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Synthesis and evaluation of the anticancer activity of albiziabioside A and its analogues as apoptosis inducers against human melanoma cells†

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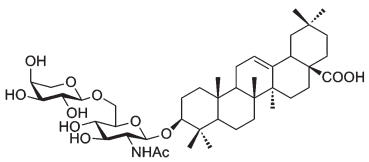
We have efficiently synthesized albiziabioside A (**1**) together with its six disaccharide analogues through a linear synthesis, and evaluated their cytotoxicity against six different skin cancer cells. All of the analogues showed weak cytotoxicity, with the exception of compound **1**, which exhibited strong cytotoxicity against A375 cells. Albiziabioside A can induce cell cycle arrest in both the S and G_2/M phases. Moreover, albiziabioside A can induce A375 cell apoptosis *via* mitochondrial pathways involving a caspase cascade. These results provide for the first time a basic mechanism for the anticancer activity of **1**.

Introduction

Skin cancer is one of the most extensively diagnosed types of cancer in Caucasians and in those living in equatorial latitudes, and currently accounts for approximately 40% of all diagnosed cancers.[1,2] Common skin cancers are classified as either melanoma or non-melanoma, and malignant melanoma is the predominant type resulting in death. Multiple reports have demonstrated that chronic exposure to ultraviolet radiation is one of the main causes of skin cancer. In addition, much effort has been focused on developing new approaches to treat skin cancer.[3,4] However, the successful treatment of skin cancer remains very challenging because of the unpleasant side effects of the therapies and drug resistance. Therefore, continuing the search for and development of alternative drugs are clinically necessary.

Oleanane triterpenoids, which are synthesized by squalene cyclization in many terrestrial plants and in low marine organisms,[5] represent an important family of natural products widely used in Asian medicine.[6] Studies have shown that oleanane triterpenoids can be used to treat most cancers through the regulation of a wide range of dysfunctional pathways, including apoptosis, the cell cycle, growth factor signaling, inflammation, drug resistance, metastasis, and angiogenesis.[7–11] Recent studies have shown that natural saponins chaining glucosamine are presenting strong *in vitro* cytotoxicity towards different tumor cell lines.[12–16] We were recently attracted to a natural oleanane triterpenoid saponin (which we have named albiziabioside A) that was originally isolated from the aerial parts of *Albizia inundata*.[17] Albiziabioside A (**1**) exhibits potent cytotoxic activity against various skin cancer cell lines, including the murine melanoma cell line B16F10, the human melanoma cell line SKMEL28, and the head and neck squamous carcinoma cell line MDA1986 and the JMAR cell line.[17]

Despite these reports, there has been no previous study on the mechanism of the cytotoxicity of **1**. Decreased rates of apoptosis in skin cancer cells are closely associated with a high risk of tumorigenesis.[18–20] Thus, induction of apoptosis in skin cancer cells is considered highly useful in therapy and for the prevention of cancer. In the present study, we report the synthesis and biological activity of **1** and its analogues as apoptosis inducers against A375 human melanoma cells.



Albiziabioside A (**1**)

Results and discussion

Synthesis of compound 1 and its analogues 12a–12f

The sugar donors used for glycosylation in this study (Fig. 1) were perbenzoylated sugar trichloroacetimidate derivatives except for **13**,[14] which are stable and easily monitored by absorption at 254 nm than those acetylated trichloroacetimidate derivatives.

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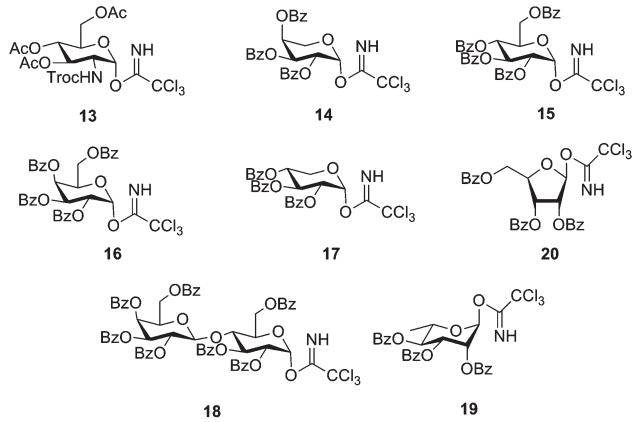
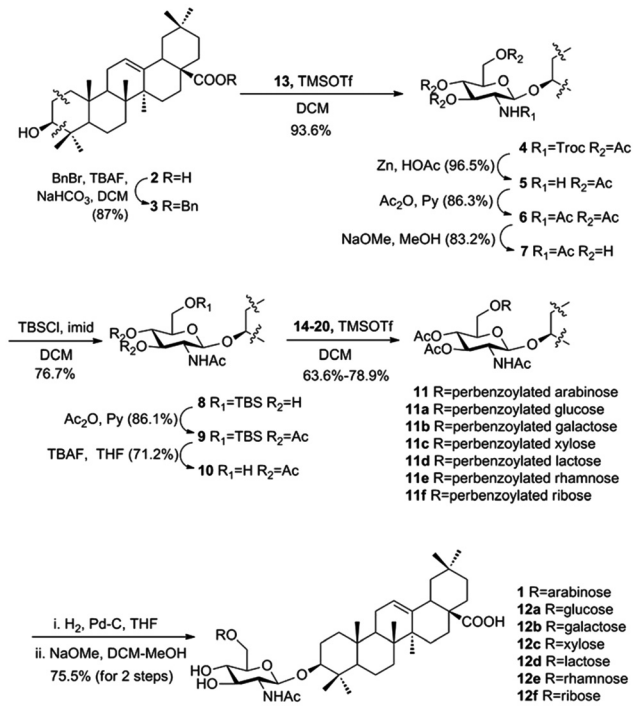


Fig. 1 Chemical structures of glycosyl trichloroacetimidates **13–20**.



Scheme 1 Synthesis of compound **1** and its analogues **12a–12f**.

