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An Intestinal Bacterial Metabolite of Ginseng Protopanaxadiol Saponins Has the Ability to Induce Apoptosis in Tumor Cells

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Our previous study demonstrated that the *in vivo* anti-metastatic effect induced by oral administration of ginseng protopanaxadiol saponins was mediated by their metabolic component M1, and that the growth, invasion and migration of tumor cells were inhibited by M1 but not by ginsenosides. Here we investigated the inhibitory mechanism of M1 on the growth of tumor cells. M1 inhibited the proliferation of B16-BL6 mouse melanoma cells in a time- and dose-dependent manner, with accompanying morphological changes at the concentration of 20 μM. In addition, at 40 μM M1 induced apoptotic cell death within 24 h. Fluorescence microscopy revealed that dansyl M1 entered the cytosol and quickly reached the nuclei (approximately 15 min). Western blot analysis revealed that M1 rapidly up-regulated the expression of $p27^{Kip1}$, but down-regulated the expression of c-Myc and cyclin D1 in a time-dependent manner. Thus, the regulation of apoptosis-related proteins by M1 is responsible for the induction of apoptotic cell death, and this probably leads to the anti-metastatic activity *in vivo*. © 1998 Academic Press

Ginseng (the root of *Panax ginseng* C. A. Meyer, Araliaceae) has been used for traditional medicine in China, Korea, Japan and other Asian countries for the treatment of various diseases including psychiatric and neurologic diseases as well as diabetes mellitus. So far, ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of ginseng. Ginsenosides are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton and have been shown to possess various biological activities including the enhancement of cholesterol biosynthesis, stimulation of serum protein synthesis, immunomodulatory effects and anti-inflammatory activity [1-5]. Several studies using ginsenosides have also reported antitumor effects, particularly the inhibition of tumor-induced angiogenesis [6], tumor invasion and metastasis [7,8], and the control of phenotypic expression and differentiation of tumor cells [9,10]. Previously, it was reported that protopanaxadiol-type ginsenisides such as Rb1, Rb2 and Rc are metabolized by intestinal bacteria after oral administration to their final derivative 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, which is referred to as M1 [11] or compound K [12,13]. Our recent study demonstrated that expression of the *in vivo* anti-metastatic effect of the ginsenosides was primarily based on their metabolite M1 [14,15]. In the present study, we investigate the anti-proliferative activity of M1 against B16-BL6 melanoma cells and its inhibitory mechanism of action. We found that M1 caused the dramatic morphological changes in B16-BL6 cells *in vitro* and consequently induced apoptotic cell death. The regulation of apoptosis-related molecules by M1 plays an important role in the anti-proliferative activity against tumor cells.

MATERIALS AND METHODS

Chemical reagents. Ginsenoside-Rb_1 was isolated from the ginseng extract according to the reported procedures [16], and their major intestinal bacterial metabolite M1 was the same as that described previously [11]. The chemical structures of ginsenoside-Rb_1 and its metabolic compound M1 are shown in Fig. 1.

Cells. A highly metastatic subline of the murine B16 melanoma, B16-BL6, was kindly provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX, USA). The B16-BL6 melanoma cells were maintained as monolayer cultures in Eagle's minimum essential medium (MEM: GIBCO BRL, Life Technologies, Inc., USA) supplemented with 5% fetal bovine serum (FBS: GIBCO) and L-glutamine (GIBCO).

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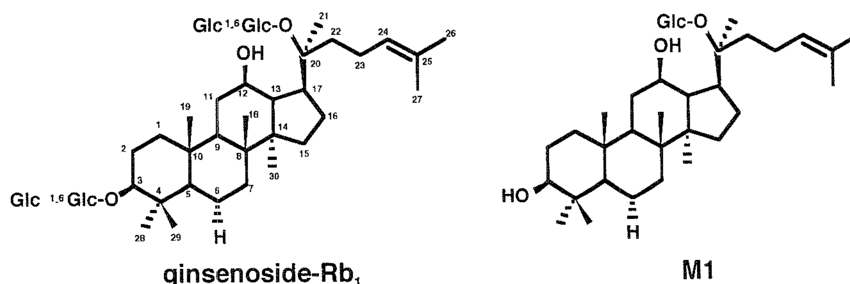

FIG. 1. Structure of ginsenoside-Rb_1 and its metabolic compound M1.

