

## Gyenosides Induced Apoptosis in Human Colon Cancer Cells through the Mitochondria-dependent Pathways and Activation of Caspase-3

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**Abstract.** The effects of the gyenosides (Gyp), a component of *Gynostemma pentaphyllum* Makino, on the cell viability, cell cycle and induction of apoptosis were investigated in human colon cancer colo 205 cells. Gyp was cytotoxic to colo 205 cells with an  $IC_{50}$  of 113.5  $\mu$ g/ml. The decreasing number of viable cells appeared to be due to the induction of cell cycle arrest and apoptotic cell death, since Gyp induced morphological changes and internucleosomal DNA fragmentation and increased the sub-G1 group. The production of reactive oxygen species and  $Ca^{2+}$  and the depolarization of mitochondrial membrane potential were observed, dose- and time-dependently, after treatment with various concentrations of Gyp. Gyp treatment also gradually decreased the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl, but increased the expression of the pro-apoptotic protein Bax. Gyp increased the levels of p53 and promoted the release of cytochrome c and the activation of caspase-3 before leading to apoptosis. These results provide information towards an understanding of the mechanisms by which Gyp induces cell cycle arrest and apoptosis in human colon cancer cells.

A number of compounds purified from plants and their derivatives have been demonstrated to show antitumor activity, such as paclitaxel which is derived from *Taxus brevifolia* (1, 2). The gyenosides (Gyp) are the major

components which are extracted from *Gynostemma pentaphyllum* Makino, a popular folk medicine in China. It was reported that Gyp treated hepatitis (3), hyperlipoproteinemia (4, 5), cardiovascular disease (6) and cancer (7). Gyp also had anti-inflammatory (8), antithrombotic (9), antioxidative (10) and anticancer (11-14) characteristics. Recently, this laboratory reported that Gyp induced apoptosis in human hepatoma cells through the activation of the caspase cascade and regulation of the Bcl-2 family (14). We also discovered that Gyp affected N-acetyltransferase activity and gene expression in human cervical cancer cells (15). However, the mechanism by which Gyp exert their cytotoxicity, in human colon cancer cells, is still unclear. To date, the major strategy for anticancer agents in killing cancer cells has been to induce cell cycle arrest and apoptosis, but with minimal effects on normal cells. Therefore, in order to gain a better understanding of Gyp toxicity, this study characterized the pattern of cell death induced by Gyp in human colon cancer colo 205 cells. The mechanisms underlying the induction of cell cycle arrest and apoptosis were investigated, with particular focus on the production of reactive oxygen species,  $Ca^{2+}$ , and mitochondrial membrane potential, the expressions of Bcl-2, Bax, cytochrome c, p53 and the activation of caspase-3, with the resulting DNA fragmentation.

### Materials and Methods

**Chemicals and reagents.** Gyp, aprotinin, antipain, triton X-100, leupeptin, propidium iodide (PI), ribonuclease-A, sodium deoxycholate, sodium orthovanadate, Tris-HCl and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

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**Key Words:** Gyenosides, apoptosis, mitochondria-dependent pathways, caspase-3, bcl-2, Bax, p53, cell cycle, ROS, cyclin E, cyclin D3, CDK4.

*Human colon cancer cell line (colo 205).* The human colon adenocarcinoma cell line, colo 205, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C). The cells were immediately placed into 75-cm<sup>3</sup> tissue culture flasks with RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% glutamine, and grown at 37°C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere (16).

*Cell morphology and viability was determined by using contrast-phase microscopy and flow cytometry.* The colo 205 cells were placed in 12-well plates at a density of 5x10<sup>3</sup> cells/well and incubated at 37°C for 24 h. The various concentrations of Gyp (0, 60, 90, 120, 150 and 180 µM) were added and the cells were incubated for various periods of time. DMSO (solvent) was used for the control regimen. For cell morphology, the cells in the plate were examined under a phase-contrast microscope and photographed (17). To determine cell viability, the flow cytometric protocol was used, as previously described (16, 17).

*Flow cytometry analysis of cell cycle and apoptosis in colo 205 cells co-treated with different concentrations of Gyp.* Approximately 5x10<sup>5</sup> colo 205 cells/well/12-well plate were incubated with various concentrations of Gyp (0, 60, 90, 120, 150 and 180 µM) for various time-periods before the cells were harvested by centrifuging, and the percentage of cells in the sub-G1 (apoptosis), G0/G1-, S- and G2/M-phases were determined by flow cytometry, as described previously (16, 17).

*Fluorescence microscope examining the Gyp-induced apoptosis of colo 205 cells by using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.* Approximately 5x10<sup>4</sup> colo 205 cells/ml were treated with or without various concentrations of Gyp at 0, 60, 90, 120, 150 and 180 µM for 48 h, before isolation of the cells for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, as described previously (18).

*Comet assay examining the Gyp-induced DNA damage of colo 205 cells.* Approximately 5x10<sup>4</sup> colo 205 cells/ml were treated with or without various concentrations of Gyp at 0, 60, 90, 120, 150 and 180 µM for 24 h before cell isolation to examine the DNA damage with the Comet assay described previously (18).

*DNA gel electrophoresis to examine the Gyp-induced DNA fragmentation (apoptosis) of colo 205 cells.* Approximately 5x10<sup>6</sup> colo 205 cells/ml were treated with Gyp at 150 µM for different periods of time (0, 12, 24 and 48 h), before isolating the cells to extract DNA to be used in DNA gel electrophoresis, as described previously (18).

*Flow cytometry examining the levels of reactive oxygen species (ROS) in colo 205 cells by using 2,7-dichlorodihydrofluorescein diacetate (DiOC<sub>6</sub>) staining.* Approximately 5x10<sup>5</sup> colo 205 cells/ml were treated with Gyp at 150 µM for various periods of time (0, 1, 2, 4, 6 and 8 h). The cells were harvested and washed twice, re-suspended in 500 µl of 2,7-dichlorodihydrofluorescein diacetate (10 µM) (DCFH-DA, Sigma) and incubated at 37°C for 30 min to detect the changes of ROS before flow cytometry, as described previously (Becton Dickinson FACS Calibur) (18, 19).

*Flow cytometry examining the levels of Ca<sup>+2</sup> in colo 205 cells by using Indo 1/AM staining.* Approximately 5x10<sup>5</sup> colo 205 cells/ml were treated with Gyp at 150 µM for various time-periods (0, 0.5, 1, 2, 3,

3.5 and 4 h) before isolating the cells to detect any changes in Ca<sup>+2</sup> concentration. The cells were harvested by centrifuging and washed twice for re-suspension in Indo 1/AM (3 µg/ml) (fluorescent dye for staining of Ca<sup>+2</sup>; Calbiochem, La Jolla, CA, USA) incubated at 37°C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (18, 20).

*Flow cytometry examining the levels of mitochondrial membrane potential in colo 205 cells by using DiOC<sub>6</sub> staining.* Approximately 5x10<sup>5</sup> colo 205 cells/ml were treated with Gyp at 150 µM for various time-periods (0, 0.5, 1, 2, 3, 4 and 6 h) to detect changes in the mitochondrial membrane potential. The cells were harvested and washed twice, re-suspended in 500 µl of DiOC<sub>6</sub> (4 mol/L), incubated at 37°C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (18, 21).

