Gypenosides Suppress Growth of Human Oral Cancer SAS Cells In Vitro and in a Murine Xenograft Model: The Role of Apoptosis Mediated by Caspase-Dependent and Caspase-Independent Pathways

Kung-Wen Lu, MD, PHD1, Jung-Chou Chen, MD, PHD1, Tung-Yuan Lai, MD, PHD1,2, Jai-Sing Yang, PHD2, Shu-Wen Weng, MD1, Yi-Shih Ma, MD1, Hui-Yi Lin, PHD1, Rick Sai-Chuan Wu, MD, PHD2, King-Chuen Wu, MD, PHD2, W. Gibson Wood, PHD3, and Jing-Gung Chung, PhD1,4

Abstract

Purpose. Gypenosides (Gyp) are the major components of Gynostemma pentaphyllum Makino. The authors investigated the effects of Gyp on cell morphology, viability, cell cycle distribution, and induction of apoptosis in human oral cancer SAS cells and the determination of murine SAS xenograft model in vivo. Experimental design. Flow cytometry was used to quantify the percentage of viable cells; cell cycle distribution; sub-G1 phase (apoptosis); caspase-3, -8, and -9 activity; reactive oxygen species (ROS) production, intracellular Ca²⁺ determination; and the level of mitochondrial membrane potential (ΔΨm). Western blotting was used to examine levels of apoptosis-associated proteins, and confocal laser microscopy was used to examine the translocation of proteins in cells. Results. Gyp induced morphological changes, decreased the percentage of viable cells, caused G0/G1 phase arrest, and triggered apoptotic cell death in SAS cells. Cell cycle arrest induced by Gyp was associated with apoptosis. The production of ROS, increased intracellular Ca²⁺ levels, and the depolarization of ΔΨm were observed. Gyp increased levels of the proapoptotic protein Bax but inhibited the levels of the antiapoptotic proteins Bcl-2 and Bcl-xl. Gyp also stimulated the release of cytochrome c and Endo G. Translocation of GADD153 to the nucleus was stimulated by Gyp. Gyp in vivo attenuated the size and volume of solid tumors in a murine xenograft model of oral cancer. Conclusions. Gyp-induced cell death occurs through caspase-dependent and caspase-independent apoptotic signaling pathways, and the compound reduced tumor size in a xenograft nu/nu mouse model of oral cancer. Keywords
gypenosides, traditional Chinese medicine, caspase cascade, caspase independent, murine xenograft model, human oral cancer SAS cells
on a report from the National Institute of Health’s National Center for Alternative and Complementary Medicine. Cancer patients use CAM extensively as a mode of treatment or as a means to reduce the side effects of conventional treatments.8-10

Among the Chinese population, Gynostemma pentaphyl- lum Makino has been a popular folk medicine for centuries, and gypenosides (Gyp) in this plant species have been used to treat hepatitis,11 hyperlipoproteinemia,12,13 cardiovascular disease,14 and cancer.15 Gyp has biological activities that include anti-inflammatory,16 antithrombotic,17 antioxidative,18 and anticancer1,19,20 actions. We have reported that Gyp induced apoptosis in human lung cancer A549 cells,21 colon cancer colo 205 cells,22 and tongue cancer SCC-4 cells23 and also inhibited migration and invasion of human tongue cancer SCC-4 cells.24 Other investigators found that Gyp induced apoptosis in human hepatoma cells.1 However, there have not been any reports on Gyp-induced apoptosis in human oral cancer SAS cells in vitro or in vivo. Therefore, we investigated the effects of Gyp on induction of apoptosis in vitro and the determination of the SAS murine xenograft model in vivo.

