AG4, a compound isolated from *Radix Ardisiae Gigantifolii*, induces apoptosis in human nasopharyngeal cancer CNE cells through intrinsic and extrinsic apoptosis pathways

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3\(^\beta\)-O-[(\(\alpha\)-L-pyran rhamnose-\((1\rightarrow3)-(\beta\)-D-xylopyranose-\((1\rightarrow2)-(\beta\)-D-glucopyranose-\((1\rightarrow4)-(\beta\)-D-lucopyranose-\((1\rightarrow2)\)-\(\alpha\)-L-pyran arabinose)-cyclamiretin A (AG4) is a saponin component obtained from the Giantleaf Ardisia rhizome (*Rhizoma Ardisiae Gigantifolii*). The present study aimed to investigate the antitumor potential of AG4 and its possible mechanisms in human nasopharyngeal carcinoma cells (CNE). We exposed tumor cells to AG4 to investigate which cell line was the most sensitive to AG4. Cell viability was assessed using the MTT reduction assay, and the effects of AG4 on apoptosis, reactive oxygen species (ROS) content, mitochondrial membrane potential (MMP), and cell cycle were detected using a flow cytometer; the glutathione, superoxide dismutase and malondialdehyde activities were measured using colorimetric methods. The relative expressions of Bax, Bad, Bcl-2, and Fas mRNA were calculated using the \(^2\Delta\Delta C_t\) comparative method by real-time PCR studies and protein was detected by western blotting. AG4 markedly inhibited the growth of CNE cells by decreasing cell proliferation, inducing apoptosis, and blocking the cell cycle in the S phase. The release of caspase-3, caspase-8, and caspase-9 was stimulated by AG4 in CNE, and the decreased proliferation induced by AG4 was blocked by the inhibitor of pan caspase (Z-VAD-FMK). Moreover, the MMP was decreased in AG4-treated cells, and AG4-induced cell apoptosis was accompanied by a rapid and lasting increase in ROS, which was abolished by N-acetyl-L-cysteine (NAC); glutathione, superoxide dismutase, and malondialdehyde were regulated by AG4. AG4 inhibited Bcl-2 mRNA and protein expression and stimulated Bax, Bad, Bid, Fas mRNA, and protein expression in CNE cultures, suggesting an effect at the transcriptional and protein level. In addition, both the FasL inhibitor (AF-016) and the Bcl-2 family inhibitor (GX15-070) could prevent the cell apoptosis induced by AG4. The findings suggested that AG4-induced apoptosis in CNE cells involved a death receptor pathway and a Bcl-2 family-mediated mitochondrial signaling pathway by decreasing the MMPs in an ROS-dependent manner and regulating genes and proteins relative to apoptosis; also, regulation of cell cycles may also play a role in the antitumor mechanism of AG4. *Anti-Cancer Drugs* 26:331–342 Copyright \(\text{©}\ 2015\) Wolters Kluwer Health, Inc. All rights reserved.

**Keywords:** apoptosis, caspases, CNE cells, *Radix Ardisiae Gigantifolii*, redox system, signal pathway

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**Introduction**

*Rhizoma Ardisiae Gigantifolii* is a traditional herbal remedy that is produced in the eastern and southern provinces and the long-river basin of China, and is used to treat rheumatism, activating blood circulation to dissipate blood stasis and inflammation. 3\(^\beta\)-O-[(\(\alpha\)-L-pyran rhamnose-\((1\rightarrow3)-(\beta\)-D-xylopyranose-\((1\rightarrow2)-(\beta\)-D-glucopyranose-\((1\rightarrow4)-(\beta\)-D-lucopyranose-\((1\rightarrow2)\)-\(\alpha\)-L-pyran arabinose)-cyclamiretin A (AG4) is a saponin component obtained from the Giantleaf Ardisia rhizome (*Ardisia densipodiflora*), with the chemical constitution as in Fig. 1a, and has beneficial effects on pain of rheumatism, wound, blood stasis of postpartum, carbuncle-abscess, and ulcer disorders. Previous reports have shown that AG4 also has anticancer properties. Extensive work over the past decade has shown that AG4 can induce obvious cytotoxicity in many tumor cell lines, such as BGC (human gastric cancer), HeLa (human cervical), HepG2 (human hepatocellular carcinoma), and EJ (human bladder cancer), and the method of isolation was as described previously [1,2].