In vitro growth inhibiton test. B16-BL6 cells (5 × 10^3/well) in MEM containing 10% FBS were seeded into 24-well culture plates (Costar, USA). After a 24 h incubation, various concentrations of M1 in 5% FBS-MEM were added to the plates and the culture continued at 37 °C. The media were replaced every day and the cell numbers were manually counted as a function of time. The assay was performed on trplicate cultures.

Observation of morphological changes. B16-BL6 cells (5 × 10^4/well) in MEM containing 10% FBS were seeded into 6-well culture plates (Becton Dickinson, USA). M1 (20 μM) and ginsenoside-Rb_1 (40 μM) in 5% FBS-MEM were added to the adhered cells, and the culture maintained according to the conditions described above. Morphological changes of cells were photographed using phase contrast microscopy on day 1, 3 and 5 (Olympus, Japan).

DNA extraction and detection of DNA fragmentation. B16-BL6 cells (>1 × 10^6 cells) were collected by centrifugation at 1,500 rpm for 5 min. The cell pellet was suspended in 600 μl cell lysis buffer (10 mM Tris-HCl buffer, pH 7.5; 10 mM EDTA, and 0.2% Triton X-100) and kept on ice for 10 min. The lysate was centrifuged at 14,000 rpm for 10 min and the supernatant collected. After TE-saturated phenol (Wako Pure Chemical Industries, Co., Ltd., Japan) was added to the supernatant, the mixture was vortexed, and then centrifuged at 14,000 rpm for 10 min. The supernatant was mixed with an equal volume of CIAA solution (chloroform : isoamylalcohol = 24 : 1). DNA in the upper aqueous phase was precipitated in 3M NaCl and cold ethanol by an overnight incubation at −20 °C. After drying, DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 7.5) and incubated with 10 μg/ml RNase (Nippon gene, Japan) at 37 °C for 30 min. Following the addition of loading buffer (Nippon gene, Japan), fragmented DNA was separated by 2% agarose gel electrophoresis at 50 V for 1 h, and visualized by staining with 100 ng/ml ethidium bromide. The morphology of apoptotic cells was photographed using phase contrast microscopy (Carl Zeiss, Co., Ltd., Germany).

Analysis of M1-distribution in B16-BL6 cells. B16-BL6 cells (1 × 10^4/well) were cultured for 15 minutes with dansyl M1 which was prepared by the condensation of M1-25, 26, 27-trisnor-24-aldehyde with dansyl hydrazine and was biologically as active at inhibiting tumor cell proliferation as M1 [17]. The cells were then washed with medium and fixed with methanol. Photograph was taken under fluorescence microscopy (Carl Zeiss, Co., Ltd., Germany).

Western blot analysis. B16-BL6 cells (3 × 10^6/dish) were cultured for appropriate time periods with M1 (40 μM). Cell nuclear extracts were prepared according to the method previously reported [18]. Briefly, 7.5 μg nuclear extracts were separated by polyacrylamide gel electrophoresis (MINI-PROTEAN II READY GELS J, Bio-Rad, USA), and the proteins electrophoretically transferred to nitrocellulose filter (Bio-Rad, USA). The filters were washed with Tris-buffered saline, and treated with block ace (Dainippon Pharmaceutical Co., Ltd., Japan) for 2 h at room temperature before being incubated overnight with specific polyclonal antibody (Santa Cruz Biotech, Inc., USA) in Tween-Tris-buffered saline containing 0.1% Tween 20 (T-TBS) at 4 °C. The filters were washed three times with T-TBS and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham LIFE SCIENCE, UK) for 2 h at room temperature. After washing with T-TBS, immunolabelled bands were detected by the SuperSignal detection system (Pierce Inc., USA). The density of the signals was quantified by the MasterScan Gel Analysis System (Scanalytics, Billerica, USA), and calculated as follows: Density = $-\log(I/I_m)$, where "I" is the pixel intensity and "I_m" is the maximal pixel intensity value handled by the image acquisition device. Molecular sizes were determined by comparison with the relative mobilities of prestained molecular weight markers.

Statistical analysis. Student's t-test for *in vitro* growth inhibiton test was performed as a two-tailed test.

