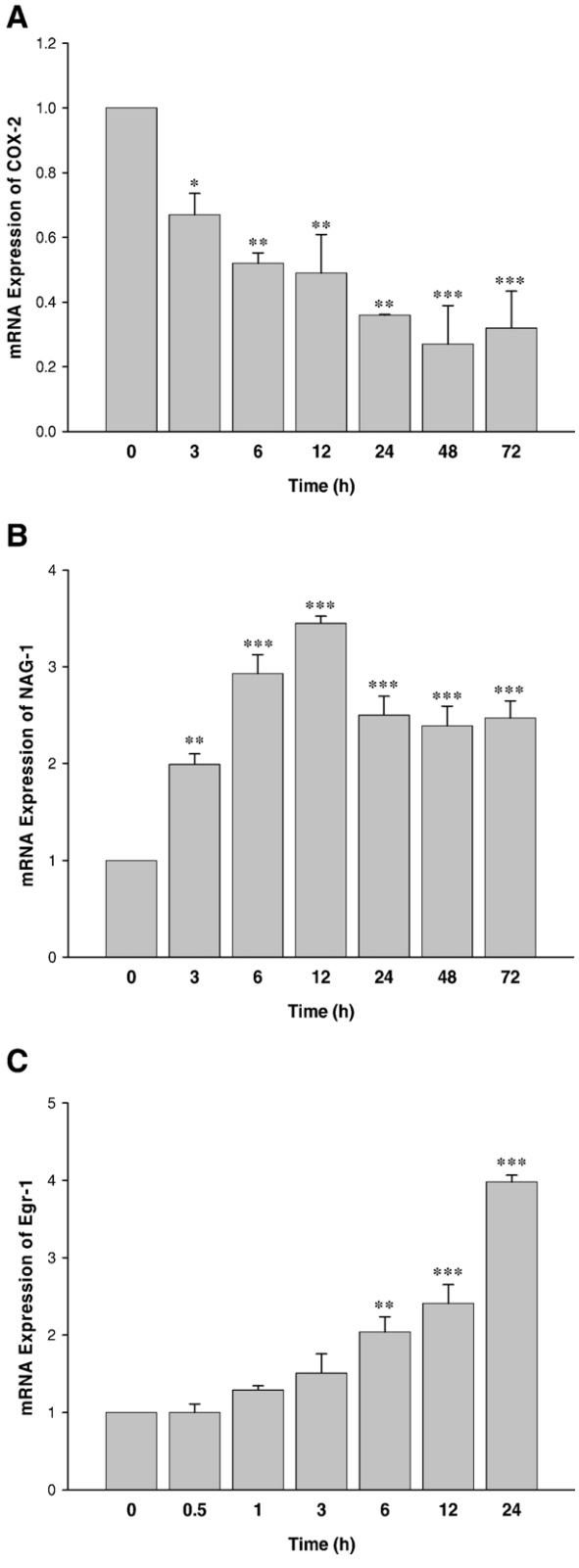


Fig. 7. Expression of COX-2, NAG-1 and PPAR-γ proteins in HT-29 cells treated with PAB (1, 5, 25 μM) or vehicle control (0 μM) for 24-72 h was detected by Western blotting. β-Actin was used as internal control. Data shown are representative immunoblots from at least 3 independent experiments with similar findings. Arrows indicate the target bands in the immunoblots.

cDNA (0.2 μl) in a final PCR reaction volume of 20 μl. COX-2 was amplified using the primers 5′-GTACCCGGACAG-GATTCTATGGA-3′ (forward) and 5′-TGTGTT-TGGAGTGGGTTT-CAGAAAT-3′ (reverse), with the MGB probe being 5′-[FAM]-CAACACCGGAATTTT-[NFQ]-3′. NAG-1 was amplified using the primers 5′-CCCTGCA-GTCCGGATACTC-3′ (forward) and 5′-GGTG-GCCGCCGGAT-3′ (reverse), with the MGB probe being 5′-[FAM]-CAGCCGCACTTCTG-[NFQ]-3′. EGR-1 was amplified using the primers provided by Taqman gene expression assays-on-demand™ (Assay ID: Hs00152928 m1), with the MGB probe being 5′-[FAM]-CACCTGACCGCAGAG TCTTTTCCTG-[NFQ]-3′. GAPDH was amplified using primers 5′-CTGGCCAAGGTCATCCATGA-3′ (forward) and 5′-GGGCCATCCACAGTCTTCTG-3′ (reverse), with the MGB probe being 5′-[FAM]-CATGGACTG TGGTCATGAG-[NFQ]-3′. The amplification parameters were the following: optimization steps at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and extension). Amplifications were normalized to GAPDH of the respective treatment, allowing relative quantitation using the 7500 Real-time PCR System software.



2.10. Reversibility study

In order to determine whether the effects of PAB on HT-29 cells could be reverted, drug-treated cells for 48 or 72 h were washed 3 times with 2 ml of PBS and placed in fresh culture medium in the absence of drug (Gajate et al., 2000). The cells were allowed to be incubated for another 24 or 48 h before they were harvested for MTT assay and Western analysis of NAG-1 protein expression. Any reversal effects on PAB-induced growth inhibition and NAG-1 overexpression would be noted.