*Examination of proteins associated with the cell cycle and apoptosis in Gyp-treated cells.* Approximately 5x10<sup>6</sup> colo 205 cells/ml were treated with Gyp at 150 µM for various time-periods (0, 6, 12, 24, 48 and 72 h), before isolating the cells to detect the proteins associated with the cell cycle and apoptosis. Isolated cells with or without Gyp treatment will be lysed and quantified protein levels. All samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, as described previously (16-18).

## Results

*Gyp effects on the cell morphology and the viability of human colon cancer colo 205 cells.* After the cells had been treated with various concentrations of Gyp for various time-periods, the cells were photographed by phase-contrast microscope, before being collected for propidium iodine staining and flow cytometric analysis. The results indicated that the cells were increasingly morphologically-changed by Gyp treatment and there were fewer viable cells as the time and concentration increased, suggesting that Gyp induced morphological changes and cell death on the colo 205 cells (Figures 1 and 2).

*Gyp effects on the cell cycle arrest and apoptosis in human colon cancer colo 205 cells.* The flow cytometry results for cell cycle and apoptosis analysis indicated that, during 48-h Gyp treatment, 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 S. The sub-G1 groups also appeared in the cell cycle, which meant that Gyp induced apoptosis in these examined cells (Figure 3). Increased concentrations of Gyp led to an increase in G0/G1- and sub-G1-phases in the colo 205 cells.

*Gyp induced apoptosis in human colon cancer colo 205 cells.* Fluorescent (DAPI) microscopy showed that Gyp induced apoptosis in the colo 205 cells. Increasing the concentration of Gyp led to more apoptotic cells (Figure 4). DNA gel electrophoresis indicated that Gyp induced DNA

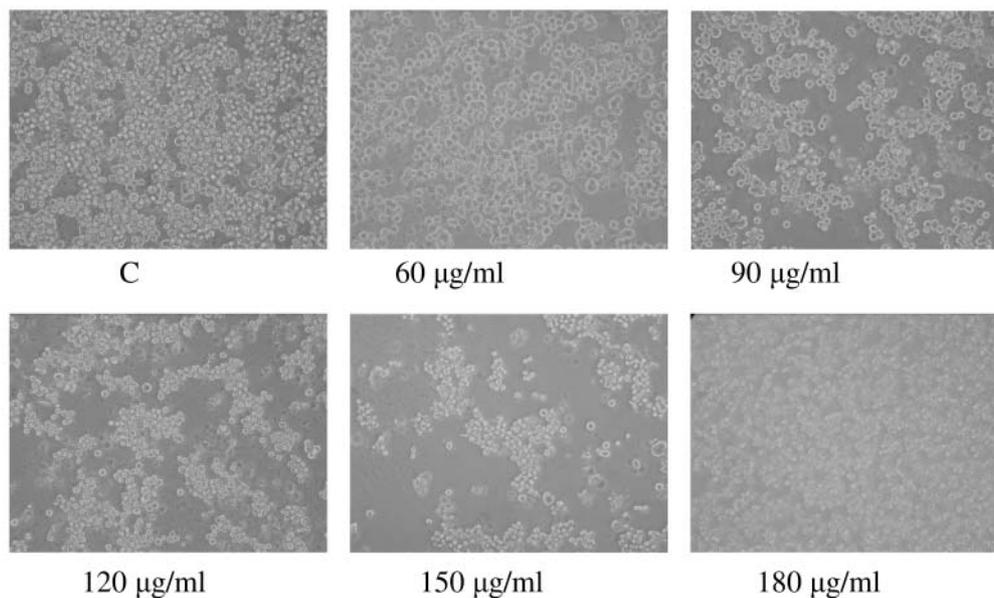


Figure 1. Gyp effects on cell morphology of human colon cancer colo 205 cells. Cells ( $5 \times 10^3$  cells/well; 12-well plates) were cultured in RPMI 1640 + 10% FBS with various concentrations of Gyp (panel A) for 24 h. The cells were examined and photographed by phase-contrast microscope, as described in Materials and Methods. Each point is mean  $\pm$  S.D. of three experiments. \* $p < 0.05$ .

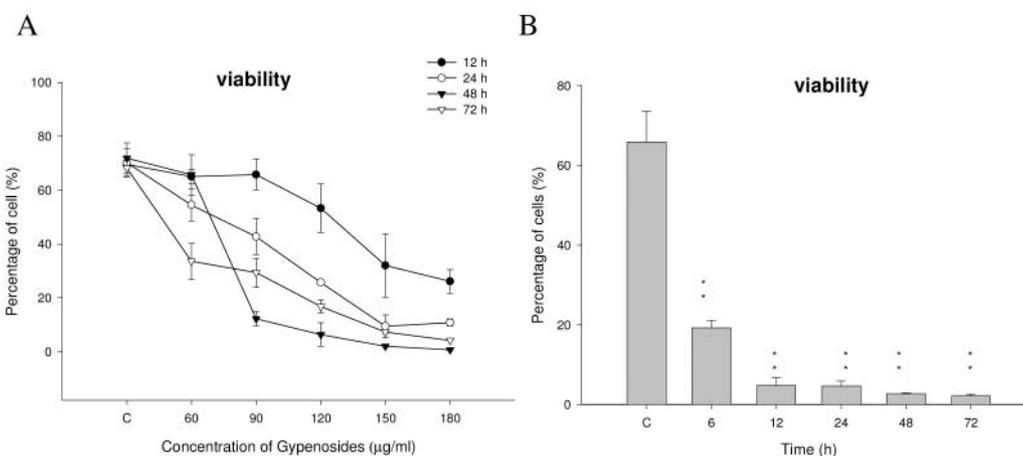


Figure 2. Gyp effects on the viability of human colon cancer colo 205 cells. Cells ( $5 \times 10^3$  cells/well; 12-well plates) were cultured in RPMI 1640 + 10% FBS with various concentrations of Gyp (panel A) for various time-periods or 150 µM Gyp for 0, 6, 12, 24, 48 and 72 h (panel B). The cells were collected by centrifuging and the viable cells were determined by flow cytometry, as described in Materials and Methods. Each point is mean  $\pm$  S.D. of three experiments. \* $p < 0.05$ .

fragmentation, therefore, apoptosis too were dose-dependent (Figure 5).

**Gyp-induced DNA damage in human colon cancer colo 205 cells examined by Comet assay.** The Comet assay showed that Gyp induced DNA damage in the colo 205 cells by fragmentation. Higher concentrations of Gyp led to more apoptotic cells being stained (Figure 6).

**Gyp-induced production of reactive oxygen species (ROS) in human colon cancer colo 205 cells.** After the colo 205 cells had been treated with Gyp for various time-periods, ROS production was analyzed and quantitated by flow cytometry. The data demonstrated that Gyp induced ROS production quite early and time-dependently (Figure 7). The representative profiles of DAPI staining are shown in Figure 7.