RESULTS AND DISCUSSION

We have recently reported that protopanxadiol-type ginsenosides and their major metabolite M1 markedly inhibited the lung metastasis of B16-BL6 melanoma cells when they were administered p.o., whereas only M1 showed the inhibitiory effect on the proliferation, migration and invasion *in vitro* [14]. These findings clearly indicate that induction of the *in vivo* anti-metastatic effect by oral administration of ginsenosides is

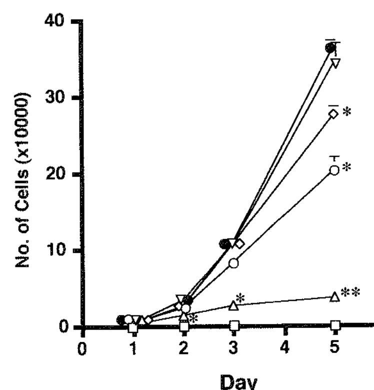

FIG. 2. Effect of M1 on the growth of B16-BL6 melanoma cells. B16-BL6 cells (5 × 10^3/well) were incubated with or without various concentrations of M1 for 3 days at 37 °C. The cell numbers were counted manually at various time periods. Control (●), M1: 5 μM (◇), 10 μM (○), 20 μM (△), 40 μM (□) and ginsenoside-Rb1: 40 μM (▽), respectively. *, $p<0.001$; **, $p<0.0001$ as compared with the untreated control on each day by two-tailed Student's t-test.

