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Inhibition of tumor cell proliferation and induction of apoptosis in human lung carcinoma 95-D cells by a new sesquiterpene from hairy root cultures of *Artemisia annua*

D.-D. Zhai[a], K. Supaibulwatana[b], J.-J. Zhong[a,c,*]

[a] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, PR China
[b] Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[c] Key Laboratory of Microbial Metabolism, Ministry of Education, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, PR China

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ABSTRACT

Artemisia annua is a rich source of many bioactive substances, and in our recent work, a new sesquiterpene, (*Z*)-7-acetoxy-methyl-11-methyl-3-methylene-dodeca-1,6,10-triene (AMDT), was isolated and identified from hairy roots culture of *A. annua*, and its bioactivity was characterized in this work. AMDT showed moderate cytotoxic activities against the human tumor cell lines of HO8910 (ovary), 95-D (lung), QGY (liver) and HeLa (cervix) by MTT assay, whose IC_{50} values were ranged within 52.44–73.3 μM. As lung cancer is the No. 1 killer of global cancer patients, our interest is to investigate the ability of AMDT in inducing apoptosis of 95-D tumor cells. The 95-D cell growth was inhibited by AMDT, and the flow cytometry analysis showed its cell cycle was arrested in the G1 phase. The apoptotic rate of the cells increased in a dose-dependent manner. AMDT lowered the mitochondrial membrane potential and increased the expression of caspase-9 and -3. These results revealed that AMDT could efficiently induce 95-D cell apoptosis through mitochondrial dependent pathway, and it may be a potential chemotherapeutic agent.

Introduction

The Compositae plant *Artemisia annua* is a good source of biologically active compounds including sesquiterpenoids, coumarins and polyacetylenes (Tan et al. 1998; Bhakuni et al. 2001). Plant cell and tissue culture is a useful technology for producing plant-specific bioactive compounds, and many hairy root cultures of *A. annua*, which present genetic and biochemical stabilities similar to those of the mother plant, are established for more stable and efficient production of their active constituents (Liu et al. 2006). Besides artemisinin, the most well-known bioactive compound of *A. annua* for treating multidrug-resistant malaria, its coumarins, flavonoids and other terpenoids constituents are also reported to have significant bioactivities such as antitumor and antibacterial activities which contribute to the therapeutic effects of the medicinal plant (Bhakuni et al. 2001; Zheng 1994).

Lung carcinoma is the No. 1 cause of cancer-related death worldwide and continuous efforts are being made for search of novel bioactive compounds to prevent it. Bioactive compounds of natural origin, particularly from medicinal herbs, have been receiving interest.

In our recent work, a new sesquiterpenoid, (*Z*)-7-acetoxy-methyl-11-methyl-3-methylene-dodeca-1,6,10-triene (AMDT), was isolated and purified from hairy root cultures of *A. annua* (Zhai et al., 2010). Its chemical structure was identified based on spectroscopic analysis and was displayed in Fig. 1. To our knowledge, there are no reports on biological activities of this new compound.

In this paper, the cytotoxic activity of AMDT against various human tumor cell lines was studied. Then, to elucidate the mechanism of its antitumor activity, its growth inhibitory effects on human lung cancer cell line 95-D and molecular events in the induction of the cancer cell apoptosis were also examined.

Materials and methods

Plant materials

The *A. annua* hairy root line induced by the leaf-disc method was used. And a voucher was deposited in the School of Life Sciences and

* Corresponding author at: The School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, 800 Dong-Chuan Road, Shanghai 200240, PR China. Tel.: +86 21 34206968; fax: +86 21 34204831.
E-mail addresses: jjzhong@sjtu.edu.cn, daneszh@hotmail.com, daneszh@126.com (J.-J. Zhong).

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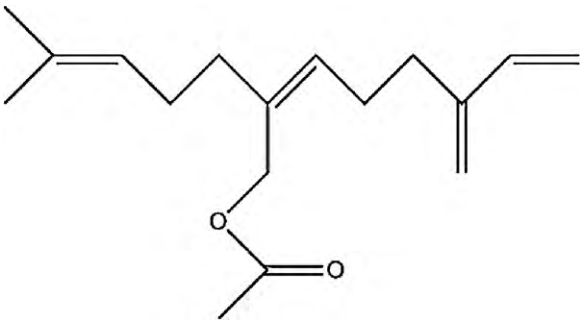

Fig. 1. Chemical structures of (*Z*)-7-acetoxy-methyl-11-methyl-3-methylene-dodeca-1,6,10-triene (AMDT).