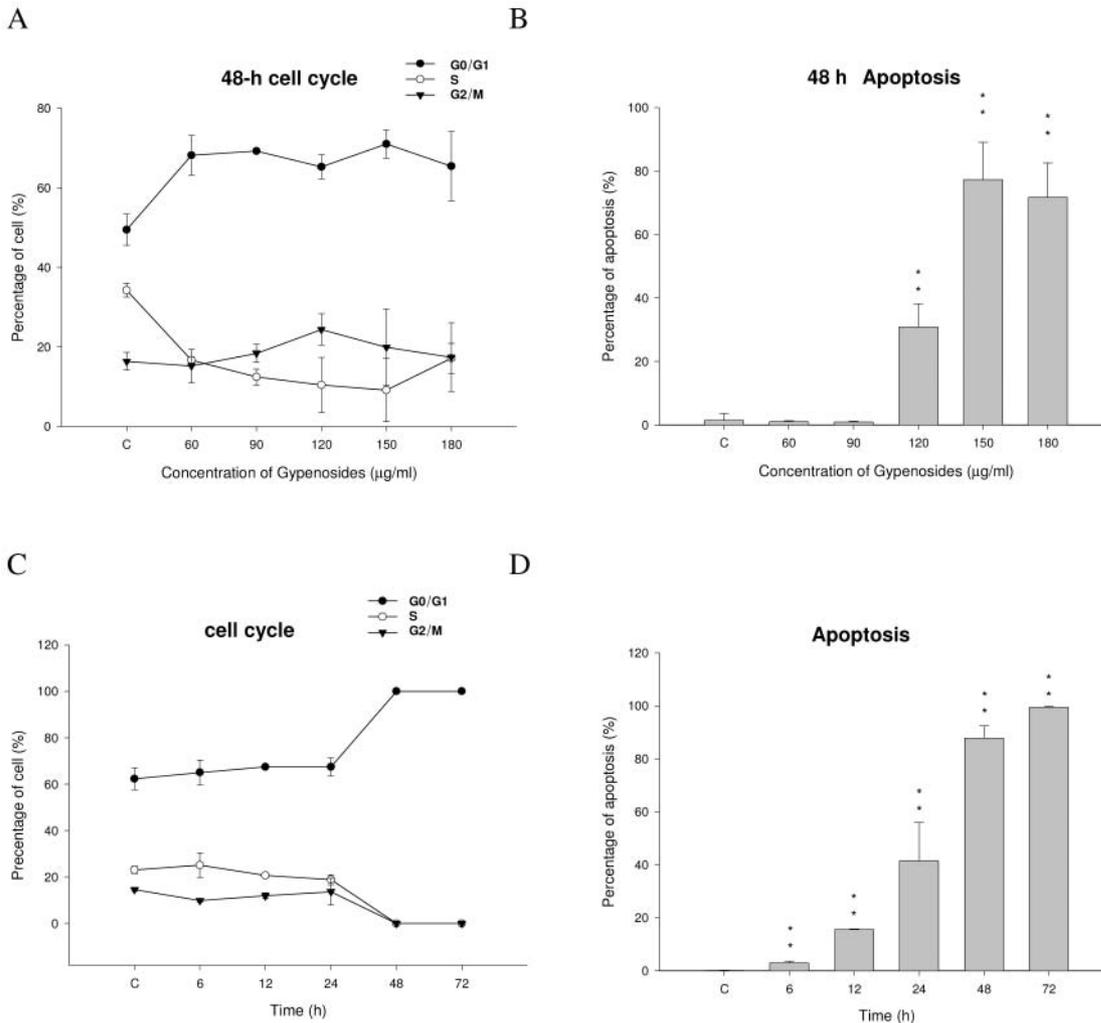


Figure 3. Gyp effects on the cell cycle arrest and apoptosis in human colon cancer colo 205 cells. Cells ( $5 \times 10^5$  cells/well; 12-well plates) were exposed to various concentrations of Gyp for 48 h or 150 µM Gyp for 0, 6, 12, 24, 48 and 72 h, and the cells were harvested and analyzed for cell cycle (panel A: representative profiles; panel B: percent of cells per phase) and the sub-G1 group (panel C: percent of cells in apoptosis) by flow cytometry, as described in Materials and Methods. Data represents mean  $\pm$  S.D. of three experiments. \* $p < 0.05$ .

Gyp-induced production was of  $Ca^{2+}$  in human colon cancer colo 205 cells. After the colo 205 cells had been treated with Gyp for various periods of time, the cells were harvested and  $Ca^{2+}$  production was analyzed and quantitated by flow cytometry. The data demonstrated that Gyp induced  $Ca^{2+}$  production time-dependently (Figure 8). The representative profiles of DiOC<sub>6</sub> staining from the flow cytometry are given in Figure 7 and indicate that increased time led to increased  $Ca^{2+}$  production after the cells had been treated with 150 µM Gyp.

Gyp-induced levels of mitochondrial membrane potential ( $\Delta\Psi_m$ ) in human colon cancer colo 205 cells. After the colo 205 cells had been treated with Gyp for various time-periods, the levels of  $\Delta\Psi_m$  were analyzed and quantitated by flow

cytometry. The data demonstrated that Gyp increased the loss of mitochondrial  $\Delta\Psi_m$  in a time-dependent manner (Figure 9). The representative profiles of DiOC<sub>6</sub> staining from flow cytometry are shown in Figure 9 and indicate that increased time led to the loss of mitochondrial  $\Delta\Psi_m$  after the cells had been treated with 150 µM Gyp.

Gyp effects on the expressions of cyclin D3, cyclin E, CDK4, CDK6, CDK2, p15, p16, p21, p27, p53, CDC25A, Bax, Bcl-2, Bcl-xl, cytochrome c, caspase-9, caspase-3, NFκB (p50) and NFκB (p65) of colo 205 cells. In order to understand the molecular mechanism of Gyp-induced G0/G1 arrest and apoptosis in the colo 205 cells, the expressions of cell cycle- and apoptosis-associated proteins during the treatment with

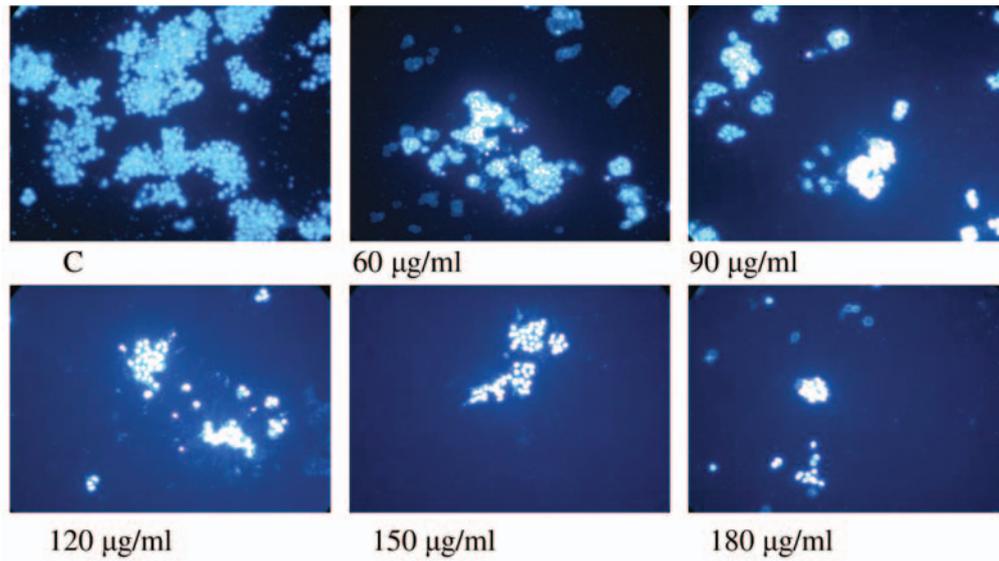


Figure 4. Gyp-induced apoptosis in human colon cancer colo 205 cells examined by DAPI staining. Cells ( $5 \times 10^4$  cells/well; 12-well plates) were incubated with various concentrations of Gyp for 48 h and apoptosis was determined by DAPI staining, as described in Materials and Methods.

