

Berberine Induces G1 Arrest and Apoptosis in Human Glioblastoma T98G Cells through Mitochondrial/Caspases Pathway

Ki-Seong EOM,^{a,#} Ji-Myoung HONG,^{a,#} Myung-Ja YOUN,^{b,#} Hong-Seob SO,^b Raekil PARK,^b Jong-Moon KIM,^a and Tae-Young KIM^{*,a}

^a Department of Neurosurgery, Wonkwang University Hospital; 344–2 Shinyoung-dong, Iksan, Jeonbuk 570–711, Korea; and ^b VestibuloCochlear Reserch Center and Department of Microbiology, School of Medicine, Wonkwang University; 344–2 Shinyoung-dong, Iksan, Jeonbuk 570–749, Korea.

Received September 25, 2007; accepted December 19, 2007; published online January 10, 2008

Berberine is an isoquinoline plant alkaloid with a long history of being used for the treatment of many diseases in Chinese herbal medicine. Berberine has a wide range of biochemical and pharmacological effects, including antitumor activities, but its mechanism of action is not clearly understood. In this study, we investigated that the relationship between the antiproliferative activities of berberine and the apoptotic pathway associated with its molecular mechanism of action in human glioblastoma T98G cells. Berberine treatment of T98G cell lines inhibited cell proliferation and induced cell death in a dose (50–200 $\mu\text{g/ml}$) dependent manner with an IC_{50} value of 134 $\mu\text{g/ml}$, which was associated with an increase in G1 arrest. Western blot analysis showed that the berberine-induced G1 arrest was mediated through the increased expression of P27 and the decreased expression of cyclin-dependent kinase (CDK) 2, CDK4, cyclin D, and cyclin E proteins. Berberine treatment also markedly enhanced apoptosis in T98G cells through the induction of a higher ratio of the Bax/Bcl-2 proteins, the disruption of mitochondrial membrane potential, and the activation of procaspase-9, caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP). Berberine can inhibit T98G cell proliferation by inducing G1 arrest and apoptosis. These results demonstrate that the berberine-induced apoptosis of T98G cells is primarily mediated through the mitochondrial/caspases-dependent pathway.

Key words berberine; glioblastoma; T98G cell; G1 arrest; apoptosis; mitochondrial

Glioblastoma is the most common and most aggressive of the primary brain tumors.^{1,2)} With its highly invasive phenotype, glioblastoma diffusely infiltrates various regions of the normal brain, making total surgical removal impossible, and thus patients diagnosed with glioblastoma have a poor prognosis, even in response to multidisciplinary treatment strategies, including surgery, radiotherapy and chemotherapy.³⁾

Unlike normal cells, the growth of tumor cells is uncontrolled. A strategy of chemotherapy is to change the biological properties of the cancerous cells that lead to apoptosis or to kill the cancer cells.⁴⁾ Apoptosis is a physiological mode of cell death that can be selectively triggered by cells in response to a stimulus. Therefore, the induction of apoptosis is a key target of anticancer drugs.

Berberine, a well-known alkaloid, was found to be initially isolated from herbs used in traditional Chinese herbal medicine, such as *Coptis chinensis* and *Hydrastis Canadensis*. Currently, the predominant clinical uses of berberine preparations include the treatment of bacterial diarrhea, intestinal parasite infections and ocular trachoma infections.⁵⁾ Other pharmacological effects of berberine, such as its antiarrhythmic, anti-inflammatory, anticancer, immunosuppressive, vasorelaxant and antiproliferative effects, have also been reported.^{6–10)} The chemical structure of berberine chloride, which has a molecular weight of 371.8, is shown in Fig. 1. In recent years, berberine has been examined for anticancer activity, following evidence of its antineoplastic properties.¹¹⁾ Berberine has also been shown to interact with nucleic acids, especially DNA, *in vitro*.¹²⁾ Berberine has the ability to induce apoptosis in human cancer cells, and promyelocytic leukemia HL-60 cells can form berberine complexes with DNA.¹³⁾ Cell cycle studies showed that berberine induces

rapid apoptosis during the S phase of the cell cycle.¹³⁾ It has also been reported that berberine has dose-dependent effects on the G2/M phase and on the process of apoptosis in Balb/c 3T3 cells.¹⁴⁾ However, the effects of berberine on human glioblastoma cells remain unclear. Therefore, we recently focused on human glioblastoma, one of the tumor incidences that has increased extensively worldwide and has become one of the most frequent malignant neoplasms. The purpose of the present study was to investigate the relationship between the antiproliferative activities of berberine and the apoptotic pathway with its molecular mechanism of action in human glioblastoma T98G cells.

