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Induction of apoptosis by penta-acetyl geniposide in rat C6 glioma cells

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Abstract

Penta-acetyl geniposide, (Ac)₅-GP, was produced by acetylation of a glycoside, isolated from an extract of *Gardenia fructus*. Previously, we have reported that C6 glioma cells could be inhibited in culturing as well as in bearing rats by treating with (Ac)₅-GP. In this study, the effect and action of (Ac)₅-GP on inducing cell death was examined in rat C6 glioma cells. Treatment of C6 glioma cells with (Ac)₅-GP caused cell death, chromatin condensation, and internucleosomal DNA ladder. Also, cell cycle arrest at G₀/G₁ phase revealed that (Ac)₅-GP-induced cell death appears to be mediated by apoptosis. In addition, the results also showed that p53 and c-Myc increased due to treatment of (Ac)₅-GP in a dose–response and time-dependent manner. Concomitant with the expression of p53 and c-Myc, decreased level of Bcl-2 and increased level of Bax protein were observed. These results suggest that cell death caused by (Ac)₅-GP through apoptosis and cell cycle arrest at G₀/G₁ may be associated with the induction of p53, c-Myc and may be mediated with apoptosis-related Bcl-2 family proteins.

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Keywords: Penta-acetyl geniposide; C6 glioma cell apoptosis; Cell cycle arrest; p53; c-Myc; Bcl-2; Bax

Abbreviations: (Ac)₅-GP, penta-acetyl geniposide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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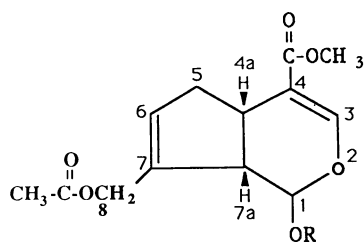
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1. Introduction

Gardenia fructus (San-jee-chee in Chinese), the fruit of *G. jasminoides* Ellis (Rubiaceae), has been used for many years in Chinese medicine for the treatment of various inflammatory diseases and hepatic disorders. However, the pharmacological basis of this medication is completely unknown. Numerous previous studies [1–7] have suggested the inhibitory effects of its compounds on tumorigenesis. Penta-acetyl geniposide, (Ac)₅-GP, 1-(β-D-2',3',4',6'-tetraacetylglucopyranosyloxy)-1,4a,5,7a-tetrahydro-7-(acetomythyl)-cyclopentapyran-4-carboxylic acid methyl ester (Fig. 1), was produced by acetylation of a iridoid glycoside isolated from an extract of *G. Fructus* [8]. Previously, we reported that C6 glioma cells can be inhibited in culturing [8], as well as in bearing rats [9] through the treatment with (Ac)₅-GP. (Ac)₅-GP has also been shown to inhibit aflatoxin B₁-induced genotoxicity and tumor progression in rats [10,11].

The biochemical actions of various cytotoxic agents have been extensively investigated, although little is known about the precise mechanisms by which they kill normal and malignant cells. Recent studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, cytarabine, camptothecin, amsacrine, etoposide and teniposide [12–18]. There is an accumulating evidence that the efficacy of antitumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [12–15,19–21]. Apoptosis, a physiological mode of cell death, is characterized by reduced cell volume, condensed chromatin in the nucleus, formation of internucleosomal DNA fragmentation and loss of membrane integrity, and generation of apoptotic bodies [22,23].

Previous evidence suggested that two cellular responses to p53 activation are well described growth arrest and apoptosis [19,24]. Many studies also indicate that apoptosis involves in the regulation of oncogenes. One of the first oncogenes demonstrated to have proapoptotic activity was c-Myc. Ectopic expression of c-Myc is sufficient to drive many cells into the cycle and also promotes apoptosis [25–27]. In a previous study [8,9], we have reported that (Ac)₅-GP inhibits the growth of C6 glioma cells in culturing and in bearing rats. The exact mechanisms responsible for the antitumor effect of (Ac)₅-GP, however, are not yet thoroughly understood. In



R=β-D-2, 3, 4, 6-tetra-acetyl-glucose

Fig. 1. The structure of (Ac)₅-GP.

this paper, we further investigate whether (Ac)₅-GP exerts cytotoxic activity against tumor cells by inducing apoptosis and cell cycle arrest, also examining the roles of p53, c-Myc and Bcl-2 in the phenomenon.

2. Materials and methods

2.1. Cell culture

The rat C6 glioma cell line was originally derived from a *N*-nitrosomethylurea-induced rat brain tumor [28]. The rat C6 glioma cell line was grown in minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin). Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. All experiments were performed in plastic tissue culture flasks, dish or in microplates (Nunc, Naperville, Denmark).