Biotechnology of Shanghai Jiao Tong University (Shanghai, China). The cell cultures were maintained by subculturing at a 14-day interval in Murashige and Skoog mineral nutrient medium (Murashige and Skoog 1962) supplemented with 3% (m/v) sucrose at pH 5.8. The cultivation was carried out at 25 ± 1 °C under 16 h light irradiation per day. About 0.5 g of fresh weight tips (2 cm long or so) of 14-day-old hairy roots were inoculated into a 250 ml Erlenmeyer flask containing 50 ml medium. The Erlenmeyer flasks were incubated on a rotary shaker at 110 rounds per minute under cool-white fluorescent lamps (Royal Philips Electronics, Netherlands) at an intensity of 5.4 W m^{-2}.

Reagents

AMDT was isolated from the hairy root culture of *A. annua* and its chemical structure was identified based on spectroscopic studies. The purity of AMDT was more than 95% by high performance liquid chromatography (HPLC) analysis. AMDT was dissolved with DMSO and diluted with RPMI1640 medium till the final concentration of DMSO was less than 0.3% (v/v). 10-Hydroxycamptothecin (HCPT), as positive control, was purchased from Winherb Medical Inc. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and other chemicals were purchased from Sigma–Aldrich Inc. (St. Louis, USA). RPMI1640 medium and fetal bovine serum were obtained from Gibco (Grand Island, USA).

Cell lines

The cell lines HO8910 (human high metastatic ovarian cancer), 95-D (human high metastatic lung carcinoma), QGY (human hepatoma), HeLa (human cervical carcinoma) and human normal cell line LO2 (human liver cell) were obtained from Shanghai Cellular Institute of Chinese Academy of Sciences (Shanghai, China). Another normal cell line HF (human dermal fibroblast) is a gift from Associate Prof. Yan Zhou of East China University of Science and Technology. Those cell lines were maintained in RPMI1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin, and were kept at 37 °C in a 5% CO_2 incubator.

MTT assay

The MTT assay was performed as described by Hansen et al. (1989). Briefly, exponentially growing cells at 1×10^5 cells/ml were incubated in 96-well microplates and treated with different concentrations of AMDT at 19.1 (corresponding to 5 μg/ml), 38.1, 76.3, and 152.6 μM or of HCPT (as positive control) at 13.7 (corresponding to 5 μg/ml), 27.4, 54.9, and 109.8 μM. Cells were routinely cultured for another 24, 48, and 72 h. At the end of each incubation, media were added with 10 μl of MTT (5 g/l) and incubated for another 4 h. The medium was then discarded and 100 μl of DMSO was added to each well. The reaction products were colorimetrically quantified at 570 nm with subtraction of the background absorbance at 630 nm by scanning with a MK3 microplate reader (Thermo, USA). And the cells treated with DMSO at same concentrations as in the drugs were used as controls. For the determination of IC_{50} (the concentration of each substance required for 50% inhibition of tumor activity), each sample was measured at four different concentrations by the MTT test. IC_{50} values of each sample were collected from three replicates, and then mean ± S.D. values for the IC_{50} assay were obtained.

Microscopic observation of cellular morphology and nuclear fragmentation

The 95-D cells at 1×10^5 cells/ml were seeded in 24-well plates and treated with different concentrations of AMDT (38.1–114.4 μM) for 24 h. HCPT (27.4 μM) was used as a positive control. Then the cells were fixed in 4% paraformaldehyde for 30 min, and then stained with 10 mg/ml Hoechst 33258, a DNA-specific fluorescent dye, for 10 min at 37 °C. The stained cells were then observed under a Nikon fluorescence microscope (Nikon Inc., Japan).

Cell cycle analysis and apoptosis evaluation by flow cytometry

Flow cytometry was employed to evaluate cell cycle distribution and inducing-apoptosis activity (Tang et al., 2006). The 95-D cells treated with different concentrations of AMDT or HCPT (as positive control) for 24 h were harvested by centrifugation at 230 × *g* for 5 min, washed with ice-cold PBS, and then fixed with 70% cold ethanol at 4 °C for 1 h. The fixed cells were washed with PBS and resuspended in a staining solution containing PI (20 μg/ml) and DNase-free RNase (100 μg/ml). The cells suspension were incubated at 37 °C for 30 min in the dark and quantitatively analyzed by a flow cytometer (BD FACScan TM, CA, USA).