*Ardisia pusilla* A. (DC) is a whole grass plant of Myrsinaceae, with a detoxifying and anti-inflammatory analgesic effect, and is effective for rheumatism and various inflammatory disorders, and for the treatment of bruise and snake bites. In recent years, Ardipusilloside-I (ADS-I) and-II isolated from *A. pusilla* have been reported. ADS-I is a saponin that has a structure similar to that of AG4; it can inhibit tumor growth in mice transplanted models, promote spleen T lymphocyte proliferation in S180, H22, EAC, and L1210 tumor-bearing mouse,
enhance macrophage phagocytic capability, and improve immune function in tumor-bearing mice. It can significantly improve immune function in CTX suppression mice, and inhibit metastasis of Lewis lung carcinoma and hepatocellular carcinoma in nude mice. On the basis of the antitumor effects of saponin compounds, the aim of the present study was to clarify the related mechanisms of AG4 in human nasopharyngeal carcinoma CNE cells [3–5].

Materials and methods

Chemicals and drugs
AG4 was isolated from *Rhizoma Ardisiae Gigantifoliae*, identified by a combination of spectra methods ( ultraviolet, infrared, mass spectrometry, and nuclear magnetic resonance) and purified by high-performance liquid chromatography (purity >95%) (Fig. 1b). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Hoechst 33258, propidium iodide (PI), fura-2 acetoxymethyl ester (Fura-2AM), rhodamine 123 (Rh123), inhibitors of caspase-3 (Ac-DEVD-CHO), caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-EMK·TFA), and pan caspase inhibitor Z-VAD-FMK were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Dulbecco’s modified Eagle’s medium (DMEM), glucose-free DMEM, and fetal bovine serum were obtained from Gibco (Carlsbad, California, USA). The Annexin V–fluorescein isothiocyanate (FTTC) apoptosis detection kit was obtained from Beckmann Coulter Inc. (Indianapolis, Indiana, USA). Caspase-3, caspase-8, and caspase-9 activity kits were obtained from Promega (Madison, Wisconsin, USA). Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) test kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).
All other chemicals were of analytic grade and commercially available. The antibodies were purchased from (Abcam, California, USA). Obatoclax mesylate (GX15-070) was purchased from Selleck Chemicals LLC (Houston, Texas, USA), and dissolved in DMSO and stored at −80°C, as recommended by the supplier; FasL inhibitor (AF-016) was purchased from Kamiya Biomedical Company (Seattle, Washington, USA).

Cell culture and treatment
The human nasopharyngeal cancer cells CNE were obtained from the Cancer Institute and Hospital of the Chinese Academy of Medical Science and maintained in DMEM supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 30 min), and 100 U/ml of penicillin and 100 U/ml streptomycin, in a 37°C incubator under a humidified, 5% CO₂ atmosphere. Confluent CNE cells were seeded into 96-well plates at a density of 1 x 10⁴ cells/well. After 24 h, the cells were exposed to various concentrations of AG4 and incubated for 24 h. AG4 was dissolved in dimethyl sulfoxide (DMSO) before they were added in cells. The final concentrations of DMSO were always less than 0.01%, which was found to exert no effects on cell viability. The absorbance was read at 570 nm with DMSO as the blank.

Cell viability
After the treatment of various concentrations of AG4 (2.03, 4.06, 8.13 μmol/l) for 24 h, or in the presence of substrate peptides Ac-DEVD-CHO (1 μmol/l), Z-IETD-FMK (20 μmol/l), Z-LEHD-FMK-TFA (20 μmol/l) [6], Z-VAD-FMK (20 μmol/l), or FasL inhibitor AF-016 (50 μg/ml) and Bcl-2 family inhibitor GX15-070 (500 nmol/l) [7,8], cell viability was evaluated using the MTT assay. Briefly, 20 μl of MTT solution (2 mg/ml in PBS) was added to the culture medium at a final concentration of 0.5 mg/ml and incubated at 37°C for 4 h. Then, the supernatants were aspirated carefully, 150 μl of DMSO was added to each well to dissolve the reaction product of MTT, and the OD was measured spectrophotometrically at 570 nm, with DMSO as a blank [9]. Viability was expressed as the percentage of the values in vehicle-treated (basal) cultures, set to 100%. Another method was used to evaluate the cell viability: reversed microscopy. Following the above cell treatment protocol, cells were stained with Hoechst; the morphology of the cells was observed and recorded as a photo under an Olympus microscope (Tokyo, Japan).

