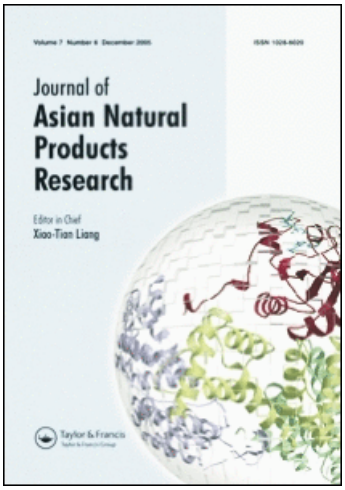

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ORIGINAL ARTICLE

Aesculetin-induced apoptosis through a ROS-mediated mitochondrial dysfunction pathway in human cervical cancer cells

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Aesculetin (**1**) is an important coumarin found in various plant materials. It has been shown to have antiproliferative effects on several types of human cancer cells, but its effect on cervical cancer cells *in vitro* is unknown. In this study, we investigated that the cytotoxic effect of **1** on a non-cancer cell line (293) was smaller than on a tumor cell line (HeLa). This is the first report showing the possible mechanism of antiproliferative effect of **1** for the prevention of cervical cancer in cell culture models. It was found that **1** inhibited cell viability by inducing apoptosis, as evidenced by the formation of apoptotic bodies, generation of reactive oxygen species (ROS), and the accumulation of cells in the sub-G1 phase. Treatment with compound **1** decreased the cell growth in a dose-dependent manner with an IC_{50} value of 37.8 μM. Aesculetin-induced apoptosis was correlated with mitochondrial dysfunction ($\Delta\Psi_m$), leading to the release of cytochrome *c* from the mitochondria to the cytosol, as well as the proteolytic activation of caspases in HeLa cells. These results indicate that **1** induces apoptosis in HeLa cells through a ROS-mediated mitochondrial dysfunction pathway.

Keywords: aesculetin; apoptosis; mitochondrial; HeLa cells

1. Introduction

Medicinal plants have been well-known natural sources for the treatment of various diseases since antiquity. Aesculetin (**1**) is a coumarin derivative found in various plant materials and has been reported to have beneficial pharmacological and biochemical activities [1–7]. Compound **1** has been shown to have an anti-inflammatory effect in the croton oil ear test [1], antiproliferative effects on vascular smooth muscle cells [2], and inhibitory action on *N*-methyl-*N*-nitrosourea-induced mammary carcinoma [3,4]. The free radical scavenging and lipid peroxidation assays revealed that **1** from four Korean medicinal plants possessed considerable antioxidant activities [5]. Aesculin can be transferred into **1** by human gut bacteria and is further modified by the host *in vivo* [6]. The extracellular signal-regulated kinase (ERK) cascade was proven to be involved in the enhancement of **1** on the Taxol-induced apoptosis in human hepatoma HepG2 cells [7], and the JNK and ERK pathways were key regulators of apoptosis in response to **1** in human leukemia U937 cells [8].

Cervical cancer, a slow-growing squamous cell carcinoma, is the second most common cancer in women, particularly in developing countries where it is the

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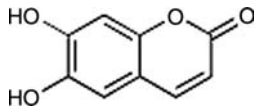

Figure 1. Chemical structure of **1**.

leading cause of cancer death among women [9]. Apoptosis can be initiated through either the death receptor or the mitochondrial pathway. Thus, identifying the mode of cell death has been recognized as a novel strategy for the screening of anticancer drugs [10].

The present study is the first report on the antitumor activity of **1** against human cervical cancer HeLa cells. The objectives were to investigate the effect of **1** on proliferation and apoptosis *in vitro* and to study the possible mechanisms of action (Figure 1).

2. Results and discussion

2.1 Compound 1 inhibited cell proliferation of HeLa cells

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay suggested that **1** induced growth inhibition of HeLa cells in a dose-dependent manner (Figure 2(A)). The concentration that induced a 50% inhibition of cell proliferation (IC_{50}) as compared to controls was 37.8 μM for 72 h. As shown in Figure 2(B), treatment with 100 μM of **1** could not induce a significant decrease in the percentage of 293 cells. Compound **1** inhibited the viability of HeLa cancer cells more effectively than that of non-cancer cells.