MATERIALS AND METHODS

Chemicals and Reagents Minimal essential medium (MEM) was purchased from Gibco Life Technologies (Gaithersburg, MD, U.S.A.). Fetal bovine serum (FBS) was purchased from Hyclone. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), trypsin, streptomycin, penicillin, and berberine were obtained from Sigma Co. (St. Louis, MO, U.S.A.). 4',6-Diamidine-2-phenylindole (DAPI) and Rhodamine 123 were purchased from Molecular Probes (Eugene, OR, U.S.A.). The antibodies used in this study were

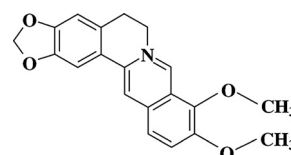


Fig. 1. Chemical Structure of Berberine Chloride

* To whom correspondence should be addressed. e-mail: tykim@wonkwang.ac.kr

These authors contributed equally to this work.

purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). The colorimetric kit used to measure caspase-3 activity was purchased from R&D systems. All other chemicals used were of the highest grade commercially available.

Cell Culture Human glioblastoma T98G cells obtained from the Korean Collection for Type Cultures (KCTC) were cultured as monolayers in MEM supplemented with 10% heat inactivated FBS and 100 $\mu\text{g/ml}$ penicillin/streptomycin. The cells were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. All experiments were carried out 24 h after the cells were seeded onto plates or dishes.

Cell Viability Assay The effect of berberine on cell viability was quantified using MTT assay. Briefly, 5×10^4 cells were plated in 48-well culture plates and allowed to adhere at 37 °C for 12 h. The following day, the cells were treated with varying concentrations of berberine (0, 50, 75, 100, 150, 200 $\mu\text{g/ml}$) for 24 h. The cells were treated with 50 μl of 5 mg/ml MTT and the resulting formazan crystals were dissolved in dimethylsulfoxide (200 μl). The absorbance was then measured at 595 nm using a Digiscan Microplate Reader (Assys Hitech, Kornenbunrg, Austria). An empty well was used as a blank. The results were assessed as the percent cell viability as compared to the vehicle-treated control cells, which were arbitrarily assigned 100% viability.

Cell Cycle Analysis Flow cytometric analysis was used to measure the cellular DNA content. Briefly, 5×10^4 cells were seeded in six-well plates and allowed to attach overnight. The cells were treated with different concentrations of berberine (50, 75, 100, 150 $\mu\text{g/ml}$) for 48 h and then harvested by trypsin treatment, washed with cold PBS, and stained with propidium iodide (PI) solution (50 $\mu\text{g/ml}$ of PI, 100 $\mu\text{g/ml}$ RNase, and 0.1% Triton X-100 in PBS). The stained cells were analyzed for DNA histograms and cell cycle phase distribution by flow cytometry (FACSCalibur, BD Bioscience). Data from 10000 cells per sample were collected and analyzed with CellQuestTM software (Becton Dickinson).

Determination of Mitochondrial Membrane Potential Mitochondrial membrane potential was quantified using the ratio metric probe Rhodamine 123. Briefly, cells in 6 cm tissue culture plates were treated with berberine (150 $\mu\text{g/ml}$) for 24 h. Twenty minutes before the cells were harvested, Rhodamine 123 was added directly to the culture medium to a final concentration of 30 nM. The cells were harvested by trypsinization, washed with 5 ml PBS at 37 °C, pelleted by centrifugation, resuspended in 500 μl of fluorescence-activated cell sorting (FACS) buffer, and analyzed immediately for Rhodamine 123 fluorescence intensity by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, U.S.A.).