Cells apoptotic assay
Cell apoptosis was measured using an Annexin-V–FITC/PI apoptosis detection kit. 5 x 10⁶ cells/well were plated in six-well plates. After treatment with AG4 for 24 h, cells were harvested and washed in PBS, and then centrifuged at 1000g for 5 min. The cell pellet was resuspended in the Annexin-V–FITC/PI labeling solution, mixed gently, and incubated for 15 min at room temperature in the dark. Cells were then analyzed in a Becton Dickinson flow cytometer (Franklin Lakes, New Jersey, USA) equipped with the analysis software, and each sample collected 10,000 cells [10].

Hoechst 33 258 staining was used to further analyze cell apoptosis. Cells were cultured in 12-well culture plates and treated with AG4 for 24 h. Afterwards, the cells were washed with PBS and incubated with 500 μl 4% methanol for 10 min, followed by staining with Hoechst 33 258 at room temperature for 10 min, and then observed with filters for blue fluorescence under fluorescence microscopy.

Measurement of cell cycle
The cell cycle was measured using a PI according to the manufacturer’s instructions. Briefly, cells were plated in 12-well plates at a density of 1 x 10⁶ cells/well. After treatment with AG4 for 24 h, cells were harvested and washed with 500 μl PBS and then fixed in ice-cold 70% ethanol for 12 h at −20°C. Then, the cells were collected and resuspended in 100 μl of RNaseA (1 mg/ml) and stained with 400 μl PI (50 μg/ml) at 4°C for 30 min in the dark. Cells were analyzed by FACS Becton Dickinson flow cytometry immediately.

Intracellular ROS quantification
The level of intracellular reactive oxygen species (ROS) was determined by the change in the fluorescent probe dichlorofluorescein diacetate (DCFH-DA). Briefly, 6 x 10⁴ CNE cells were cultured into a six-well plate and were treated with indicated concentrations of AG4 for 24 h. Cells were trypsinized and washed with PBS, and then incubated with 10 mmol/l DCFH-DA for 30 min at 37°C. Subsequently, cells were washed twice with PBS and analyzed using a flow cytometer. The protective effect of N-acetyl-L-cysteine (NAC) was determined by adding NAC with AG4 to the CNE cells before the ROS measurement.

Measurement of SOD, MDA, and GSH activity
CNE cells were plated in 96-well plates with 7 x 10⁴ cells/ml allowed to attach for 24 h. AG4 was added at different final concentrations; after 48 h of incubation, cells were thawed three times at a temperature of −80°C. The supernatant was collected to measure the intracellular SOD, MDA, and GSH activity according to the instructions provided with the kits using an automatic microplate reader.

Measurement of mitochondrial membrane potential
Diversities of mitochondrial membrane potential (MMP) were assessed using the fluorescent cationic dye Rh123, which accumulates in the mitochondria as a direct function of the membrane potential and is released upon membrane depolarization. The CNE cells (5 x 10⁶ cells/well) were plated in six-well plates; after treatment with
AG4 for 24 h, cells were harvested and washed with PBS, and then incubated with 2 μmol/l Rh123 for 30 min at 37°C. Then, the Rh123 fluorescence intensity was measured by flow cytometry, with the excitation filter wave set at 488 nm and the emission filter wave set at 535 nm. The mean fluorescence intensity in the cells represented the state of depolarization of MMP.

