20*S*-Protopanaxadiol-Induced Programmed Cell Death in Glioma Cells through Caspase-Dependent and -Independent Pathways

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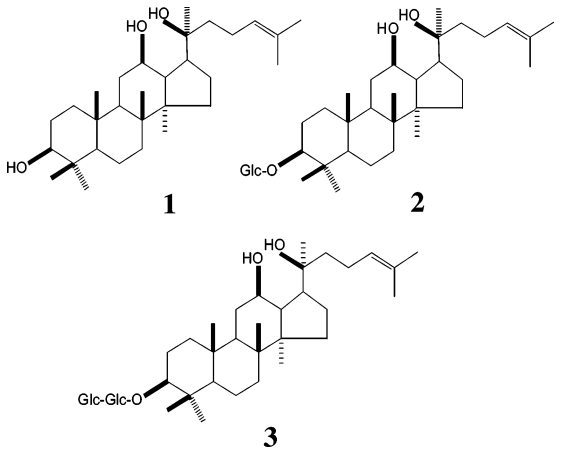
Received July 3, 2006

20*S*-Protopanaxadiol (**1**) is an aglycon metabolic derivative of the protopanaxadiol-type ginseng saponins. In the present study, **1** was used to induce cytotoxicity for two human glioma cell lines, SF188 and U87MG. For the SF188 cells, **1** activated caspases-3, -8, -7, and -9 within 3 h and induced rapid apoptosis, which could be partially inhibited by a general caspase blocker and completely abolished when the caspase blocker was used in combination with an antioxidant. Compound **1** also induced cell death in U87MG cells but did not activate any caspases in these cells. Monodansylcadaverine staining showed that **1** induced dramatic autophagy in both cell lines. Elevated levels of superoxide anion in both cells and reduced levels of phosphorylated Akt in U87MG cells were also demonstrated. These results showed that 20*S*-protopanaxadiol (**1**) induces different forms of programmed cell death, including both typical apoptosis and autophagy through both caspase-dependent and -independent mechanisms.

The most commonly used cancer therapeutic agents eliminate tumors by inducing apoptosis and inhibiting proliferation of tumor cells. Although the cellular targets of different cytotoxic agents vary, most of the signaling pathways activated by anticancer drugs ultimately lead to the activation of caspases.[1,2] In some cell types, caspase-8 activation fully triggers the caspase activation cascade and induces apoptosis by proteolytic activation of pro-caspase-3,[3,4] the principal effector of caspase. However, in some cell types, death-receptor cross-linking fails to cause a caspase-8 activation, leading to activation of caspase-3. In this case, the pathway involves mitochondrial membrane permeabilization and release of cytochrome *c* from mitochondria, which causes cytochrome *c*-dependent formation of a caspase-3 activation complex (also called the apoptosome).[5] Although caspases are the principal mediators of apoptotic cell death in many developmental and physiological settings,[6,7] they are not the single cause of execution of the death command. In fact, recent data indicate that other forms of programmed cell death can occur in the complete absence of caspases.[8]



1 **2** **3**

Protopanaxadiol compounds such as Rh_2 (**2**) and Rg_3 (**3**), which are found in ginseng (*Panax ginseng* C. A. Meyer; Araliaceae) and other related plants, have been shown to be cytotoxic to tumor cells without any detectable toxicity toward normal tissues.[9−12] A number of studies have indicated that **2**-induced apoptosis of tumor cells is dependent on activation of caspase-3.[9,11,12] In addition, **2**-induced tumor growth inhibition and/or apoptosis is related to reactive oxygen species (ROS) generation[13] and p21 activity.[10] We previously reported that while **2** caused apoptosis, it was not accomplished through steroid receptors and was independent of p53 in various tumor cell lines.[9] In addition, we found that although **2** can activate caspases, caspase activation is not absolutely required for apoptosis.

All ginsenosides are believed to be metabolized in the gastrointestinal system through a series of deglycosylation steps into aglycon derivatives, including 20*S*-protopanaxadiol (**1**) and 20*S*-protopanaxatriol.[14,15] Some of these metabolites have demonstrated a cytotoxic potency on tumor cells equivalent to that of **2**.[15−17] However, the pharmacological mechanism of **1**-induced apoptosis has not been studied in detail. In the current study, we have investigated the signal transduction pathways involved in **1**-induced apoptosis in two glioma cell lines, SF188 and U87MG, with p53- and PTEN- status, respectively. Our results demonstrate that **1** can cause cell death through a variety of mechanisms, including caspase-related and caspase-unrelated pathways.