2.11. Statistical analysis

The results of at least three independent experiments were expressed as the means±S.E.M. for most tests. For real-time PCR experiments, calculations were based on the level of significance on Ct values following adjustment for GAPDH, and results were expressed as the means±S.D. Statistical significance of at least $P<0.05$ was determined by one-way analysis of variance (ANOVA) followed by a post-hoc Turkey's test using the SPSS version 10.0 software.

3. Results

3.1. PAB induced cytotoxicity and inhibition of HT-29 cell proliferation

HT-29 cells were treated with various concentrations of PAB (0.8–50 μM). PAB-induced cytotoxicity was demonstrated

Fig. 8. mRNA expression of (A) COX-2, (B) NAG-1 and (C) Egr-1 in HT-29 cells was determined by real-time PCR after exposure to 25 μM of PAB for 3-72 h (COX-2 and NAG-1) or 0.5-24 h (Egr-1). Calculations were based on the level of significance on Ct values following adjustment for GAPDH (housekeeping gene). Results are expressed as means±S.D. of 2–3 independent experiments and analyzed by one-way ANOVA with Turkey's post-hoc test. $P<0.05$ (*), $P<0.01$ (**) and $P<0.01$ (***) indicate significant difference between PAB-treated samples at different time points and that at zero time (before drug treatment).

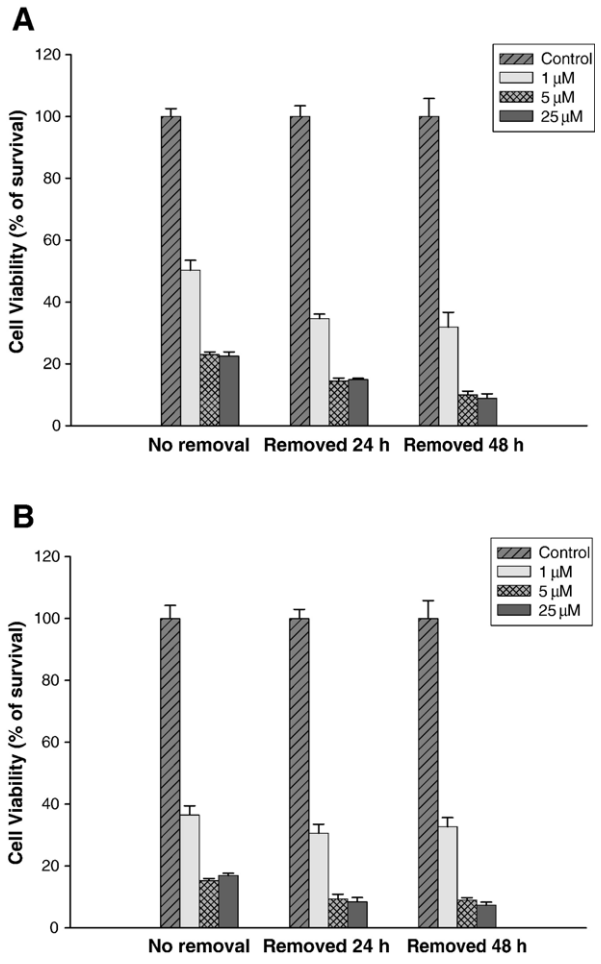


Fig. 9. Reversibility study on the growth-inhibitory effect of PAB in HT-29 cells. Cells cultured in D-MEM supplemented with 10% FBS were incubated with vehicle control (0 μM) or PAB (1, 5, 25 μM) for (A) 48 or (B) 72 h. In the first batch of treatment groups, MTT assay was performed right after the incubation period. In additional batches of treatment groups, the drug-containing culture medium was removed following the incubation period with replacement of fresh medium, and the cells were harvested 24 or 48 h later for MTT assay. Data represent the percent reduction in the number of viable cells after drug treatment relative to that of vehicle-treated control cells (100%). Data were obtained from 3 independent experiments.

following 24, 48 and 72 h of drug incubation (Fig. 2A). Growth inhibition was observed with PAB concentrations higher than 0.8 μM after 24 and 48 h of drug treatment, while with 72 h of incubation, even 0.8 μM of the drug could induce almost 25% reduction of the number of viable cells. The ED_{50} of PAB after 48 h of incubation in HT-29 cells was found to be 2.14 μM. We had also examined the effect of PAB in HT-29 cell proliferation using a BrdU-labeling method. Results indicated a dose-dependent inhibition in cell proliferation by 24, 48 and 72 h of drug incubation (Fig. 2B). At the highest concentration of 25 μM, PAB induced a 75–90% reduction in the number of proliferating cells.