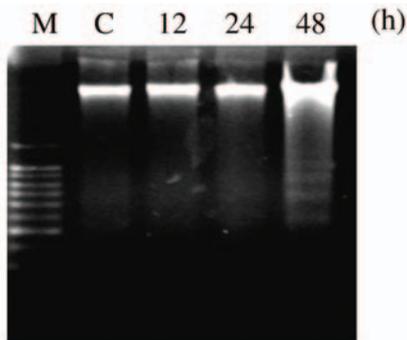


Figure 5. Gyp-induced DNA fragmentation in human colon cancer colo 205 cells examined by DNA gel electrophoresis. Cells ( $5 \times 10^6$  cells/well; 12-well plates) were incubated with various concentrations of Gyp for 48 h, the DNA extracted, then DNA fragmentation was examined by DNA gel electrophoresis, as described in Materials and Methods.

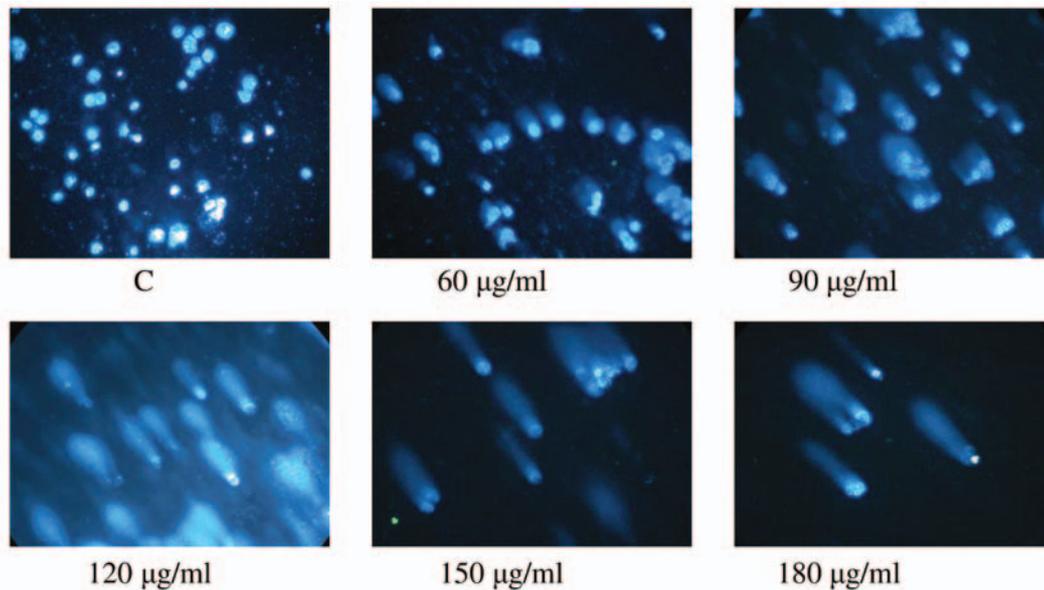


Figure 6. Gyp-induced DNA damage in human colon cancer colo 205 cells examined by Comet assay. Cells ( $5 \times 10^4$  cells/well; 12-well plates) were incubated with various concentrations of Gyp 24 h and the DNA damage was examined by Comet assay, as described in Materials and Methods.

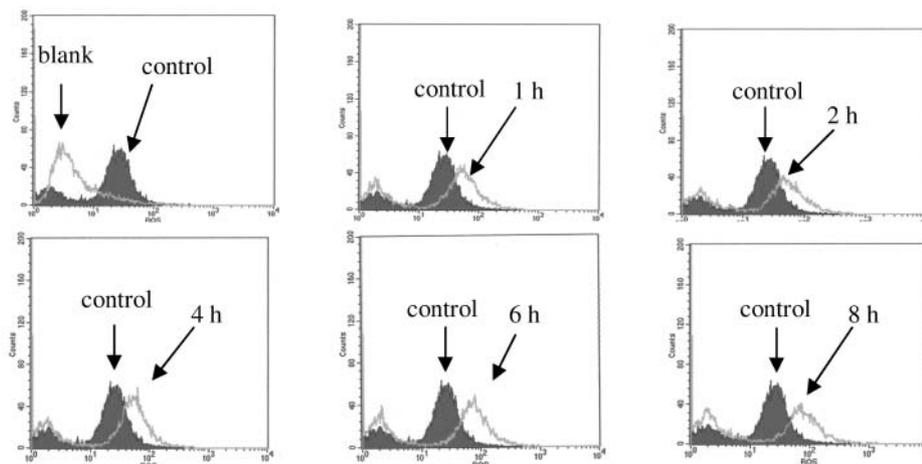


Figure 7. Gyp-induced production of reactive oxygen species (ROS) in human colon cancer colo 205 cells. Cells ( $5 \times 10^5$  cells/well ) were treated with  $150 \mu\text{M}$  Gyp for 1, 2, 4, 6 and 8 h, before being collected, stained by 2,7-dichlorodihydrofluorescein diacetate and the ROS levels were determined, as described in Materials and Methods.

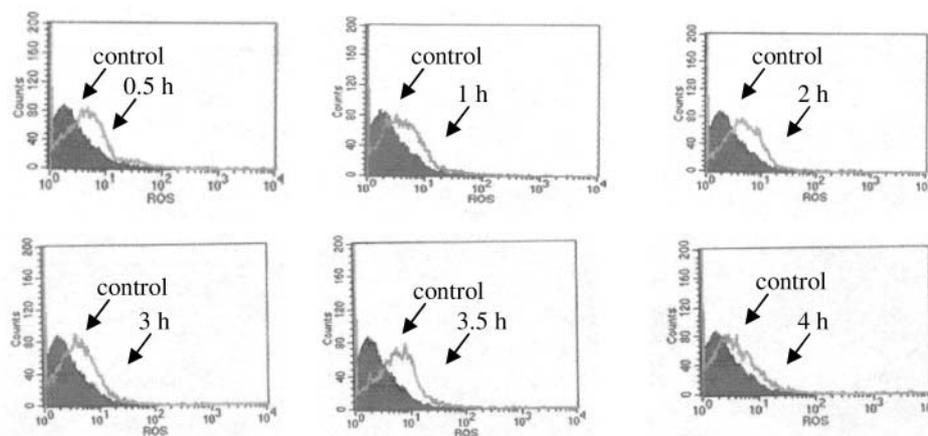


Figure 8. Gyp-induced production of  $\text{Ca}^{2+}$  in human colon cancer colo 205 cells. Cells ( $5 \times 10^5$  cells/well ) were treated with  $150 \mu\text{M}$  Gyp for 0.5, 1, 2, 3, 3.5 and 4 h, before cells being collected, stained by Indo 1/AM and the  $\text{Ca}^{2+}$  levels were determined, as described in Materials and Methods.