2.2 Compound 1 induced apoptosis in HeLa cells

The treatment of HeLa cells with 50 μM of **1** for 12 h led to the generation of plasma membrane blebs, while treatment with the solvent alone (control) did not (Figure 3(A)). Both control and aesculetin-treated cells were stained with the fluorescent dye Hoechst 33258 and visualized by a fluorescent microscope. As shown in Figure 3(B), control cells were normal and the nuclei were round and homogeneous; in contrast, nuclei with condensed chromatin and apoptotic bodies, typical characteristics of apoptosis, were observed in HeLa cells treated with **1**.

After the HeLa cells were treated with 50 μM of **1** for 12, 24, and 48 h, they were harvested and stained with propidium

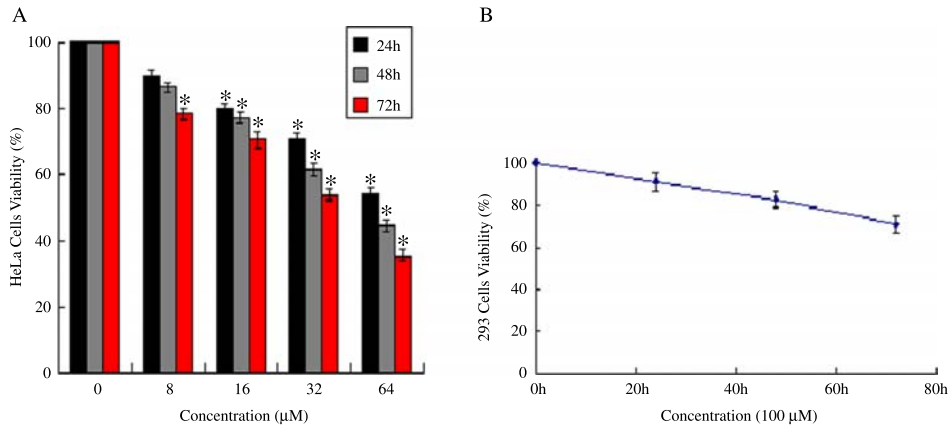

Figure 2. (A) HeLa cells were treated with 8, 16, 32, or 64 μM of **1** for 24, 48, or 72 h, and control cells were maintained in the vehicle for the indicated time periods. (B) 293 cells were treated with 100 μM of **1** for 24, 48, or 72 h. *Statistically significant difference compared with controls ($p < 0.05$).