3.2. PAB caused HT-29 cell cycle arrest by modulation of growth-related cyclins and protooncogene

HT-29 cells were harvested after 48 h exposure to 1, 5 or 25 μM of PAB, followed by PI staining. The effect of PAB in cell cycle distribution was assessed by flow cytometric analysis (Fig. 3A). The typical diploid peak pattern of the DNA histogram was lost due to the drastic reduction of cells in the G_0/G_1 phase and accumulation of cells in the G_2/M phase following PAB treatment (at 5 and 25 μM). Cell population in the G_2/M phase had increased from 4.60% in the control to nearly 100% in the two higher concentrations of PAB treatment groups, while those in the G_0/G_1 and S phases were dropped accordingly (from 53.28% to 0% and from 43.66% to 1.64%, respectively) (Fig. 3B). The same concentrations of PAB also caused inhibition of the protooncogene c-*myc* (which promotes cell cycle progression) as well as cyclin A protein expression after 24, 48 and 72 h of treatment, whereas the expression of cyclin B1 was upregulated after 24 h of drug treatment (Fig. 4).

3.3. PAB caused HT-29 cell death via DNA fragmentation

Fig. 4 shows the pro-apoptotic effects of PAB in HT-29 cells. Incubation of 5 and 25 μM of PAB for 48 h had caused the formation of internucleosomal DNA fragmentation (Fig. 5A). This in turn led to a concentration-dependent cell death (with 21.13 and 46.85% of apoptotic cells after treatment with 5 and 25 μM of PAB, respectively), which was reflected by the cell population located at the sub-G_1 region when determined by flow cytometric analysis (Fig. 5B). To determine the mode of cell death induced by PAB, morphologic alterations in HT-29 cells after drug treatment for 48 h were examined under fluorescence microscopy using DAPI staining (Fig. 5C). In the control group, HT-29 cells were round and with homogeneous staining. In the PAB treatment group (particularly 5 and 25 μM), nuclear chromatin condensation and presence of granular apoptotic bodies were observed. In order to investigate the mechanism underlying PAB-induced apoptosis, the effects of the drug on the expression of the anti-apoptotic protein Bcl-x_L, activation of caspase-3 and cleavage of the downstream protein PARP were assessed (Fig. 6A). It was found that PAB concentration-dependently reduced the immunoreactivity of Bcl-x_L, which was accompanied by a proteolytic processing of procaspase-3 and cleavage of the caspase-3 substrate PARP to its 85 kDa product at all time points being tested (Fig. 6A and B).

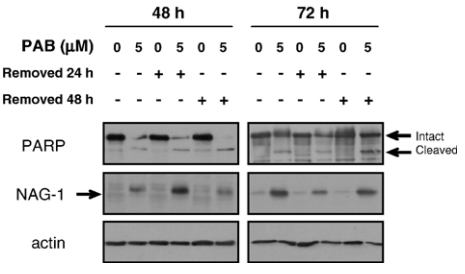


Fig. 10. Expression of PARP and NAG-1 proteins in HT-29 cells treated with 5 μM of PAB or vehicle control (0 μM) for 48–72 h, with or without the removal of drug-contained medium followed by further incubation in fresh medium for 24 or 48 h, was detected by Western blotting. β-Actin was used as internal control. Data shown are representative immunoblots from at least 3 independent experiments with similar findings. Arrows indicate the target bands in the immunoblots.

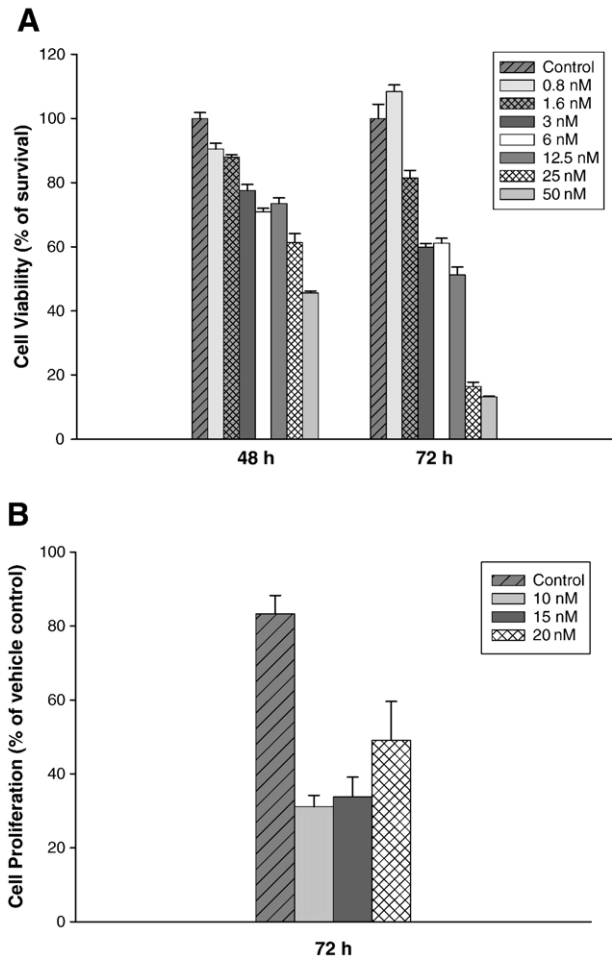


Fig. 11. Effects of triptolide on (A) cell growth inhibition and (B) cell proliferation in HT-29 cells. Cells were incubated with various concentrations of triptolide for 48 or 72 h. The cytotoxic effect and number of proliferating cells were determined by MTT assay and BrdU ELISA, respectively. Data represent the percent reduction in the number of viable/proliferating cells after drug treatment relative to that of vehicle-treated control cells (100%). Data were obtained from 3–5 independent experiments.