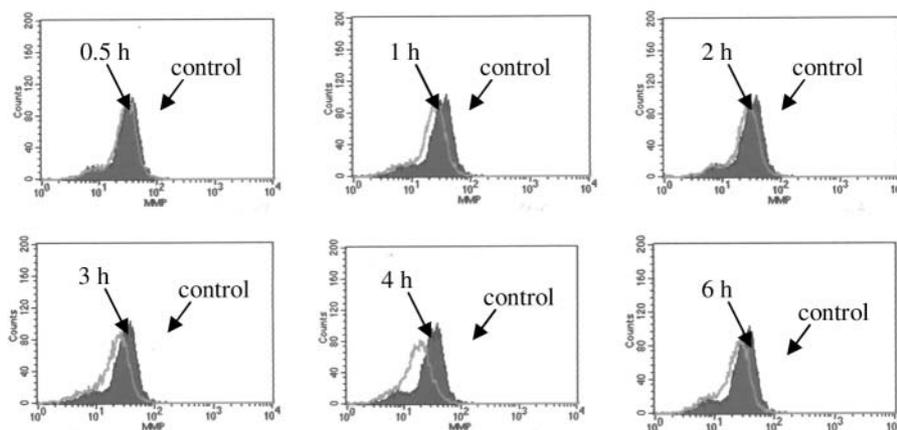
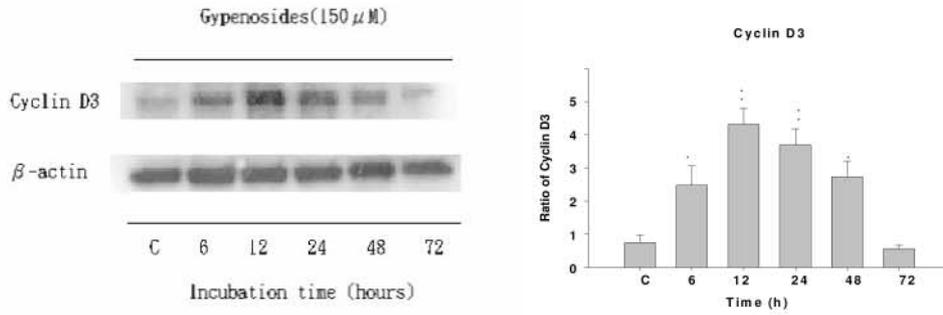


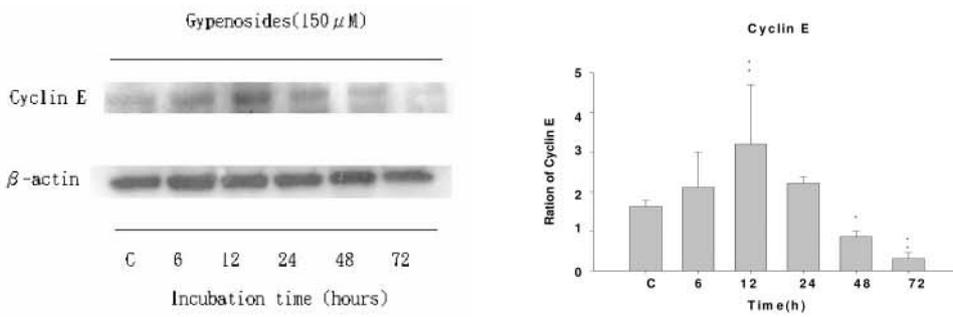
Figure 9. Gyp-induced levels of mitochondria membrane potential ( $\Delta\Psi_m$ ) in human colon cancer colo 205 cells. Cells ( $5 \times 10^5$  cells/well) were treated with  $150 \mu\text{M}$  Gyp for 0.5, 1, 2, 3, 4 and 6 h, before being collected, stained with DiOC<sub>6</sub> and the  $\Delta\Psi_m$  levels were determined, as described in Materials and Methods.

Figure 10. *continued*

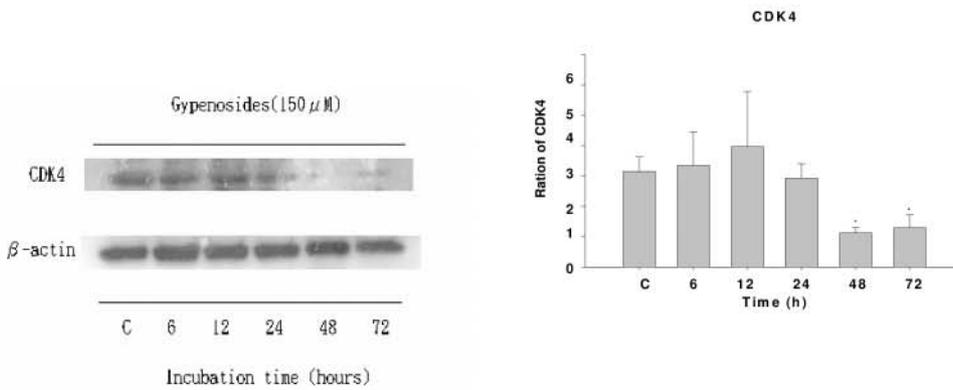
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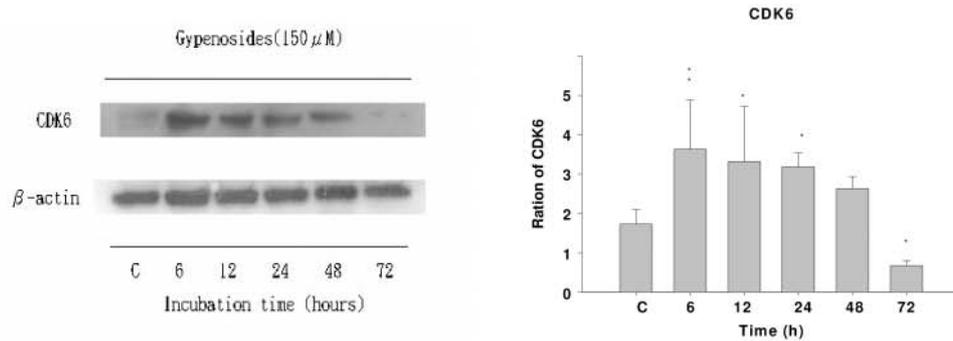
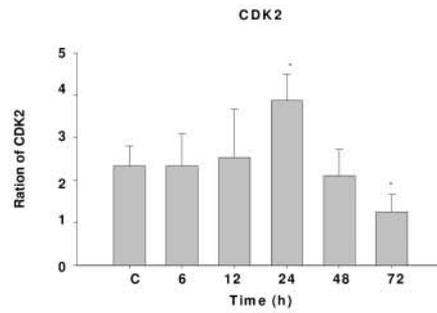
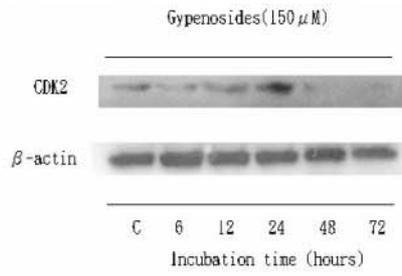
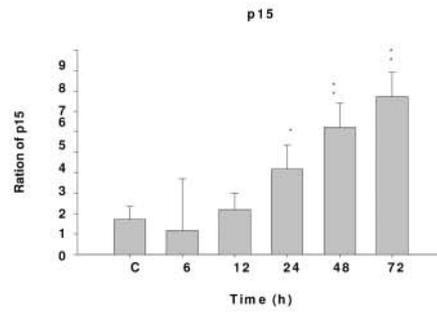
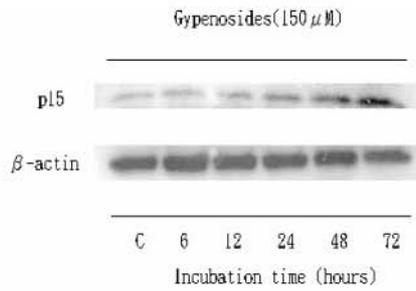