As shown in Scheme 1, the naturally abundant oleanolic acid was used as the starting material to afford **3** in good yield after treatment with BnBr and TBAF in dry CH_2Cl_2. Glycosylation of **3** using the trichloroacetimidate **13** in dry CH_2Cl_2 in the presence of TMSOTf at 0 °C gave **4** in excellent yield. The addition of zinc to a solution of **4** in acetic acid resulted in the selective removal of the Troc group.[21] This was followed by acetylation with acetic anhydride in pyridine to afford **6**. Deacetylation of **6** with MeONa in CH_2Cl_2–MeOH furnished **7**. Then, the primary 6-OH group on **7** was selectively protected with a TBS group using TBSCl and imidazole,[22] and acetyl protection of the 3,4-OH groups was performed to provide **9**. A catalytic amount of TBAF in acetic acid was slowly added to **9** in dry THF at 0 °C, and the key intermediate **10** was obtained after stirring.[23] Condensation of **10** with various sugar donors (**14–20**), as described for the preparation of **3**, generated **11** and **11a–11f**, which were subjected to hydrogenation with H_2 over Pd/C. Finally debenzoylation and deacetylation with a catalytic amount of NaOMe in DCM–MeOH furnished the target compound albiziabioside A (**1**) and its analogues **12a–12f**.

Table 1 Effects of **1** and **12a–12f** on the growth of six skin cancer cell lines *in vitro*

	IC_{50} (μM)					
	A431	B16BL6	OCM1	SKMEL24	HT144	A375
1	12.19	7.34	9.64	8.02	8.27	5.47
12a	19.84	19.16	18.1	19.74	24.1	14.69
12b	16.84	16.98	19.76	18.1	27.42	15.91
12c	16.04	18.12	16.21	18.72	25.89	18.64
12d	22.01	24.42	27.21	15.61	26.37	20.82
12e	>50	>50	40.93	44.31	>50	21.53
12f	8.45	8.28	6.27	9.48	26.05	26.09
5-FU	26.18	28.14	49.02	38.30	>50	35.28

Cytotoxic effects in six skin cancer lines

To evaluate the effects of **1** and **12a–12f** on the growth of skin cancer cells, the growth inhibitory potential was evaluated using an MTT assay. Compared with derivatives **12a–12f**, **1** exhibited the strongest growth inhibition in each cell line, suggesting that the 6-OH arabinose of acetylglucosamine is more effective than other monosaccharides, as shown in Table 1. In addition, albiziabioside A exhibited outstanding growth inhibition of all tested skin cancer cell lines with IC_{50} values below 15 μM. The lowest IC_{50} values for **1**, 5.47 μM, was obtained in A375 cells compared to other cell lines. Concentrations of **1** up to 10 μM primarily resulted in cell-cycle arrest; concentrations exceeding 20 μM of **1** showed cytotoxicity, and floating dead cells were observed (Fig. 2A). As shown in Fig. 2B, cells treated with **1** exhibited morphological changes and showed distinctly rounded shapes and detachment in a dose-dependent manner compared to the vehicle-treated control cells. This result inspired us to investigate the mechanism of action of **1** against A375.

Albiziabioside A (1) induces S-phase and G_2/M-phase cell cycle arrest

Cell proliferation and growth generally occur as a progression through three phases (G_0/G_1, S, and G_2/M) of the cell cycle, which is an important chemotherapeutic target. To investigate whether growth inhibition induced by **1** was associated with regulation of the cell cycle, the cell cycle distribution in the presence of **1** was analyzed by flow cytometry. As shown in Fig. 3, following treatment with **1** at 0.31, 1.25 and 5.00 μM for 72 h, there was an increase in the S fraction of cells (37.87%, 42.42% and 43.32%, compared to 37.28% in untreated cells) and in G_2/M (9.62%, 10.42% and 16.10%, compared to 4.59% in untreated cells), and a decrease in the G_0/G_1 phases (52.52%, 47.16% and 41.58%, compared to 58.12% in

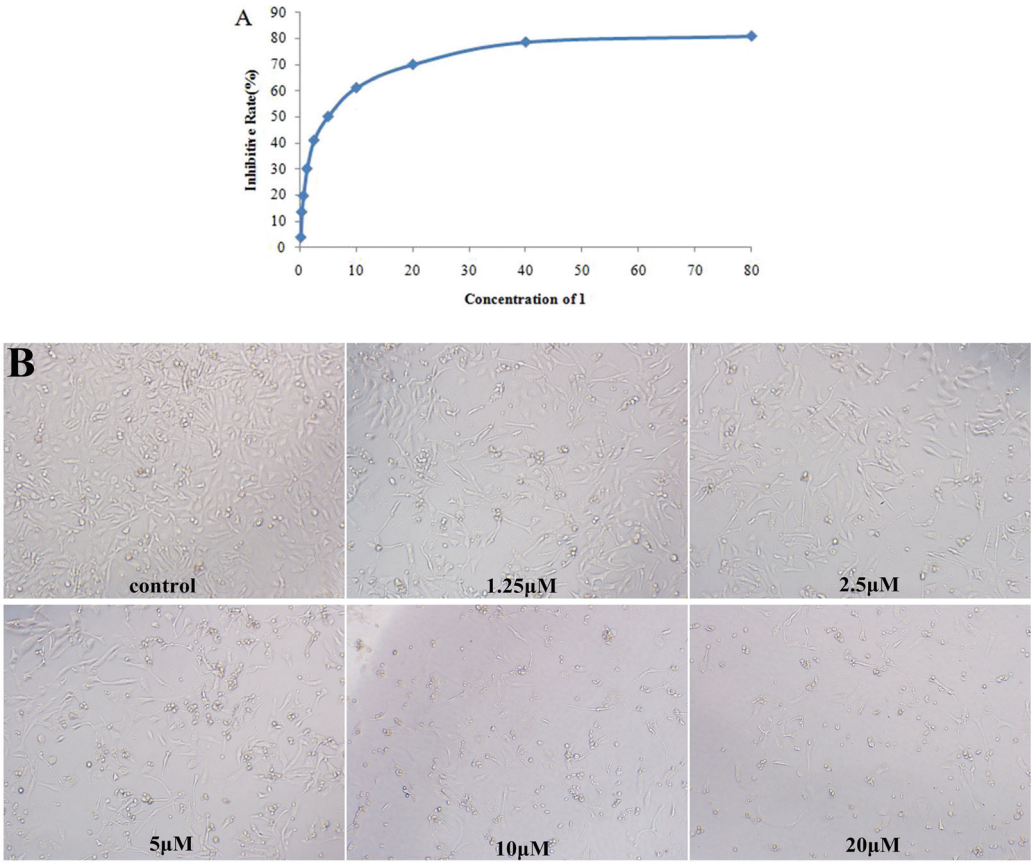


Fig. 2 Inhibitory effects of **1** on A375 cell growth. (A) A375 cells were treated with **1** (80, 40, 20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0.16 μM) for 72 h. (B) Morphological changes by **1** with **1** (20, 10, 5, 2.5 and 1.25 μM) in A375 cells.

untreated cells). These data show that **1** can induce blockade of the cell cycle at both the S and G_2/M phases.