Results and Discussion

The glioma cell lines, SF188 and U87MG, were treated with **1**, and cytotoxicity was determined using a MTT assay. Figures 1A and 1B show that **1** induced cytotoxicity more readily in SF188 cells than in U87MG cells ($p < 0.001$). When both cell lines were treated for 24 h, 43.4 μM **1** was lethal to nearly 100% of SF188 cells, while 108.4 μM was required to achieve 90% cell death of U87MG cells in 24 h (Figure 1A). These different sensitivities to **1**-induced cytotoxicity were also evident from the results of the time-course experiment shown in Figure 1B. Nearly 90% of SF188 cells died within 10 h of treatment with 108.4 μM **1**, but 24 h were required to reach similar levels of cell death with U87MG cells ($p < 0.001$). At 24 h, the IC_{50} values of **1**-treated SF188 and U87MG cells were about 24 and 38 μM, respectively.

Both SF188 and U87MG cells treated with **1** (>40 μM) showed positive TUNEL staining in an ApopTag assay (Figures 1D and 1F). The apoptotic nature of the cell death was also confirmed by morphology under a light microscope and using DNA laddering, a mitochondrial membrane permeability assay, and Hoecht staining

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10.1021/np060313t CCC: $37.00
Published on Web 01/30/2007

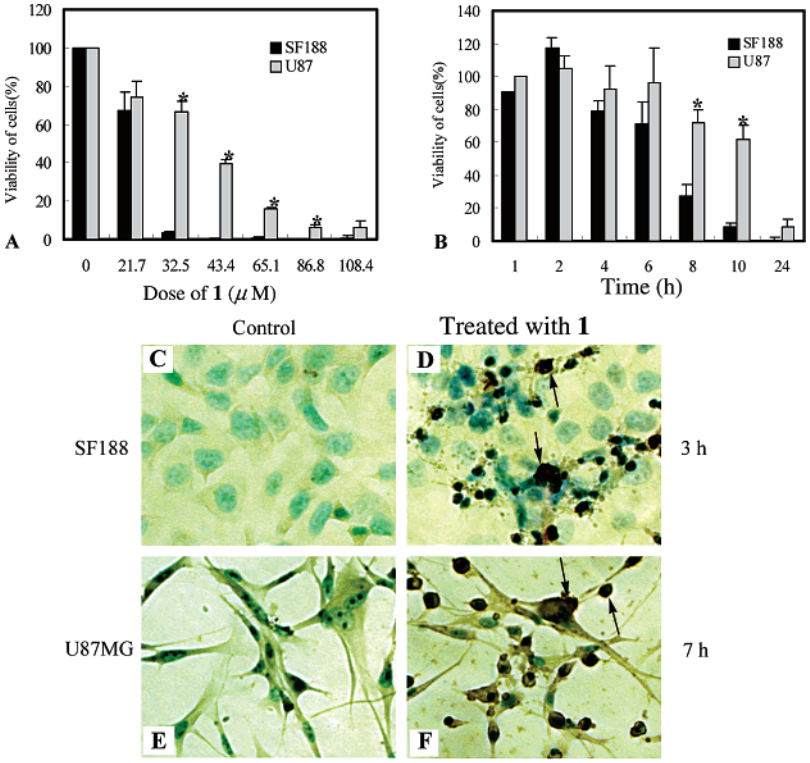

Figure 1. Cytotoxicity of **1** for SF188 and U87MG glioma cells. For this viability assay, cells were seeded in 96-well plates 24 h prior to each experiment at 2.5×10^5/mL cell density. (A) Dose−response of **1**-induced cell death determined by MTT assay measured at 24 h after treatment with different concentrations of **1**. (B) Viability of cells at 1, 4, 6, 8, 10, and 24 h after treatment with 108 μM **1**. It shows that even at a high concentration of **1** (108.4 μM), a longer treatment time was required to cause cell death for U87MG cells (*$p < 0.001$). (C−F) TUNEL-stained SF188 and U87MG cells, among which C and E are controls; D and F are cells treated with **1** (108 μM); and the arrows indicate apoptotic cells.

for nuclear condensation (data not shown). Again, the apoptotic markers appeared much earlier in SF188 cells than in U87MG cells. Positive TUNEL staining was seen within 3 h in SF188 cells (42.67 ± 1.80%) (Figure 1D) and in 7 h in U87MG cells (51.01 ± 4.15%) with the same concentration of **1** (Figure 1F).