3.4. PAB induced upregulation of NAG-1 and inhibition of COX-2 protein and gene expression as well as suppressed PPARγ protein expression

The modulation of COX-2 and the novel protein NAG-1 by PAB was studied in HT-29 cells. Results had shown that NAG-1 protein was prominently overexpressed by PAB treatment (5 and 25 μM) for 24, 48 and 72 h, with a simultaneous downregulation of COX-2 and PPARγ protein expression (Fig. 7). In order to examine whether the induction of NAG-1 protein requires *de novo* synthesis at the transcriptional level and to reveal their correlation with the regulation of an upstream transcriptional factor early growth response gene Egr-1 in HT-29 cells, quantitative real-time PCR was performed using GAPDH as the housekeeping gene. Data at each time point was compared with respect to that obtained at time zero when drug had not been added. NAG-1 mRNA synthesis was significantly induced by 25 μM of PAB in a time-dependent manner from 3 to 12 h (peak) of incubation, and maintained at high levels until 72 h (Fig. 8B). A concurrent concentration-dependent inhibition of COX-2 mRNA synthesis was also achieved by the same concentration of PAB from 3 to 72 h of drug incubation (Fig. 8A). It is interesting to note that PAB-induced upregulation of Egr-1 mRNA became significant after 6 h of drug incubation and kept increasing until 24 h (Fig. 8C). Since the

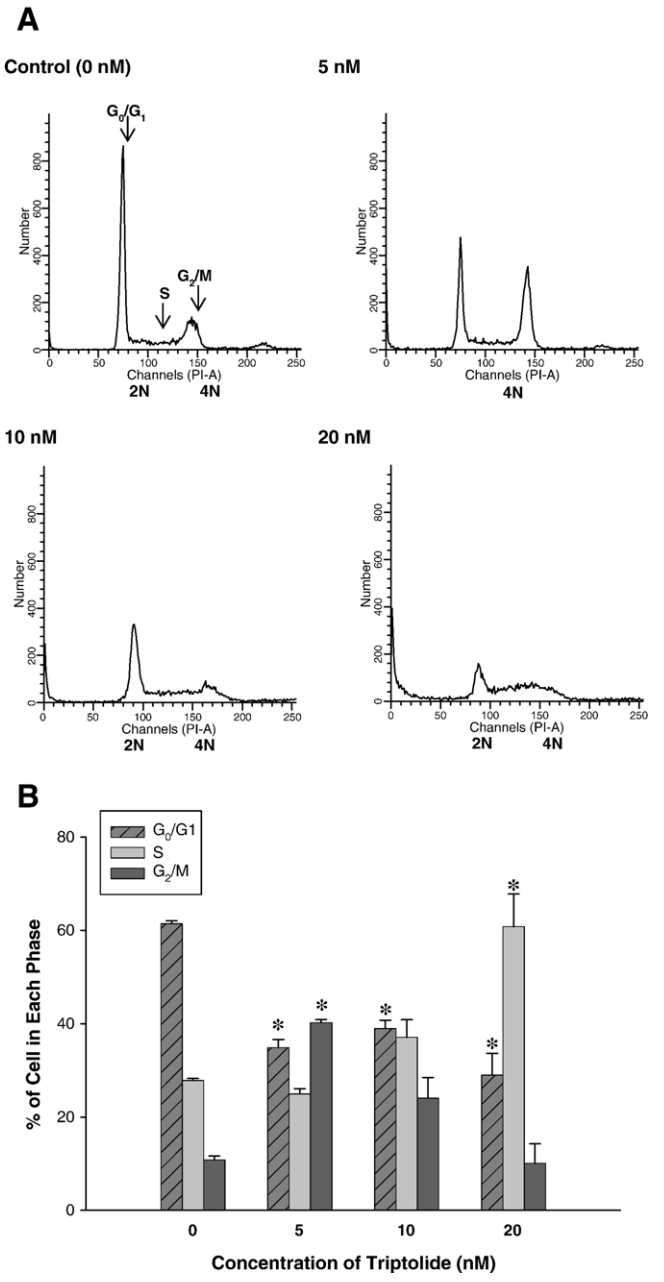


Fig. 12. Cell cycle analysis of HT-29 cells treated with triptolide. Cells were stained with PI after 72 h exposure to triptolide (5, 10, 20 nM) and analyzed by flow cytometry. (A) DNA histograms of vehicle-(Control) or drug-treated cells are shown. Axes *X* and *Y* illustrate the DNA content and number of cells, respectively. (B) Percentages of cell population in different phases of the cell cycle were analyzed using the ModFit LT software version 3.0 (Beckton Dickinson). Data were obtained from 3 independent experiments and analyzed by one-way ANOVA with Turkey's post-hoc test. $P<0.05$ (*) indicates significant difference between control and triptolide-treated cells.

onset time of Egr-1 gene upregulation by PAB was later than that of NAG-1, the induction of NAG-1 might not be due to transcriptional activation of Egr-1.