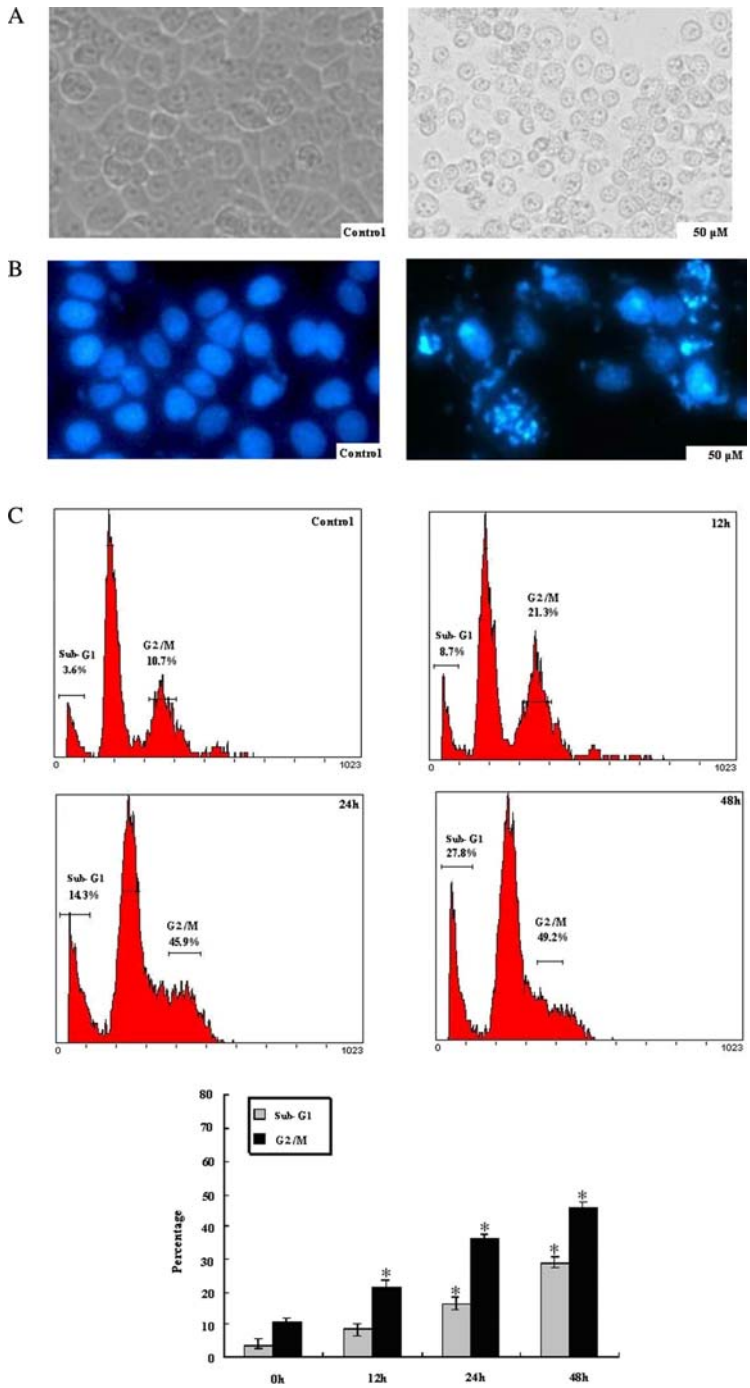

Figure 3. (A) Morphological changes of HeLa cells after treatment with 50 μM of **1** for 48 h. HeLa cells were observed under phase contrast microscope. Magnification: 200 ×. (B) HeLa cells were stained by Hoechst 33258 and observed under an inverted Leica fluorescence 40×10 microscope. (C) Compared to control, **1** could induce apoptosis (increasing the sub-G1 population) and G2/M arrest in HeLa cells. *Statistically significant difference compared with controls ($p < 0.05$).

iodide (PI), and the cell populations of each phase were counted by flow cytometry. As shown in Figure 3(C), the sub-G1 population, which indicated apoptotic cells, increased in a time-dependent manner from 3.6% at 0 μM (control) to 8.7% at 50 μM for 12 h, 14.3% at 50 μM for 24 h, and 27.8% at 50 μM for 48 h. These results clearly indicated that **1** could induce cell cycle arrest in the G2/M phase and apoptosis in HeLa cells in a time-dependent manner ($p < 0.05$).

2.3 Compound 1 induced loss of mitochondrial membrane potential

The loss of mitochondrial membrane potential ($\Delta\Psi_m$) is an important event in apoptosis [11]. To determine whether early loss of mitochondrial membrane potential occurred during treatment with **1**, HeLa cells were grown in the absence (control) or the presence of **1** (50 μM) for 6, 12, 24, and 48 h, and stained with rhodamine 123 (Rh123). As shown in Figure 4, **1** induced mitochondrial dysfunctions in HeLa cells; the maximum loss of $\Delta\Psi_m$ was observed after 48 h of treatment ($p < 0.05$). These results suggest the direct role of mitochondria in aesculetin-induced apoptosis.

2.4 Activation determination of caspase

To elucidate the role of caspases in aesculetin-induced apoptosis, whole cell lysates were prepared from HeLa cells that were stimulated with 50 μM of **1** for 4, 8, and 12 h, and the proteolytic activity of the executioner caspase-3 and the initiators caspase-7 and -9 were measured. As shown in Figure 5, catalytic activity of caspase-9 was detected as early as 4 h after toxin exposure and reached its maximal level after the cell had been incubated for 8 h with **1** ($p < 0.05$). The caspase-3/7 activity was also modulated by **1** and reached its maximum after 12 h ($p < 0.05$).