Western Blot Analysis Cytosolic protein extracts were prepared according to the previously described methods.¹⁵⁾ Briefly, cells were collected by centrifugation at $300 \times g$ for 5 min at 4 °C and washed with cold PBS. The cell pellet was then resuspended in 500 μl of lysis buffer (20 mM HEPES-KOH, pH 7.5, 250 mM sucrose, 70 mM mannitol, 1.5 mM MgCl_2 , 10 mM KCl_2 , 10 $\mu\text{g/ml}$ leupeptin, and 10 μM digitonin). After 10 min incubation at 25 °C, the sample was centrifuged at $14000 \times g$ for 15 min, and the supernatant containing cytosolic proteins was stored at -70 °C until analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). The pro-

tein extract was subjected to standard SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore) and probed with the appropriate antibodies as described individually in the figure legends. The bound primary antibody was detected by using an appropriate horseradish peroxidase-conjugated secondary antibody, and the reaction band was visualized using an enhanced chemiluminescence detection kit. Western blot analysis was performed using primary antibodies against p27, CDK2, CDK4, cyclin D, cyclin E, Bcl-2, Bax, procaspase-9, caspase-9, caspase-3, poly(ADP-ribose) polymerase (PARP), and β -actin (SantaCruz Biotechnology) at optimal dilution. β -actin was used as an internal control to confirm that the amount of loaded protein was equal.

Nuclear Morphology The nuclei of T98G cells were stained with DAPI in order to assess the degree of apoptosis. The cells were fixed in phosphate buffered saline (PBS) containing 3.7% paraformaldehyde for 15 min. After fixation, the cells were washed twice with PBS and then treated with DAPI (1 : 5000 dilutions) in PBS at room temperature for 15 min. After three washes, the cells were observed under a fluorescence microscope.

Statistical Analysis The data shown are a summary of the results from at least three experiments and are presented as the means \pm S.D. Statistical evaluation of the results was performed by One-way analysis of variance (ANOVA). The results were considered significant at a value of $p < 0.05$, $p < 0.01$.

RESULTS

Berberine Decreases Cell Viability in T98G Cells We first examined the effect of berberine on the proliferation and cell viability of T98G cells using MTT assay. The treatment of T98G cells with varying concentrations of berberine (0, 50, 75, 100, 150, 200 $\mu\text{g/ml}$) led to a significant reduction in cell viability. The dose-dependent reduction in the viability of the cells ranged from 18 to 60% after 24 h of berberine treatment, and the IC_{50} value was found to be 134 $\mu\text{g/ml}$ of berberine (Fig. 2).

Berberine Induces G1 Arrest in T98G Cells The cell cycle distribution was evaluated using flow cytometric analysis in order to examine the possible mechanism of anti-proliferative activity of berberine. As shown in Fig. 3A, treatment of T98G cells with berberine for 24 h resulted in a clear

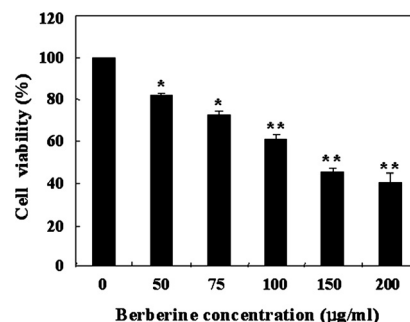


Fig. 2. Cytotoxic Effect of Berberine in T98G Cells

Cell viability was determined by MTT assay. The treatment of T98G cells with varying concentrations of berberine (0, 50, 75, 100, 150, 200 $\mu\text{g/ml}$) for 24 h resulted in a significant dose-dependent reduction in the viability of the cells. The data are represented as the means \pm S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with control cells.