3.5. PAB-induced PARP cleavage and NAG-1 overexpression was not reversible in HT-29 cells

For reversibility study, when HT-29 cells were treated with PAB for 48 or 72 h and then removed, no significant reversion could be observed. Following removal of PAB from the culture medium for either 24 or 48 h, the reduction in the number of viable cells in all treatment groups remained at similar level of inhibition as that before reversion (Fig. 9A and B). In addition, PAB-induced PARP cleavage and NAG-1 overexpression remained visible after 24 or 48 h of drug removal (Fig. 10).

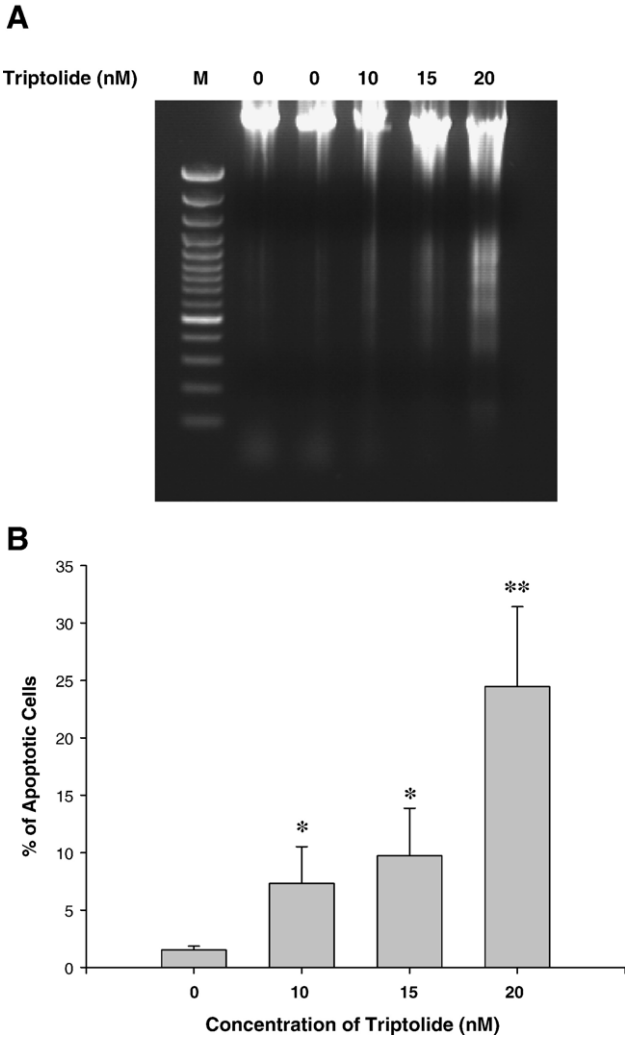


Fig. 13. Induction of apoptosis in HT-29 cells after 72 h exposure to triptolide (10, 15, 20 nM). (A) DNA laddering pattern formation in 1.8% agarose gel indicates that the two higher triptolide concentrations had caused DNA fragmentation [*M*=DNA marker; vehicle-treated control: 0 nM triptolide]. (B) The amount of apoptotic cells was quantified by PI staining and analyzed by flow cytometry using the ModFit LT software version 3.0 (Beckton Dickinson). Apoptotic cells were defined as those distributed in the sub-G_1 region of the DNA histograms. Results are expressed as means±S.E.M. of 3 independent experiments and analyzed by one-way ANOVA with Turkey's post-hoc test. $P<0.05$ (*) and $P<0.01$ (**) indicate significant difference between control (0 nM) and triptolide-treated cells.