2.5 Release of cytochrome c from mitochondria

Release of the apoptogenic protein cytochrome *c* from the mitochondria into the cytosol was a hallmark event of cells undergoing apoptosis. We further evaluated whether the apoptotic cell death was induced by **1** in the expression of cytochrome *c* by Western blot analysis employing mouse monoclonal cytochrome *c* antibodies. As shown in Figure 6, a significant increase in the cytosolic level of cytochrome *c* was obtained after 6, 12, and 24 h of treatment with **1**, indicating its release from mitochondria and the levels of cytochrome *c* that remained in the mitochondria decreased in a time-dependent manner.

2.6 Reactive oxygen species generation in aesculetin-treated HeLa cells

Reactive oxygen species (ROS) production is implicated in apoptosis and has been described as an early event. The role of ROS in aesculetin-mediated apoptosis was further evaluated. The exposure to 2, 4, and 6 h with 50 μM of **1** caused a significant increase in fluorescence response ($p < 0.05$), which indicated the production of $O_2^{-\cdot}$ in the mitochondria of HeLa cells (Figure 7). These results indicated that **1** induced an early increase in mitochondrial ROS production upstream of caspase activation.

2.7 Discussion

Compound **1**, a coumarin derivative, is found in various traditional herbal medicines such as *Artemisia scoparia*, *Artemisia capillaris* (Compositae), *Ceratostigma willmottianum* (Plumbaginaceae), and *Citrus limonia* (Rutaceae) [12–20]. Previous studies showed that **1** could exert an anti-proliferation effect together with an inhibitory effect on the activation of p42 and p44 ERKs and p38 MAPK in HepG2 cells *in vitro*, and the SP600125 (a specific