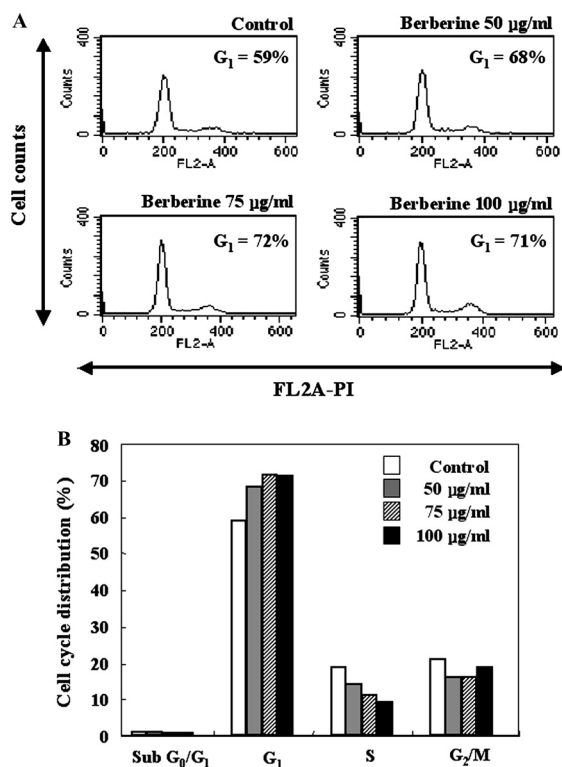


Fig. 3. Effect of Berberine on the Cell Cycle in T98G Cells

T98G cells were treated with varying concentrations (0, 50, 75, 100 µg/ml) of berberine for 24 h. The cells were then harvested and washed with cold PBS buffer. Cellular DNA was stained with propidium iodide solution and flow cytometric analysis was performed in order to analyze the cell cycle distribution. (A) Cell cycle distribution in T98G cells after treatment with varying concentrations of berberine. (B) A summary of the cell cycle distribution data in histograms.

increase in the percentage of cells in the G₁ phase at all concentrations used: 50 µg/ml (68%), 75 µg/ml (72%), and 100 µg/ml (71%), as compared with the non-berberine-treated control cells (59%). The increase in the percentage of cells in the G₁ phase was accompanied by a concomitant reduction in the number of cells in the S and G₂/M phases, which suggested that berberine induces G₁ phase cell cycle arrest in T98G cells (Fig. 3B).

Effect of Berberine on Cell Cycle-Related Molecules in T98G Cells Since the treatment of T98G cells with berberine is known to induce G₁ arrest, we evaluated the effect of berberine on cell cycle regulatory molecules involved in the G₁ phase of the cell cycle progression. The treatment of T98G cells with varying concentrations of berberine (0, 50, 75, 100, 150, 200 µg/ml) led to an increase in the expression of the p27 proteins (Fig. 4A) and a marked reduction in the expression of CDK2, CDK4, cyclin D, and cyclin E in a dose-dependent manner after 24 h of treatment (Fig. 4B). The berberine-induced increase in inhibitory proteins may play a role in the blockade of T98G cells in the G₁ phase. These results suggest that the down-regulation of the levels of the cyclin/CDK proteins in berberine-treated T98G cells is closely related to its induction and regulatory role in the berberine-induced G₁ arrest of cell cycle progression.

Berberine Decreased the Expression of the Anti-apoptotic Protein Bcl-2 But Increased the Expression of the Pro-apoptotic Protein Bax in T98G Cells To investigate the mitochondrial apoptotic events involved in berberine-induced apoptosis, we first analyzed the changes in the levels

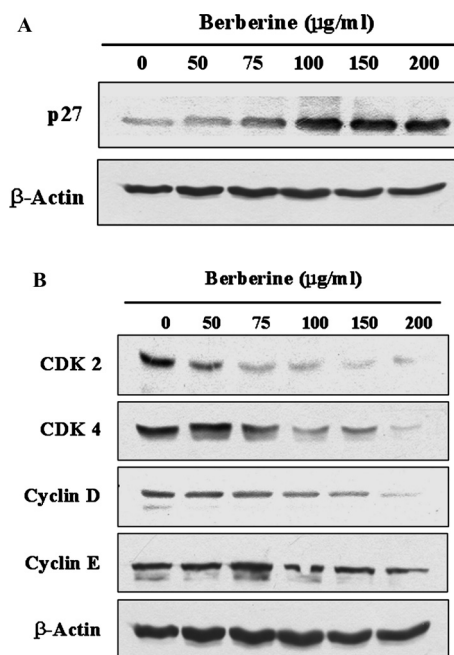


Fig. 4. Effect of Berberine on Cell Cycle-Related Molecules in T98G Cells

The T98G cells were cultured in complete medium and treated with varying concentrations of berberine (0, 50, 75, 100, 150, 200 µg/ml) for 24 h and then subjected to SDS-PAGE followed by Western blot analysis with antibodies for p27 (A) as well as CDK2, CDK4, cyclin D, and cyclin E (B). Berberine treatment resulted in the increased expression of p27 proteins and a marked reduction in the expression of CDK2, CDK4, cyclin D, and cyclin E in a dose-dependent manner.

