Induction of Apoptosis in Human Hepatocellular Carcinoma Cells by Synthetic Antineoplaston A10

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Abstract. *Antineoplaston A10 (3-phenylacetylamino-2,6-piperidinedion) is a naturally occurring substance and was the first antineoplaston in the human body to be chemically identified. The effect of antineoplaston A10 on human hepatocellular carcinoma cell lines HepG2 and HLE has been examined. Antineoplaston A10 displayed anti-proliferative action inhibiting cell growth in a dose- and time-dependent manner in vitro as measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays. Incubation with antineoplaston A10 for 48 h induced apoptotic events such as a typical apoptotic morphology, formation of a characteristic ladder pattern of DNA migration and accumulation of sub-G1 phase cells. Next, hepatoma xenografts in nude mice were employed to study the antitumor effects of antineoplaston A10 in vivo. Oral administration of antineoplaston A10 delayed the growth of HepG2 and HLE cells in the mice without a reduction in body weight. A higher proportion of apoptotic cells in xenografts was observed by means of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. In addition, the level of expression of apoptotic marker p53 increased while that of anti-apoptotic protein bcl-2 decreased, as evaluated with immunohistochemical staining in the xenografts. These results suggested that antineoplaston A10 may inhibit the growth of human hepatoma cells through the induction of apoptosis.*

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Antineoplastons are naturally occurring polypeptides and amino acid derivatives found in blood and urine. The compounds are believed to act as agents for the biochemical defense system and to control neoplastic growth (1, 2), although the mechanisms underlying their antitumor effects have not been fully investigated. The chemical structure of antineoplaston A10, 3-phenylacetylamino-2,6-piperidinedione, was the first to be determined (3). Antineoplaston A10 has previously been synthesized and the chemical and physiological characteristics of the compound have been investigated (4). The current study examined the effects of synthetic antineoplaston A10 on human hepatocellular carcinoma cells.

Materials and Methods

Chemicals. Antineoplaston A10 was synthesized by condensation of L-glutamine with phenylacetyl chloride and the subsequent cyclization of phenylacetylglutamine (4, 5). The chemical was dissolved in dimethylsulfoxide for *in vitro* experimentation or in 5% amylum for *in vivo* study.

Cell lines and cell culture. Human hepatoma cell lines, HepG2 and HLE, were obtained from the Institute of National Cancer Research of China (Beijing, China). The cells were grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin-streptomycin (100 IU/ml–100 μg/ml), 2 mM glutamine, and 10 mM Hepes buffer at 37˚C in a humid atmosphere (5% CO_2-95% air) and were harvested by brief incubation in 0.02% trypsin-EDTA in phosphate-buffered saline (PBS). Cell growth and viability were evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, clonogenic assay and trypan blue exclusion (6, 7).

Antitumor assay in vivo. The *in vivo* antitumor effects of antineoplaston A10 were assessed in nude mice bearing tumors (8). Balb/c athymic (nu+/nu+) female mice, 4-6 weeks of age, were purchased from the Animal Experimental Central of Beijing

(Beijing, China) and housed under pathogen-free conditions. The cells ($1x10^7$) were suspended in 100 μl of Matrigel (Collaborative Biomedical, Bedford, MA, USA) and injected subcutaneously into the right anterior flank of nude mice. After 7 days, when the tumor volume reached approximately 0.1-0.2 cm^3, the mice were orally administered 0, 200, 300 or 600 mg/kg of antineoplaston A10 in 0.8 ml of 5% amylum. The administrations were performed 6 days per week for five consecutive weeks. The tumor growth inhibition rates were defined in proportion to the control tumor weight. The specimens were subjected to the assays of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method and immunohistochemical staining.

Acridine orange/ethidium bromide staining. Twenty-five microliters of cell suspension (10^6 cells/ml) treated with various concentrations of A10 for different periods of time were incubated with 1 μl of acridine orange/ethidium bromide (AO/EB) solution (50 μg/ml each of AO and EB in PBS) followed by observation with a fluorescence microscope (9). The apoptotic cells were detected by the staining characteristics of cell morphology.