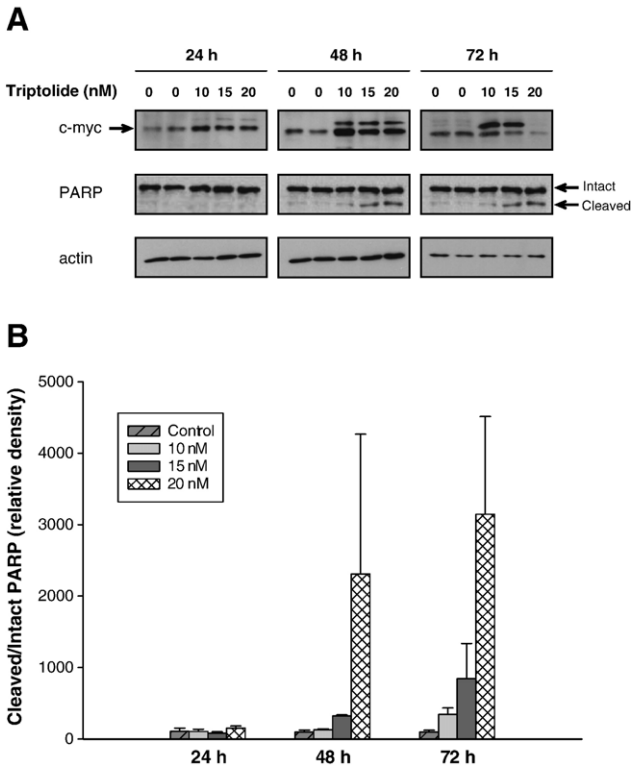


Fig. 14. (A) Expression of c-*myc* protein and PARP cleavage after treatment of HT-29 cells with triptolide (10, 15, 20 nM) or vehicle control (0 nM) for 24–72 h was detected by Western blotting. β-Actin was used as internal control. Data shown are representative immunoblots from at least 3 independent experiments with similar findings. Arrows indicate the target bands in the immunoblots. (B) Increased cleavage of PARP was induced by triptolide treatment concentration-dependently.

3.6. Effects of triptolide on HT-29 cells

In order to compare the mode of anti-carcinogenic action of PAB with other known herbal diterpenoids, the effects on triptolide had been tested. Triptolide-induced growth inhibition was observed after 48 or 72 h of drug treatment (0.8-50 nM) in HT-29 cells. Its optimal cytotoxic effect was achieved after 72 h of incubation with an ED_{50} of 13.8 nM (Fig. 11A), together with the inhibition of cell proliferation which was concentration-independent (Fig. 11B). Nevertheless, unlike PAB, the effect of triptolide on HT-29 cell cycle progression was inconsistent, with G_2/M phase arrest at low concentrations and S phase arrest at high concentrations, respectively (Fig. 12A and B), which was difficult to explain. On the contrary, DNA fragmentation and the subsequent concentration-dependent induction of apoptosis were detected in HT-29 cells following 72 h of triptolide treatment (Fig. 13A and B), which was associated with PARP cleavage (Fig. 14A and B). Nonetheless, expression of the protooncogene c-*myc* was unexpectedly upregulated (Fig. 14A). Although inhibition of COX-2 and induction of Egr-1 gene expression were not demonstrated after triptolide

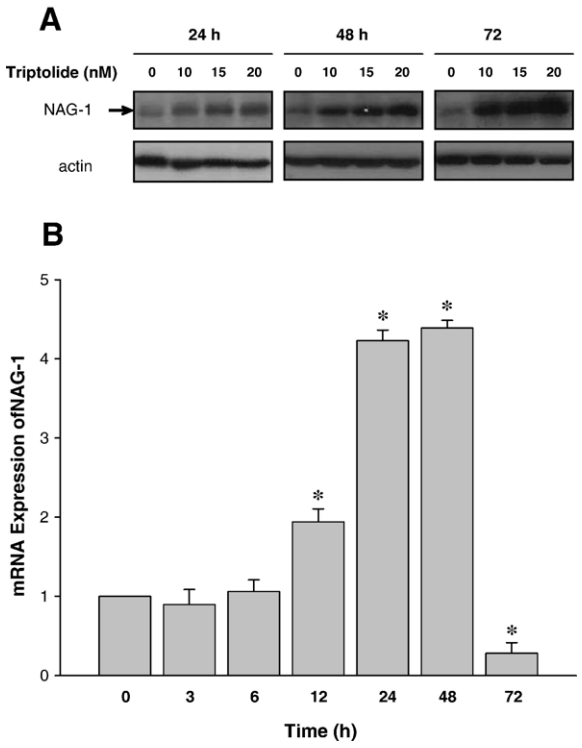


Fig. 15. Expression of NAG-1 protein and mRNA after treatment of HT-29 cells with triptolide. (A) Protein expression of NAG-1 after treatment of triptolide (10, 15, 20 nM) or vehicle control (0 nM) for 24–72 h was detected by Western blotting. β-Actin was used as internal control. Data shown are representative immunoblots from at least 3 independent experiments with similar findings. Arrows indicate the level of target bands in immunoblots with multiple bands. (B) mRNA expression of NAG-1 in HT-29 cells was determined by real-time PCR after exposure to 20 nM of triptolide for 3–72 h. Calculations were based on the level of significance on Ct values following adjustment for GAPDH (housekeeping gene). Results are expressed as means ± S.D. of 2–3 independent experiments and analyzed by one-way ANOVA with Turkey's post-hoc test. $P<0.001$ (*) indicates significant difference between triptolide-treated samples at different time points and that at zero time (before drug treatment).

treatment (unpublished findings), upregulation of both NAG-1 protein and gene expression had been resulted (Fig. 15A and B). However, the sudden drop of the NAG-1 mRNA expression at 72 h after 20 nM of triptolide treatment to a level beyond the baseline at 0 h was again not explainable.