of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. The results of Western blot analysis showed that the treatment of T98G cells with berberine resulted in a dose-dependent reduction in the levels of anti-apoptotic proteins of the Bcl-2 family. At the same time, the level of the pro-apoptotic protein Bax increased with increasing doses of berberine under identical conditions in comparison with the cells that were not treated with berberine (Fig. 5A). These results suggested that berberine has the ability to alter the levels of pro- and anti-apoptotic proteins of the Bcl-2 family in a manner that contributes to the susceptibility of T98G cells to berberine-induced apoptosis.

Berberine Induces the Disruption of Mitochondrial Membrane Potential in T98G Cells Changes in the mitochondrial membrane potential ($\Delta\psi/m$) have been linked to the initiation and activation of the apoptotic cascade.¹⁶ This event is induced by a variety of stimuli, including the translocation of Bax from the cytosol to the mitochondria, which triggers the release of cytochrome c from the mitochondria to the cytosol.^{16,17} This then contributes to the activation of caspases and subsequent induction of apoptotic cell death. The T98G cells treated with 150 µg/ml of berberine for 6, 18, and 24 h showed clear changes in mitochondrial membrane potential, which confirmed the disruption of berberine treatment (Fig. 5B).

Berberine Induces the Activation or Cleavage of Caspases and Poly(ADP-ribose) Polymerase (PARP) in T98G Cells Once in the cytosol, cytochrome c binds to Apaf-1 and recruits and activates procaspase-9 in the apoptosome, active caspase-9 cleaves and activates executioner caspases, including caspase-3, which cleave a broad spectrum of cellular target proteins, including PARP, thus leading to cell