The substantial difference seen in the time course of **1**-induced apoptosis between SF188 and U87MG cells led us to further investigate the mechanisms involved. Since **2**, an analogue of **1**, was previously reported to activate caspase-3,[9,12] the active forms of various caspases were measured by western blot analyses. Figure 2 shows that cleaved fragments of caspases-3, -7, -8, and -9 were all present in SF188 cells treated with 108.4 μM **1** within 2 or 3 h (Figures 2A to 2C and 2E to 2G), indicating the activation of these caspases. In addition, the amount of activated caspases increased with the concentration of **1** and the duration of treatment, suggesting that activation was time- and dose-dependent. A general caspase inhibitor, Z-VAD-fluoromethylketone (Z-VAD-FMK), partially blocked the **1**-induced apoptosis by 44.72 ± 6.9% ($p < 0.001$) (Figure 2L). Interestingly, **1** did not activate caspases in U87MG cells at all. Despite the presence of caspases-3 and -8 in their procaspase forms, cleaved fragments were absent in U87MG cells treated with **1** for up to 9 h (Figures 2J and 2K). In addition, the same levels of cell death were seen in **1**-treated U87MG cells with and without Z-VAD-FMK (Figure 2M), suggesting that caspases were not involved in the **1**-induced cell death processes in U87MG.

The incomplete protection by caspase inhibitors for **1**-induced apoptosis in SF188 cells and the absence of caspase activation in U87MG cells suggested a caspase-independent mechanism. Since it is known that overproduction of reactive oxygen species (ROS) can induce apoptosis in a caspase-independent manner in some cells,[18,19] the levels of the superoxide anion ($O_2^{\bullet -}$) in both SF188 and U87MG cells treated with **1** were determined by measuring intracellular hydroethidine (HE) fluorescence. Compound **1** markedly increased the percentage of HE positive cells in a dose−response fashion ($p < 0.001$) (Figure 3A). To determine the relationship between caspase activation and overproduction of ROS in **1**-induced cell death, viability was measured in SF188 cells treated with *tert*-butylhydroquinone (tBHQ), an activator of the antioxidant response element (ARE), the general caspase inhibitor Z-VAD-FMK, or both of them. As shown in Figure 3B, the viability of **1**-treated cells in the presence of tBHQ (10 μM) or Z-VAD-FMK (50 μM) was 30.46 ± 3.47% and 36.0 ± 9.6%, respectively. Compared to viability using **1** (16.5 ± 2.2%), there was a partial protection by either agent alone ($p < 0.01$). Interestingly, treatment with both tBHQ (10 μM) *and* Z-VAD-FMK (50 μM) together almost completely inhibited the cell death induced by **1** (89.4 + 14.7% cell viability, Figure 3B). Therefore, it is apparent that both the caspases and oxidant stress are involved in **1**-induced cell death in SF188 cells.

To further investigate the mechanism of **1**-induced caspase-independent cell death, especially in U87MG cells, monodansylcadaverine (MDC) was used to measure the levels of autophagic vacuoles (AVs).[20] The results showed that SF188 cells (Figure 4A, a−c) and U87MG (Figure 4A, d−f) treated with 43.4 and 108.4 μM of **1** separately for 4 h both demonstrated strong positive staining for autophagic vacuoles. Thus, it is clear that besides a typical apoptosis, autophagy is another form of programmed cell death induced by **1** in both glioma cell lines.

A significant difference between SF188 and U87MG is that SF188 is PTEN+ while U87MG is PTEN−. Akt is constitutively activated in cells that lack PTEN expression.[21] Inhibition of Akt phosphorylation blocks its downstream survival pathway, which can induce apoptosis[22,23] and autophagy[24,25] in a manner independent of caspase activation. Therefore, the status of Akt phosphorylation in PTEN− U87MG cells was investigated. Phosphorylated-Akt (P-Akt) was detected by probing western blots with an antibody specifically recognizing P-Akt on Ser 473. Seven hours after