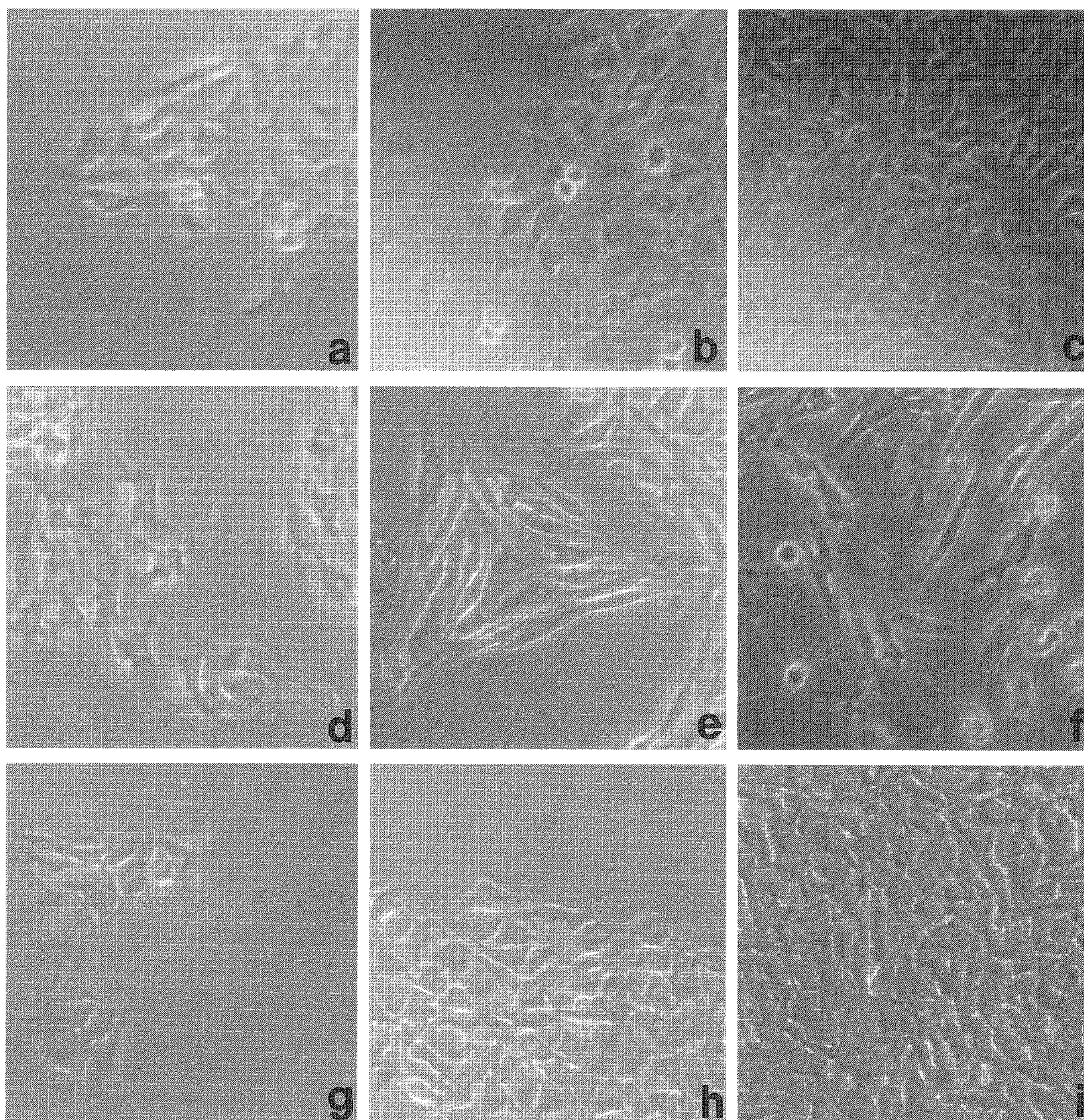

FIG. 3. Morphological changes of B16-BL6 melanoma cells treated with ginsenoside-Rb_1 or its metabolite M1. B16-BL6 cells (5×10^4/well) were seeded in each well, and the cultures incubated with 5% FBS-MEM in the absence (a, b, c) or presence of 20 μM M1 (d, e, f) and 40 μM ginsenoside- Rb_1 (g, h, i) for 1 day (a, d, g), 3 days (b, e, h) or 5 days (c, f, i). Original magnification: $\times$ 200.

primarily mediated by their metabolic component M1. However, the detail of how the active metabolite M1 affects the growth of tumor cells has not been clear. We therefore examined the inhibitory mechanism of M1 on the proliferation of tumor cells *in vitro*. Co-incubation of tumor cells with M1 at concentrations ranging from 5 to 40 μM resulted in a time- and concentration-dependent inhibition of tumor cell proliferation (Fig. 2). When tumor cells were cultured for 5 days with M1 (20 μM), marked morphological changes were observed as compared with the untreated control (Fig. 3). M1-treated cells exhibited a spindle-shape morphology after a 3-day incubation and partly induced cell death with a swelling-shape at 5 day. In addition, M1 at a concentration of 40 μM caused the cytotoxic response in tumor cells at an earlier time period (within 24 h) in the culture (Fig. 2). Our previous study indicated that M1 at the concentration of more than 16 μM possessed relatively selective cytotoxicity against B16-BL6 murine melanoma and HT-1080 human fibrosarcoma