Albiziabioside A (1) causes A375 cell death by apoptosis

Apoptosis is programmed cell death and involves a highly organized physiological mechanism to destroy injured or abnormal cells in multicellular organisms. To investigate whether A375 cell death induced by **1** is mediated by apoptosis, flow cytometric analysis after Annexin V-FITC antibody binding and propidium iodide staining of A375 cells was performed after treatment with **1** at various concentrations. Following treatments with vehicle or **1** (0.31, 1.25 or 5 μM) for 72 h, the proportions of cells displaying early stage (A^+P^-)/late stage (A^+P^+) apoptosis were 3.18%/0.10%, 9.55%/2.83%, 24.58%/10.31% and 31.2/22.90%, respectively (Fig. 4). These results show that **1** can induce A375 cell apoptosis, especially early apoptosis, efficiently and in a dose-dependent manner.

Albiziabioside A (1) induces A375 cell apoptosis *via* mitochondrial pathways involving a caspase cascade

In general, apoptosis occurs *via* either an intrinsic or an extrinsic pathway, which involves the induction of pro-apoptotic proteins or the suppression of anti-apoptotic proteins, respectively.

Mitochondria are necessary mediators that play a vital role in the execution of both intrinsic and extrinsic apoptotic pathways. To estimate the role of mitochondria in **1**-induced A375 cell apoptosis, we explored mitochondrial membrane potential (MMP) changes by flow cytometric analysis after JC-1 staining experiments. After treatment with 0 to 5.0 μM of compound **1**, MMP marked losing was observed relative to controls (Fig. 5). So we can conclude that **1** induced the membrane permeability of mitochondria to increase.

Subsequent experiments were undertaken to analyze changes in the expression of apoptosis-related proteins that result from **1**; these effects were assayed using Western blotting. The release of mitochondrial cytochrome c into the cytoplasm is a crucial event preceding the activation of a caspase cascade involving caspase-9, -3, and -8.[24] As shown in Fig. 6, the levels of cytochrome c released from mitochondria increased from 0.11 to 0.83 in a dose-dependent manner in the presence of **1**. This increase was correlated with an increase in the levels of cytochrome c. Furthermore caspase-3, caspase-9 and caspase-8 activation was also detected, and the levels increased from 0.11 to 0.6, 0.13 to 0.53 and 0.34 to 0.74, respectively. Compound **1** also significantly down-regulates the expression of Bcl-2, which prevents mitochondrial protein release.[25] In contrast, the level of the Bax anti-apoptotic

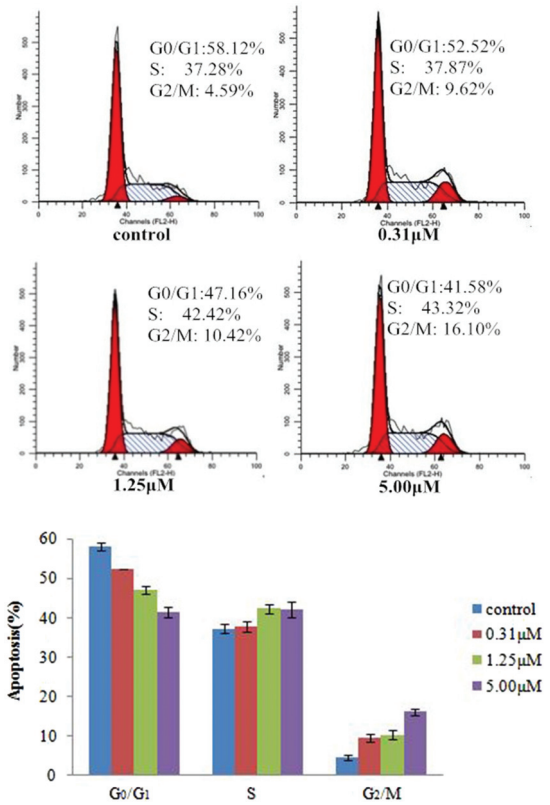


Fig. 3 Effects of **1** on A375 cell cycle progression. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.00 μM) for 72 h, then harvested, stained with PI, and analyzed by flow cytometry.

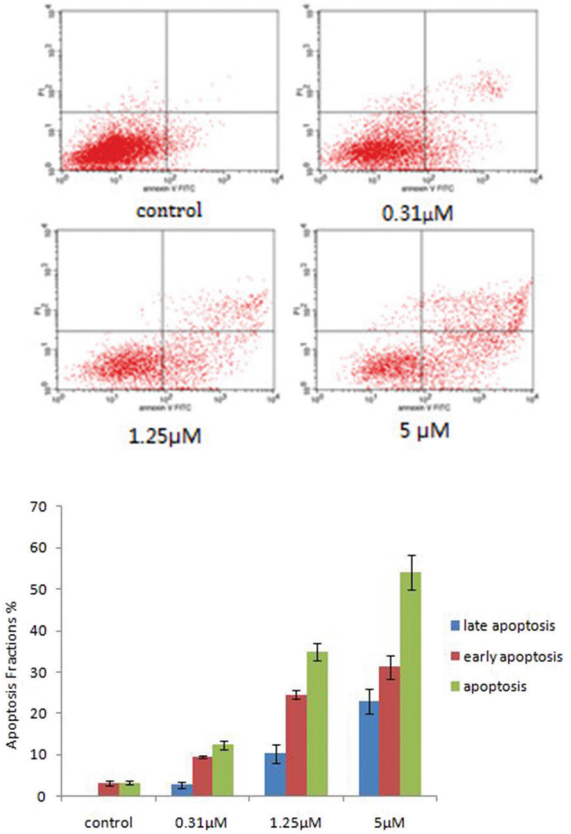


Fig. 4 Apoptosis in A375 cells induced by **1**. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.0 μM) for 72 h and then harvested, stained with Annexin V/PI, and analyzed by flow cytometry.

protein increased from 0.36 to 0.93 in a dose-dependent manner.