Apoptosis was detected by annexin V-FITC binding assay. Normal, apoptotic and necrotic cells were distinguished by using the annexin V-propidium iodide (PI) kit according to the manufacturer's instructions (BIPEC, USA). Briefly, after treatment with different concentrations of AMDT or HCPT, the 95-D cells were harvested and processed for 24 h after treatment. The cells were washed in ice-cold PBS and resuspended in binding buffer. 5 μl of annexin V-FITC staining solution was added and the cells were incubated in the dark for 10 min, then 10 μl of propidium iodide (PI) was added to each sample and the cells were incubated in the dark for another 5 min before flow cytometric analysis. Data analysis was

Table 1
Growth inhibition of AMDT on various human cell lines (24 h).

Compounds	IC_{50}[a] (μM)					
	LO2	HF	HO8910	95-D	HeLa	QGY
AMDT	115.45 ± 7.86	124.68 ± 11.86	54.77 ± 2.48	52.44 ± 5.16	81.35 ± 5.07	73.31 ± 1.49

[a] All data are expressed as means ± S.D. (n = 3).

performed with standard Cell Quest software (BD FACScan™, CA, USA). The percentages of viable and dead cells were determined with about 1×10^4 cells/sample.

Measurement of caspase-9 and -3 activities

Caspase-9 and -3 assay kits (Nanjing Kaiji Bio-Tek Corporation, Jiangsu, China) were used for their activity assay (Li et al. 2007), which is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after its cleavage from the labeled substrate DEVD-pNA. Briefly, 24 h after drug treatment, 95-D cells (1×10^6 cells) were harvested and caspase-9 and -3 activities were determined according to the manufacturer's instruction. Absorbance of the chromophore p-nitroanilide produced was measured using a Thermo MK3 microplate reader (Thermo Labsystems Co., USA) at 405 nm. Activities of caspase-9 and -3 were expressed relative to the amount of protein in the cell extracts, which was determined by the method of Bradford (1976).

Analysis of mitochondrial membrane potential

Mitochondrial transmembrane potential ($\Delta\psi_m$) was estimated using the fluorescent cationic dye rhodamine123 (Rh123) (Zamzami et al. 2001; Li et al. 2007). Briefly, 1×10^5 cells/ml 95-D cells were harvested and incubated for 15 min at 37 °C with 5 μM Rh123 in the dark. Cells were then washed twice with PBS and analyzed immediately by flow cytometry (BD FACScan™, USA). Loss in Rh123 staining indicates an association to the disruption of mitochondrial membrane integrity.

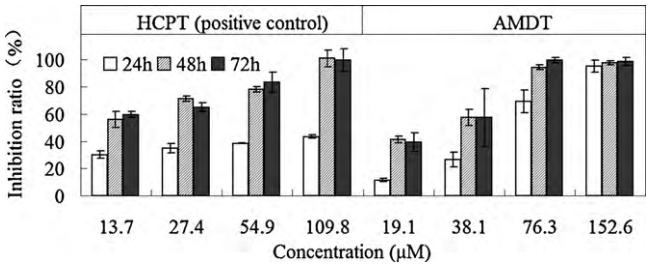

Fig. 2. Growth inhibition effects of AMDT or 10-hydroxycamptothecin (HCPT, as positive control) against 95-D cells. Data are shown in the mean ± S.D. ($n = 3$).

Statistical analysis

All experiments were repeated at least three times. Data were expressed as means ± S.D. Significance was assessed by Student's *t*-test. $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered as statistically significant.

Results

Effect of AMDT on proliferation of various human tumor cell lines

To evaluate the growth inhibition effect of AMDT on various human tumor cells, the cells were treated with AMDT for 24 h. The results in Table 1 indicated that AMDT inhibited the proliferation of these cancer cells. Meanwhile, it showed a relatively low cytotoxicity to the normal cell lines LO2 and HF. As shown in Fig. 2, AMDT inhibited the proliferation of human lung carcinoma 95-D cells in a concentration- and time-dependent manner. The highest tested