**Measurement of caspase-3, caspase-8, and caspase-9 activities**

Caspase-3, caspase-8, and caspase-9 activities were measured using the Caspase Activity Kit according to the manufacturer’s instructions. We exposed CNE cells to various concentrations of AG4 (2.03, 4.06, 8.13 μmol/l) for 0, 6, 12, and 24 h, respectively. Briefly, cells were lysed, and then the supernatant was mixed with buffer containing the substrate peptides for caspase attached to p-nitroanilide. The release of p-nitroanilide was qualified by determining the absorbance using an ELISA reader (Bio-Tek, Shoreline, Washington, USA) at 405 nm. The caspase activities were expressed as percentages compared with the controls.

**Reverse transcriptase-polymerase chain reaction**

Total RNA was extracted from cultured cells with Trizol (Gibco BRL) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of purified RNA with random primer using the First Strand Synthesis Kit (ReverTra Ace-a-, Toyobo Co., Japan). Human β-actin and Bax, Bad, Bid, Bcl-2, and Fas primer pairs used were synthesized by Biomed (Beijing, China) and described as follows: β-actin: forward 5'-GGACATCCGGCAAGACCTGTA-3', reverse 5'-ACATCTGCTGGAAGGTGACA-3'; Bcl-2: forward 5'-CTCTGTGGATGACTGAGTACC-3', reverse 5'-GAGACAGCCAGGAGGAAATCGAC-3'; Bad: forward 5'-CCAGGATGAGTACACCCCTTCGCTGA-3'; Bax: forward 5'-CAGCGAAGACCTGCTGACG-3'; Bf: forward 5'-CACGACTTGGAGGTCAACAGC-3', reverse 5'-TCGTCGTCTGGATGTACAG-3'. The PCR reaction was performed using the SYBR Green real-time PCR Master Mix (Toyobo Co.) to detect the abundance of PCR products among samples. The cycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 62°C for 20 min, and 72°C for 20 s. β-Actin was chosen as an internal control to normalize the gene expressions of mRNA were determined using the 2^(-ΔΔCt) comparative method. All quantities were expressed as n-fold relative to the calibrator (control value, which was defined as a value of ‘1.0’).

**Western blot analysis**

For western blot analysis, total protein was extracted first, cells were washed twice with cold PBS, and lysed in the radioimmunoprecipitation assay buffer (20 mmol/l Tris-HCl, pH 7.5, 150 mmol/l sodium chloride, 1% Triton, 1 mmol/l EDTA, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l sodium orthovanadate, 1 μg/ml leupeptin, 1 mmol/l PMSF) for 20 min on the ice. After centrifugation at 12 000g for 10 min, protein was then quantified using a bicinchoninic acid Protein Assay Kit (Bioworld Technology, St Louis, Missouri, USA). Membranes were blocked 2 h in TBST [10 mmol/l Tris base (pH 8.0), 150 mmol/l NaCl, and 0.1% Tween-20] containing 5% (w/v) BSA (Sigma-Aldrich, St Louis, Missouri, USA) at room temperature, and then probed with each primary antibody at 4°C overnight. After three washes with TBST, the membranes were incubated with the appropriate horseradish peroxidase-labeled secondary antibody for 2 h at room temperature. For quantitative analysis of the immunoblot bands, the densities of the bands were measured by scanning densitometry (Bio-Rad, Hercules, California, USA). All the results of western blot include three independent experiments, and each independent experiment has three replicates.

**Statistical analysis**

All the data obtained in the experiments were represented as mean ± SD. Statistical analysis was carried out using the software package SPSS, v. 10.0 (IBM Corp., Armonk, New York, USA). Differences were determined using one-way analysis of variance. P values less than 0.05 and 0.01 were considered statistically significant.

**Results**

**Effect of AG4 on cell proliferation in tumor cell lines**

We determined the antitumor effect of AG4 on a variety of cancer cell lines using the MTT assay to investigate which cancer cell line was the most sensitive to AG4 under the same condition. The cell lines Bel-7402 (human liver cancer), A549 (human lung cancer), HCT-8 and HT-29 (human colon carcinoma), BGC, LS180 (human colorectal), HeLa, HepG2, EJ, SYSY (human neuroblastoma), CNE (human nasopharyngeal carcinoma), and MCF-7 (human breast cancer) were included. The results showed that the CNE cell line was the most sensitive cell line to the antitumor effect of AG4, the IC50 (50% inhibition concentration) of AG4 was only 3.8 μmol/l, and AG4 inhibited the proliferation of CNE cells in a time-dependent manner (Fig. 2).