4. Discussion

Recent studies have shown that herbal terpenoids possess anti-tumor activities and suggest that these compounds constitute a new class of cancer chemopreventive agents (Shishodia et al., 2006). In the present investigation, we had demonstrated that the herbal diterpenoid PAB could induce cytotoxicity, growth inhibition and apoptosis in HT-29 human colon cancer cells, which were associated with a prominent cell cycle arrest at the G_2/M phase. Other than that, we also compared these effects with those caused by another herbal diterpenoid triptolide, which is a known cytotoxic and pro-apoptotic agent that has been investigated for some years. Triptolide was initially established as a promising immunosuppressive agent from herbal medicine (Luk et al., 2000). This compound had previously been shown to inhibit primary and secondary tumors derived from the breast, bladder, stomach as well as melanomas, but the molecular mechanisms responsible for its anti-neoplastic effect on human malignant cells remain poorly understood. Despite the suggestion that triptolide acts more readily in the wild-type p53 cell lines (e.g. AGS gastric cancer cells), it is generally believed that it could suppress tumor cell growth regardless of their p53 status. We confirmed in the present study that triptolide could induce cytotoxic and pro-apoptotic effect in HT-29 colonic cancer cells − the cell line that possesses mutation in codon 273 of the p53 gene.

There are a lot of similarities between PAB and triptolide. PAB is a novel diterpenoid compound from plant origin, which caused cytotoxicity in several human tumor cell lines (Pan et al., 1990). Like triptolide, it has also demonstrated pro-apoptotic activity independent of the p53 status. The induction of cell cycle arrest by PAB in other cell lines was generally reported at the G_2/M transition (Gong et al., 2005; Wong et al., 2005), which is consistent with our findings in HT-29 cells. In the cell cycle, cyclin A/cyclin-dependence kinase (cdk)2 activation is necessary for the initiation and progression through S phase as well as the onset of mitosis (Vermeulen et al., 2003). In late G_2 and early M phases, cyclin A complexes with cdk1 (also known as cdc2) to facilitate entry into the mitotic phase (Ford and Pardee, 1998). Cdc2 further drives the cell into M phase through its association with cyclin B (O'Connell and Cimprich, 2005). Cyclin A has a function in regulating cyclin B/cdc2, of which ablation of cyclin A may activate cyclin B/cdc2 complex by dephosphorylation of Tyr 15 (Walker and Maller, 1991). In turn, entry into mitosis is tightly regulated by the phosphorylation status of the cyclin B/cdc2 complexes. The initiation of mitosis begins with the binding of cdc2 to cyclin B1 and phosphorylation of Thr-161 site of cdc2 (Solomon et al., 1990). G_2/M arrest in human breast cancer cell MCF-7 has been shown by inhibiting the activity of the cyclin B1/cdc2 complex through its association either with cyclin B1 or cdc2 at the Tyr-15 phosphorylation site (Barboule et al., 1998). In our study, the associated inhibition of cyclin A by PAB treatment could progress the cell cycle to the G_2/M phase through initial activation of the cyclin B/cdc2 complex. However, persistent high expression of cyclin B1 (indicating the lack of cyclin B1/cdc2 binding) arrests the cells at the G_2/M transition without exiting to mitotic switch. Similar overexpression of cyclin B1 along with G_2/M arrest has also been reported in a study on prostate cancer cells (Ray et al., 2006).

Beside its anti-proliferative property, PAB was found to induce apoptosis through caspase activation in human melanoma cells (Gong et al., 2005). In the present study, in addition to the associated nuclear damage, DNA fragmentation, cleavage of procaspase-3 and PARP, as well as inhibition of Bcl-x_L, we had also demonstrated a significant down-regulation of the growth-related c-*myc* protein, an action that was not achieved by triptolide. Our findings that cell-cycle arrest detected in HT-29 cells can be accompanied by apoptosis had been confirmed by other reports (Sačková et al., 2006; Shih and Stutman, 1996). It was suggested that human colon cancer cells lacking p53 can still initiate G_2/M arrest, but they are unable to remain arrested

in G_2/M and eventually enter mitosis (Bunz et al., 1998). Mueller et al. (2006) also stated that some cases of drug-induced tumor cell death are highly dependent on cell-cycle phase, of which the apoptotic process is initiated and eventually executed in the G_2/M phase of the cell cycle.

Although triptolide was found to arrest cells in the G_0/G_1 phase of the gastric cancer cell cycle (Jiang et al., 2001) and an S-phase arrest was reported in another study on prostate cancer cells (Kiviharju et al., 2002), we had discovered here that triptolide inconsistently caused G_2/M and S phase arrest in HT-29 cells by using various concentrations of the drug. It had been suggested that triptolide may directly inhibit growth-promoting cyclins such as cyclin A, cyclin D, and cyclin E or block cdk4 or cdk6 activity (Chang et al., 2001). These factors could in turn be activated by the protooncogene c-*myc*. However, according to our findings in Western analysis, overexpression of c-*myc* was caused by triptolide, which implicates that this protooncogene may not be relevant to the observed growth-inhibitory effects in HT-29 cells. It had been suggested in a parallel study that the moderate growth-inhibitory activity of triptolide at low concentration might be suitable for chemopreventive strategies, of which the prominent pro-apoptotic activity of high concentration of the drug would make it a good choice as a chemotherapeutic agent (Kiviharju et al., 2002).