2.2. Preparation of (Ac)₅-GP solution

The chemical structure of (Ac)₅-GP is shown in Fig. 1. (Ac)₅-GP was isolated and acetylated from *G. fructus* in accordance with our previous method [8]. A stock solution of (Ac)₅-GP was prepared in DMSO, protected from light and stored at –20 °C. Prior to use, the (Ac)₅-GP solution was freshly prepared in a medium at the desired concentration. Control experiments were carried out in 0.05 ml of medium containing 0.02% (v/v) DMSO.

2.3. Assessment of cytotoxicity

The cytotoxicity assay was performed according to the MTT colorimetric assay [29]. The cells (5×10^4 cells/well) were seeded in 12-well plates (Nunc) and treated with (Ac)₅-GP at 0.015–0.225 mM concentration for 24 h at 37 °C. After the exposure period, media from all treatments were withdrawn, followed by washing of the cells with PBS solution. Thereafter the medium was changed and incubated with 20 µl MTT (5 mg/ml) for 4 h. The viable cell number/dish is directly proportional to the production of formazan, which following solubilization with isopropanol, can be measured spectrophotometrically at 563 nm.

2.4. Determination of DNA fragmentation

Cultured cells grown on a 100 mm Petri dish were treated with various concentrations (0.15–0.6 mM) of (Ac)₅-GP for 24 h. The cells were harvested by scraping and then centrifuged at $800 \times g$ for 10 min at 4 °C. The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described in [16,18] with slight modification. Briefly, the cells were first lysed in a solution containing 0.5% SDS, 100 mM EDTA and 10 mM Tris–HCl (pH 8.0). RNase A (Sigma) (20 µg/ml)

was then added to the lysate followed by the mixture being incubated for 1 h at 37 °C. Cells were then treated with 100 µg proteinase K/ml for an additional 3 h at 50 °C. The DNA was extracted with phenol, chloroform and isoamylalcohol (1:24:25, v/v). Two volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 7.2) was added to the supernatant and left undisturbed at –20 °C overnight. The precipitate containing the DNA fragment was centrifuged at 14,700 rpm for 30 min. DNA derived from 1×10^6 cells was suspended in 10 µl DNase-free RNase for 1 h at 37 °C. The sample DNA was suspended in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and loaded onto a 1.5% agarose gel.

2.5. Cellular DNA fragmentation ELISA

Quantification of the internucleosomal DNA fragmentation was based on the immunoassay with a commercial kit (Boehringer Mannheim GmbH, Germany). Exponentially growing C6 glioma cells were incubated with Brdu 10 µmol/l overnight at 37 °C in a humidified atmosphere. After labeling, the cells were centrifuged at $250 \times g$ for 10 min and suspended in a culture medium. The cell concentration was adjusted to 1×10^5 cells/ml and 100 µl/well were transferred to a microtiter plate (Nunc) to replicate wells containing either (Ac)₅-GP solution or culture medium (to measure spontaneous release of Brdu labeled DNA) to yield a final volume of 200 µl/well. The cells were then incubated for 6 h at 37 °C in a humidified atmosphere. After incubation, the cells were centrifuged at $250 \times g$ for 10 min and 100 µl/well of supernatant removed and tested by ELISA. The cells were then lysed with 100 µl/well incubation buffer containing BSA, EDTA and Tween-20, for 30 min at room temperature. The lysed cells were centrifuged at $250 \times g$ for 10 min followed by the removal of 100 µl of supernatant for ELISA. The samples were transferred directly without an additional dilution step to the wells of a precoated plate. The samples were then incubated for 90 min at room temperature. After washing, the samples were denatured and fixed by continuous microwave irradiation (275Z, 650W) for 5 min. After cooling the plate for 10 min at –20 °C, anti-Brdu peroxidase conjugated solution was added and incubated for an additional 90 min at room temperature. After washing, immunocomplexed anti-Brdu peroxidase was detected by the TMB substrate (10 min reaction time, at room temperature in the darkness). DNA fragmentation is directly proportional to the absorbance at 450 nm.

2.6. Analysis of cell cycle and quantification of apoptosis

Flow cytometric analysis of C6 glioma cells was performed using a FACScan [21] (Becton Dickinson Immunocytometry Systems, UK) from 0 to 48 h of culture. Thereafter, cells were washed twice with buffer solution. Then the cell suspension was performed centrifugation at $400 \times g$ for 5 min at room temperature. Decanting of all the supernatant was followed by adding 250 µl of trypsin buffer to the pellet. After incubation for 10 min at room temperature, 200 µl of trypsin inhibitor and RNase buffer were added at room temperature. Prior to the samples being analyzed by the flow cytometry, 200 µl of cold propidium iodide stain solution was added to

the mixture and it was incubated for 10 min in the darkness on ice. Propidium iodide (PI) was excited at 488 nm and the fluorescence signal was subjected to logarithmic amplification with PI fluorescence (red) being detected above 600 nm. Cell cycle distribution are presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence, and the extent of apoptosis was determined by counting cells of DNA content below the G₀/G₁ peak.