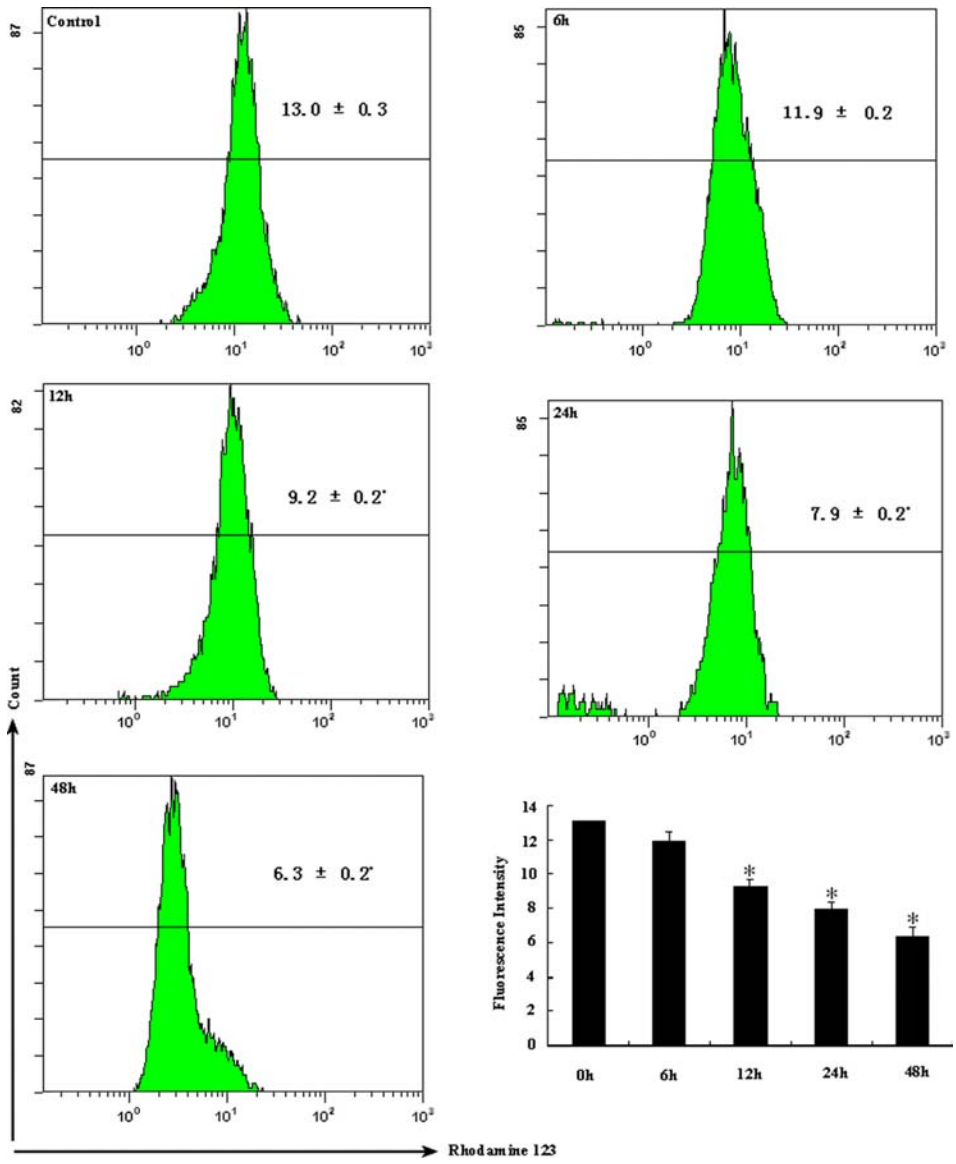

Figure 4. HeLa cell population showed time-dependent loss of mitochondrial membrane potential following exposure to **1**. *Statistically significant difference compared with controls ($p < 0.05$).

inhibitor of the JNK MAP kinase pathway) reduced aesculetin-induced apoptosis by inhibiting the release of cytochrome *c*, suggesting that aesculetin-induced apoptosis is associated with the ERK and JNK signaling pathways [7]. Compound **1** significantly induced apoptosis in U937 cells *in vitro* via regulation of Bcl-2 family proteins and caspase activity, and the induction was associated with caspase-3 activation, PARP and PLCγ1 degradation, and ERK and JNK pathways [8].

In the current report, we use **1** in the human cervical cancer cell line as a model system to investigate the effects of **1**-induced apoptosis *in vitro*. HeLa cells

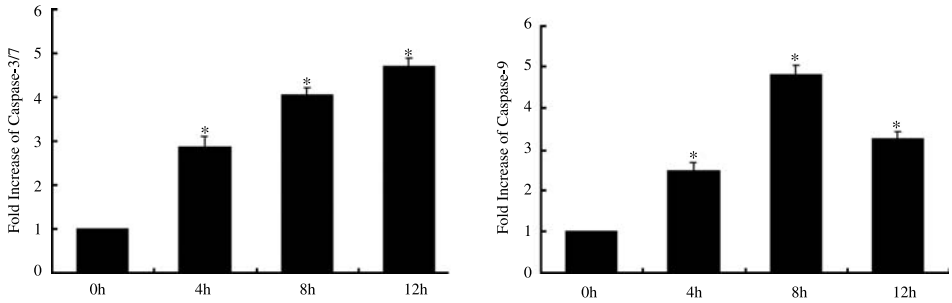

Figure 5. HeLa cells were incubated for 4, 8, or 12 h in the absence (control) or presence of 50 μM of **1**. Fold increases in caspase activity were determined by comparing the results at indicated times with control. The asterisk indicates a significant difference between control and aesculetin-treated cells (*$p < 0.05$). *Statistically significant difference compared with controls ($p < 0.05$).

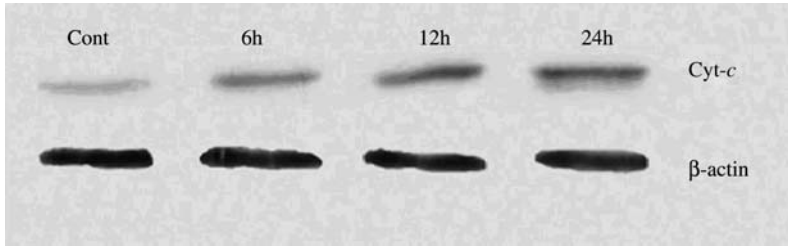

Figure 6. Cells were incubated for different times in the presence or absence of 50 μM of **1**. Cytosolic fraction was analyzed to detect the release of cytochrome *c* in the cytosol by Western blotting. β-Actin was used for normalization and verification of protein loading.