Materials and Methods

Chemicals, Reagents, and Cell Culture

Gyp was kindly provided by Dr Jung-Chou Chen (Department of Chinese Medicine, China Medical University, Taichung, Taiwan).25 Dimethyl sulfoxide (DMSO), propidium iodide (PI), potassium phosphates, ribonuclease-A, Triton X-100, Tris–HCl, and trypan blue were obtained from Sigma Chemical Co (St Louis, MO). 2,7-Dichlorodihydrofluorescein diacetate, DiOC6, and Fluo-3/AM were obtained from Molecular Probes/Invitrogen Corp (Eugene, OR). Dulbecco’s modified Eagle’s medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from GIBCO BRL/Invitrogen Corp (Grand Island, NY). The SAS cell line (human oral squamous cell carcinoma) was obtained from Dr Pei-Jung Lu (Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan). Cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in 75 cm2 tissue culture flasks at 37°C under a humidified 5% CO2 and 95% air atmosphere as we have previously reported.22

In Vitro Studies

Assessment of cell morphology and viability. Gyp was prepared and dissolved in DMSO. Cells (2 × 10^5 cells/well) were plated in 12-well plates in 2 mL DMEM and incubated at 37°C for 24 hours. Cells were then treated with 0, 60, 90, 120, 150, and 180 µg/mL Gyp for 24 hours. DMSO was used as a vehicle control. At the end of the incubation period, cells were photographed with a phase-contrast microscope. They were then harvested, stained with PI (5 µg/mL) and analyzed by flow cytometry (Becton-Dickinson, San Jose, CA) as previously described.22,26,27

Flow cytometry analysis of sub-G1 phase in SAS cells. Cells were incubated with 0, 60, 90, 120, 150, and 180 µg/mL Gyp for different time periods, after which time they were harvested by centrifugation and stained with PI (5 µg/mL). The percentage of cells in the sub-G1 (apoptosis), G0/G1, S, and G2/M phases were determined by flow cytometry as previously described.22,28

4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining for apoptotic cell death. SAS cells were treated with or without Gyp (0, 60, 90, 120, 150, and 180 µg/mL) for 48 hours. They were then isolated, stained with DAPI, and photographed using a fluorescence microscope, as previously described.28,29

Detections of reactive oxygen species (ROS), intracellular Ca2+ levels, and mitochondrial membrane potential (ΔΨm) in SAS cells. SAS cells were treated with 180 µg/mL Gyp for 0.5, 1, 3, 6, 12, 24, and 48 hours. The cells were harvested and washed twice, resuspended in 500 µL of 2,7-dichlorodihydrofluorescein diacetate (10 µM) for determining changes of ROS, in 500 µL of Fluo-3/AM (2.5 µg/mL) for staining of intracellular Ca2+, and in 500 µL of DiOC6 (1 µmol/L) for determining ΔΨm. The cells were incubated at 37°C for 30 minutes and were analyzed by flow cytometry.28,30,31

Confocal laser scanning microscopy for protein translocation in SAS cells. SAS cells in DMEM were plated on 4-well chamber slides and treated without or with 180 µg/mL Gyp for 24 hours. Cells were then fixed in 4% formaldehyde in PBS (phosphate-buffered saline) for 15 minutes and permeabilized with 0.3% Triton-X 100 in PBS for 1 hour, with nonspecific binding sites being blocked using 2% BSA. Fixed cells were then incubated with primary antibodies to AIF, Endo G, cytochrome c, and GADD153 (1:100 dilution; green fluorescence) overnight and were then stained by a secondary antibody (FITC-conjugated goat antimouse IgG at 1:100 dilution), followed by DNA and mitochondria staining with PI and rhodamine 123 (red fluorescence). Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope.22,28

Determination of levels of certain proteins associated with apoptotic cell death. Cells were incubated with 180 µg/mL Gyp for 0, 6, 12, 24, 48, and 72 hours, isolated, and lysed, and specific protein levels associated with cell cycle arrest and apoptosis were determined. All samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis and Western blotting as previously described.22,28,29

Real-time polymerase chain reaction (PCR) for caspase-3, -8, -9 mRNA levels. Cells were incubated with 180 µg/mL Gyp for 0 and 48 hours. Total RNA was extracted using the
Qiagen RNeasy Mini Kit (Qiagen, Inc, Valencia, CA), and RNA samples were then reverse transcribed with the High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems, Carlsbad, CA) as previously described.27 Quantitative PCR was performed on 200 nM of forward (F) and reverse (R) primers (caspase-3, F-CAGTGGAGGCCGACTTCTTG, R-TGGCACAAGGCGACTGGAAT; caspase-8, F-GGATGGCACCTGTGAAACTGG, R-TGGGAGGATGCACTCTCTCTTT, R-GTGAGCCCACTGCTCAAAGAT; GAPDH, F-ACCTCA; caspase-9, F-TGTCCTACTCTACTTTCCAGGTGCCACTGTGAATAACTG, R-TCGAGGACATCGCTCTTCT). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicate, and fold changes in expression were derived using the comparative \( C_{\Delta\Delta} \) method.27,33