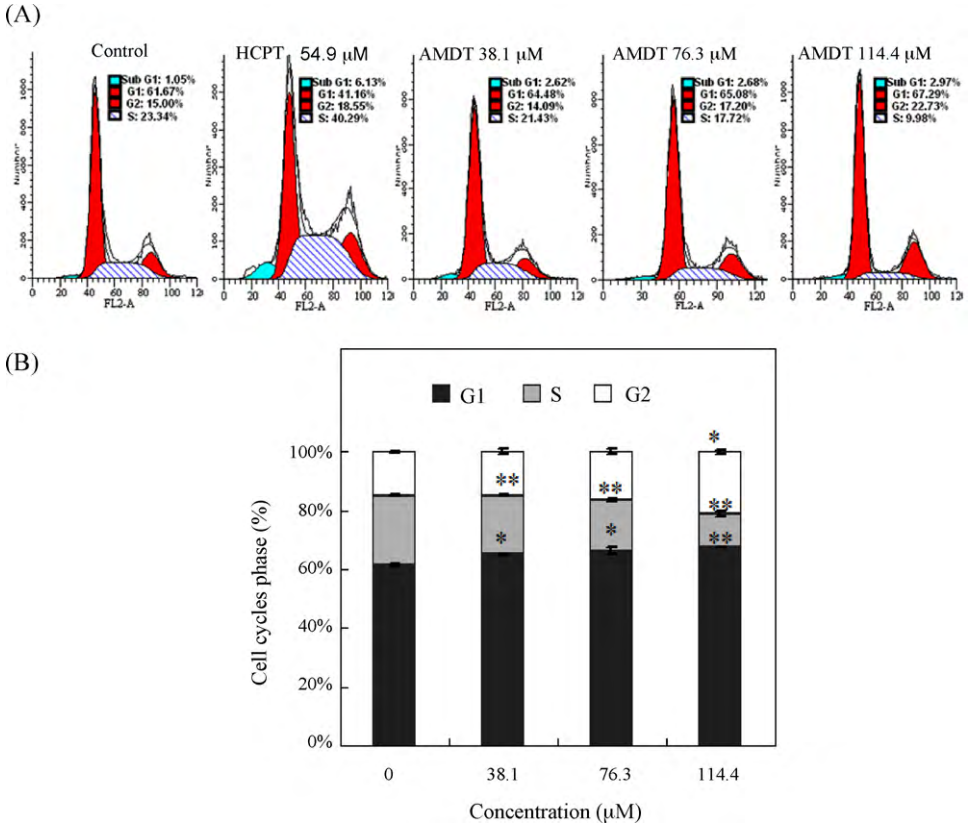

Fig. 3. Effects of AMDT or HCPT (positive control) acted on cell cycle distributions in 95-D cell line. (A) Cell cycle analysis of 95-D cells treated with AMDT or HCPT for 24 h and measured by flow cytometry. (B) Quantitation of cell cycle distribution using standard Modifit and CellQuest analysis software (BD FACScan™, USA). Data in the histograms are means ± S.D. of three independent experiments in triplicate. For the percentage of cell cycle distribution, it takes the total percentage of cells at G1 phase, S phase, and G2 phase as 100%. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control.

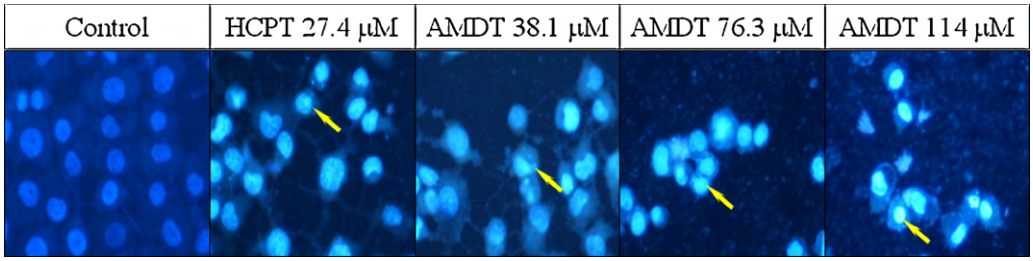

Fig. 4. The morphologic changes of 95-D cells treated with AMDT or HCPT (positive control) for 24 h observed by fluorescence microscopy (200×) after nuclei staining with Hoechst 33258. Cells with characteristic apoptotic morphology were marked by arrow.