**Effect of AG4 on cell apoptosis and cell cycle**

The effects of AG4 could also be confirmed by morphological observation. Distinct chromatin condensation and nuclear fragmentation were identified in the AG4 treatment groups, whereas the nuclei of the control cells stained a weak homogeneous blue. There was a
significant injury in CNE cells after treatment with AG4, including the disappearance of cellular processes and falling to pieces; cell nuclei were stained as high light blue by Hoechst 33 258 (Fig. 3a). The Annexin-V–FITC/PI staining assay was used to evaluate the apoptosis in CNE cells. As shown in Fig. 3b, control cells without the treatment with AG4 showed uniformly dispersed chromatin and an intact cell membrane. In the cells treated with AG4 (4.06 and 8.13 μmol/l), the percentage of apoptotic cells was increased \( (P < 0.01) \). The nuclear staining assay by PI was used to evaluate the effect of AG4 on the cell cycle in CNE cells. As shown in Fig. 3c, 4.06 and 8.13 μmol/l AG4 could decrease the G1-phase cells and increase the percentage of S phase cells from 20 to 34 and 68%, respectively.

**ROS are responsible for AG4-induced cell apoptosis in CNE cells**

To investigate whether AG4 could increase ROS levels, we examined the intracellular ROS production using a fluorescent probe, DCFH-DA. As shown in Fig. 4a, after AG4 treatment, the fluorescence intensity increased markedly compared with the control group \( (P < 0.01) \). In addition, the antioxidants, 5, 10, and 20 mmol/l of NAC, were added together with AG4, respectively; the fluorescence intensity reduced markedly compared with groups treated with AG4 alone. NAC (20 mmol/l) induced total blockage, which showed that the NAC can block the effect of AG4 in enhancing intracellular ROS levels. Also, we found that AG4 increased the ROS content in a time-dependent manner within 12 h. The SOD and GSH activities were suppressed by AG4, and the MDA content was increased after AG4 treatment in a dose-dependent manner (Fig. 4b). The level of MMP was determined using a flow cytometer after staining with Rh123. As shown in Fig. 4c, the fluorescence intensity of the cells treated with AG4 was significantly decreased compared with the control group and reversed by 10 mmol/l NAC.

To further verify whether AG4-induced apoptosis was correlated with ROS, we used NAC, the inhibitors of ROS. As shown in Fig. 4d, AG4, either alone or in combination with NAC, exerted quite different effects in inducing cell apoptosis in CNE cells. The cell viability rate decreased significantly when the CNE cells were treated with 4.06 and 8.13 μmol/l AG4, and blocked by 10 mmol/l NAC.

**AG4 increased the activities of caspase-3, caspase-8, and caspase-9**

The process of apoptosis involves a cascade of proteolytic activity, much of it carried out by caspases; thus, we investigated whether AG4 can activate the caspases. As shown in Fig. 5a and b, after treatment with AG4 for 24 h, the activities of caspase-3, caspase-8, and caspase-9 all obviously increased in a dose-dependent manner (2.03–8.13 μmol/l) and time-dependent manner (within 12 h) compared with the control group. Activation of caspase-8, caspase-9, and the subsequent activation of caspase-3 indicated that AG4 may be inducing intrinsic and extrinsic apoptosis pathways.

To further verify whether the effect of apoptosis was correlated with caspases, we used the inhibitors of caspase-3 (Ac-DEVD-CHO), caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-EMK·TFA), and pan caspase inhibitor (Z-VAD-FMK). As shown in Fig. 5c, the cell viability rate decreased significantly when the CNE cells were treated with AG4 and reversed by Z-VAD-FMK. However, Ac-DEVD-CHO, Z-IETD-FMK, and Z-LEHD-EMK·TFA did not exert effects on cell viability.