**Determinations of caspase-3, -8, and -9 activity.** Cells were treated with 180 µg/mL of Gyp for 0, 6, 12, 24, 48, and 72 hours. They were then collected by centrifugation; 50 µL of each 10 µM substrate solution (PhiPhiLux-G D12, for caspase-3, CaspLUX8-L D4 for caspase-8, and CaspLUX-M D2 for caspase-9; Oncolimmunin, Inc, Gaithersburg, MD) was added, and individual caspase activity was analyzed according to the manufacturer’s instructions using flow cytometry as previously described.34,35 Cells were also treated with 180 µg/mL of Gyp in the presence or absence of 10 µM of caspase-9 inhibitor (Z-IETD-FMK), 10 µM of caspase-8 inhibitor (Z-IETD-FMK), 10 µM of caspase-3 inhibitor (Z-DEVD-FMK), and 10 µM of general caspase inhibitor (Z-VAD-FMK, R&D Systems, Minneapolis, MN), or 5 mM of NAC (Sigma Chemical Co) for ROS and 5 µM of BAPTA (Ca2+ chelator, Molecular Probes/Invitrogen Corp.) for intracellular Ca2+. Cell viability was determined as previously described.21,36

**In Vivo Studies**

**SAS mouse xenograft model.** A total of 18 male athymic BALB/c nude mice 4 to 6 weeks of age were purchased from the National Laboratory Animal Center of National Applied Research Laboratories (Taipei, Taiwan). Mice were maintained on a commercial diet and water ad libitum. For the injections, SAS cells were resuspended in serum-free DMEM. The cell suspension (5 \( \times \) 10^6 cells/0.2 mL) was subcutaneously injected into the flanks of mice for an 8-day incubation period. After xenografts reached volumes of 200 mm^3, treatment was initiated. Body weight and tumor size were measured every 2 days. Mice bearing tumors were randomly divided into 3 treatment groups and intraperitoneally injected every 2 days in the morning with 30 µL of DMSO control vehicle, 2 mg/kg doxorubicin, and 20 mg/kg Gyp. The measurement of tumor volume was based on the following formula: Tumor volume (mm^3) = \( L \times W^2/2 \) (\( L \) is the length and \( W \) the width). At the end of the 28 days of treatment, mice were killed, and tumors were measured, and weighted as previously described.33,37,38 All animal studies were conducted according to institutional guidelines (Affidavit of Approval of Animal Use Protocol) approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

**Statistical Analysis**

Data of control and experimental groups were expressed as mean ± standard deviation for at least 3 separate experiments. Statistical analyses of the data were performed using Student’s t test and 1-way analysis of variance (ANOVA). Statistical significance was set at \( P < .05 \).

**Results**

**In Vitro Studies**

**Effects of Gyp on morphology, viability, cell cycle distribution, and sub-G1 phase of SAS cells.** Cells were morphologically altered by Gyp treatment as shown in Figure 1A. There were fewer viable cells as incubation time and Gyp concentration increased (Figure 1B). It can be seen in Figure 1C that there was an increase in the percentage of cells in G0/G1 (enhanced G0/G1 peak) and a decrease in the percentage of cells in the S phase. The sub-G1 group also appeared in the cell cycle (Figure 1C), and increasing concentrations of Gyp led to an increase in G0/G1- and sub-G1 phases.

**Gyp-induced apoptosis in SAS cells.** Induction of apoptosis by Gyp in SAS cells was confirmed by DAPI staining, as seen in Figure 2A, which showed that Gyp induced nuclei condensation. These effects were time dependent as noted in Figure 2B. Higher concentrations of Gyp resulted in a greater number of apoptotic cells being stained.