2.7. Electrophoresis and Immunoblotting

Analysis of p53, c-Myc, Bcl-2 and Bax proteins were performed with the method of SDS-PAGE and immunoblotting. For the analysis of the expression of proteins, the (Ac)₅-GP (0.015–0.30 mM) was added to the culture for the indicated time. The medium was removed and rinsed with PBS at room temperature. Then 0.1 ml of cold RIPA buffer with freshly added leupeptin (10 µg/ml) and sodium orthovanadate (1 mM) was added. Scraping of cells and the transfer of lysate into a eppendorf was performed prior to incubation for 30 min on ice and the addition of 5 µl of 10 mg/ml PMSF stock. Microfuge (7,000 rpm) of cell lysate was performed for 20 min at 4 °C. Cell lysate (0.5 µg purified protein) [30] was mixed with an equal volume of electrophoresis sample buffer and then boiled for 90 s was then performed followed by analysis using SDS-PAGE and transfer of protein from the gel to nitrocellulose membranes using electroblotting apparatus (Bio-Rad, California, USA). Thereafter, blocking of non-specific binding by soaking membrane in the PBS solution containing 1% non-fat milk and 1% bovine serum albumin at room temperature was executed for 30 min. The membrane was then incubated with p53 monoclonal antibody (Oncogene), Bcl-2 polyclonal antibody (Oncogene), Bax polyclonal antibody (Santa Cruz Biotechnology, California, USA) or c-Myc monoclonal antibody (Santa Cruz Biotechnology, California, USA) at 1 µg/ml in PBS for 1 h with PBS washing in between. Incubation with Horseradish Peroxidase conjugate anti-mouse IgG (Amersham Life Science) diluted to 1:2000 in PBS was followed by washing three times for 5 min. Finally, the membrane was incubated in Amersham ECL reagents for 1 min and exposed to ECL hyperfilm in a darkroom for 15 min.

3. Results

3.1. Effect of (Ac)₅-GP on viability on C6 glioma cells

For most anticancer agents, cytotoxicity is measured using a standard MTT assay following a brief drug exposure. Fig. 2 illustrates the results of MTT assays performed with various concentrations of (Ac)₅-GP administered to logarithmically growing C6 glioma cells for 24 h. The resulting survival curve shows that the (Ac)₅-GP had a dose-dependent effect on the cytotoxicity of cells. Within 24 h of the addition of 0.20 mM (Ac)₅-GP, viability was reduced by 52%.

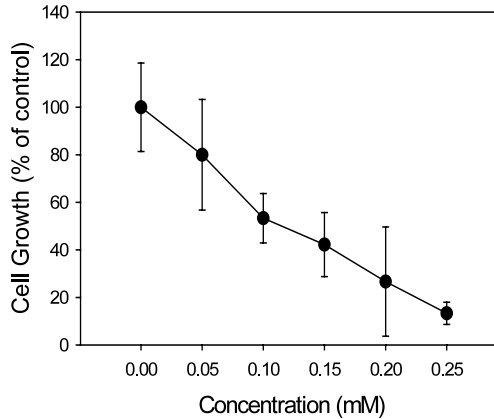


Fig. 2. Dose–response of the viability of C6 glioma cells after treatment with (Ac)₅-GP. After overnight attachment of cells, the culture was exposed to indicated concentration of (Ac)₅-GP for 24 h. Then the medium was removed and isopropanol was added to dissolve the formazan crystal for MTT assay. The viable cell number is directly proportional to the production of formazan. Results are shown as mean ± SD from three independent experiments.