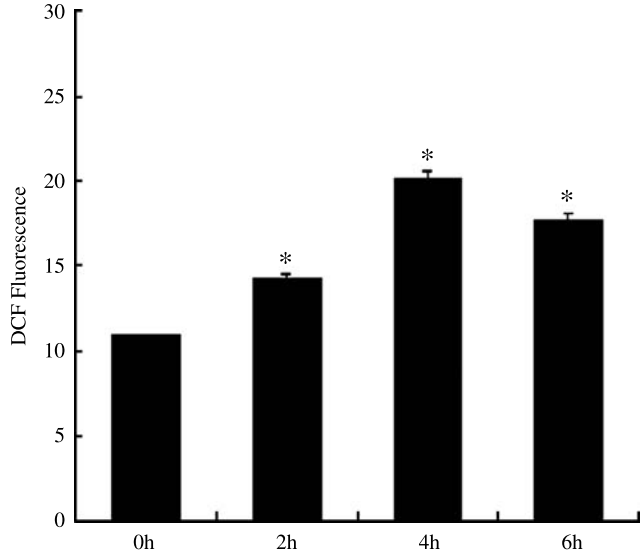

Figure 7. After treatment, cells were treated with 10 μM DCFH-DA for 30 min, and ROS was measured using DCF-DA by flow cytometry analysis. *Significant differences from the untreated group were $p < 0.05$.

treated with **1** (50 μM) showed cytotoxic effects such as rounding of cells with increased intercellular spaces, altered morphology, and decrease in viability as seen by Hoechst 33258 staining. The survival of HeLa cells was decreased in a dose- and time-dependent manner ($p < 0.05$) [21]. Kidney is an important organ for excreting various toxic substances and drugs. HeLa cells as well as 293 cells belong to epithelial cellular morphology. It is worth to note that the toxicity did not occur in aesculetin-treated non-cancer cell lines, 293 cells (Figure 2(B)).

Our observations demonstrated that excessive generation of ROS could lead to opening of the mitochondrial permeability transition pore with decline in $\Delta\Psi_m$ and consequent release of cytochrome *c* from the intermembrane space into the cytosol, culminating in activation of the caspase cascade and apoptotic cell death. The present study strongly proposes that **1** may possess potential anti-cancer effects and supports its use as a treatment modality for cancers.

3. Experimental

3.1 Chemicals and reagents

Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), MTT, 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA), Hoechst 33258, and ribonuclease (RNase A), Rh123, and PI were obtained from Sigma Chemical (St Louis, MO, USA). Caspase-Glo®-3/7 Assay and Caspase-Glo®-9 Assay were from Promega, Inc. (Madison, WI, USA). The mouse monoclonal antibodies against cytochrome *c* were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and the protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Compound **1** (6,7-dihydroxycoumarin, 98% purity) was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in DMSO (vehicle). All cell culture supplies were purchased from GIBCO BRL (Grand Island, NY, USA).

3.2 Cell lines and culture

Human cervical adenocarcinoma cell line (HeLa) and embryonal kidney 293 cells, used in this work, were purchased from China Centre for Type Culture Collection (Wuhan, China). HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing 5% CO_2 at 37°C. All experiments were performed in triplicate for each treatment condition. Fresh medium was given every second day and on the day before the experiments were done. Cells were passaged at preconfluent densities, using a solution containing 0.05% trypsin and 0.5 mM EDTA. Compound **1** was dissolved in DMSO, and all cells received DMSO to a final concentration of 2% (v/v). Controls were always treated with the same amount of DMSO.

3.3 Cell growth inhibition assay

This assay detected the reduction of MTT by mitochondrial dehydrogenase to a blue formazan product, which reflected the normal functioning of mitochondrial and cell viability [21]. Cells with logarithmic growth phase (1×10^5 cells/ml) were seeded independently in a 96-well plate with a final volume of 100 μl containing 1×10^4 cells per well, and were allowed to attach for 12 h before treatment. Cells were treated with **1** ranging from 8 to 64 μM. After a certain period of time, cells in each well were then incubated at 37°C in 50 μl of MTT (5 mg/ml) for 3 h. After the medium and MTT were removed, 200 μl of DMSO was added to each well. Absorbance in the control (treated with

0.1% DMSO) and in the aesculetin-treated cells at 570 nm was detected using a microplate ELISA reader (MRX II, DYNEX Technologies, Chantilly, VA, USA).