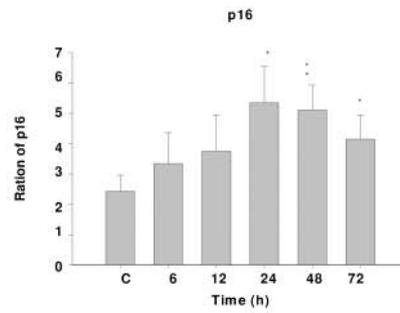
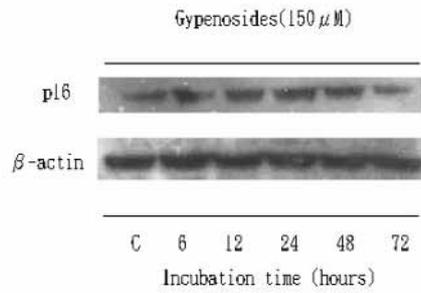
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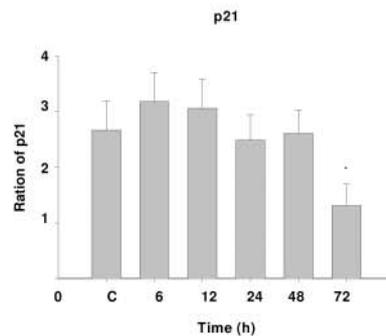
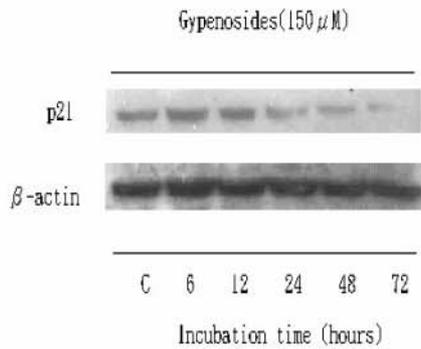
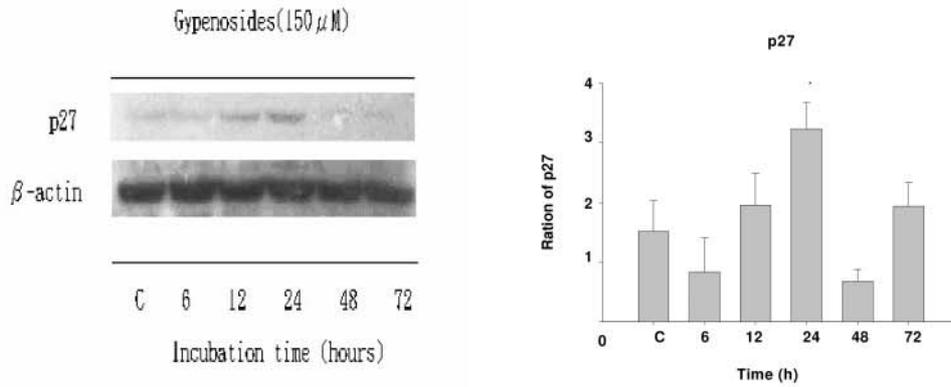
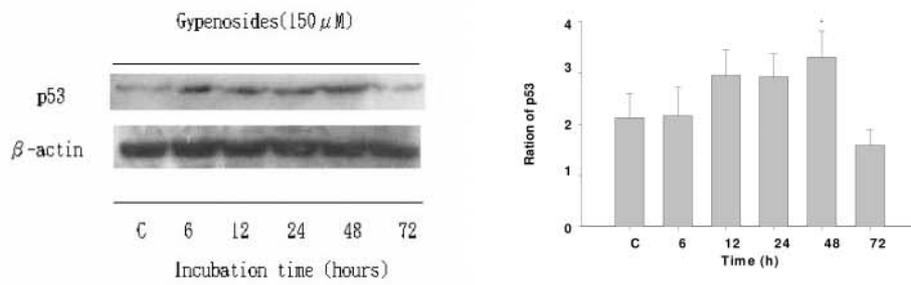


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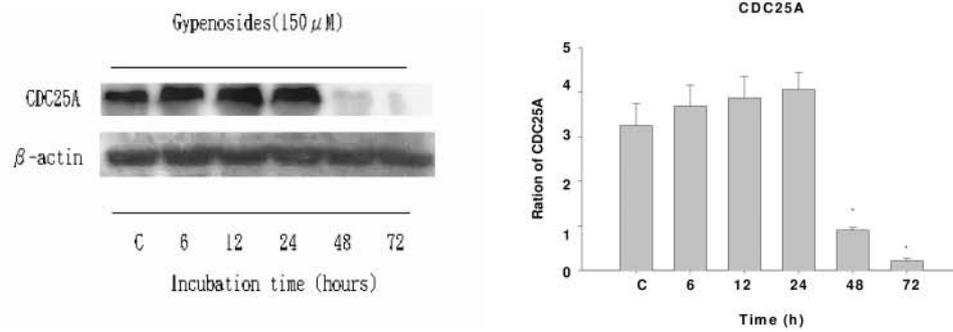
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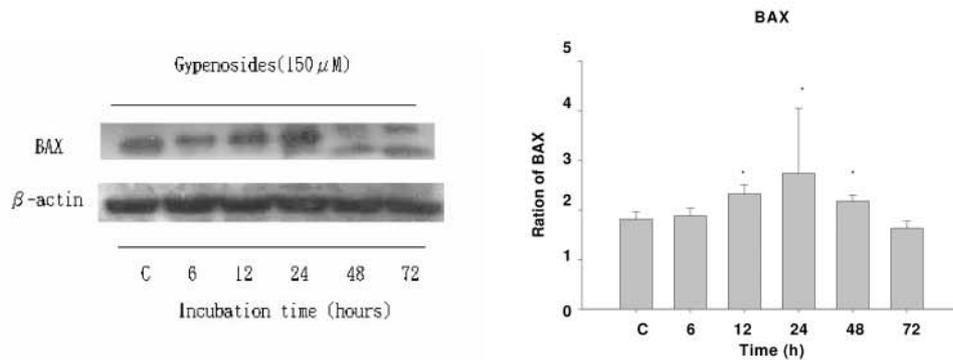
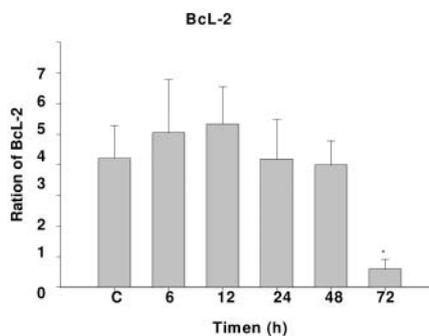
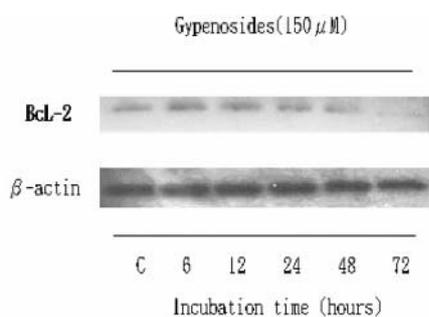
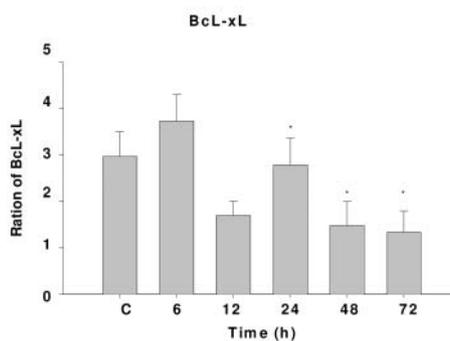
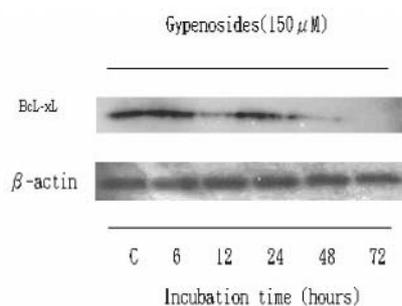