3.2. (Ac)₅-GP-induced apoptotic death

The effect of treatment with (Ac)₅-GP (0.15–0.60 mM) for 24 h on internucleosomal DNA fragmentation in C6 glioma cells is shown in Fig. 3. Exposure to (Ac)₅-GP produced the characteristic ladder of oligonucleosomal DNA fragments that were 180 bp integer multiples in size (Fig. 3A). Fig. 3B shows quantitative analyzes of the fragmented DNA in the supernatant of the lysed C6 cells, expressed as the mean percent of the total starting DNA. DNA fragmentation is directly propor-

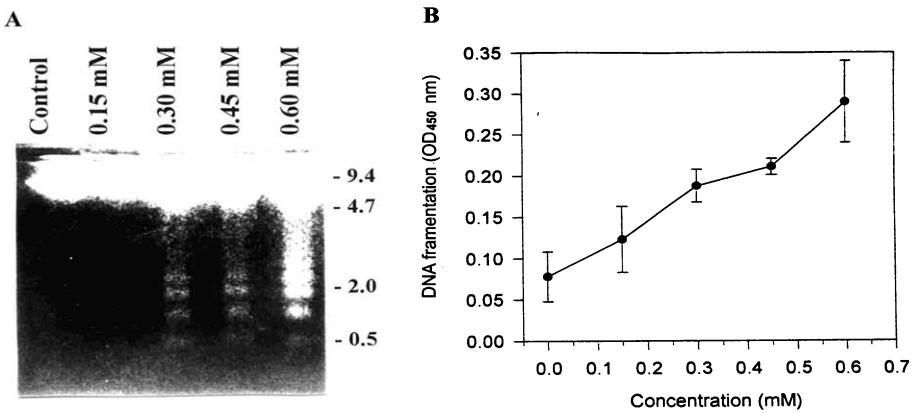


Fig. 3. Agarose gel electrophoretic (A) and quantitative (B) analysis of the fragmented DNA from (Ac)₅-GP-treated glioma cells. Cells were incubated for 24 h in the presence of indicated concentration of (Ac)₅-GP. DNA fragmentation is measured by anti-BrdU and is directly proportional to the absorbance at 450 nm.

tional to the absorption at 450 nm by ELISA using a monoclonal antibody against Brdu. Fig. 3B shows that a marked increase in the DNA fragmentation was observed when C6 glioma cells were treated with (Ac)₅-GP. The resulting DNA fragmentation shows that the (Ac)₅-GP had a dose-dependent effect on the C6 cells. The DNA histogram of the PI-stained cells in Fig. 4 shows that various concentrations of (Ac)₅-GP-treated cells (24 h) had hypodiploid DNA, indicative of apoptosis. The higher concentration of (Ac)₅-GP-treated cells contained more hypodiploid DNA, indicating that (Ac)₅-GP significantly induced apoptosis.