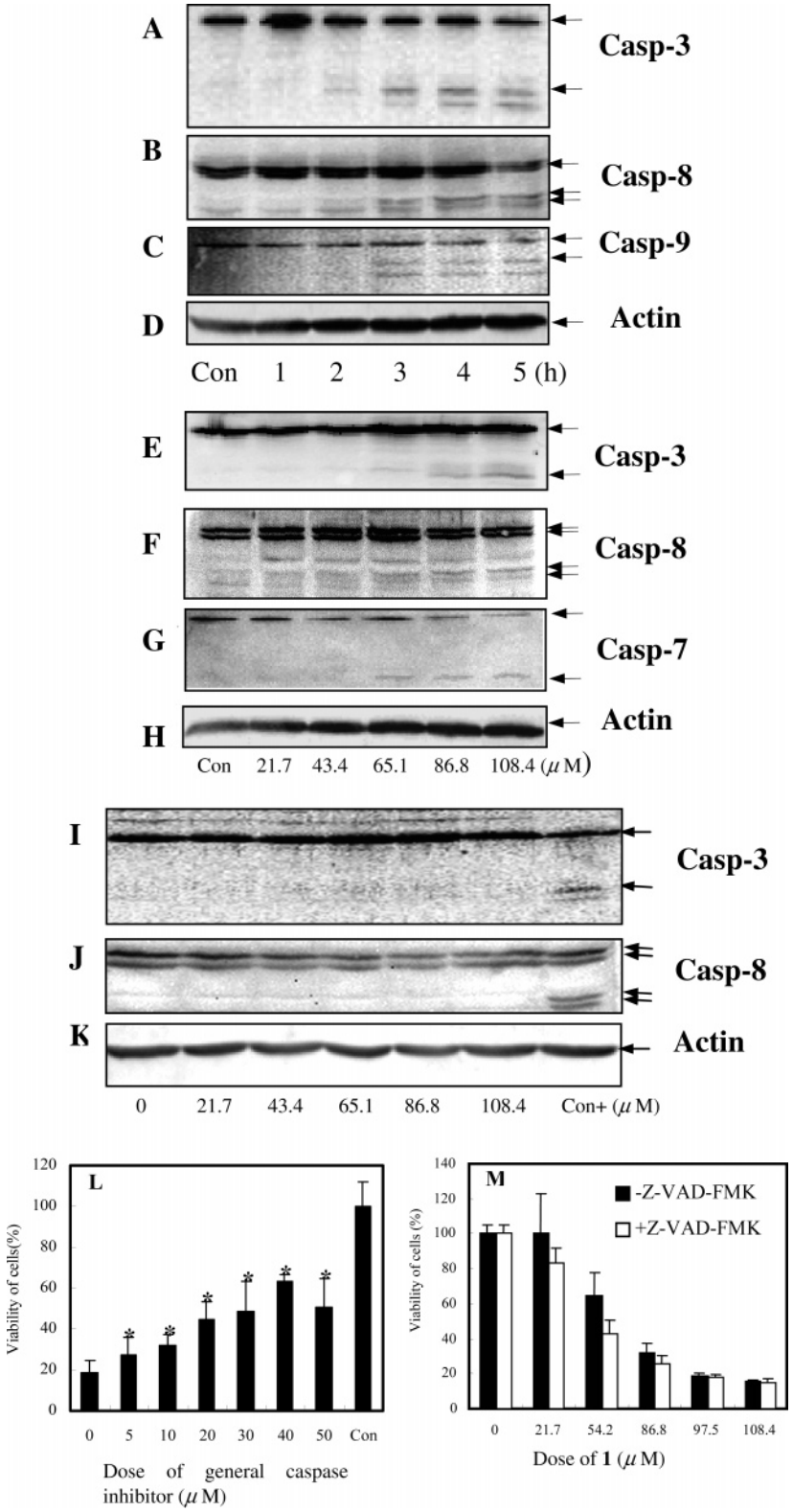

Figure 2. Effects of **1** treatment on activities of caspases. Western blot analyses for caspase activities in SF188 cells treated with **1** (108.4 μM) at different time points (A−C) or concentrations (E−G). (J and K) Activities of caspases-8 and -3 in U87MG cells treated with **1** (108.4 μM) for 9 h; the last lane (Con+) in each panel is a positive control (**1**-treated SF188 cell lysate). 50 μg of total protein was loaded in each lane. In each panel, the top arrow indicates inactive pro-caspases, while the lower arrow points to cleaved (activated) caspases. (L) SF188 cells were pretreated with various concentrations of Z-VAD-FMK for 2 h followed by adding 43.4 μM **1**. Cell viability was determined with an MTT assay at 8 h after **1** treatment. Z-VAD-FMK partially blocked **1**-induced apoptosis by 44.7 ± 6.9% ($p < 0.001$). (M) Effect of the general caspase inhibitor on the viability of **1**-treated U87MG cells. U87MG cells were pretreated with 40 μM Z-VAD-FMA (+Z-VAD-FMA) or without (−Z-VAD-FMK) for 2 h followed by adding different concentrations of **1**. Cell viability was determined with an MTT assay at 24 h after **1** treatment. (D, H, and K) Actin loading controls (actin).

treatment with **1**, P-Akt levels declined in U87MG cells in a dose-dependent fashion (Figure 5A). The level of total Akt in SF188 cells was similar to that of U87MC cells (Figure 5E). However, the amount of phosphorylated Akt was low (Figure 5D), and there