DNA fragmentation analysis by agarose gel electrophoresis. The analysis of the DNA ladder was performed with an apoptotic DNA ladder kit (Roche Diagnostics, Basel, Switzerland). The cells were lysed on ice using a lysis buffer (10 mM Tris-HCl, 50 mM EDTA, 0.5% NP-40), and the lysate was treated with RNase A (50 μg/ml) and proteinase K (100 μg/ml). The DNA was precipitated and electrophoresed on 1% agarose gel for analysis of fragmentation. The gel was stained with ethidium bromide and photographed under a UV transilluminator.

Flow cytometric analysis. The cells were subjected to propidium iodide (PI) staining and then the sub-G1 population was analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) as described previously (8).

TUNEL assay for apoptosis detection. An *in situ* cell death detection kit (Boster Biological Technology Ltd, Wuhan, China) was used to measure the apoptotic cells in xenografts. Serial 5-μm sections were cut from formalin-fixed xenografts of HepG2 and HLE in nude mice. Staining was performed according to the manufacturer's instructions. The TUNEL-positive cells were recognized by their purple/black nuclear staining, as visualized with a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. The proportion of apoptotic cells in at least 3 different mice in randomly chosen fields under a light microscope was scored (10).

Immunohistochemical staining. The expression of the tumor suppressor and apoptotic mediator p53 and anti-apoptotic bcl-2 proteins was examined by immunohistochemical staining (11). Sections were cut from the same tissues as described above and deparaffinized in xylene, dehydrated in ethanol and heated in a microwave oven. Immunohistochemical staining was performed employing a three-step streptavidin biotin immunoperoxidase staining system (Boster Biological Technology) using 3,3'-diaminobenzidine (Sigma-Aldrich China, Shanghai, China) as a chromogenic substrate. The endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol and sections were rinsed in Tris-buffered saline. Anti-bcl-2 antibody (clone 124, dilution 1:25; Dako, Glostrup, Denmark) and anti-p53 antibody (DO7, dilution 1:100; Novocastra, Newcastle, UK) were used as primary antibodies. The expression was evaluated by scoring the stained cells in randomly chosen fields in at least 3 different mice in one group (12).

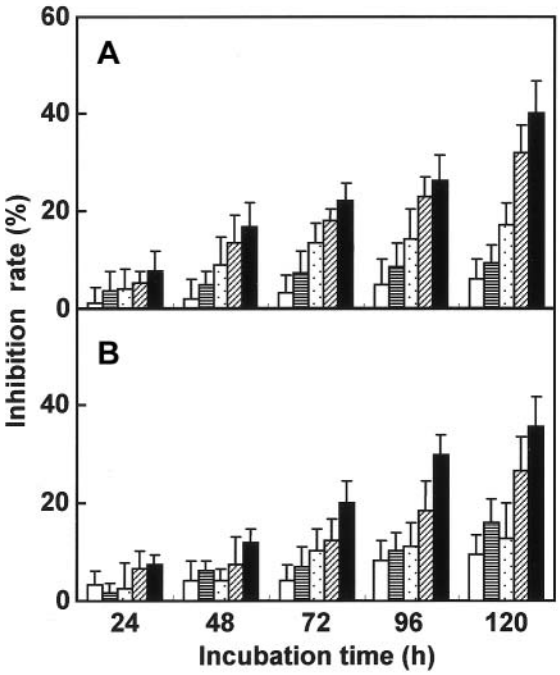

Figure 1. *Inhibition rates of HepG2 (A) and HLE (B) cell growth in the presence of antineoplaston A10 in vitro. Cells were treated with various concentrations of antineoplaston A10 for the indicated periods and subjected to MTT assay. Inhibition rates were denoted as a percentage of untreated controls at the concurrent time point. Open column, 10 μg/ml; horizontal lined column, 50 μg/ml; dotted column, 100 μg/ml; diagonal lined column, 200 μg/ml; solid column, 400 μg/ml of antineoplaston A10. Bars, SD (n=3).*