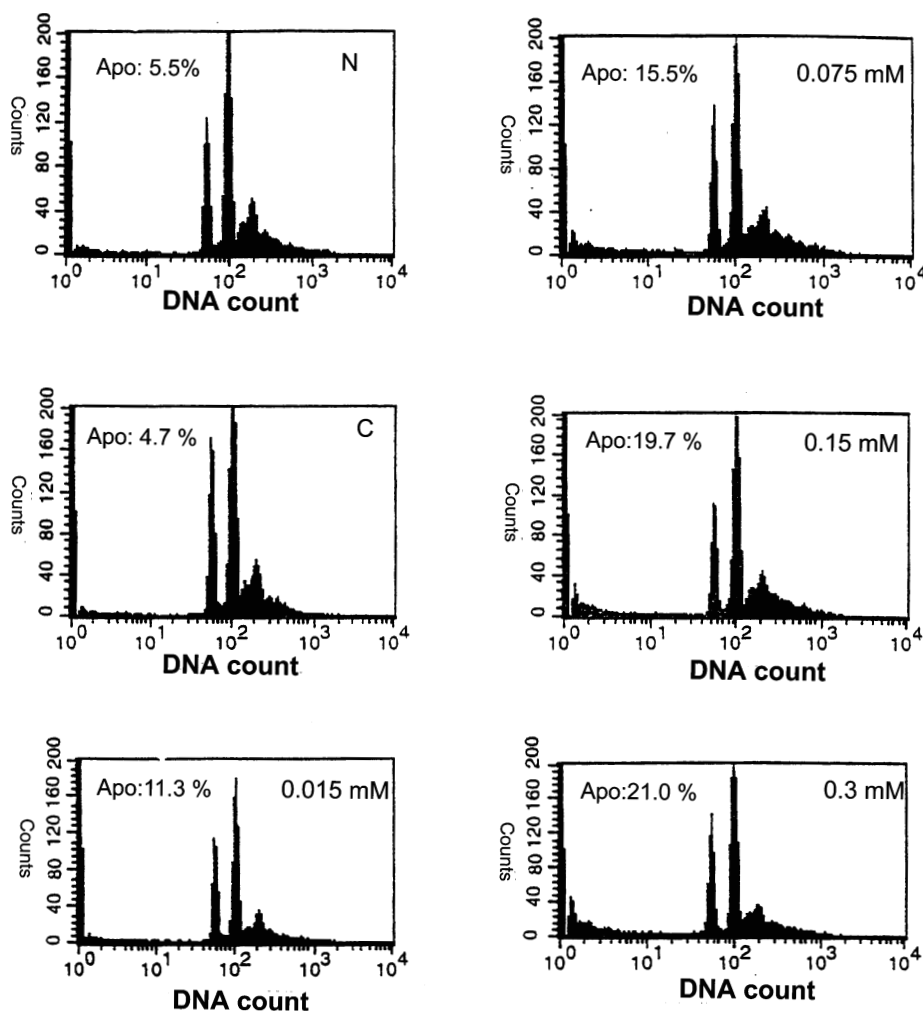


Fig. 4. Determination of pro-apoptotic bodies in (Ac)₅-GP-treated C6 glioma cell by flow cytometry. Cells were incubated with (Ac)₅-GP as indicated concentration of (Ac)₅-GP for 24 h. Apo indicates the cells with hypodiploid DNA. N, control; C, cells in 0.02% DMSO.

3.3. Induction of cell cycle arrest by (Ac)₅-GP

In order to investigate (Ac)₅-GP-mediated inhibition of cell growth, we have examined the cell cycle progression alterations and analyzed the relative DNA content of (Ac)₅-GP-treated cells. The cells were treated with 0.3 mM (Ac)₅-GP for various time periods (0, 12, 24 and 48 h) and analyzed by flow cytometry. (Ac)₅-GP caused a marked increase from 59 to 80% in the proportion of G₀/G₁-phase cells (Fig. 5), whereas the S phase decreased compared with the control.

3.4. Effect of (Ac)₅-GP on p53, c-Myc, Bcl-2 and Bax

We next examined whether (Ac)₅-GP-induced apoptosis and cell cycle arrest at G₀/G₁ phase are modulated by the p53, c-Myc and Bcl-2 family proteins. C6 glioma cells

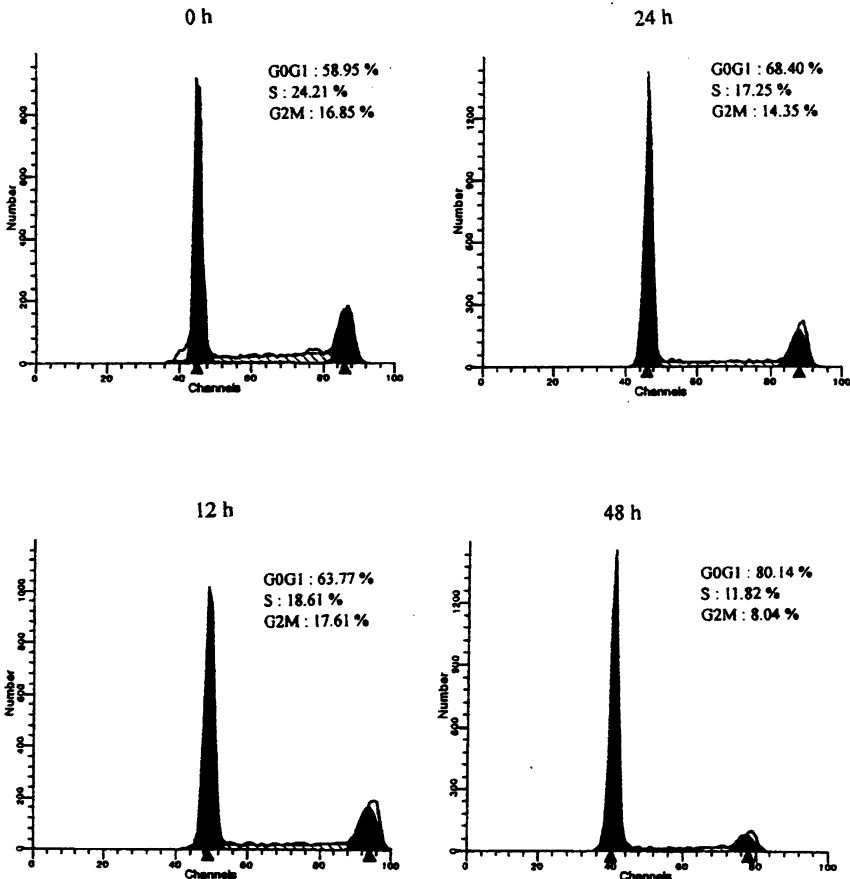


Fig. 5. Flow cytometric DNA fluorescence profiles of C6 glioma cells. PI-stained DNA histograms of (Ac)₅-GP-treated cells are shown. Cells were treated with 0.3 mM for 0–48 h.