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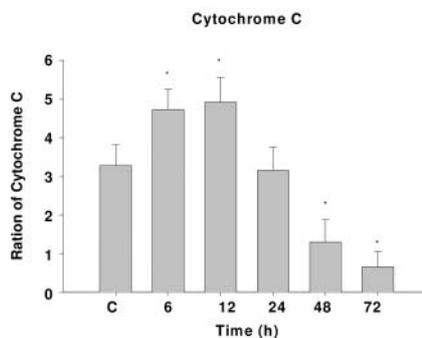
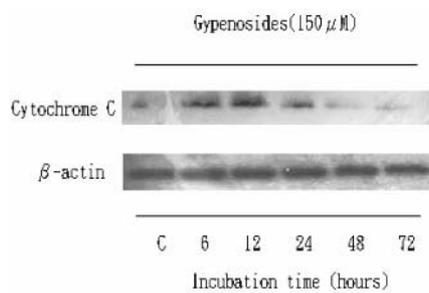
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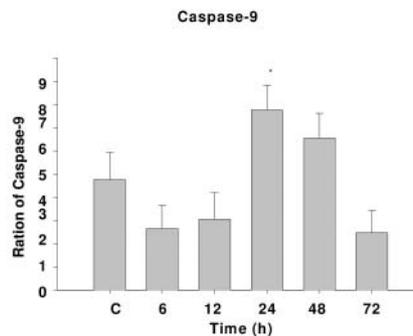
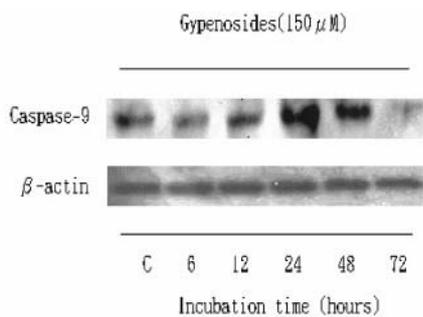
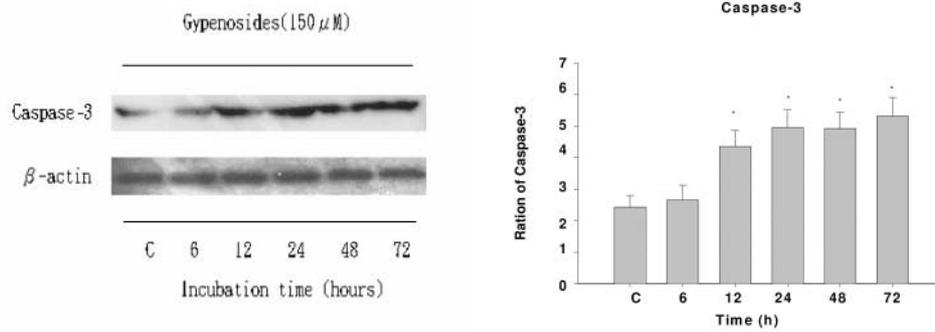
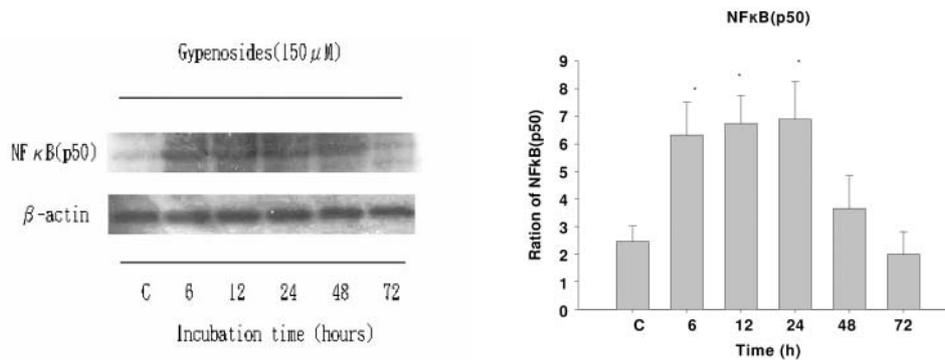


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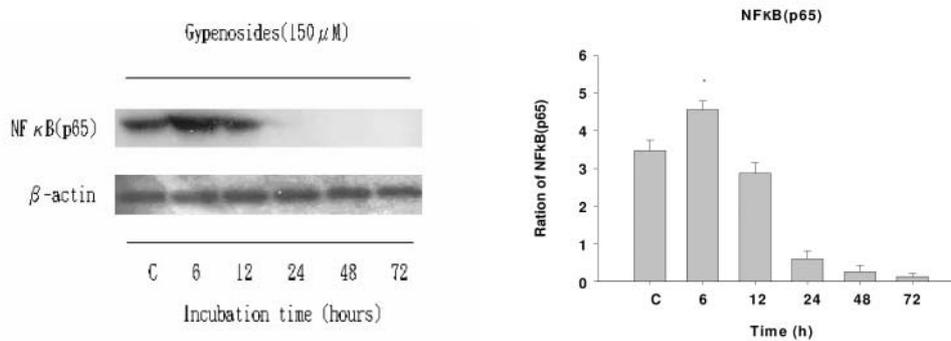


Figure 10. Representative Western blot showing changes in the levels of cyclin D3, cyclin E, CDK4, CDK6, CDK2, p15, p16, p21, p27, p53, CDC25A, Bax, Bcl-2, Bcl-xl, cytochrome c, caspase-9, caspase-3, NFκB (p50) and NFκB (p65) in colo 205 cells after exposure to Gyp. The colo 205 cells ( $5 \times 10^6$  cells/well) were treated with Gyp at  $150 \mu\text{M}$  for various periods of time (0, 6, 12, 24, 48 and 72 h), then the cytosolic fraction and total protein were prepared and determined, as described in Materials and Methods. The levels of cyclin D3 (panel A), cyclin E (panel B), CDK4 (panel C), CDK6 (panel D), CDK2 (panel E), p15 (panel F), p16 (panel G), p21 (panel H), p27 (panel I), p53 (panel J), CDC25A (panel K), Bax (panel L), Bcl-2 (panel M), Bcl-xl (panel N), cytochrome c (panel O), caspase-9 (panel P), caspase-3 (panel Q), NFκB (p50) (panel R) and NFκB (p65) (panel S) expressions were estimated by Western blotting, as described in Materials and Methods.