**Gyp induced production of ROS and intracellular Ca2+ and reduction of mitochondrial membrane potential (\( \Delta \Psi_m \)) in SAS cells.** Gyp induced ROS production quite early, and this was time dependent, as seen in Figure 3A. After 0.5 hours of incubation, there was a decrease in ROS levels in the Gyp group. Gyp stimulated intracellular Ca2+ release, which was time dependent (Figure 3B), and reduced the levels of \( \Delta \Psi_m \) in a time-dependent manner (Figure 3C). Both ROS and intracellular Ca2+ were increased by Gyp (Figure 3). We next determined if the ROS scavenger (NAC) and Ca2+ chelator (BAPTA) could inhibit effects of Gyp on cell viability and apoptosis. It can be seen in Figure 3 (panels A, B, C, and D) that Gyp increased the levels of ROS, but NAC protected Gyp-treated SAS cells against induction of cell death (Figure 4A) and reduction of apoptotic cells (Figure 4C). Gyp increased intracellular Ca2+, but BAPTA was protective (Figure 4B) and reduced apoptotic SAS cell numbers (Figure 4D). NAC and BAPTA significantly blocked Gyp-induced cell death.
Gyp increased the translocation of proteins in SAS cells. The results from confocal laser microscopy indicated that the protein levels of AIF (Figure 5A), Endo G (Figure 5B), cytochrome c (Figure 5C), and GADD153 (Figure 5D) were increased when compared with the levels in controls. It can be seen that cytochrome c is released from mitochondria, as seen by the higher green fluorescence intensity. AIF, Endo G, and GADD153 trafficked to nuclei, which indicated higher fluorescence in SAS cells.

Effects of Gyp on levels of proteins associated with cell cycle and apoptosis. Results are presented in Figures 6A, 6B, 6C, and 6D (A: chk2, p53, p21, Cdc25A, Cyclin E, Cyclin A, Cdk2; B: Bax, Bcl-2, puma, cytochrome c, Endo G, AIF, XIAP, CAD, Caspase-9, Caspase-3, caspase-7; C: TRAIL, Bid, Fas, FasL; D: ATF-4, GRP78, and GADD153). Gyp treatment reduced the levels of cdc25A, cyclin E and A and cdk2 (Figure 6A), Bcl-2 and XIAP (Figure 6B), and Bid.
Lu et al (Figure 6C), whereas the levels of p53 and p21 (Figure 6A) were increased. Gyp increased protein levels of Bax, puma, cytochrome c, Endo G, AIF, CAD, caspase-9 and caspase-3 (Figure 6B), TRAIL, Fas and FasL (Figure 6C), ATF-4, GRP78, and GADD153 (Figure 6D) but reduced levels of Bcl-2 and XIAP (Figure 6B) and Bid (Figure 6C). Expression levels of mRNA of caspase-3, -8, and -9 were increased after Gyp treatment (Figure 6E).

Gyp increased productions of reactive oxygen species (ROS) and intercellular Ca$^{2+}$ and decreased the level of mitochondria membrane potential ($\Delta\Psi_m$) in human oral cancer SAS cells. Cells were cultured in DMEM + 10% FBS with 150 μg/ml Gyp for 0.5, 1, 3, 6, 9, 12, 24 and 48 h, before being collected, stained by DCFH-DA and the ROS levels determined (A and B), stained by Fluo-3/AM and the intracellular Ca$^{2+}$ levels determined (C and D) and stained with DiOC6 and the $\Delta\Psi_m$ levels determined (E and F) as described in Materials and Methods. *$P < 0.05$ was considered statistically significant when compared with control.

**Figure 3.** Gypenosides (Gyp) increased productions of reactive oxygen species (ROS) and intercellular Ca$^{2+}$ and decreased the level of mitochondria membrane potential ($\Delta\Psi_m$) in human oral cancer SAS cells. Cells were cultured in DMEM + 10% FBS with 150 μg/ml Gyp for 0.5, 1, 3, 6, 9, 12, 24 and 48 h, before being collected, stained by DCFH-DA and the ROS levels determined (A and B), stained by Fluo-3/AM and the intracellular Ca$^{2+}$ levels determined (C and D) and stained with DiOC6 and the $\Delta\Psi_m$ levels determined (E and F) as described in Materials and Methods. *$P < 0.05$ was considered statistically significant when compared with control.