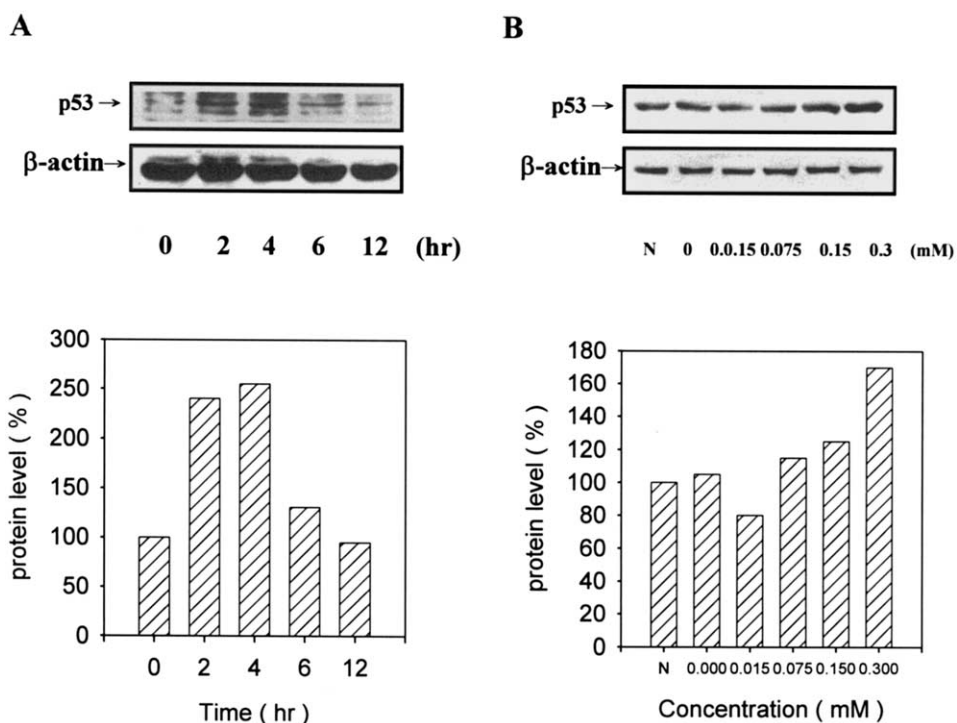


Fig. 6. Expression of p53 protein in the (Ac)₅-GP-treated C6 glioma cells. Cells were treated with 0.3 mM for indicated time (A) and treated with various concentration for 4 h (B), then lysed and analyzed by Western blotting using anti-p53 Ab. Three independent experiments were conducted and showed the same pattern of changes in the levels of p53, a representative one shown here. N, control; 0.0, cells in 0.02% DMSO.

express wild type p53. The expression of p53 was gradually increased resulting in a peak at 4 h after (Ac)₅-GP treatment and then decreased to basal, as shown in Fig. 6A. Concomitantly, the expression of c-Myc and Bax proteins were gradually increased during the period of treatment of (Ac)₅-GP (Fig. 7A and Fig. 8B). However, the expression of Bcl-2 was gradually decreased at the same period of (Ac)₅-GP treatment (Fig. 8A). The expression of all these proteins showed dose-dependence in (Ac)₅-GP-treated C6 glioma cells (Fig. 6B and Fig. 7B). These results indicated that (Ac)₅-GP-induced apoptosis and cell cycle arrest at G₀/G₁ was associated with the induction of p53, c-Myc and Bax and inhibition of Bcl-2.

4. Discussion

(Ac)₅-GP was produced by acetylation of a glycoside isolated from an extract of *G. fructus* [8]. We have found that significant inhibition can be achieved in rat C6 glioma cells, in culture and in bearing rat through extensive administration of this