**AG4 regulated the mRNA and protein levels related to apoptosis**

CNE cell cultures were exposed to AG4 for 24 h. Total RNAs were extracted and the relative mRNA amounts
were measured by real-time PCR and normalized to β-actin mRNA. The effects of AG4 on Bax, Bad, Bid, Bel-2, and Fas mRNA are shown in Fig. 6a, Bax, Bad, Bid, and Fas mRNA were increased after AG4 treatment in a dose-dependent manner (2.03–8.13 μmol/l), and Bel-2 mRNA was suppressed by AG4. The same effects were found for protein levels (Fig. 6b). To determine whether the Fas or the Bel-2 family was functional after AG4 treatment and whether the intrinsic and extrinsic pathway was required for AG4-induced apoptosis, the FasL inhibitor and the Bel-2 family inhibitor were used. Figure 6c shows that the FasL inhibitor (AF-016) and the Bel-2 family inhibitor (GX15-070) protected CNE cells from apoptosis induced by AG4 under the conditions of the experiments.

Discussion
Human nasopharyngeal carcinoma cell CNE is a classical cell line obtained from nasopharyngeal carcinoma patients, and it is used in various kinds of studies of nasopharyngeal carcinoma [11]. However, the CNE cell line was more sensitive than other tumor cell lines to the antitumor effect of AG4 in this experiment; thus, we selected the CNE cells and expected that AG4 may be effective for the treatment of nasopharyngeal carcinoma someday clinically.

Apoptosis (or programmed cell death) is a physiological mechanism that is crucial for the normal development of organisms during embryogenesis, maintenance of tissue homeostasis in adults, and elimination of diseased or otherwise harmful cells during pathogenesis [12]. Dysregulated apoptosis has been implicated in many human diseases, including neurodegenerative diseases such as Alzheimer’s disease and Huntington disease, ischemic damage, autoimmune disorders, and several forms of cancer [13]. In this study, AG4 was shown to enhance apoptosis, cell-cycle arrest, decrease MMPs, and...
Fig. 4

(a) Effect of AG4 on levels of ROS, SOD, GSH, and MDA content on CNE cells. (b) Effect of AG4 on levels of ROS, SOD, GSH, and MDA content on CNE cells. (c) Effect of different concentrations of AG4 on MMP of CNE cells for 24 h. (d) Proliferation of CNE cells treated with NAC for 24 h. **P < 0.01 versus control, compared with AG4 treatment-alone group, #P < 0.05, ##P < 0.01. GSH, glutathione; MDA, malondialdehyde; MMP, mitochondrial membrane potential; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; SOD, superoxide dismutase.

ROS are responsible for AG4-induced cell death in CNE cells. (a, b) Effect of AG4 on levels of ROS, SOD, GSH, and MDA content on CNE cells. (c) Effect of different concentrations of AG4 on MMP of CNE cells for 24 h. (d) Proliferation of CNE cells treated with NAC for 24 h. **P < 0.01 versus control, compared with AG4 treatment-alone group, #P < 0.05, ##P < 0.01. GSH, glutathione; MDA, malondialdehyde; MMP, mitochondrial membrane potential; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; SOD, superoxide dismutase.
Fig. 4 (continued)

(c) NAC (0 mmol/l) NAC (10 mmol/l)

AG4 0 2.03 4.06 8.13 0 2.03 4.06 8.13

Concentration (μmol/l)

(d) Cell viability (% of control)

AG4 (μmol/l) 0 2.03 4.06 8.13 0 2.03 4.06 8.13

NAC (10 mmol/l) - - - - + + + +
Influence of AG4 to the enzyme activities of caspase-3, caspase-9, and caspase-8 in CNE cells. (a) Effect of AG4 on levels of caspase-3, caspase-9, and caspase-8 for 24 h in CNE cells. (b) Effect of AG4 on caspase-3, caspase-9, and caspase-8 activities at different time points. (c) Proliferation of CNE cells treated with AG4 and caspase inhibitors for 24 h. **P<0.01 versus control, compared with the AG4 treatment-alone group, #P<0.05.
AG4 regulated oncogene and anti-oncogene mRNA and protein expression. (a) Effect of AG4 on mRNA expression; the mRNA levels are normalized to β-actin. (b) Effect of AG4 on protein expression. (c) FasL inhibitor (AF-016) and Bcl-2 family inhibitor (GX15-070) protected CNE cells from apoptosis induced by AG4. Each bar is the mean ± SD of three independent experiments, statistical difference compared with the normal group (vehicle-treated) was calculated using Dunnett’s test after analysis of variance, **P < 0.01.
increase ROS content and caspase activities in CNE cells; the inhibitor of ROS (NAC), the FasL inhibitor (AF-016), and the Bcl-2 family inhibitor (GX15-070) could protect CNE cells from apoptosis.