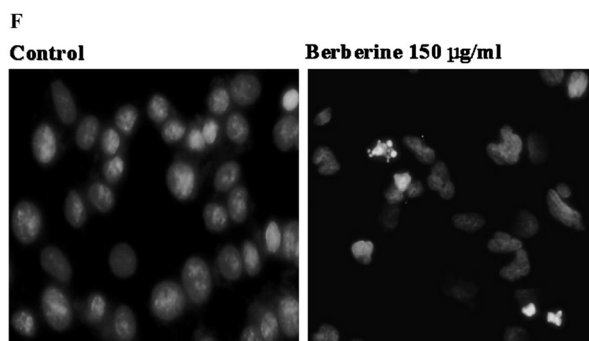
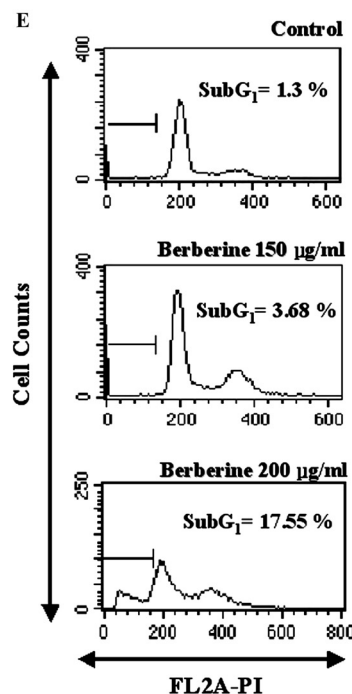
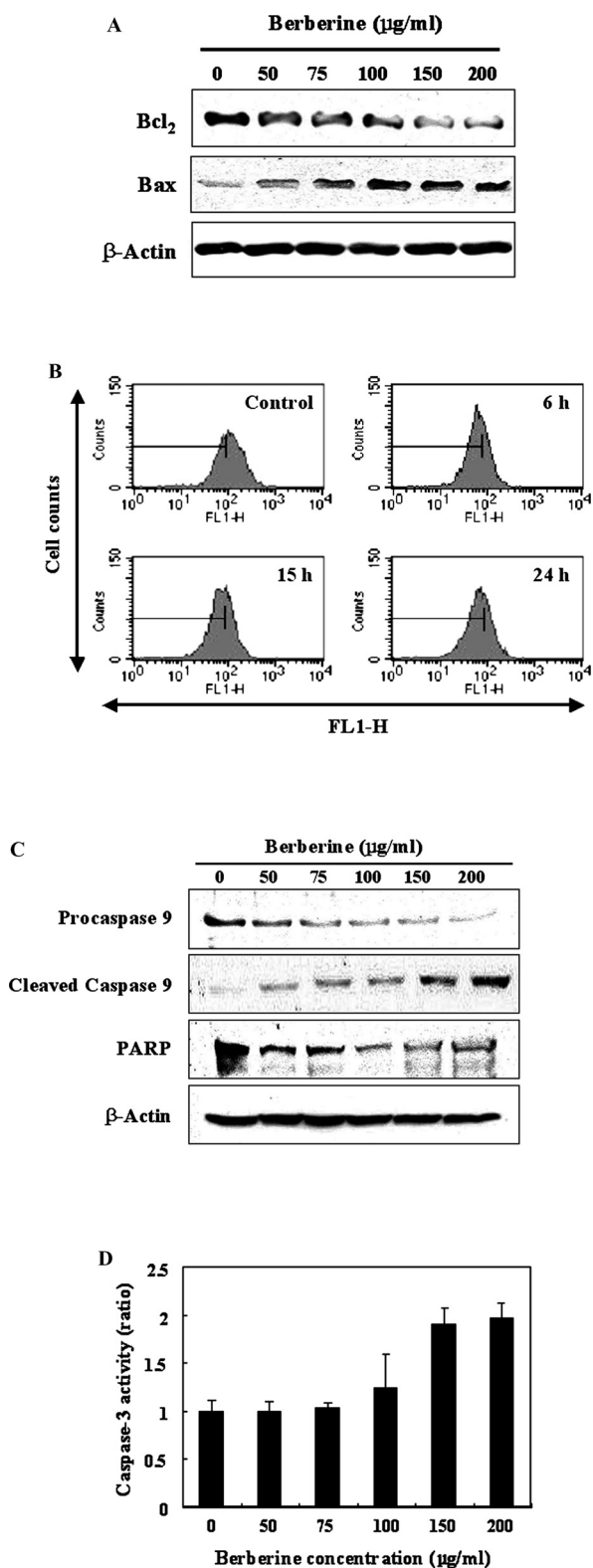


Fig. 5. Berberine Induced Apoptosis through the Mitochondrial/Caspases Pathway in T98G Cells

(A) Treatment of T98G cells with varying concentrations of berberine (0, 50, 75, 100, 150, 200 μg/ml) for 24 h led to a decrease in the expression of Bcl-2 and an increase in the expression of Bax in a dose-dependent manner as estimated by Western blot analysis. (B) Treatment of T98G cells with berberine led to a decrease in the mitochondrial membrane potential. T98G cells were treated with 150 μg/ml berberine for 6, 18, and 24 h, and the relative Δψ_m was measured by fluorescent intensity. We found clear changes in the mitochondrial membrane potential, which confirmed the disruption by berberine treatment. (C) T98G cells were treated with varying concentrations of berberine for 24 h, and the cells were then harvested and the samples were prepared for the analysis of procaspase-9, cleaved caspase-9 and poly(ADP-ribose) polymerase (PARP) using Western blot analysis. Representative blots from three independent experiments with very similar results. (D) The activity of caspase-3 was measured using a colorimetric protease assay. Berberine treatment increased the activity of caspase-3 in a dose-dependent manner in T98G cells. (E) Flow cytometric analysis also revealed that the percentage of the sub-G₁ fraction in berberine-treated cells was increased in a dose-dependent manner, which was indicative of apoptotic cell death. (F) T98G cells were treated with 150 μg/ml berberine, and their nuclear morphology was examined by 4',6-diamidino-2-phenylindole (DAPI) staining. In contrast to those of the control group, the cells in the treatment group showed heterogeneous staining, chromatin condensation, and DNA fragmentation.