Gyp were examined by Western blotting. The results (Figure 10, Panel A: cell cycle-associated proteins; Panel B: apoptosis-associated proteins) from Western blotting indicated that the levels of cyclin D3, cyclin E, CDK4, CDK6 and CDK2 were decreased, while the levels of p15,

p16, p21, p27 and p53 were increased. These effects may have led to G0/G1 arrest. Furthermore, Gyp increased the expressions of p53, Bax, cytochrome c, caspase-9 and caspase-3, but decreased the expressions of Bcl-2, Bcl-xl and NFκB (p65). These effects may have led to apoptosis.

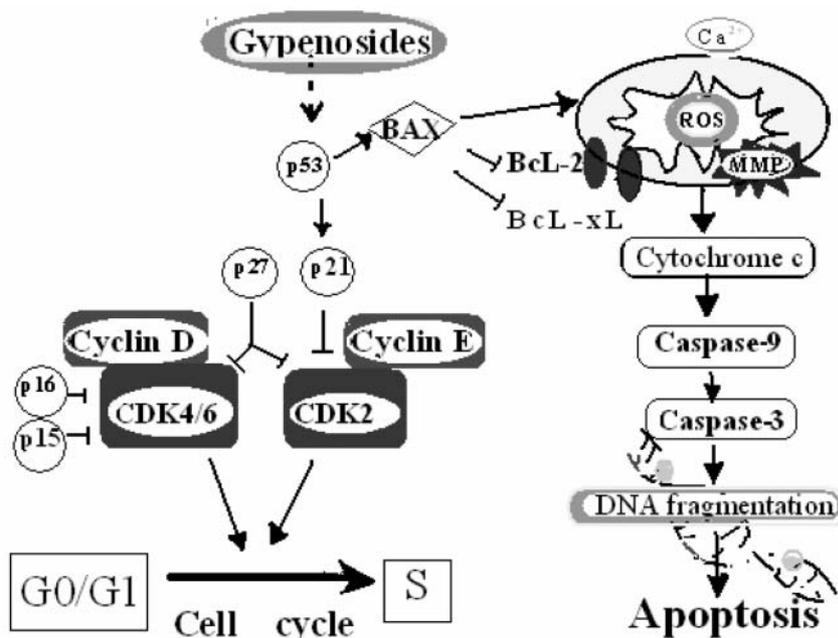


Figure 11. The proposed signal pathways of Gyp-induced apoptosis in human colon cancer colo 205 cells.

## Discussion

Previous studies performed at this laboratory showed that Gyp affected NAT activity and gene expression in human cervical cancer cells (15). In the present study, Gyp induced cell cycle arrest and apoptosis in human colon cancer colo 205 cells, thus, providing a useful model system to characterize the cytotoxic and/or apoptotic effects of various agents.

The results of flow cytometric assays indicated that treatment with Gyp increased the number of cells in the G0/G1-phase and decreased those in the S- and G2/-phase, compared to the untreated cells suggesting that Gyp inhibits cell cycle progression by blocking the transition from G1- to S-phase. To further investigate this result, Western blotting was used to examine proteins associated with the cell cycle, e.g., cyclin D1 which is expressed in G1 cells and binds to the cyclin-dependent kinases CDK4 and CDK6 to activate them (22-24). The analysis of DNA content *versus* light scatter of the Gyp-treated cells indicated that apoptosis followed Gyp-induced G0/G1-phase arrest. Cells normally progress from the G1- to the S-phase, which is regulated by CDK2 associated with cyclin E (25, 26). It was also reported that the subsequent up-regulation of p53 was sufficient to activate protein kinase c-mediated p53 gene transcription, induce G1-phase arrest and promote cellular repair mechanisms in mock cells (27, 28). Our results indicated that Gyp induced growth inhibition mainly *via* regulation of the p15 and p27 status in the colo 205 cells. p27 is a cyclin-

dependent kinase inhibitor which negatively regulates cyclin-CDK complexes at the G1-S transition, thus inhibiting entry into the S-phase of the cell cycle. Low levels of p27 have been correlated with tumor grade, recurrence rate and prognosis in several cancers (29, 30).

Alterations in morphology, DNA damage, DNA fragmentation and the appearance of the sub-G1 group all indicate Gyp-induced apoptosis in these cells. It is well-known that apoptosis is an active process which leads to the activation of endonucleases and the cleavage of DNA into fragments of about 180-200 base pairs (31). Our DNA gel electrophoresis results indicated that Gyp induced DNA fragmentation in the colo 205 cells dose-dependently. The cells in sub-G1 were observed after treatment with Gyp and analyzed by flow cytometry, based on the analysis of DNA content *versus* light scatter of the Gyp-treated cells. These results were confirmed by DAPI staining and DNA gel electrophoresis for DNA fragmentation. The Bcl-2 family is one of the major regulators of apoptosis and, being an intracellular suppressor of apoptosis, serves as a cyto-protective function in cells (3), while also functioning with its pro-apoptotic relative Bax (33). The ratio of Bcl-2 to Bax was associated with the sensitivity or resistance of a cell to apoptotic stimuli (34). Our results showed that Gyp decreased the levels of Bcl-2 but increased the levels of Bax; therefore, Gyp-induced apoptosis may be caused by a reduction in the Bcl-2:Bax ratio. Our data also showed that Gyp-treated colo 205 cells exhibited up-regulation of p53 and down-regulation of the Bcl-2 protein

levels. Therefore, cell survival and death in Gyp-treated cells may be the result of the co-operation between Bcl-2 and p53-mediated mechanisms. Other investigators have demonstrated that accumulation of cells in the G0/G1-phase at 24-120 h after transduction of the p53 or p27/kip genes was associated with an increase in early apoptosis (35-37).

It was also shown that down-regulation of Bcl-2 was ensued by p53-mediated up-regulation of Bax. Apoptosis is triggered by the activation of the family of cysteine proteases called caspases which are synthesized as latent intracellular pro-enzymes. Cleavage of the procaspase forms converts the procaspase into the biologically-active caspases. It is also well known that the apoptosis pathway can be divided into caspase-3-dependent and independent pathway. The present results also showed that Gyp promoted the activation of caspase-3, and that the inhibitor of caspase-3 (z-VAD-fmk) led to a decrease in caspase-3 activity and decreased apoptosis. So, Western blotting was used to show the caspase-3 protein levels in the colo 205 cells that were promoted by Gyp. Taken together, these findings provide new insights (Figure 11) into the possible pathway of Gyp-induced apoptosis in human colon cancer colo 205 cells.

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