To evaluate whether **1**-mediated apoptosis is caspase-dependent, A375 cells were pretreated with Z-VAD-fmk (50 μM), a potent caspase inhibitor, and then treated with **1** (0.31, 1.25, and 5.00 μM). As shown in Fig. 7, Z-VAD-fmk effectively and significantly inhibited **1**-mediated apoptosis.

To investigate the expression levels of apoptosis-related genes (Bcl-2, Bax and caspase-3) in the mitochondrial pathways, we used real-time PCR experiments. As shown in Fig. 8, we demonstrated that the Bcl-2 gene level was down-regulated, and this finding was in concordance with the decrease in the Bcl-2 protein levels from a previous experiment. Similarly, Bax and caspase-3 gene levels were up-regulated.

All of the results obtained (MMP, apoptosis-related proteins, Z-VAD-fmk on **1**-induced apoptosis, and apoptosis-related genes) suggested that **1** induces apoptosis *via* a mitochondrial pathway involving a caspase cascade.

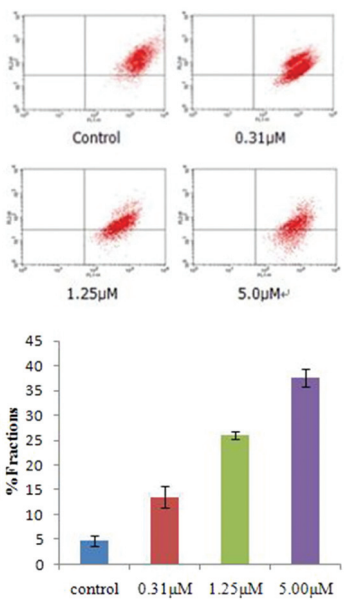


Fig. 5 Effect of **1** on the A375 cells mitochondrial membrane potential. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.0 μM) for 72 h and then harvested, stained with JC-1, and analyzed by flow cytometry.

Conclusion

We have efficiently synthesized albiziabioside A (**1**) through a linear synthesis and also obtained 6 new disaccharide ana-

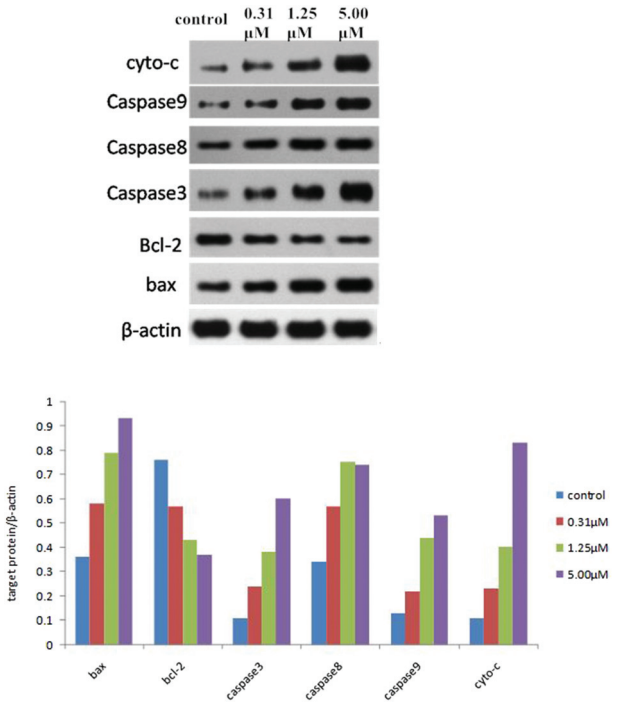


Fig. 6 Effect of **1** on expression of apoptosis-related proteins. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.00 μM) for 72 h. Then cyto-c, Bcl-2, Bax, caspase-9, caspase-8, caspase-3 and β-actin were analyzed using Western blotting. β-Actin was used as an internal control.

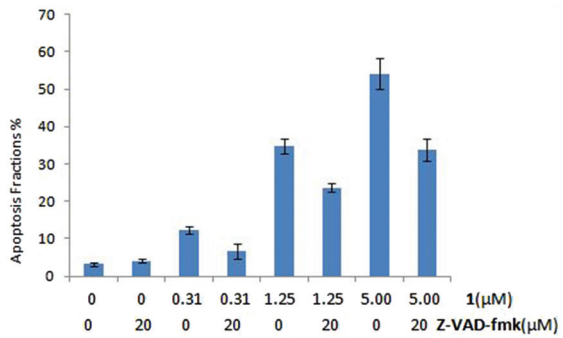


Fig. 7 Effect of Z-VAD-fmk on **1**-induced apoptosis. A375 cells were preincubated with or without 20 μM Z-VAD-fmk for 3 h, then were treated with indicated concentrations of **1**, and stained with Annexin V/PI. Cells were analyzed by flow cytometry to evaluate apoptosis.

logues. *In vitro* biological evaluation demonstrated the strong cytotoxicity of **1** in skin cancer cells. **1** induces cell cycle arrest at both the S and G_2/M phases. Moreover, **1** induces A375 cell apoptosis *via* mitochondrial pathways involving a caspase cascade. These findings support the potential of **1** as a promising natural product for the treatment of skin cancer, although additional studies are necessary to further study this compound.

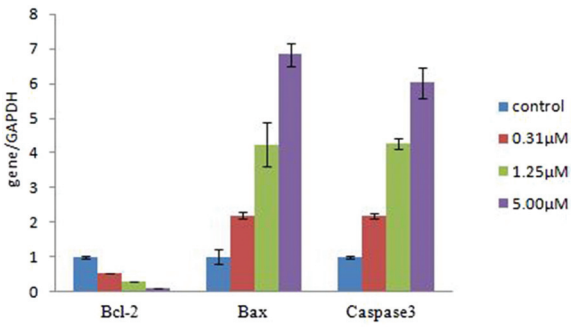


Fig. 8 Effect of **1** on expression of apoptosis-related genes. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.00 μM) for 72 h. Then Bcl-2, Bax, and caspase-3 were analyzed using real-time PCR experiments. GAPDH was used as an internal control.