**Gyp stimulated activity of caspase-3, -8, and -9.** Data seen in Figure 7 indicate that Gyp increased the activity of caspase-3, caspase-8, and caspase-9, and these effects were time dependent. To determine whether caspase activation was involved in apoptotic cell death, cells were pretreated with inhibitors of caspase-3, caspase-8, and caspase-9 and a general inhibitor of caspases. As shown in Figures 8A, 8B, 8C, and 8D, these inhibitors significantly blocked Gyp-triggered cell death.
Gyp inhibited tumor size in a xenograft mouse model. Results from our in vitro studies indicated that Gyp induced cell cycle arrest and apoptosis in SAS cells through mitochondrial-dependent and -independent pathways. We next examined the in vivo antitumor activities of Gyp in BALB/c nu/nu nude mice injected with SAS cells and treated with Gyp and doxorubicin (positive control). Representative tumors in the xenograft mice treated with or without Gyp are shown in Figure 9A. Treatment with Gyp and doxorubicin did not significantly alter body weight (Figures 9B and 9D). Gyp significantly (*P < .01) decreased the tumor weight by 34%, compared with controls, after treatment from the 10th to the 28th day, as shown in Figure 9C. In Figures 9A and 9C, it can be seen that doxorubicin (2 mg/kg) also significantly reduced tumor mass by ~49% after 28 days of treatment. Overall, the tumors in treatment groups were significantly smaller than those in the control group.

Discussion

We had previously reported that Gyp induced apoptosis in human colon cancer cells, which was mitochondria dependent and involved caspase-3 activation,23 and in human tongue cancer SCC-4 cells through endoplasmic reticulum (ER) stress and mitochondria-dependent pathways.22 Effects of Gyp on human oral cancer cells have not been examined, and the purpose of the present study was to determine whether or not Gyp induced S phase arrest and apoptosis in human oral cancer SAS cells. We found that Gyp induced cell death through cell cycle arrest and induction of apoptosis. Apoptosis followed Gyp-induced S phase arrest. Gyp may have therapeutic efficacy in the treatment of oral cancers.

Gyp-induced morphological changes and reduced viability of SAS cells were dose and time dependent. Gyp induced...
Figure 5. Effects of gypenosides (Gyp) on AIF, Endo G, cytochrome c, and GADD153 distribution in human oral cancer SAS cells. Cells were cultured in Dulbecco's modified Eagle's medium + 10% fetal bovine serum and treated with Gyp at 180 μg/mL for 24 hours. Cells were harvested, fixed, and stained with primary antibodies to AIF (A), Endo G (B), cytochrome c (C), and GADD153 (D), and then, FITC-labeled secondary antibodies were used (green fluorescence); the proteins were detected by a confocal laser microscopic system. The nuclei and mitochondria were stained by propidium iodide and rhodamine 123, respectively (red fluorescence). Areas of colocalization between AIF, Endo G, cytochrome c, and GADD153 expressions and cytoplasm and nuclei in the merged panels are yellow. Scale bar, 20 μm.
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cell cycle arrest, and the compound altered levels of proteins associated with the S phase in SAS cells. Protein levels of CDK2, Cyclin A, and Cyclin E were reduced. Furthermore, in model cells, it has been shown that upregulation of p53 was sufficient to activate protein kinase c-mediated p53 gene transcription, induce G1 phase arrest, and promote cellular repair mechanisms. Gyp-induced apoptosis in SAS cells was also confirmed by DAPI staining and DNA gel electrophoresis (data not shown). Gyp-induced apoptosis in SAS cells and these effects were dose and time dependent. It is well documented that the Bcl-2 family plays an important role in apoptosis with respect to both antiapoptotic and proapoptotic signaling. The ratio of Bcl-2 to Bax is an indicator of sensitivity or resistance to apoptotic stimuli.