ROS have recently been proposed to be involved in tumor development and metastasis, which is a complicated processes including epithelial–mesenchymal transition, migration, invasion of the tumor cells, and angiogenesis around the tumor lesion [14]. Our results suggested that the activity of ROS was increased significantly in CNE cells after the treatment of AG4, and showed that AG4 could mediate ROS production. The apoptosis rate was increased following an increase in the content of ROS and could be protected by NAC. We found that the SOD and GSH activities were inhibited and the MDA content was increased, which might be involved in the ROS production [15].

Moreover, our results showed that AG4 could activate the activities of caspase-3, caspase-8, and caspase-9 significantly in a dose-dependent and time-dependent manner. Caspases, a family of cysteine–aspartic proteases, are key regulators of apoptosis; caspase-3 is a key effecter molecule in the caspase-dependent cell apoptosis pathway that cleaves a number of cellular proteins, leading to apoptotic changes. Cleavage of caspase-3, caspase-8, and caspase-9 in this study suggested that the extrinsic and intrinsic apoptotic pathways were activated. The pathways were also found to crosstalk through the truncation of members of the Bcl-2 protein family, which transduced apoptotic signals from the cell surface to the mitochondria [16–21]. Although caspases were activated in CNE cells exposed to AG4, the addition of the caspase inhibitors, Ac-DEVD-DHO, Z-IETD-FMK, and Z-LEHD-EMK·TFA, failed to attenuate the apoptosis induction effect of AG4; only Z-VAD-FMK could reverse the decreased cell viability rate caused by treatment with AG4. These results suggest that another caspase-independent apoptotic pathway was also activated.

The mitochondria serve a critical function at the core of the apoptotic pathways, and mitochondrial depolarization is a major event in mitochondrial dysfunction; the loss of MMPs is crucial for the balance between the caspase-dependent and caspase-independent apoptotic pathways [22–25]. In the present study, we found that treatment with AG4 resulted in a decrease in MMPs and NAC.
could reverse this decrease, suggesting that ROS generation was crucial for the decrease in MMPs induced by AG4. Members of the Bcl-2 protein family, which are categorized as prosurvival (antiapoptotic) and proapoptotic (Fig. 7), act as gatekeepers of the mitochondria by controlling the permeability of the mitochondrial outer membrane [26–31]. In this study, the mRNA level of Bcl-2 was downregulated in the presence of AG4; moreover, AG4 could increase the relative expression of Bad, Bid, and Bax mRNA, the proapoptotic genes. Two distinct pathways of apoptosis have been identified as mitochondria-initiated apoptosis occurs through caspase-9 and the death receptor-mediated pathway requires caspase-8 [32]. Therefore, the Fas mRNA level was detected as a proapoptotic gene in the death receptor-mediated pathway; we found that AG4 increased the relative expression of Fas mRNA. The same effects were found in protein level detection. The FasL inhibitor and the Bcl-2 family inhibitor could protect CNE cells from apoptosis induced by AG4. Cell-cycle analysis showed that AG4 could arrest cell cycle in the S phase, and this might be attributed to the inhibition of cell growth and induction of cell apoptosis.

Conclusion
AG4-induced apoptosis in CNE cells occurs by the decrease in MMPs in a ROS-dependent manner and regulation of genes and protein relative to apoptosis. AG4-induced apoptosis in CNE cells was linked to a death receptor pathway and Bcl-2 family-mediated mitochondrial signaling pathway. Accordingly, the results of this study suggest that AG4 may be a potential candidate for nasopharyngeal carcinoma treatment.

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Conflicts of interest
There are no conflicts of interest.

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