Experimental

Synthesis

Materials and equipment. Reagents were used without further purification unless otherwise specified. Solvents were dried and redistilled prior to use in the usual way. Analytical TLC was performed using silica gel HF254. Preparative column chromatography was performed with silica gel H. Melting points were obtained on a Büchi melting point B-540 apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 600 MHz spectrometer. HR-MS were obtained on a Bruker micrOTOF_Q spectrometer.

28-*O*-Benzyl-3-*O*-[2-(2,2,2-trichloroethoxylcarbonylamino)-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl] oleanolic ester (4). A mixture of **3** (2.00 g, 3.66 mmol) and trichloroacetimidate **13** (2.74 g, 4.39 mmol) in dry CH_2Cl_2 (50 mL) was stirred at rt for 30 min. Then the mixture was cooled to 0 °C and TMSOTf (0.07 mL, 0.36 mmol) was added. After stirring at this temperature for 3 h, Et_3N was added. The resulting mixture was filtered. The filtrates were concentrated to give a residue, which was purified by a silica gel column chromatography (6 : 1 petroleum ether–EtOAc) to afford **4** (3.45 g, 93.6%) as a white foam. Mp 105.3–106.9 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38–7.27 (m, 5H), 5.31–5.23 (m, 2H), 5.18–4.99 (m, 1H), 4.72 (d, J = 12.0 Hz, 1H), 4.64 (d, J = 10.2 Hz, 2H), 4.26 (dd, J = 12.1, 5.5 Hz, 1H), 4.10 (dd, J = 12.1, 2.5 Hz, 1H), 3.71–3.63 (m, 2H), 3.10 (dd, J = 11.8, 4.5 Hz, 1H), 2.90 (dd, J = 13.9, 4.5 Hz, 1H), 2.07 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.11 (s, 3H), 0.91 (s, 3H), 0.91 (s, 3H), 0.89 (s, 3H), 0.87 (s, 3H), 0.76 (s, 3H), 0.59 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.6, 170.9, 170.8, 169.6, 154.0, 143.9, 136.6, 128.6, 128.1, 128.1, 122.6, 103.2, 95.3, 91.0, 74.8, 72.1, 71.7, 69.1, 66.1, 62.5, 57.0, 55.6, 47.8, 46.9, 46.1, 41.8, 41.5, 39.5, 39.1, 38.6, 36.9, 34.0, 33.3, 32.8, 32.5, 30.9, 28.2, 27.8, 26.0, 25.9, 23.8, 23.6, 23.2, 22.8, 20.9, 20.8, 18.3, 17.0, 16.6, 15.4; HRMS(ESI): calcd for $[M + Na]^+$ $C_{52}H_{72}Cl_3NNaO_{12}$: 1030.4018, found 1030.4012.

28-*O*-Benzyl-3-*O*-[2-amino-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl] oleanolic ester (5). To a mixture of **4** (3.00 g, 2.98 mmol) in acetic acid (50 mL), freshly activated zinc powder (8.76 g, 13.40 mmol) was added. After it was stirred for

4 h, the reaction mixture was filtered and diluted with CH_2Cl_2. Then the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to afford **5** (2.39 g, 96.5%) as a white foam. Mp 75.6–87.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.40 −7.28 (m, 5H), 5.29 (dd, J = 6.8, 2.0 Hz, 1H), 5.02 (m, 1H), 4.30 (d, J = 7.9 Hz, 1H), 4.26 (dd, J = 12.0, 5.6 Hz, 1H), 4.08 (dd, J = 12.0, 2.1 Hz, 1H), 3.70–3.62 (m, 1H), 3.16 (dd, J = 11.8, 4.4 Hz, 1H), 2.98 (dd, J = 9.7, 8.1 Hz, 1H), 2.90 (dd, J = 13.7, 4.0 Hz, 1H), 2.06 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.11 (s, 3H), 1.00 (s, 3H), 0.91 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H), 0.82 (s, 3H), 0.60 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.3, 170.6, 169.7, 143.7, 136.3, 128.3, 127.9, 127.8, 122.4, 106.0, 89.9, 75.3, 71.5, 69.1, 65.9, 62.5, 56.5, 55.5, 47.5, 46.7, 45.8, 41.6, 41.3, 39.2, 39.0, 38.4, 36.6, 33.8, 33.0, 32.6, 32.3, 30.6, 28.3, 27.5, 25.8, 25.7, 23.6, 23.3, 23.0, 20.7, 20.7, 20.6, 18.1, 16.8, 16.6, 15.2; HRMS(ESI): calcd for $[M + H]^+$ $C_{49}H_{72}NO_{10}$: 834.5156, found 834.5151.

28-*O*-Benzyl-3-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl] oleanolic ester (6). To a mixture of **5** (2.39 g, 2.87 mmol) in pyridine (20 mL), acetic anhydride (0.98 mL, 10.33 mmol) was added. After it was stirred for 1.5 h, the reaction mixture was quenched by addition of MeOH, filtered and diluted with CH_2Cl_2. Then the organic layer was washed with aqueous HCl (1 N), saturated aqueous $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to afford **6** (2.16 g, 86.3%) as a white foam. Mp 252.1–254.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38–7.28 (m, 5H), 5.58–5.49 (m, 1H), 5.32–5.24 (m, 2H), 5.12–4.98 (m, 3H), 4.67 (d, J = 8.3 Hz, 1H), 4.23 (dd, J = 12.1, 5.5 Hz, 1H), 4.14–4.03 (m, 1H), 3.89–3.82 (m, 1H), 3.70–3.64 (m, 1H), 3.07 (dd, J = 11.7, 4.5 Hz, 1H), 2.90 (dd, J = 13.7, 4.0 Hz, 1H), 2.06 (s, 3H), 2.02 (s, 3H), 2.02 (s, 3H), 1.92 (s, 3H), 1.10 (s, 3H), 0.91 (s, 3H), 0.90 (s, 3H), 0.89 (s, 3H), 0.86 (s, 3H), 0.76 (s, 3H), 0.58 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.3, 171.0, 170.6, 169.9, 169.4, 143.6, 136.3, 128.3, 127.9, 127.8, 122.4, 103.0, 90.6, 72.2, 71.4, 68.9, 65.9, 62.4, 55.4, 55.3, 47.5, 46.7, 45.8, 41.6, 41.3, 39.2, 38.8, 38.3, 36.6, 33.8, 33.0, 32.6, 32.3, 30.6, 27.8, 27.5, 25.7, 25.6, 23.6, 23.3, 23.3, 23.0, 20.7, 20.7, 20.6, 18.1, 16.8, 16.4; HRMS (ESI): calcd for $[M + Na]^+$ $C_{51}H_7$ $NNaO_{11}$: 898.5081, found 898.5076.