death.^{18,19} Treatment of T98G cells with berberine (0, 50, 75, 100, 150, 200 μg/ml) for 24 h led to a dose-dependent decrease in procaspase-9 while increases were observed in the cleavage of caspase-9 and PARP when compared with the cells that were not treated with berberine (Fig. 5C). The role of caspase-3 activation in berberine-induced apoptosis was further confirmed using a colorimetric caspase-3 activity assay. Treatment of T98G cells with berberine for 24 h re-

sulted in a dose-dependent increase in the activity of caspase-3 (Fig. 5D). In addition, flow cytometric analysis also revealed the effect of berberine on the induction of apoptosis. As shown in Fig. 5E, the percentage of the sub-G₁ fraction in berberine-treated cells increased in a dose-dependent manner, which was indicative of apoptotic cell death. In order to define the mode of cell death induced by berberine treatment, we examined the nuclear morphology and DNA fragmenta-

tion in T98G cells. After T98G cells were treated with 150 $\mu\text{g/ml}$ berberine, their nuclear morphology was examined by DAPI staining. In contrast to the control group, the group treated with berberine showed heterogeneous staining, chromatin condensation, and DNA fragmentation under fluorescence microscopy (Fig. 5F).

DISCUSSION

In the present study, we show that a naturally occurring isoquinoline alkaloid, berberine, significantly inhibited the proliferation of T98G cells and reduced their viability, which suggests that berberine may be an effective chemotherapeutic agent against malignant glioma cells. Further studies were performed to elucidate the mechanism underlying the reduction in cell viability and the induction of cell death in T98G cells by berberine treatment. Our *in vitro* data indicated that the treatment of T98G cells with berberine resulted in significant cell cycle arrest at the G1 phase, which indicates that one of the mechanisms by which berberine may act to inhibit the proliferation of cancer cells is by inhibiting cell cycle progression. Our finding of a significant decrease in the expression of cyclin D, cyclin E, CDK2, and CDK6 in T98G cells treated with berberine suggests that berberine was effective at disrupting the uncontrolled cell cycle progression in this cell and that the berberine-induced G1 arrest is mediated through the up-regulation of p27 proteins, which enhances the formation of heterotrimeric complexes with the G1/S CDKs and cyclins, thereby inhibiting their activity (Fig. 4). The increased expression of G1 cyclins in cancer cells provides an uncontrolled growth advantage because most of these cells either lack CDK inhibitors or the expression of CDK inhibitors is not at a sufficient level to control CDK-cyclin activity.²⁰ The arrest of cell cycle progression at the G1 phase provides an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway. In most of the advanced malignancies, cancer cells become resistant to apoptosis and/or do not respond to the cytotoxic effects of chemotherapeutic agents.²¹

Apoptosis is tightly regulated by antiapoptotic and proapoptotic effector molecules, including proteins of the Bcl-2 family, and it can be mediated by several different pathways. Therefore, we investigated the contribution of Bcl-2 family proteins to berberine-induced apoptosis in human glioblastoma T98G cells. The ratio of Bax/Bcl-2 is critical for the induction of apoptosis because it determines whether cells will undergo apoptosis.¹⁸ An increase in the Bax/Bcl-2 ratio stimulates the release of cytochrome c from the mitochondria into the cytosol. Cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-3 and PARP.^{18,19} We found that treatment of T98G cells with berberine resulted in an increase in the expression of the Bax protein and a decrease in the expression of Bcl-2 (Fig. 5A), which increased the ratio of Bax/Bcl-2. This may be responsible for the concomitant execution phase of apoptosis observed in these cells, which included the disruption of the mitochondrial membrane (Fig. 5B). As the level of cytochrome c increases in the cytosol, it interacts with Apaf-1, and ATP forms a complex with procaspase-9, leading to the activation of procaspase-9 and caspase-3.²² Activated caspase-3 is the key executioner of apoptosis and cleaved caspase-3 leads to the

cleavage and inactivation of key cellular proteins, such as PARP.²² We found that the treatment of T98G cells with berberine led to the dose-dependent activation of procaspase-9 and cleavage of caspase-9, and PARP (Fig. 5C). The involvement of a berberine-induced increase in caspase-3 and its effect on apoptosis were further confirmed by measuring its activity (Fig. 5D) and by analyzing the induction of apoptosis by flow cytometry (Fig. 5E). Since DNA damage is a feature of apoptotic cell death, we further confirmed DNA damage using DAPI staining (Fig. 5F).