Results

Effects of antineoplaston A10 on cell growth in vitro. The human hepatoma cell lines HepG2 and HLE were treated with various concentrations of antineoplaston A10 for up to 120 h and then subjected to MTT assay. As shown in Figure 1, although antineoplaston A10 did not have a severe anti-proliferative effect on cells in lower doses and/or shorter incubation periods, dose- and time-dependent cell growth inhibition was observed at higher doses and with longer incubation periods. The HepG2 cells, when incubated with 10 μg/ml of antineoplaston A10, had a cell growth inhibition rate of less than 10% for up to 120 h, while cells incubated with 200 μg/ml of antineoplaston A10 had an inhibition rate of 34.9% at 120 h (Figure 1). In each incubation period, cytotoxicity of antineoplaston A10 was not observed, as verified by trypan blue staining (data not shown). The

Table I. *Effects of antineoplaston A10 on HepG2 and HLE cell growth in nude mice.*

Cell lines	Dosage (mg/kg)	Body weight (g)[1]	Tumor weight (g)[1]	Inhibition of tumor growth (%)	Incidence of apoptotic cells in xenografts (%)[2]
HepG2	0	24.3±2.4	3.1±0.7	-	5.8
	150	25.1±2.9	2.4±0.6[3]	22.6	18.3[3]
	300	24.1±3.2	1.9±0.7[3]	38.7	27.2[3]
	600	23.5±2.6	1.8±0.5[3]	41.9	35.4[3]
HLE	0	23.7±1.9	2.8±0.7	-	4.3
	150	24.6±2.0	2.2±0.8[3]	21.4	19.0[3]
	300	23.2±3.1	1.9±0.9[3]	32.1	25.1[3]
	600	23.1±2.4	1.7±0.9[3]	39.3	32.6[3]

[1]Data are indicated as the mean±SD (n=8); [2]apoptotic cells in xenografts were detected by TUNEL assay; [3]$p<0.05$. Differences were analyzed using the Student's two-tailed test.

clonogenic assay indicated that the rate of clone formation inhibition was less than 50% even in the presence of a higher dose (~400 μg/ml) of antineoplaston A10 for longer incubation periods (~2 weeks) (data not shown).

Antitumor effects of antineoplaston A10 in vivo. The effects of antineoplaston A10 on human hepatoma cells xenografts in nude mice were then examined. As shown in Table I, the growth of HepG2 and HLE cells transplanted into mice was significantly suppressed by administration of antineoplaston A10 in a dose-dependent manner. In contrast, antineoplaston A10 treatment was well tolerated by mice with no significant signs of acute or delayed toxicity or reduction in body weight.

Morphological changes of cells treated with antineoplaston A10. To determine whether antineoplaston A10 induced apoptosis in the human hepatoma cells, morphological changes of the cells using AO/EB staining was examined. While untreated cells displayed uniformly green nuclei (Figure 2A) the HepG2 cells incubated with 50 μg/ml of antineoplaston A10 for 48 h had a yellowish stain and dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation in early apoptotic events (Figure 2B). The cells treated with 100 μg/ml of antineoplaston A10 had orange dots, suggesting cell shrinkage or loss of membrane integrity in late apoptotic events (Figure 2C).

Fragmentation of chromosomal DNA in cells treated with antineoplaston A10. Chromosomal DNA fragmentation was analyzed by agarose gel electrophoresis. The chromatins of the HepG2 cells underwent internucleosomal cleavage within 48 h of treatment with antineoplaston A10, as demonstrated by the formation of the characteristic ladder pattern of DNA migration (Figure 3). In addition, a marked accumulation of an apoptotic sub-G1 population was observed by means of flow cytometry in cells treated with antineoplaston A10. The incidence of the apoptotic population dose-dependently increased to 3.7, 5.3, 12.5, 20.2, 40.8 and 54.9% after 48 h of treatment with 0, 10, 50, 100, 200, and 400 μg/ml of antineoplaston A10, respectively (data not shown).