28-*O*-Benzyl-3-*O*-[2-acetamido-2-deoxy-β-D-glucopyranosyl] oleanolic ester (7). To a solution of **6** (2.16 g, 2.47 mmol) in CH_2Cl_2–MeOH (1 : 1, 40 mL), freshly prepared NaOMe in MeOH solution (1.0 mol L^{-1}, 0.25 mL) was added. After it was stirred overnight, the mixture was neutralized with Dowex H^+ resin to pH 7, and then filtered. The filtrate was concentrated and purified by a silica gel column chromatography (15 : 1, CH_2Cl_2–MeOH) to afford **7** (1.54 g, 83.2%) as a white solid. Mp 146.1–147.3 °C; ^{1}H NMR (600 MHz, CDCl3) δ 7.37–7.27 (m, 5H), 5.29 (s, 1H), 5.06 (s, 2H), 4.46 (s, 1H), 3.90–3.53 (m, 5H), 3.35 (br s, 1H), 3.05 (s, 1H), 2.89 (t, J = 7.3 Hz, 1H), 1.98 (s, 3H), 1.12 (s, 3H), 0.94–0.86 (m, 12H), 0.75 (s, 3H), 0.59 (s, 3H); 13C NMR (150 MHz, $CDCl_3$) δ 177.5, 172.2, 143.8, 136.6, 128.6, 128.1, 128.1, 122.6, 90.2, 75.3, 74.4, 72.2, 70.2, 66.0, 61.9, 55.6, 47.8, 46.9, 46.0, 41.8, 41.6, 39.5, 39.0, 38.5, 36.8, 34.0, 33.3, 32.8, 32.5, 30.8, 28.0, 28.0, 27.8, 26.2, 26.0, 25.8, 23.8, 23.6, 18.4, 17.0, 16.9, 15.5; HRMS(ESI): calcd for $[M + Na]^+$ $C_{45}H_{67}NNaO_8$: 772.4764, found 772.4759.

28-*O*-Benzyl-3-*O*-[2-acetamido-6-*O*-*tert*-butyldimethylsilyl-2-deoxy-β-D-glucopyranosyl] oleanolic ester (8). To a solution of **7** (1.54 g, 2.05 mmol) in CH_2Cl_2 (30 mL), imidazole (1.40 g, 20.50 mmol) and TBSCl (1.54 g, 10.25 mmol) were added. After it was stirred for 10 min, the mixture was filtered. Then the filtrate was concentrated and purified by a silica gel column chromatography (50 : 1, CH_2Cl_2–MeOH) to afford **8** (1.36 g, 76.7%) as a white solid. Mp 153.2–155.7 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39–7.28 (m, 5H), 5.28 (s, 1H), 5.06 (dd, J = 32.2, 12.6 Hz, 1H), 4.55–4.50 (m, 1H), 3.89 (dd, J = 10.6, 3.8 Hz, 1H), 3.84–3.69 (m, 2H), 3.50–3.39 (m, 2H), 3.10 (dd, J = 7.7, 3.3 Hz, 1H), 2.90 (dd, J = 13.6, 3.7 Hz, 1H), 1.11 (s, 3H), 0.93–0.86 (m, 21H), 0.78 (s, 3H), 0.59 (s, 3H), 0.08 (s, 3H), 0.07 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.4, 171.9, 143.6, 136.3, 128.3, 127.9, 127.8, 122.4, 102.5, 89.7, 75.1, 74.5, 72.8, 65.9, 64.1, 58.4, 55.3, 47.5, 46.7, 45.8, 41.6, 41.3, 39.2, 38.9, 38.3, 36.6, 33.8, 33.0, 32.6, 32.3, 30.6, 28.2, 27.5, 25.8, 25.6, 23.6, 23.3, 23.0, 18.2, 18.1, 16.8, 16.6, 15.2, −5.5, −5.5; HRMS (ESI): calcd for $[M + Na]^+$ $C_{51}H_{81}NNaO_8$ Si: 886.5629, found 886.5624.

28-*O*-Benzyl-3-*O*-[3,4-*O*-bi-acetyl-6-*O*-*tert*-butyldimethylsilyl-2-acetamido-2-deoxy-β-D-glucopyranosyl] oleanolic ester (9). To a solution of **8** (1.36 g, 1.57 mmol) in pyridine (20 mL), acetic anhydride (0.36 mL, 3.77 mmol) was added. After it was stirred for 2.5 h, the reaction mixture was quenched by addition of MeOH, filtered and diluted with CH_2Cl_2. Then the organic layer was washed with aqueous HCl (1 N), saturated aqueous $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to afford **9** (1.28 g, 86.1%) as a white foam. Mp 244.9–246.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 5.46 (s, 1H), 5.28 (brs, J = 3.4 Hz, 1H), 5.21 (dd, J = 10.7, 9.3 Hz, 1H), 5.06 (m, 2H), 4.92 (t, J = 9.6 Hz, 1H), 4.60 (d, J = 8.2 Hz, 1H), 3.91–3.84 (m, 2H), 3.68–3.61 (m, 1H), 3.53–3.47 (m, 1H), 3.07 (dd, J = 11.8, 4.4 Hz, 1H), 2.90 (dd, J = 13.8, 4.0 Hz, 1H), 2.02 (s, 3H), 2.01 (s, 3H), 1.91 (s, 3H), 1.11 (s, 3H), 0.93–0.85 (m, 21H), 0.76 (s, 3H), 0.59 (s, 3H), 0.04 (s, 3H), 0.03 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.4, 171.2, 169.7, 169.3, 143.6, 136.3, 128.3, 127.9, 127.8, 122.5, 103.1, 90.2, 74.6, 72.7, 69.3, 65.9, 62.8, 55.4, 55.2, 47.5, 46.7, 45.8, 41.6, 41.3, 39.2, 38.8, 38.3, 36.6, 33.8, 33.0, 32.3, 30.6, 29.9, 27.8, 27.5, 25.7, 25.6, 25.5, 23.6, 23.3, 23.0, 20.7, 18.2, 18.1, 16.8, 16.4, 15.2, −5.5, −5.6; HRMS(ESI): calcd for $[M + Na]^+$ $C_{55}H_{85}N$-$NaO_{10}Si$: 970.5840, found 970.5835.