In conclusion, these data suggest that the inhibition of cell proliferation or induction of cell death in T98G cells by berberine may be exerted by the induction of G1 phase arrest and subsequent apoptotic processes. In addition, we provide mechanistic evidence that berberine-induced apoptosis in T98G cells is mediated through the enhanced expression of Bax, disruption of the mitochondrial membrane potential, and activation of caspase pathways, although other pathways may be involved, which requires further investigation. Further *in vivo* studies are required to determine whether berberine could be an effective chemotherapeutic agent for the management of malignant gliomas.

Acknowledgements This paper was supported by funds from Wonkwang University in 2007.

REFERENCES

- 1) Kleihues P, Soylemezoglu F, Schäuble B, Scheithauer B. W., Burger P. C., *Glia*, **15**, 211—221 (1995).
- 2) Sehgal A., *Semin. Surg. Oncol.*, **14**, 3—12 (1998).
- 3) Shapiro W. R., *Arch. Neurol.*, **56**, 429—432 (1999).
- 4) Lin J. P., Yang J. S., Lee J. H., Hsieh W. T., Chung J. G., *World J. Gastroenterol.*, **12**, 21—28 (2006).
- 5) Birdsall T. C., Kelly G. S., *Altern. Med. Rev.*, **2**, 94—103 (1997).
- 6) Zhao X. Z., Guo X., *Zhonghua Xin Xue Guan Bing Za Zhi*, **17**, 159—161 (1989).
- 7) Kuo C. L., Chi C. W., Liu T. Y., *Cancer Lett.*, **203**, 127—137 (2004).
- 8) Mitani N., Murakami K., Yamaura T., Ikeda T., Saiki I., *Cancer Lett.*, **165**, 35—42 (2001).
- 9) Marinova E. K., Nikolova D. B., Popova D. N., Gallacher G. B., Ivanovska N. D., *Immunopharmacology*, **48**, 9—16 (2000).
- 10) Ko W. H., Yao X. Q., Lau C. W., Law W. I., Chen Z. Y., Kwok W., Ho K., Huang Y., *Eur. J. Pharmacol.*, **399**, 187—196 (2000).
- 11) Hoshi A., Ikekawa T., Ikeda Y., Shirakawa S., Iigo M., Karetani K., Fukoka F., *Gann*, **67**, 321—325 (1976).
- 12) Yamagishi H., *J. Cell Biol.*, **15**, 589—592 (1962).
- 13) Kuo C. L., Chou C. C., Yung B. Y., *Cancer Lett.*, **93**, 193—200 (1995).
- 14) Yang I. W., Chou C. C., Yung B. Y., *Naunyn Schmiedebergs Arch. Pharmacol.*, **354**, 102—108 (1996).
- 15) O'Malley K. L., Liu J., Lotharius J., Holtz W., *Neurobiol. Dis.*, **14**, 43—51 (2003).
- 16) Green D. R., Reed J. C., *Science*, **281**, 1309—1312 (1998).
- 17) Susin S. A., Lorenzo H. K., Zamzami N., Marzo I., Snow B. E., Brothers G. M., Mangion J., Jacotot E., Costantini P., Loeffler M., Larochette N., Goodlett D. R., Aebbersold R., Siderovski D. P., Penninger J. M., Kroemer G., *Nature (London)*, **397**, 441—446 (1999).
- 18) Yang J., Liu X., Bhalla K., Kim C. N., Ibrado A. M., Cai J., Peng T. I., Jones D. P., Wang X., *Science*, **275**, 1129—1132 (1997).
- 19) Kluck R. M., Bossy-Wetzel E., Green D. R., Newmeyer D. D., *Science*, **275**, 1132—1136 (1997).
- 20) Hunter T., Pines J., *Cell*, **79**, 573—582 (1994).
- 21) Pilat M. J., Kamradt J. M., Pienta K. J., *Cancer Metastasis Rev.*, **17**, 373—381 (1999).
- 22) Wolf B. B., Green D. R., *J. Biol. Chem.*, **274**, 20049—20052 (1999).