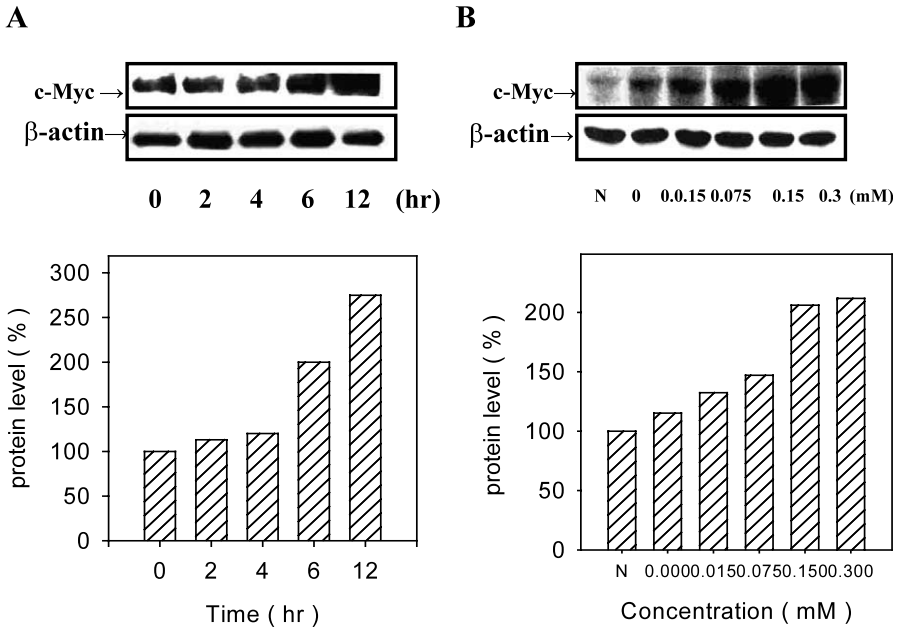


Fig. 7. Expression of c-Myc protein in the (Ac)₅-GP-treated C6-glioma cells. Cells were treated with 0.3 mM for indicated time (A) and treated with various concentration for 6 h (B), then lysed and analyzed by Western blotting using anti-c-Myc Ab. Three independent experiments were conducted and showed the same pattern of changes in the levels of c-Myc, a representative one shown here. N, control; 0.0, cells in 0.02% DMSO.

compound [8,9]. The present study is, to our knowledge, the first to demonstrate that (Ac)₅-GP triggers apoptosis in C6 glioma cells. (Ac)₅-GP was previously reported to have antitumor activity [8,9], although the mechanism responsible for the activity was not clarified. Here we demonstrate that elevated expression of p53, c-Myc and Bax and lowered expression of Bcl-2 are involved in the early events of (Ac)₅-GP-induced cell cycle progression delay/arrest as well as in the apoptosis of C6 glioma cells. Cells receiving 24 h treatment with (Ac)₅-GP showed apparent DNA fragmentation. Apoptosis has been characterized by the alteration of morphological and biochemical features of cells [22,23,31]. The apoptotic phenomena induced by (Ac)₅-GP have also been observed in the present results. The morphology of chromatin condensation and membrane surface bleb was found by phase contrast microscope (data not shown), as well as DNA fragmentation into oligomers of approximately 180 bp in C6 glioma cells were observed by gel electrophoresis (Fig. 3A), and formation of hypoploid DNA was investigated by flow cytometer (Fig. 4).

In these studies, the (Ac)₅-GP-induced apoptotic cell death and cell cycle arrest was also associated with marked induction of c-Myc and p53. The c-Myc proto-oncogene, usually implicated in the cell transformation, differentiation and cell cycle progression [32,33], also has a central role in some forms of apoptosis [34–36]. In addition, p53 has been proposed to inhibit progression through G₁ into S phase via

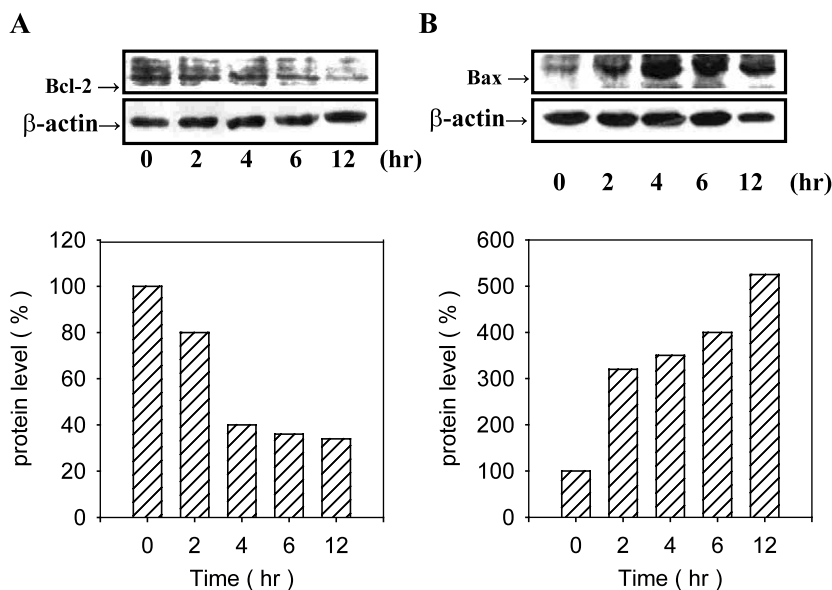


Fig. 8. Expression of Bcl-2 family (Bcl-2 and Bax) proteins in the $(Ac)_5$ -GP-treated cells. Cells were treated with 0.3 mM for indicated time, then lysed and analyzed by Western blotting using anti-Bcl-2 Ab (A) and anti-Bax Ab (B). Three independent experiments were conducted and showed the same pattern of changes in the levels of Bcl-2 and Bax, a representative one shown here.