28-*O*-Benzyl-3-*O*-[2-acetamido-3,4-*O*-bi-acetyl-6-*O*-2-deoxy-β-D-glucopyranosyl] oleanolic ester (10). To a solution of **9** (1.28 g, 1.35 mmol) in THF (25 mL), TBAF (0.39 g, 1.49 mmol) was added. After it was stirred for 11 h, the reaction mixture was concentrated and purified by a silica gel column chromatography (1 : 1, petroleum ether–EtOAc) to afford **10** (0.80 g, 71.2%) as a white solid. Mp 256.7–258.4 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 5.60–5.50 (m, 1H), 5.33–5.26 (m, 2H), 5.06 (m, 2H), 4.98 (t, J = 9.6 Hz, 1H), 4.70 (d, J = 8.3 Hz, 1H), 3.86 (dt, J = 10.7, 8.6 Hz, 1H), 3.72–3.67 (m, 1H), 3.63–3.56 (m, 1H), 3.54–3.49 (m, 1H), 3.09 (dd, J = 11.3,

5.0 Hz, 1H), 2.90 (dd, J = 13.7, 4.0 Hz, 1H), 2.04 (s, 3H), 2.03 (s, 3H), 1.92 (s, 3H), 1.10 (s, 3H), 0.94–0.84 (m, 12H), 0.76 (s, 3H), 0.58 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.4, 171.0, 170.0, 169.9, 143.6, 136.3, 128.3, 127.9, 127.8, 122.4, 103.0, 90.3, 73.8, 72.2, 69.0, 65.9, 61.6, 55.4, 55.3, 47.5, 46.7, 45.8, 41.6, 41.3, 39.2, 38.8, 38.3, 36.6, 33.8, 33.0, 32.6, 32.3, 30.6, 27.8, 27.5, 25.9, 25.8, 23.6, 23.3, 23.0, 20.7, 20.6, 18.1, 16.8, 16.4, 15.2; HRMS(ESI): calcd for $[M + Na]^+$ $C_{49}H_{71}NNaO_{10}$: 856.4976, found 856.4970.

28-*O*-Benzyl-3-*O*-[2,3,4-tri-*O*-benzoyl-α-L-arabinopyranosyl-(1→6)-2-acetamido-3,4-*O*-bi-acetyl-2-deoxy-β-D-glucopyranosy] oleanolic ester (11). A mixture of **10** (130.00 mg, 0.16 mmol) and benzoylated arabinose trichloroacetimidate **14** (188.7 mg, 0.31 mmol) in dry CH_2Cl_2 (10 mL) was stirred at rt for 30 min. Then the mixture was cooled to 0 °C and TMSOTf (6.03 μL, 0.031 mmol) was added. After stirring at this temperature for 2 h, Et_3N was added. The resulting mixture was filtered. The filtrates were concentrated to give a residue, which was purified by a silica gel column chromatography (6 : 1, petroleum ether–EtOAc) to afford **11** (126.7 mg, 63.6%) as a white foam. Mp 346.7–348.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04–7.95 (m, 6H), 7.59–7.28 (m, 15H), 5.68–5.64 (m, 1H), 5.60 (d, J = 2.8 Hz, 1H), 5.44 (d, J = 8.9 Hz, 1H), 5.31–5.26 (m, 2H), 5.18 (dd, J = 10.7, 9.4 Hz, 1H), 5.12–5.01 (m, 2H), 4.94–4.86 (m, 2H), 4.54 (d, J = 8.3 Hz, 1H), 4.33–4.28 (m, 1H), 3.92–3.82 (m, 3H), 3.77–3.64 (m, 2H), 2.96 (dd, J = 11.7, 4.4 Hz, 1H), 2.90 (dd, J = 13.7, 3.8 Hz, 1H), 2.02 (s, 3H), 2.00 (s, 3H), 1.90 (s, 3H), 1.07 (s, 3H), 0.92 (s, 3H), 0.89 (s, 3H), 0.83 (s, 3H), 0.76 (s, 3H), 0.72 (s, 3H), 0.57 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.4, 171.0, 169.7, 169.4, 165.5, 165.3, 165.1, 143.2, 136.4, 133.3, 130.8, 129.8, 129.8, 129.7, 129.3, 129.1, 128.8, 128.5, 128.4, 128.4, 128.3, 127.9, 127.8, 122.8, 103.2, 99.9, 90.3, 73.5, 72.2, 69.8, 69.1, 67.9, 67.4, 65.9, 65.5, 55.4, 55.1, 53.4, 47.4, 46.6, 45.8, 41.5, 41.3, 39.2, 38.9, 38.1, 36.6, 33.8, 33.1, 32.6, 32.3, 30.6, 30.5, 29.6, 27.7, 27.5, 25.8, 25.7, 23.8, 23.6, 23.3, 23.0, 22.6, 20.6, 20.6, 19.1, 18.1, 16.8, 16.3, 15.1, 14.1, 13.6, 7.9; HRMS (ESI): calcd for $[M + Na]^+$ $C_{75}H_{91}NNaO_{17}$: 1300.6185, found 1300.6191.