3.4 Staining of apoptotic cells with Hoechst 33258 and PI staining assay

HeLa cells from exponentially growing cultures were seeded in a 24-well culture plate and treated with 50 μM of **1** for 12 h. Cells were harvested and washed once with PBS, and then fixed with 2% glutaraldehyde solution for 1 h and stained with Hoechst 33258 (100 μg/ml) for 30 min. Stained cells were observed and photographed with a fluorescence microscope (Olympus, BX-60, Tokyo, Japan) in less than 15 min.

After 24, 48, and 72 h of incubation with or without 50 μM of **1** at 37°C, HeLa cells were harvested by trypsinization and fixed with 70% ethanol for 1 h, washed twice with PBS, and resuspended in the PI/RNase A solution for 30 min at room temperature in the dark [22]. Data acquisition and analysis were performed on a FACScan flow cytometry (Beckman, San Diego, CA, USA), and data from 1×10^4 cells were collected for each data file analysis. Cell cycle analysis was performed with CellQuest and Modfit softwares (Beckman).

3.5 Flow cytometry assay for mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by Rh123, a mitochondrial potential sensor (Molecular Probes, St Louis, MO, USA). After 6, 12, 24, and 48 h of incubation with or without 50 μM of **1** at 37°C, HeLa cells were harvested by trypsinization, washed twice with PBS, and incubated with Rh123 (final concentration 10 mg/l) for 30 min at 37°C and 5% CO_2 in the dark. Data were collected and analyzed using a FACScan (Beckman) equipped with the CELL Quest software.

3.6 Caspase activity assay

Caspase activities were assayed by using Caspase-Glo® Assays, Caspase-3/7 and -9 Assay kit (Promega), according to the manufacturer's protocol. The proluminescent substrate containing the DEVD was cleaved by caspase-3 and -9. After caspase cleavage, a substrate for luciferase was released and this results in the luciferase reaction and the production of a luminescent signal [23]. In brief, 50 μl of a caspase reagent was added to each well of a 96-well plate containing 50 μl of blank, negative control cells, or treated cells (50 μM of **1**) in culture medium. After incubation at 37°C for 1 h, the luminescence of each sample was measured using a plate-reading luminometer (Turner Designs, Sunnyvale, CA, USA).

3.7 Western blotting

The cytochrome *c* leakage from mitochondria to the cytosol was measured by Western blot analysis. HeLa cells were incubated for different times in the presence or absence of 50 μM of **1**, harvested, and washed twice with cold PBS, pH 7.2, followed by centrifugation at 200 *g* for 5 min. The cell pellet was then resuspended in ice-cold cell extraction buffer (20 mM HEPES-KOH, pH 7.5; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 100 μM PMSF; protease inhibitor cocktail) for 30 min on ice. The protein content was determined using a Bio-Rad protein assay reagent with bovine serum albumin as the standard. The extracts were electrophoresed using 15% SDS-polyacrylamide gels along with protein molecular weight standards and transferred to a 0.45 μm PVDF membrane. The membranes were blocked with 5% (w/v) non-fat milk

powder solution and incubated with the monoclonal mouse anti-human cytochrome *c* antibody (IgG) using gentle agitation at 4°C for 12 h and then incubated with alkaline phosphate-conjugated goat anti-mouse antibody (IgG). Detection of the proteins was achieved using a Western blot chemiluminescence reagent kit (Amersham Pharmacia Biotech).

3.8 Determination of intracellular ROS production

Intracellular ROS production was detected by flow cytometry using DCFH-DA [24]. After 2, 4, and 6 h of incubation with or without 50 μM of **1** at 37°C, HeLa cells were harvested by trypsinization, washed twice with PBS, and incubated with 10 μM DCFH-DA in culture medium for 30 min at 37°C. The fluorescence emission from DCF was analyzed via flow cytometry (Beckman) with excitation and emission spectra set at 488 and 525 nm, respectively.

3.9 Statistical analysis

Data obtained represented mean values of at least three different experiments and are expressed as mean ± SD. Statistical analysis was determined by a *t*-test. A p-value < 0.05 was considered statistically significant.

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