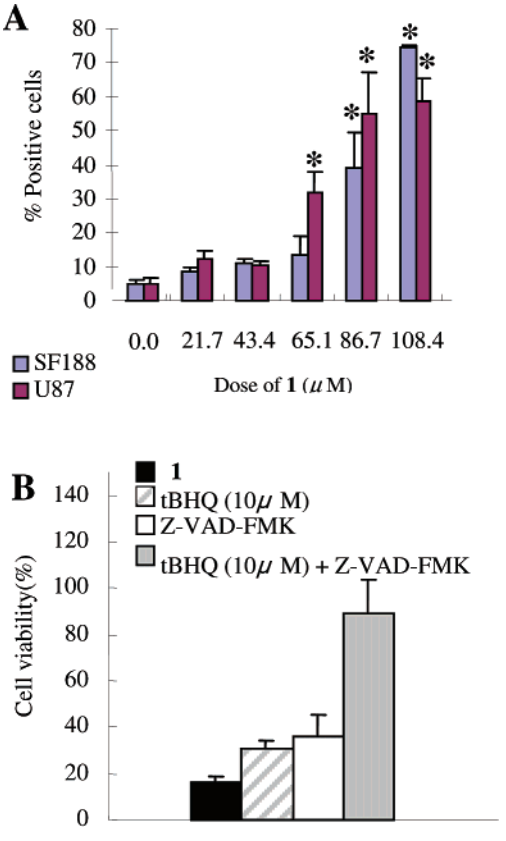

Figure 3. Generation and function of superoxide anion in **1**-treated glioma cells. Levels of superoxide anion ($O_2^{\bullet -}$) were determined by dihydroethidine (HE) fluorescence with flow cytometry in **1**-treated SF188 or U87MG cells at 2 h post-treatment. (A) Percentages of HE positive cells in **1**-treated SF188 and U87MG cells (*****$p < 0.01$). (B) Effects of general caspase inhibitor Z-VAD-FMK and *tert*-butylhydroquinone (tBHQ) in **1**-treated SF188 cells. SF188 cells were treated with 10 μM tBHQ or without tBHQ for 48 h prior to the treatment with **1** (86.8 μM for 8 h). The viability of SF188 cells was measured in cells treated with **1** only (**1**); **1** with 10 μM tBHQ (tBHQ); **1** with Z-VAD-FMK (Z-VAD-FMK) (50 μM); or **1** with both tBHQ and Z-VAD-FMK (tBHQ+Z-VAD-FMK) (*****$p < 0.01$). The viability is 89.4 ± 14.7% ($p < 0.001$) for **1**-treated cells in the presence of both tBHQ (10 μM) and Z-VAD-FMK.

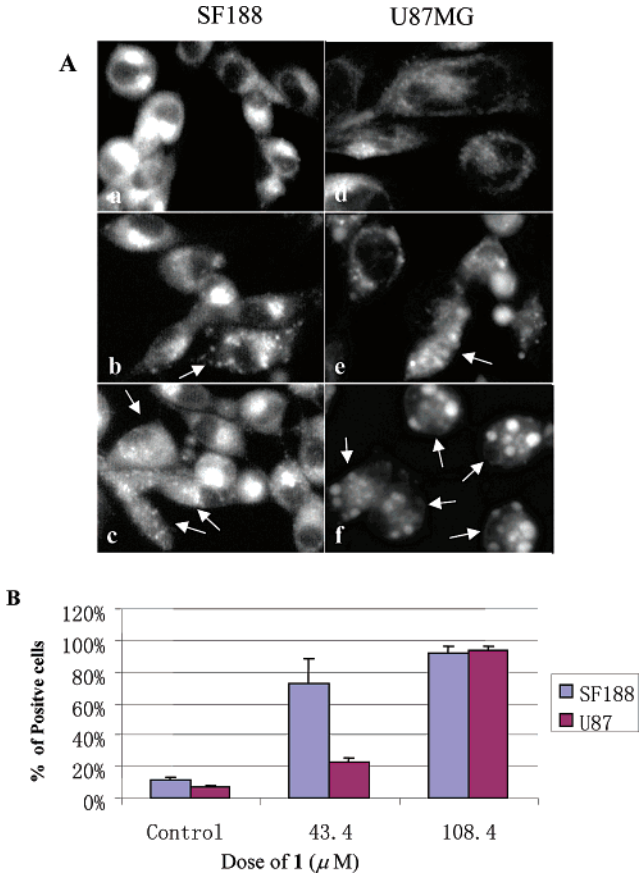

Figure 4. Autophagy in glioma cells induced by **1**. Autophagic cells were evident in both SF188 and U87MG cells treated with **1** for 4 h. (A) Monodansylcadaverine (MDC)-stained cells treated with **1**: (a and d) **1**-untreated controls; (b and e) cells treated with 43.4 μM (20 μg/mL) **1**; (c and f) cells treated with 108.4 μM **1**. Arrows point to autophagic vacuoles. (B) Percentages of autophagic cells treated with **1** for 4 h. The values were averages of positive cells in three microscopic fields counting a total of more than 200 cells within each sample.

was no change after treatment with **1**, which is consistent with the PTEN-positive status of this cell line.