3-*O*-[α-L-Arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] oleanolic ester (1). A mixture of **11** (126.7 mg, 0.099 mmol) and 10% Pd/C (25.3 mg) in EtOAc (5 mL) was stirred at 50 °C under H_2 at atmospheric pressure. After 6 h, the reaction mixture was filtered and concentrated *in vacuo*. Then the residue was dissolved in CH_2Cl_2–MeOH (1 : 1, 5 mL). The resulting solution was added freshly prepared NaOMe in MeOH solution (1.0 mol L^{-1}, 0.1 mL). After it was stirred overnight, the mixture was neutralized with Dowex H^+ resin to pH 7, then filtered. The filtrate was concentrated and purified by a silica gel column chromatography (7 : 2 : 1, CH_2Cl_2–MeOH–H_2O) to afford **1** (59.2 mg, 75.5% in two steps) as a white solid. ^{1}H NMR (600 MHz, Pyr) δ 8.92 (d, J = 9.0 Hz, 1H), 5.45 (d, J = 3.9 Hz, 1H), 5.00 (d, J = 8.7 Hz, 1H), 4.95 (d, J = 6.6 Hz, 1H), 4.63–4.51 (m, 2H), 4.48 (dd, J = 8.3, 6.7 Hz, 1H), 4.40–4.30 (m, 3H), 4.28–4.22 (m, 1H), 4.21–4.17 (m, 1H), 4.13–4.07 (m, 2H), 3.77 (d, J = 10.4 Hz, 1H), 3.32 (d, J = 13.7 Hz, 1H), 3.19 (dd, J = 11.7, 4.4 Hz, 1H), 1.29 (s, 3H), 1.15 (s, 3H), 1.04 (s, 3H), 1.00 (s, 9H), 0.80 (s, 3H); HRMS(ESI): calcd for $[M + Na]^+$ $C_{43}H_{69}NNaO_{12}$: 814.4717, found 814.4712.

Cell viability assay

Cell viability was evaluated using an MTT assay. A375 cells were seeded into 96-well plates at a density of 5 × 104 cells per well and stabilized at 37 °C for 24 h. Compounds **1** and **12a–12f** were added to each well at various concentrations (80, 40, 20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0.16 μM), and then the cells were incubated for 72 h. The MTT solution (20 μL 5 mg mL^{-1}) was added to each well, and the cells were incubated for another 4 h. Formazan crystals were dissolved in 150 μL of DMSO. Cell viability was assessed by measuring the absorbance at 540 nm wavelength using a microplate reader (BioTek ELx800, USA).

Cell cycle analysis

Cell cycle was assessed using the PI staining assay. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.0 μM) for 72 h. Then, the cells were collected, washed with ice-cold PBS buffer, fixed with 80% alcohol at 4 °C for 12 h, and stained with propidium iodide in the presence of 1% RNAase A at RT (25 °C) for 15 min before analysis by flow cytometry (BD FACSCalibur USA).

Cell apoptosis analysis

Cells apoptosis was assessed using Annexin V/PI staining assay. A375 cells were treated with **1** at various concentrations (0, 0.31, 1.25 and 5.0 μM) for 72 h. Then, cells were collected, washed with Annexin-binding buffer, and stained with Annexin V fluorescein isothiocyanate (FITC) and PI for 15 min at RT (25 °C). After that, the samples were analyzed by flow cytometry (BD FACSCalibur, USA).

Mitochondrial membrane potential assay

The mitochondrial membrane potential was assessed using the JC-1 dye. A375 cells were plated at 1 × 10^6 cells per well in 24-well plates and incubated with **1** at various concentrations (0, 0.31, 1.25 and 5.0 μM) for 72 h. The cells were subsequently incubated with the JC-1 dye, and finally analyzed by flow cytometry (BD FACSCalibur, USA).

Western blotting

A375 cells were seeded at a density of 4 × 10^5 cells per well and treated with various concentrations of **1** (0, 0.31, 1.25 and 5.0 μM) for 72 h. After this, cells were collected and washed twice with ice-cold DPBS. The pellets were resuspended in a total protein extraction buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF) containing a protease inhibitor cocktail and incubated on ice for 30 min with intermittent mixing. The protein concentration was measured using the Bradford reagent (Bio-Rad Laboratories Inc, CA, USA). An equal amount (20 μg) of protein was loaded on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk, the membrane

was incubated at 4 °C overnight with specific primary antibodies. The membrane was washed and incubated at room temperature for 1 h with secondary antibodies conjugated with horseradish peroxidase (HRP). Finally, the immunoblot was developed for visualization using a chemiluminescence kit. Primary antibodies for cytochrome c, caspase-9, caspase-3, caspase-8, Bax, Bcl-2, and β-actin and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Quantitative RT-PCR

Total RNA was extracted from A375 cells with various concentrations (0, 0.31, 1.25 and 5.0 μM) using the Trizol reagent (Invitrogen). 2 μg of total RNA was used for reverse transcription using RevertAid H Minus first strand cDNA synthesis kit (Fermentas, MA, USA) following the manufacturer' s instructions. For real-time qPCR, the ABI PRISM 7900 sequence detection system (ABI) was used. Nine microliters of master mix (2 × Maxima SYBR Green/ROXqPCR master mix, 0.3 μM forward pri mer, 0.3 μM reverse primer) and 1 μL of 100 ng cDNA were added to the 96-well plates and amplified using a suitable program. At the completion of cycling, melting curve analysis was performed to establish the specificity of the amplicon production. Data were analyzed according to the comparative Ct method and were normalized by GAPDH expression. The primers used for qRT-PCR were as follows.

Homo-GAPDH primer (115 bp):
Forward 5-CATCTTCTTTTGCGTCGCCA-3,
Reverse 5-TTAAAAGCAGCCCTGGTGACC-3;
Homo-Bcl2 primer (81 bp):
Forward 5-ATCCAGGATAACGGAGGC-3,
Reverse 5- CAGCCAGGAGAAATCAAAC-3;
Homo-Bax primer (117 bp):
Forward 5-GACCCGGTGCCTCAGGATGC-3,
Reverse 5-GTCTGTGTCCACGGCGGCAA-3;
Homo-caspase3 primer (139 bp):
Forward 5-CATGGAAGCGAATCAATGGACT-3 ,
Reverse 5-CTGTACCAGACCGAGATGTCA-3.

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