Figure 6. Gypenosides (Gyp) altered the associated protein levels in human oral cancer SAS cells. Representative Western blotting showing changes in the levels of associated proteins in S-phase arrest and apoptosis of human oral cancer SAS cells after exposure to Gyp. SAS cells were cultured in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and treated with Gyp at 180 μg/mL for various periods of time (0, 6, 12, 24, 48, and 72 hours). The total protein was prepared and determined as described in the Materials and Methods section. The levels of associated protein expression (A: chk2, p53, p21, Cdc25A, Cyclin E, Cyclin A, Cdk2; B: Bax, Bcl-2, puma, cytochrome c, Endo G, AIF, XIAP, CAD, Caspase-9, Caspase-3, Caspase-7; C: TRAIL, Bid, Fas, FasL; D: ATF-4, GRP78, and GADD153) were estimated by Western blotting. E: The mRNA levels of caspase-3, -8 and -9 were shown and measured by real-time PCR as described in the Materials and Methods section.
Figure 7. Gypenosides (Gyp) stimulated the activities of caspase-3, -8, and -9 of SAS cells. Cells were plated onto 12-well plates in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and were then incubated with 180 μg/mL Gyp for 24 hours. Cells were harvested from each sample for caspase-3 (A), -8 (C), and -9 (E) as described in the Materials and Methods section. Each experiment was done with triplicate sets. *P < .05, significantly different comparing Gyp treatment with control.

Figure 8. Gypenosides (Gyp) reduced the percentage of viable cells via caspase-3, -8, and -9 of SAS cells. Cells were plated onto 12-well plates in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum, which were preincubated with or without inhibitors of caspase-3, -8, and -9 and general inhibitor of caspases. They were then incubated with 180 μg/mL Gyp for 24 hours. Cells were harvested from each sample for percentage of viable SAS cells as described in the Materials and Methods section. A, caspase-3 inhibitor; B, caspase-8 inhibitor; C, caspase-9 inhibitor; D, a general caspase inhibitor. *P < .05, significantly different comparing Gyp treatment with control.
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Figure 9. Gypenosides (Gyp) inhibited tumor growth in the xenograft animal model: 18 athymic BALB/c nu/nu nude mice were subcutaneously implanted with $5 \times 10^6$ SAS cells for 8 days and then randomly divided into 3 groups. Group 1 was treated with dimethyl sulfoxide (DMSO) only. Group 2 was treated with 2 mg/kg doxorubicin, and group 3 was treated with 20 mg/kg Gyp. At 28 days, all mice from each group were killed. (A) Representative animal solid tumor and tumor weight; (C) percentage inhibition of tumor volume and (B and D) of body weight. Data presented are mean ± standard deviation at 8 to 28 days after tumor implantation, and the tumor volumes observed in DMSO, Gyp, and doxorubicin groups were compared as analyzed by 1-way ANOVA. Data represent mean ± standard deviation of 6 animals.

Abbreviation: N.S., not significant.

*P < .05, significantly different compared with control.

Figure 3A indicates that Gyp promoted ROS production in the earlier time periods of treatment of SAS cells. Much evidence suggests that ROS play an important role in apoptosis induced by certain chemopreventive agents via the engagement of downstream proteins involved in the execution of apoptosis. The intracellular generation of $H_2O_2$ (the most stable ROS) has been shown to be an important mediator of apoptosis, including exogenous addition of $H_2O_2$. $H_2O_2$ can diffuse into the mitochondria and may cause damage to the mitochondrial membrane. Results from Figure 3B indicate that Gyp increased $Ca^{2+}$ levels at earlier time points, suggesting that Gyp treatment induced ER stress, which elicited a rise in intracellular $Ca^{2+}$ and subsequent mitochondrial membrane depolarization (decreased the levels of $\Delta \Psi_m$) in SAS cells. This finding is in agreement with other reports that $Ca^{2+}$-mediated signaling is involved in Gyp-induced apoptosis.

In the present study, Gyp increased Bax levels but reduced Bcl-2 levels. Gyp decreased the levels of $\Delta \Psi_m$ and promoted the release of cytochrome c, AIF, and Endo G and increased both the levels and activity of caspase-9 and -3 resulting in apoptosis. Gyp-induced cell survival or death may occur through the interaction of Bcl-2- and p53-mediated mechanisms followed by caspase-9 and -3 activation. One conclusion is that Gyp-induced apoptosis involves multisignaling pathways that are caspase dependent and independent. To further understand the role of Gyp we used an in vivo SAS xenograft mouse model and found that Gyp decreased the weight and size of SAS cells tumors, which is certainly in agreement with our in vitro results. Findings of the present study provide new insight into possible multiple signaling pathways of Gyp-induced apoptosis in human oral cancer SAS cells. It is well known that certain products from plants are known to induce apoptosis in tumor cells. Understanding the mech-
anisms of Gyp on tumor cells may provide valuable information that can be used in cancer therapy and prevention.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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