transactivation of WAF1/CIP1 which inhibit cyclin-dependent kinases [37,38]. p53 stabilization may be also involved in the initiation of apoptosis [39–43], and has been proposed to be a c-Myc-responsive gene. Recently it has been reported that c-Myc-induced apoptosis of fibroblasts requires p53 and is associated with p53 stabilization [44], demonstrating that c-Myc stimulated p53-induced apoptosis. In the present report, we shows that the apoptosis of C6 cells caused by $(Ac)_5$ -GP is associated with a marked induction of c-Myc. Also, $(Ac)_5$ -GP produced a marked increase in p53 expression after 4 h treatment and then gradually declined. These results indicated that $(Ac)_5$ -GP-induced apoptosis is associated with induction of p53 and c-Myc.

Bcl-2 is an oncogene that contributes to malignancy by preventing apoptosis [45–47]. Subsequent in vitro studies confirmed that Bcl-2 overexpression promotes cell survival by inhibiting apoptosis induced by a variety of stimuli including radiation, hyperthermia, and glucocorticoids [48,49]. Proto-oncogene Bcl-2, which encodes an inner mitochondrial protein that reportedly, antagonizes apoptosis in many tumor cells. Decreased expression of this protein might contribute to drug-mediated lethality. Recently, the induction of apoptosis in HL-60 cells by taxol [50,51], curcumin [52], and retinoid *N*-(4-hydroxyphenyl) retinamide [53] has been associated temporally with the down-regulation of Bcl-2. Consistent with these reports, our study also showed that $(Ac)_5$ -GP-induced apoptosis was associated with the down-regulation of Bcl-2 after 2 h treatment. In contrast, overexpressed Bax accelerates

apoptotic death induced by different stress in many cell lines. Overexpressed Bax also counters the death repressor activity of Bcl-2 [54]. It has also been previously reported that in cells expressing low levels of Bcl-2, a continuous decline c-Myc expression may be responsible for the arrest of their growth and apoptosis [55]. However, gene transfer experiments have shown that Bcl-2 blocks apoptosis induced by c-Myc [56,57]. Our findings show that C6 glioma cells express relatively low levels of Bcl-2 and high levels of Bax after (Ac)₅-GP treatment. (Ac)₅-GP also produced a marked induction in c-Myc expression with a concomitant effect both in the expression of Bcl-2 and Bax thereby facilitating the induction of apoptosis.

In addition, c-fos and c-jun expression were found to be induced during apoptosis of lymphoid cells [58] and c-fos in epithelial cells of the rat ventral prostate undergoing apoptosis [59]. In our unpublished data, no effect of (Ac)₅-GP-induced C6 glioma apoptosis on the expression of c-fos and c-jun was observed. (Ac)₅-GP also activate protein kinase C (PKC) in C6 glioma cells. However, no effect on (Ac)₅-GP-induced apoptosis in the presence of PKC activator (TPA) or inhibitor (H7) (unpublished data). These results indicate that C6 glioma cells apoptosis induced by (Ac)₅-GP is not associated with PKC signals and the expression of c-fos and c-jun.

Enhanced apoptosis is responsible for many of the adverse effects of chemotherapy and for tumor regression. (Ac)₅-GP, a potential anticancer compound developed in our laboratory, has demonstrated [8,9] excellent antitumor activity in culturing and bearing C6 glioma cells. In view of these encouraging results, it will be worthwhile to investigate the (Ac)₅-GP-induced cell death by apoptosis through induction of p53 and c-Myc and modulation of Bcl-2 family proteins. In the previous studies, we had treated several cell lines with (Ac)₅-GP, including NIH3T3, Hep G2, MCF-7, KB, HL-60, C6 and AGS. The data showed that NIH3T3 had less effect than all other tumor cell lines, indicating that tumor cells are more sensitive to the (Ac)₅-GP treatment. We also found that (Ac)₅-GP is not toxic in treated animals [11]. All of the above results reflect the therapeutic potential of this new compound.

In conclusion, the data presented in this paper indicate that (Ac)₅-GP, a potential chemotherapy agent, inhibits C6 glioma cells proliferation by inducing apoptotic death and cell cycle arrest at G₀/G₁, which is accompanied, at least partly, by the induction of expression of p53 and c-Myc and mediated Bcl-2 family proteins.

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