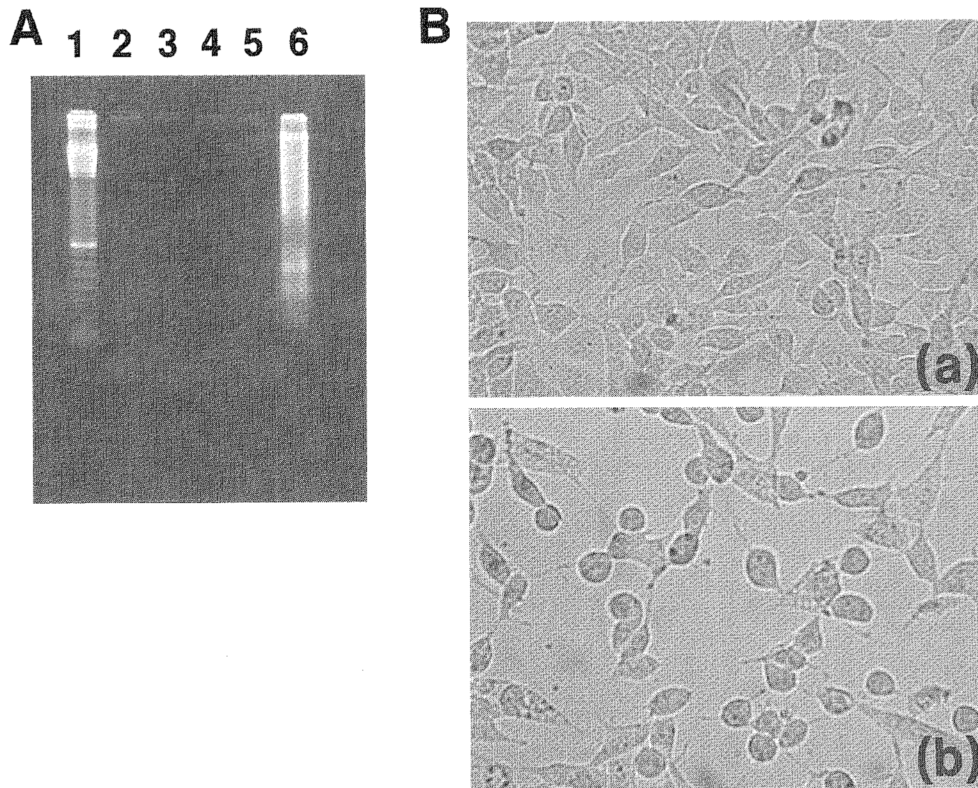

FIG. 4. M1-induced DNA fragmentation of B16-BL6 melanoma cells and their morphology. (A) B16-BL6 cells were cultured for 24 h in the presence or absence of 40 μM M1. The DNA was isolated, and electrophoresed on a 2% agarose gel. The fragmented DNA was detected by ethidium bromide staining. 100 bp ladder (lane 1), Control (lane 2), and M1: 5 μM (lane 3), 10 μM (lane 4), 20 μM (lane 5) and 40 μM (lane 6), respectively. (B) The apoptotic morphology of the cells untreated (a) or treated with 40 μM M1 (b). Original magnification: $\times$ 200.

cells compared with that of normal fibroblasts [14]. In contrast, the incubation with ginsenoside-Rb_1 (40 μM) did not affect the morphology of tumor cells (Fig. 3) or cell proliferation [14]. Since the swelling-shape morphology of tumor cells is considered to be an apoptotic character, we next investigated whether the cell death by treatment with 40 μM was due to the induction of apoptosis. The ladder fragmentation of the extracted DNA and swollen-round morphology indicated that the cell death was caused by apoptosis (Fig. 4a and 4b).

Although the molecular events that drive the apoptotic signaling pathway are not entirely clear, some apoptosis-related proteins such as cyclin D1, c-Myc or cyclin-dependent kinase (CDK) inhibitors have been reported to be associated with cell division and proliferation [19-21]. Therefore in order to clarify the mechanism of M1-induced apoptosis, we investigated the effect of M1 on expression of the apoptosis-related proteins, p21, $p27^{Kip1}$, c-Myc and cyclin D1. As shown in Fig. 5, M1 treatment (40 μM) markedly increased the expression of $p27^{Kip1}$ as compared with the untreated control. No expression of the other CDK inhibitor, p21 was detected in B16-BL6 cells in this experiment (data not shown). The up-regulation of $p27^{Kip1}$, which is known to inhibit the CDK activity, was observed during the apoptotic process caused by anti-cancer agents including etoposide and camptotecin [22]. On the other hand, a proto-oncogene product c-Myc as well as cyclin D1 have been reported to be overexpressed in the proliferative phase of various types of tumor cells [19-21]. The expression of c-Myc and cyclin D1 was down-regulated by M1 treatment in a time-dependent manner. Thus, M1 might cause the cell-cycle arrest in tumor cells through the up/down-regulation of these cell-growth related molecules, and consequently induce apoptosis.

It has been reported that various molecules such as Bcl-2 (an inhibitor of apoptotic cell death), Bax (promotion of apoptosis by antagonizing the function of Bcl-2) and caspases (interleukin-1β converting enzymes to trigger the execution of cell death) are involved in positively or negatively regulating apoptosis signaling [23-25]. Recent studies have proposed some signaling pathways for apoptosis mediated by different regulatory molecules [23, 26]. Therefore, the possibility that M1 inhibits or promotes these apoptosis-related molecules is under investigation.

We also examined the intra-cellular distribution of M1 after the incubation of tumor cells with dansyl M1. Figure 6 showed that the fluorescent signal of dansyl