Tumor apoptosis induced by antineoplaston A10 in vivo. TUNEL assay of the xenograft sections from the hepatoma cell transplanted mice revealed that 5 weeks of treatment with antineoplaston A10 induced a significantly higher proportion of apoptotic cells in the xenografts compared to the controls (Figure 4). The incidence of apoptotic cells in xenografts increased in a dose-dependent manner (Table I).

Expression of p53 and bcl-2 in hepatocellular carcinoma xenografts. As shown in Figure 5A and B, p53 expression was chiefly observed in nuclei of HepG2 cells and the level of expression evidently increased as a result of administration of antineoplaston A10. In contrast, as shown in Figure 5C and D, bcl-2 expression was mostly observed in the cytoplasm and the level of expression apparently decreased as a result of administration of antineoplaston A10. At doses of 150, 300 and 600 mg/kg, the rates of p53 positive cells rose to 20.6, 36.2, and 60.5%, respectively and those of bcl-2 positive cells decreased to 82.9, 69.4, and 56.3%, respectively.

Discussion

The present study examined the effects of synthetic antineoplaston A10 on human hepatocellular carcinoma cell lines HepG2 and HLE *in vitro* and *in vivo*. Antineoplaston A10 displayed anti-proliferative effects, inhibiting cell growth in a dose- and time-dependent manner *in vitro* (Figure 1). Morphological changes, DNA ladder formation, and accumulation of cells in the sub-G1 phase suggested that apoptotic events occured in cells treated with antineoplaston A10 (Figures 2 and 3). Notably, oral

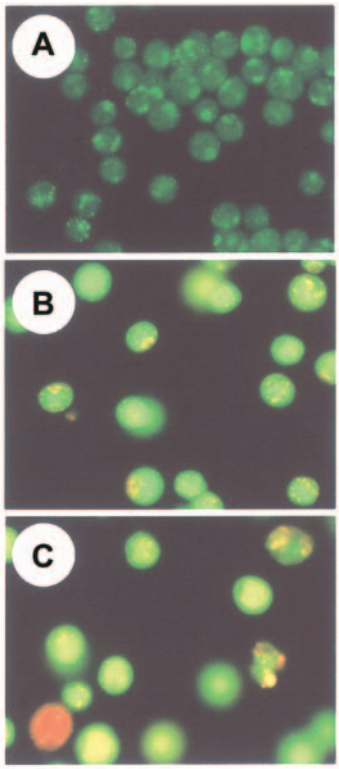

Figure 2. *Morphological changes in HepG2 cells treated with antineoplaston A10. HepG2 cells were treated with 0 (A), 50 (B), or 100 μg/ml (C) of antineoplaston A10 for 48 h and then subjected to AO/EB staining. Original magnification, x400.*

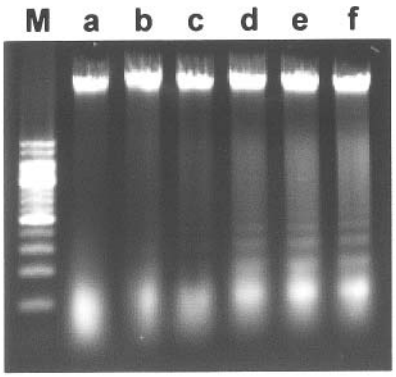

Figure 3. *DNA fragmentation in antineoplaston A10-treated HepG2 cells. DNA obtained from HepG2 cells treated with antineoplaston A10 for 48 h was fractionated in a 1% agarose gel and stained with ethidium bromide. M, marker; a-f, cells treated with 0, 10, 50, 100, 200 and 400 μg/ml of antineoplaston A10, respectively.*

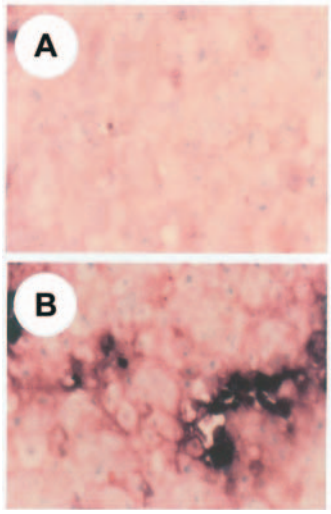

Figure 4. *TUNEL labeled HepG2 xenografts sections. HepG2 cells were transplanted into nude mice and the mice were orally administered 0 (A) or 600 mg/kg (B) of antineoplaston A10 for five weeks. Sections of the xenografts were subjected to TUNEL staining and viewed under a microscope. Original magnification, x400.*