As one of the major metabolic products of ginseng, it is important to better understand the biological activity of ginsenoside aglycons, such as 20*S*-protopanaxadiol (**1**). In the present study, it has been shown that **1** effectively induced cell death in human glioma cell lines regardless of their p53 and PTEN status. Simultaneously, **1** induced two types of programmed cell death, apoptosis and autophagy, in both SF188 and U87MG cells. **1**-induced cell death may be mediated by multiple pathways, including both caspase-dependent (such as in SF188 cells) and -independent (such as in U87MG cells), and the latter may involve oxidant stress and inhibition of Akt phosphorylation, which may be responsible for the autophagy induced by **1**.

Previously, Fei et al.[12] demonstrated that inhibitors of caspases-3 and -8 did not entirely block apoptosis of human malignant melanoma cells treated with **2**. This is consistent with the present results with SF188 cells. To exclude the possibility that the incomplete inhibition of apoptosis was due to incomplete blocking of caspase activation, caspase-3 and -8 activation was tested after SF188 cells were treated with the general caspase inhibitor Z-VAD-FMK. The results showed that Z-VAD-FMK completely inhibited activation of both caspases in **1**-treated cells by western blot assay (data not shown). However, this treatment only partially blocked apoptosis in SF188 cells. In addition, the same concentration of Z-VAD-FMK completely blocked SF188 cell apoptosis induced by TNF-α and cycloheximide (data not shown). Together with the fact that none of the caspases were activated in **1**-treated U87MG cells, but apoptosis still occurred at higher concentrations (Figure 1), it was concluded that both caspase-dependent and -independent mechanisms participate in **1**-induced cell death. Both mechanisms are involved in the cell death of **1**-treated SF188 cells, but only the caspase-independent mechanism is responsible for the death of U87MG cells. This explains why SF188 is more susceptible to **1** treatment than U87MG. The lack of caspase activation in U87MG might be related to the negative status of PTEN, a possibility currently under investigation.

In parallel to manifested apoptotic cell death, significant numbers of autophagic vacuoles were evident in **1**-treated SF188 and U87MG cells. This is the first report suggesting autophagy may be an alternative mechanism for cell death caused by a protopanaxadiol saponin aglycon. Oxidant stress is well documented for causing caspase-independent cell death including autophagy in various systems.[18,19,26−28] It was reported that **1** plays a pro-oxidative role in 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)-induced hemolysis of erythrocytes.[29] In the present study, levels of superoxide anion were significantly elevated in glioma cells treated with **1** at the concentrations used, at which significant autophagy occurred. Consistent with high levels of superoxide anion found in **1**-treated U87MG cells, inhibition of phosphorylation of Akt by **1**

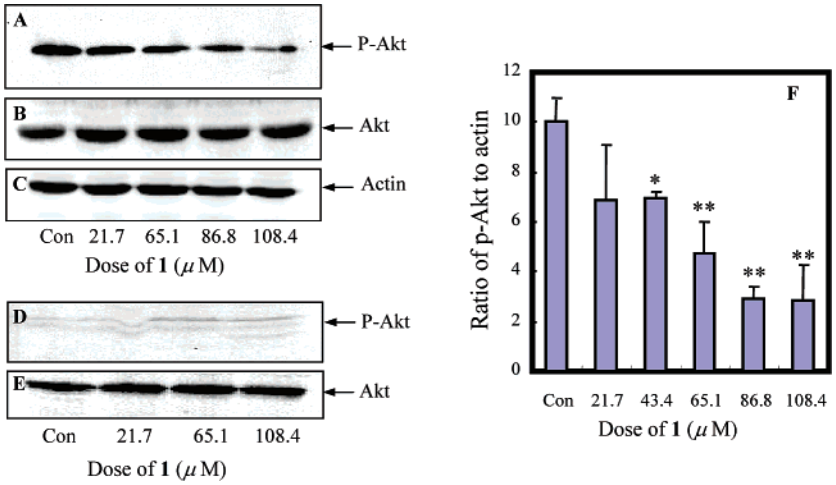

Figure 5. Akt phosphorylation in **1**-treated cells. U87MG and SF188 cells were treated with **1** at indicated concentrations for 7 and 5 h, respectively. Levels of phosphorylated Akt (P-Akt) or total Akt (Akt) were determined by western blotting and normalized with the amount of actin. (A and B) P-Akt and Akt levels in U87MG cells treated with **1**. (D and E) P-Akt and Akt levels in SF188 cells treated with **1**. (F) Normalized P-Akt levels in U87MG cells.