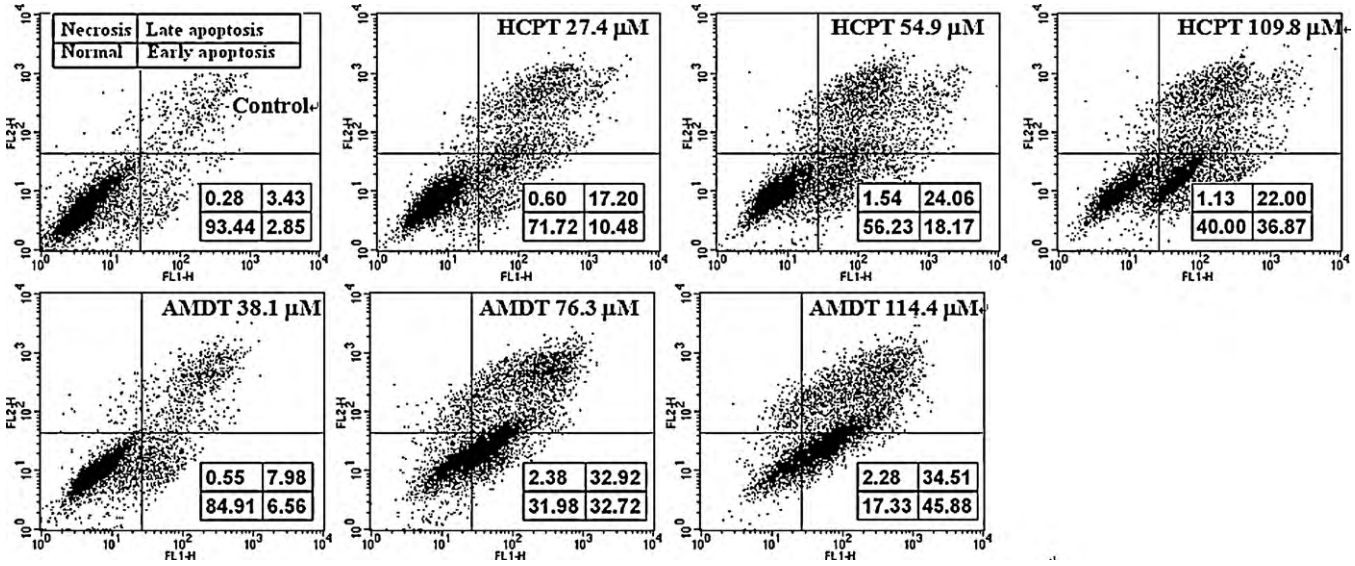

Fig. 5. Effects of AMDT or HCPT (positive control) acted on cell death (apoptosis and necrosis) in 95-D cells. Shown are typical data from one of three independent experiments with similar results. For the apoptosis percentage, it takes the total percentage of normal cells, apoptotic cells and necrotic cells as 100%.

concentration (152.6 μM) of AMDT had an acute cytotoxic effect on the 95-D cells.

Cell cycle distribution and induction of apoptosis of 95-D cells under AMDT treatment

Cell cycle phase distribution was analyzed by flow cytometry (Fig. 3). Compared with control, an accumulation of 95-D cells in the G1 phase (61.4–67.5%) was noticed in various AMDT concentrations. These results suggested that the cell cycle of 95-D cells was arrested in the G1 phase by AMDT.

To elucidate whether the decrease in cell viability induced by AMDT was attributable to apoptosis, nuclear staining with Hoechst 33258 and annexin V/PI staining were conducted.

It was noticed that the 95-D cells of control had regular and round-shaped nuclei revealed by nuclear staining with Hoechst 33258 (Fig. 4A). By contrast, the condensation of nuclei and blebbing characteristic of apoptotic cells were evident in 95-D cells treated with different concentrations AMDT or positive control HCPT (Fig. 4B–H), which was different from that in the control cells.

Flow cytometric analysis using annexin V/PI double-staining (Fig. 5; Table 2) revealed that the percentage of annexin V-FITC-positive 95-D cells that were apoptotic exceeded the control level (early apoptosis, 2.54%; late apoptosis, 3.51%) after 24 h of treatment with AMDT (114.4 μM: early apoptosis, 42.35%; late apoptosis, 32.3%). Taken together, these data indicated that AMDT-induced apoptosis in 95-D cells.