It is plausible that there are multiple target molecules critical to colonic cancer cell survival, which in turn activates various transcriptional factors leading to suppression of the cell cycle and/or induction of apoptosis that inhibit tumor cell growth. However, the specific upstream target(s) of PAB and triptolide remain(s) unidentified. Among the postulated pathways that may facilitate the apoptotic effects of these diterpenoids, activation of NF-κB and ERK, a member of the MAPK family, had been documented (Frese et al., 2003; Gong et al., 2004; Lee et al., 2002). Furthermore, transcriptional activation of PPAR isoforms was also a distinctive proposed mode of PAB's action (Jardat et al., 2002). Our study has shown that PAB could induce a concentration-dependent downregulation of PPARγ protein expression. Nonetheless, it is still unclear how PPARγ impacts on colon cancer, thus a definite conclusion cannot be drawn at the present moment (Auwerx, 2002). On the other hand, NAG-1 is the TGF-β superfamily member that had shown vital anti-tumorigenic activity and was profoundly involved in the pro-apoptotic effect of COX-2 inhibitors in human colonic cancer cells (Baek et al., 2001b). It had also been reported that genistein, a naturally occurring isoflavanoid, possesses anti-carcinogenic properties through a p53-dependent induction of NAG-1 (Wilson et al., 2003). Despite that, it is known that NAG-1 contains p53 binding sites as well as several other transcriptional binding sites in its promoter, indicating that its induction may involve multiple mechanisms (Baek et al., 2001a). It was suggested that overexpression of the death receptor would couple with NAG-1 induction to cause apoptosis (Jang et al., 2004), which is consistent to our observation of reduced procaspase-3 expression and PARP cleavage. In the post-translational level, we discovered that NAG-1 protein could be induced by treating the HT-29 cells with PAB and triptolide. The follow-up time-course study of NAG-1 gene expression also indicated a time-dependent induction of NAG-1 mRNA synthesis by the two herbal diterpenoids. Prospective study using siRNA would be useful to verify the importance of NAG-1 in apoptosis induced by the herbal terpenoids. Although COX-2 is often highly expressed in colonic tumors, alteration of its expression by drugs did not always modulate NAG-1 expression (Baek et al., 2002). In our study, a coherent correlation between NAG-1 induction and COX-2 inhibition was only observed in the PAB-treated HT-29 cells. It is remarkable that the pro-apoptotic effects and NAG-1 overexpression caused by PAB could not be reversible upon removal of the drug from the culture medium. It has been suggested that some anti-neoplastic agent actually induces reversible cell-cycle arrest, while causing later events such as internucleosomal DNA degradation that eventually leads to irreversible apoptosis (Gajate et al., 2000). This idea seems to be in concert with our observations in the action of PAB in HT-29 cells.

In spite of the knowledge that NAG-1 is an important target of NSAID and other novel chemotherapeutic agents, the mechanism of its regulation is not clearly defined. Several upstream transcriptional factors had been suspected to cause induction of NAG-1 in response to drug treatments. Epicatechin gallate was reported to induce NAG-1 expression in colorectal cancer cells via a p53-independent activation of the activating transcription factor ATF-3, which led to a G_1 to S phase growth arrest and induction of apoptosis (Baek et al., 2004a). Other than that, Egr-1 is a nuclear phosphoprotein that is involved in the regulation of cell growth and differentiation in response to signals such as mitogens, growth factors and stress stimuli. It is also a pro-apoptotic protein that could be induced following treatment with PPARγ ligands against colorectal cancers. Two Egr-1 binding sites have been identified to transactivate NAG-1 expression, of which mutation of these sites could cause dramatic reduction of NAG-1 promotor activity (Baek et al., 2004b). Based on our results, although there is not enough evidence to prove that increased expression of Egr-1 mRNA directly leads to NAG-1 induction in the action of PAB, it is still possible that Egr-1 activation could be closely related to the transcriptional activity of NAG-1 − a phenomenon that is important in the effects of COX inhibitors and novel PPAR ligands (Baek et al., 2005; Chintharlapalli et al., 2005). In contrast, triptolide-induced NAG-1 gene expression was not associated with modulation of Egr-1 transcription (data not shown). Since Egr-1 is generally considered as a tumor suppressor gene that is capable of inducing transcriptional up-regulation of other anti-tumorigenic genes, its precise down-stream pathway to induce apoptosis and/or anti-tumorigenesis requires further investigation.

Taken together, we have demonstrated marked growth-inhibitory and pro-apoptotic effects in HT-29 colon cancer cells treated with PAB. PAB caused a G_2/M phase arrest along with suppression of the growth-related protein c-*myc* and modulation of the associated cyclins. More importantly, the novel NAG-1 pathway had been proven to be one of the molecular targets that mediate the cytotoxic actions of PAB. Egr-1 may be another transcriptional factor that is responsible for the PAB-induced anti-tumorigenic action, although its correlation with NAG-1

regulation could not be confirmed. In spite of the inconsistent and somewhat ambiguous actions of triptolide, it is also well known for its high toxicity in human cells, resulting in a narrow therapeutic index. Thus, the comparatively safer drug PAB would have a higher value for prospective study of its anti-tumor effects.

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