probably played an important role in the autophagic reaction by the cells. The PTEN-negative status in U87MG cells resulted in the constitutive activation of Akt and ILK.[21] It has been reported that the PI3K-dependent pathway might regulate toxic levels of ROS generated by oxidative stress[30] and that the PI3K/Akt pathway has been found to be important in regulating autophagy in various tumor cells including U87MG glioma cells.[25,31,32] Thus, suppressed Akt activity and elevated oxidative stress may result in the caspase-independent mechanism of **1** that induces programmed cell death in an autophagic fashion in U87MG cells. On the other hand, compound **1** was able to activate both caspase-dependent and -independent mechanisms in SF188 cells, resulting in both typical apoptotic and autophagic cell death, which is the reason for this cell line being more susceptible to cell death induced by **1**.

Our study has shown clearly that 20*S*-protopanaxadiol (**1**) causes programmed cell death through multiple pathways. We have applied this compound to many different cancer cells of various tissue origins and have yet to encounter a cell line that is not sensitive to it (data will be published separately). Given the genetic instability and heterogenic nature of cancer cell populations, inducing cell death through multiple pathways is obviously advantageous in cancer therapy. Our results from animal tumor models (to be published separately) have shown that a LD_{50} at the maximum dose of the compound could not be achieved in animals and an oral dose of 25 mg/kg was sufficient to cause complete inhibition in an intracranial rat glioma model, suggesting a reasonable bioavailability of this compound. Thus, compound **1** may be a potential lead compound for further structural modification for anticancer drug development. In addition, the different status of caspase activity in SF188 and U87MG treated with **1** also provides an interesting model for study of pathways responsible for caspase activation.

Experimental Section

General Experimental Procedures. Compound **1** (20*S*-protopanaxadiol) was provided by Pegasus Pharmaceuticals Inc. (Vancouver, BC, Canada). The compound was 99.7% pure as measured by HPLC analysis. An ApopTag Plus peroxidase *in situ* apoptosis detection kit was purchased from Chemicon International, Inc. (Temecula, CA). Antibodies for caspase-8 (polyclonal), caspase-3 (CPP32), and caspase-7 (monoclonal) were purchased from BD Biosciences (Mississauga, Ontario, Canada). Purified mouse anti-human caspase-9 antibody was purchased from Calbiochem (San Diego, CA). Phospho-Akt (Ser473) and Akt antibodies were purchased from Cell Signalling Technology, Inc. (Mississauga, Ontario, Canada). HRP-conjugated anti-rabbit or anti-mouse IgG was purchased from Perkin-Elmer Life Sciences (Boston, MA). Dihydroethidium (hydroethidine) was purchased from Molecular Probes (Leiden, The Netherlands). Z-VAD-FMK (general caspase inhibitor) was purchased from BD Biosciences. Protease inhibitor cocktails were obtained from Roche Molecular Biochemicals (Mannheim, Germany). A modified DC protein assay kit and nitrocellulose membranes were purchased from BIO-RAD (Hercules, CA). Thiazolyl blue tetrazolium bromide (MTT) and other chemicals were purchased from Sigma (St. Louis, MO). SF188 glioma cells were obtained from the Brain Tumor Research Center at the University of California at San Francisco. U87MG cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Media (Dulbecco's modified Eagle's medium, DMEM), PBS, and cell culture supplements (fetal bovine serum (FBS), antibiotics) were obtained from GIBCO BRL.

Cell Cultures and Treatments. Human glioma cells, SF188 and U87MG, were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2 and fed every 3−4 days. Cells were seeded in 96-well or six-well plates 24 h prior to each experiment, at a specific cell density for each experiment. A stock solution of 108 mM (50 mg/mL) **1** was prepared in 100% ethanol. Compound **1** was diluted in DMEM (supplemented with 2% FBS) immediately before each experiment (final ethanol concentration was 0.25% v/v). At selected time points, the treatments were terminated with the addition of MTT (for cell-viability assays) or 1× sample buffer (for western blotting). For caspase inhibition, Z-VAD-FMK was dissolved in DMSO to make a 10 mM stock solution and stored at −20 °C. Before use, the stock solutions were diluted with DMEM (with 2% FBS) to the desired concentrations. Cells were pretreated with 100 μL of inhibitor for 2 h at 37 °C in a 5% CO_2 incubator, followed by the addition of **1** at varying concentrations. Cell viability was determined with MTT assay.