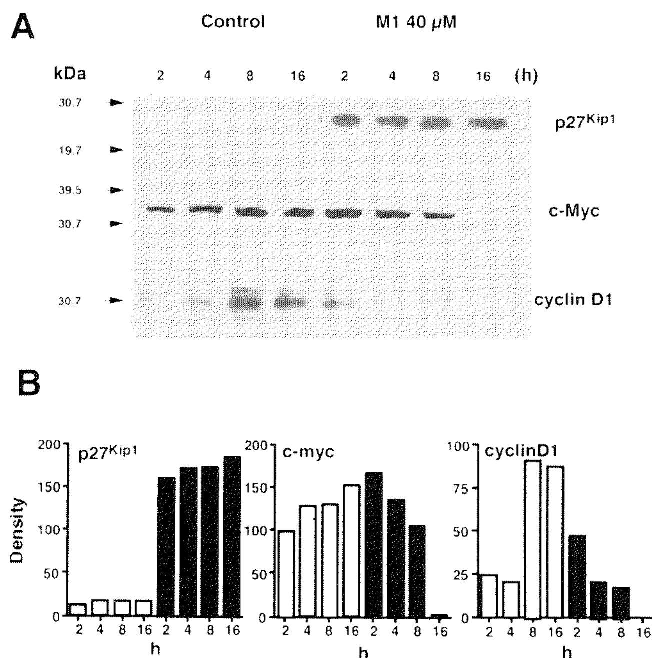

FIG. 5. Western blot analysis of $p27^{Kip1}$, c-Myc and cyclin D1 in B16-BL6 cells treated with M1. (A) Result of Western blotting. B16-BL6 cells were cultured with or without 40 μM M1 for the time periods indicated. $p27^{Kip1}$, c-Myc and cyclin D1 were detected using specific antibodies. (B) The density of the bands at each time point was densitometrically quantified. Control (□), M1 treated (■).

M1 was detected in the cytosol and nuclei 15-min after incubation, and thereafter was observed predominantly in the nuclei. These findings suggest that the apoptotic cell death is induced by intra-cellular M1 through the transcriptional regulation of several cell-growth associated proteins. Since M1 has a steroid-like chemical structure, it may interact with some intracellular receptors including a steroid receptor, which are known to be involved in the rapid regulation of nuclear proto-oncogene transcription [23]. The regulatory mechanisms of M1 at the transcriptional level will need to be investigated in detail.

In conclusion, the present study demonstrated that a metabolite of ginseng protopanaxadiol saponin (M1), with anti-metastatic property [14, 15], inhibited the proliferation of tumor cells in a time- and concentration-dependent manner, and in addition induced apoptotic cell death. The induction of apoptosis by M1 involved the up-regulation of the CDK-inhibtor $p27^{Kip1}$ as well as the down-regulation of c-Myc and cyclin D1. The nucleosomal distribution of M1 suggests that the modification of these molecules is induced by transcriptional regulation.

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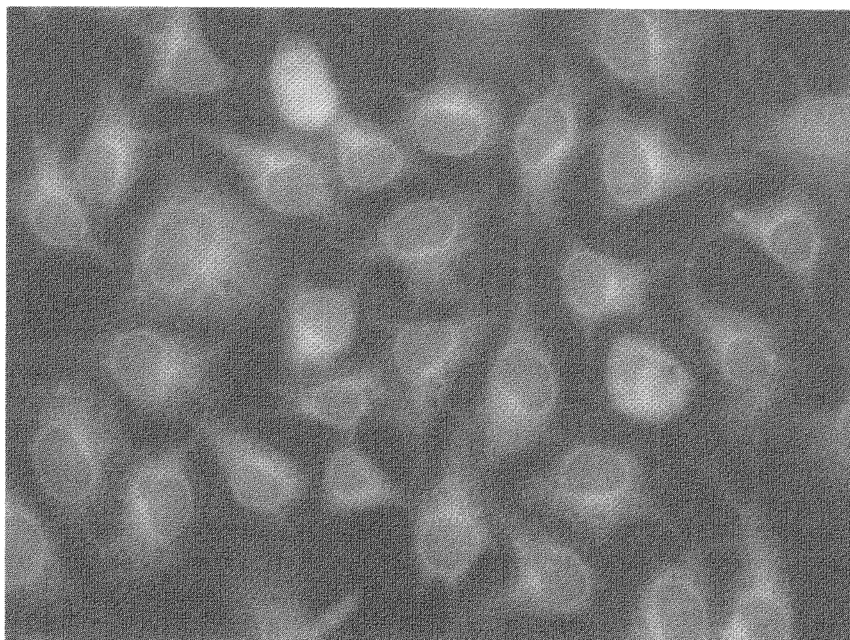

FIG. 6. Fluorescent microscopy of B16-BL6 cells treated with dansyl M1. B16-BL6 cells were treated with dansyl M1 for 15 min. The cells were fixed with methanol and the photograph was taken under fluorescent microscopy. Original magnification: × 400.

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