Effect of AMDT on mitochondrial transmembrane potential ($\Delta\psi_m$) and caspase-3 and -9 activities

To evaluate the influence of AMDT on the function of mitochondria, alterations in mitochondrial potential were analyzed by employing a fluorescent dye Rh123. As shown in Fig. 6, AMDT

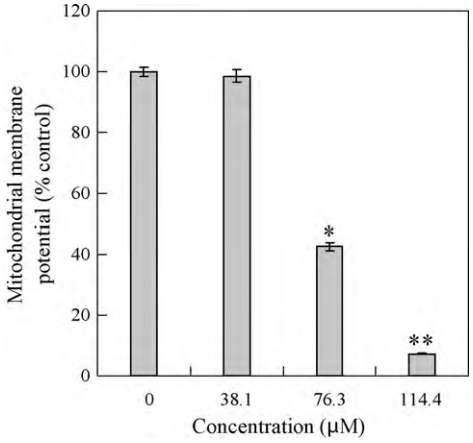

Fig. 6. Effects of AMDT on mitochondrial transmembrane potential in 95-D cells. Values are expressed as means ± S.D. obtained from three culture wells per experiment, determined in three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with control.

Table 2
Effects of AMDT on apoptosis and necrosis of 95-D human lung tumor cells (HCPT as positive control).

Compound	Concentration (μM)	Normal[a] (%)	Early apoptosis[a] (%)	Late apoptosis[a] (%)	Necrosis[a] (%)
Control	0	93.55 ± 0.93	2.54 ± 0.72	3.51 ± 0.30	0.4 ± 0.11
HCPT (positive control)	27.4	72.79 ± 1.51*	10 ± 0.68*	16.51 ± 0.98*	0.71 ± 0.16
	54.9	55.2 ± 1.46*	18.57 ± 0.57**	24.67 ± 0.86*	1.56 ± 0.03*
	109.8	35.46 ± 6.43*	40.10 ± 4.56*	23.31 ± 1.85*	1.15 ± 0.02*
AMDT	38.1	86.36 ± 1.44*	5.21 ± 1.49	7.78 ± 0.18**	0.64 ± 0.17
	76.3	37.42 ± 8.72**	28.61 ± 7.51*	30.83 ± 2.49*	3.14 ± 1.18
	114.4	22.41 ± 8.35**	42.35 ± 6.21**	32.3 ± 3.52*	2.94 ± 1.39

[a] The values are expressed as mean ± S.D. ($n = 3$).
* Significant differences with the corresponding controls were designated as $P < 0.05$.
** Significant differences with the corresponding controls were designated as $P < 0.01$.

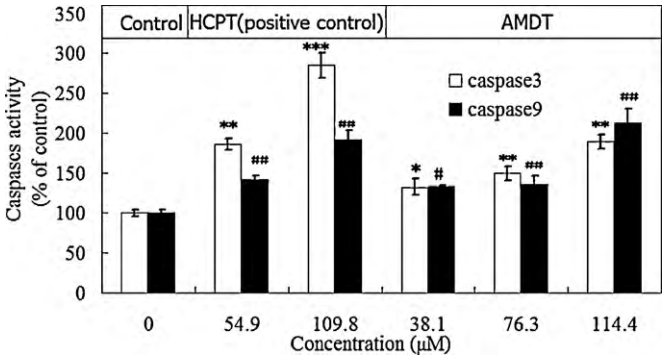

Fig. 7. Effects of AMDT or HCPT (positive control) on caspase-3 and -9 in 95-D cells. Values represent the means ± S.D. obtained from three culture wells per experiment, determined in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* caspase-3 control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* caspase-9 control.

dose-dependently caused the collapse of mitochondrial membrane potential of 95-D cells. The data indicated that AMDT-induced apoptosis accompanied by the alterations in the mitochondrial membrane potential.

Following 24 h treatment of 95-D cells with various concentrations of AMDT, significant increases of both caspase-9 and -3 activities were detected and found to be in a dose-dependent manner compared with the control (Fig. 7). The results suggested that AMDT-induced apoptosis occurred through the activation of common executors of apoptosis – caspase-3 and caspase-9.

Discussion and conclusion

Medicinal herbs are considered as a significant source for new drug discovery (Wagner and Ulrich-Merzenich 2009). Many drugs in clinical today have botanical origins such as aspirin, lanoxin and artemisinin. In this study, the new acyclic sesquiterpene AMDT from the hairy root cultures of *A. annua* was investigated for its antitumor activity. MTT assay showed that the purified sesquiterpene AMDT inhibited the cell growth of various human carcinoma cell lines, while its cytotoxicity was less pronounced on the normal cell lines LO2 and HF. Some acyclic sesquiterpenes with similar structure to AMDT were documented with their cytotoxicity against tumor cell lines P-388, A-549, HT-29 and MEL-28, whose IC_{50} ranged within 9.5–23.8 μM (Rueda et al. 2001). These facts suggested the common part of their chemical structures might serve as an active site or play an important role in the interaction with tumor cells. Alternatively, those similar structure compounds only were reported the cytotoxicities of purified compounds against cancer cells, but the antitumor mechanism was not understood fully.