MTT Assay. The viability of cultured cells was determined with an assay based on the reduction of MTT by viable cells. Cells were seeded in 96-well plates at a density of 2.5×10^4/well, 1 day prior to experiments. Cultures were then incubated with 2% FBS DMEM containing **1** for various periods from 3 to 24 h. At the end of each time point, the culture medium was removed and 100 μL of MTT (0.05 mg/mL in serum-free medium) was added to each well. Following incubation at 37 °C and 5% CO_2 for 4 h, 100 μL of lysis buffer (50 v/v *N*,*N*-dimethylformamide (Sigma), 20% SDS (BIO-RAD), and 0.4% (v/v) glacial acetic acid in distilled water (pH 4.8) were added and the plates were incubated overnight in a humidified atmosphere (37 °C with 5% CO_2). The optical density of each well was determined with a microplate reader (BIO-TEK, Winooski, VT) at 595 nm.

DNA Staining with ApopTag. The SF188 and U87MG cells were seeded on coverslips in six-well plates 24 h prior to treatment and then treated with **1** (108.4 μM) for 3 or 7 h, respectively. The ApopTag peroxidase in situ apoptosis detection kit, which detects DNA strand breaks as biochemical markers of apoptosis, was used as described by

the manufacturer. After staining, the slides counterstained by 0.5% (w/v) methyl green were mounted with Permount and viewed under a microscope (Zeiss, Oberkochen, Germany). Cells were considered apoptotic if they exhibited brown staining and characteristic apoptotic morphology such as cell shrinkage, pyknotic nuclei, and cell blebs or apoptotic bodies.

Protein Extraction and Western Blotting Assay. Cells were treated with **1** in 2% FBS DMEM for different times and concentrations. Both detached and attached cells (collected by scraping) were pooled and collected by centrifugation. The cells were washed with ice-cold PBS, lysed with the addition of 1× sample buffer (62.5 mM Tris-HCl at pH 6.8 and 25 °C, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue), and heated to 95−100 °C for 5 min. The protein concentration was measured using a modified DC protein assay (Bio-Rad). An equal amount of total protein (50 μg/lane) from each sample was used for immunoblot analysis. The proteins were resolved using SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semidry transfer system (BIO-RAD) for caspases and a wet transfer system (BIO-RAD) for Akt and Phospho-Akt. The membranes were blocked with 5% nonfat milk (BIO-RAD) for 1 h at room temperature and probed with primary antibodies at 4 °C overnight. The membranes were then washed three times with PBST (phosphate-buffered saline with 0.05% Tween 20) and incubated with HRP-conjugated anti-rabbit or mouse IgG (1:5000) for 2 h at room temperature. The reaction products were detected by enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA) using a Kodak image system (Kodak, Rochester, NY).

Superoxide Anion Staining Assay. Hydroethidine (HE) is a dye that specifically stains for the superoxide anion.[33] HE was dissolved in DMSO to make a 20 mM stock solution and stored at −20 °C in the dark. Cells were seeded in six-well plates 24 h before experiments. They were then treated with **1** for 2 h, followed by the addition of 2 mL of 5 μM HE (diluted in PBS) to each well. The cells were incubated for another 20 min at 37 °C and harvested with a plastic scraper. The cells were centrifuged at 1000*g* and washed twice with PBS, then resuspended in 500 μL of PBS for analysis with flow cytometry (excitation, 480 nM; emission, 567 nM).

Monodansylcadaverine (MDC) Staining Assay. The SF188 or U87MG cells were treated with different concentrations of **1** (0, 43.4, and 108.6 μM). After 4 h of treatment, the cells were stained with 50 μM monodansylcadaverine (MDC) (Sigma) in DMEM for 10 min at 37 °C, washed once with PBS, and viewed using an Axiovert 200 microscope under phase contrast and fluorescent filters equipped with Northern Eclipse software (Carl Zeiss, Oberkochen, Germany). For fluorescence measurement of MDC, the excitation wavelength was set to 335 nm and the emission wavelength was 525 nm. MDC-positive cells were those with accumulation of large MDC-stained red vesicles.

Statistical Analysis. Data were obtained from at least two independent experiments, each performed in triplicate or more. Data were analyzed for statistical significance by a two-tailed Student's *t*-test. Differences with *P*-values < 0.01 were regarded as statistically significant and are indicated by an asterisk (*) in the figures.

Acknowledgment. We thank Pegasus Pharmaceuticals Inc. (Vancouver, BC, Canada) for providing us the ginsenosides utilized in the present study. We appreciate Dr. S. Dadhar for his helpful suggestions in AKT assay. We also thank A. Shih for help with the tBHQ assay. This study was supported by The National Research Council of Canada.

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NP060313T