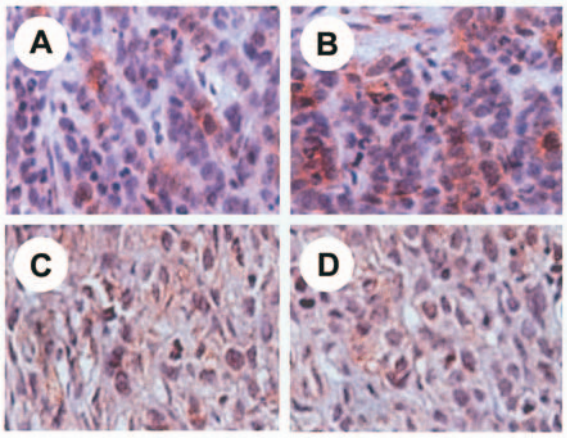

Figure 5. *Immunohistochemical staining of p53 (A and B) and bcl-2 (C and D) in HepG2 xenografts. HepG2 cells were transplanted into nude mice and the mice were orally administered 0 (A and C) or 600 mg/kg (B and D) of antineoplaston A10 for five weeks. The sections of xenografts were subjected to immunohistochemistry to detect p53 and bcl-2 as described in Materials and Methods. Original magnification, x400.*

administration of antineoplaston A10 delayed the growth of HepG2 and HLE cells in nude mice without significantly reducing body weight (Table I). A higher proportion of apoptotic cells was also observed in the xenografts of the mice administered antineoplaston A10 (Figure 4). These results suggest that antineoplaston A10 treatment induced apoptosis in human hepatocellular carcinoma cell lines *in vitro* and *in vivo*.

Antineoplaston A10 may yield phenylacetylglutamine and phenylacetate in the body (5). Phenylacetylglutamine can use the same membrane carrier as glutamine, a growth-critical

amino acid. Therefore, phenylacetylglutamine derived from antineoplaston A10 may decrease the uptake of and thus deplete glutamine in cancer cells (13), which would trigger cancer cell apoptosis *via* many pathways, including a decrease in the expression of antiapoptotic proteins such as bcl-2, survivin, and phosphorylated Bad (14). Concomitantly, phenylacetate derived from antineoplaston A10 may work as a molecular switch that turns off the bcl-2 pathway and activates the silenced tumor suppressor and apoptosis mediator p53 through demethylation of the promoter sequences (15, 16). In addition, the activated p53 may also increase the expression of p21 to synthesize $p21^{WAF1/Cipl}$ protein, an inhibitor of proliferating cell nuclear antigen (PCNA), to inhibit cancer cell growth (17-19). The present study indicated a significant increase in p53 expression and a decrease in bcl-2 in hepatoma xenografts in mice administered synthetic antineoplaston A10 (Figure 4). The apoptosis induced by antineoplaston A10 may have been triggered by the activity of p53 and inhibition of bcl-2.

Most cancer chemotherapies thus far have poor specificity and numerous side-effects. Antineoplaston A10 may induce inhibition of the growth of cancer cells rather than normal cells because of the different levels and activity of oncogenes and tumor suppressor genes found in neoplastic and normal cells. In fact, antineoplaston A10 is reported not to display evident cytotoxicity and inhibit the growth of normal cells (20). Therefore, antineoplaston A10 may exhibit a targeted cancer treatment *via* signal transduction and/or molecular switches that control neoplastic growth by activation of silenced tumor suppressors and/or normalization of overexpressed oncogenes (15).

In summary, antineoplaston A10 had an anti-proliferative effect on human hepatoma cells without significant toxicity in mice. Apoptosis regulated by p53 and bcl-2 expression may be responsible for the antitumor effect of antineoplaston A10.

Acknowledgements

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