To have an insight into the mechanism of the cytotoxicity and with our great interest in treating the most fatal cancer in the world, a human lung tumor cell line 95-D was chosen for further investigation. The growth inhibitory effect of AMDT on 95-D cells (Fig. 2) displayed a distinct dose- and time-dependent manner.

Cell cycle progression and apoptosis are two pivotal signaling mechanisms used to maintain homeostasis in healthy tissues. Many anti-cancer agents and DNA-damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce cell apoptosis (Kessel and Luo 2000; Hu et al. 2009). Further experiments revealed that apoptosis and cell cycle progression block were both responsible for the inhibition of the tumor cell proliferation by AMDT.

Data from flow cytometry analysis (Fig. 3) showed that the cell cycle was arrested in G1 phase by AMDT. The results implied that AMDT might affect the G1/S cell-cycle checkpoint (Waldman et al. 1997).

Apoptosis involves a series of cellular biochemical events leading to a variety of characteristic morphological changes such as membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation (Hickman 1992; Kerr et al. 1994). Microscopic observation showed the 95-D cells under drug treatment displayed the characteristic apoptosis morphology. From the result of double staining (Fig. 5 and Table 2), the proportion of cells reactive with the annexin V-FITC conjugate suggested that target cells entered apoptosis after the treatment. In the AMDT-treated and the positive control HCPT-treated 95-D cells, only the proportion of apoptosis cell increased significantly in a dose-dependent manner as the percentage of the necrosis cell did not show an obvious difference. These results demonstrated that AMDT efficiently induced apoptosis of 95-D cells.

To date, two major apoptotic signaling pathways of mammalian cells are well known. One is the intrinsic pathway and another is the extrinsic pathway (Chen and Wang 2003). The intrinsic pathway involves an increase of outer mitochondrial membrane permeability that leads to the release of various proteins from the intermembrane space into the cytoplasm, such as cytochrome *c* and DNaseG (Green and Reed 1998; Tsujimoto and Shimizu 2007). The extrinsic pathway involves ligation of death receptors, resulting in the recruitment of the adaptor protein FADD through interaction between the death domains (DD) of both molecules. In both pathways, the stress-mediated apoptosis is often triggered by mitochondrial function loss. Accordingly, mitochondrion in AMDT-mediated apoptosis was also explored in this work. The results indicated AMDT-induced a subsequent drop in mitochondria potential. Therefore, the apoptosis induced by AMDT was considered through intrinsic pathway related to mitochondrial dysfunction. However, further studies of mitochondria mechanism of AMDT in detail are necessary to obtain definite conclusions.

Caspases, a family of cysteinyl aspartate-specific proteases, play an essential role in the regulation and the execution of apoptotic cell death (Hengartner 2000; Lavrik et al. 2005). All caspases are produced in cells as inactive zymogens and require a proteolytic cleavage and then convert to active form during apoptosis. The mitochondria-mediated pathway involves the release of cytochrome *c*, and then followed by the formation of apoptosome,

a 140-kDa cytoplasmic complex, consisting of Apaf-1 (apoptotic protease-activation factor-1), dATP, cytochrome *c*, and procaspase-9, which results in activation of caspase-9 and in turn, activating caspase-3 and final fragmentation of DNA. From our results (Fig. 7), both caspases-9 and caspase-3 were activated in the AMDT-treated cells. It revealed that AMDT could activate caspase-9 and -3, which correspondingly initiated the death cascade and finally induced apoptosis. Therefore, the AMDT-induced apoptosis was mediated through activation of caspase cascade.

Taken together, in this work, the bioactivities of the new compound AMDT were studied. AMDT was found to possess significant cytotoxicities to various human cancer cells as tested. In particular it could effectively induce the apoptosis and cell cycle arrest of the human lung carcinoma 95-D cells. The apoptosis induction was revealed to be through the mitochondria dependent pathway and activation of caspase cascade. These results indicated that AMDT would deserve further study as a potential chemotherapeutic drug candidate.

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