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Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line

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Abstract

AIM: To investigate the relationship between the inhibited growth (cytotoxic activity) of berberine and apoptotic pathway with its molecular mechanism of action.

METHODS: The *in vitro* cytotoxic techniques were complemented by cell cycle analysis and determination of sub-G_1 for apoptosis in human gastric carcinoma SNU-5 cells. Percentage of viable cells, cell cycle, and sub-G_1 group (apoptosis) were examined and determined by the flow cytometric methods. The associated proteins for cell cycle arrest and apoptosis were examined by Western blotting.

RESULTS: For SNU-5 cell line, the IC (50) was found to be 48 μmol/L of berberine. In SNU-5 cells treated with 25-200 μmol/L berberine, G_2/M cell cycle arrest was observed which was associated with a marked increment of the expression of p53, Wee1 and CDk1 proteins and decreased cyclin B. A concentration-dependent decrease of cells in G_0/G_1 phase and an increase in G_2/M phase were detected. In addition, apoptosis detected as sub-G_0 cell population in cell cycle measurement was proved in 25-200 μmol/L berberine-treated cells by monitoring the apoptotic pathway. Apoptosis was identified by sub-G_0 cell population, and upregulation of Bax, downregulation of Bcl-2, release of Ca^{2+}, decreased the mitochondrial membrane potential and then led to the release of mitochondrial cytochrome C into the cytoplasm and caused the activation of caspase-3, and finally led to the occurrence of apoptosis.

CONCLUSION: Berberine induces p53 expression and leads to the decrease of the mitochondrial membrane potential, Cytochrome C release and activation of caspase-3 for the induction of apoptosis.

Key words: Berberine; Cell cycle; Apoptosis; Caspase-3; ROS; MMP; SNU-5 cells

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INTRODUCTION

The growth of tumor cells not like normal cells is uncontrolled. It is a strategy to change biological properties of cancer cells that lead to apoptosis of killing cancer cells in order to reach chemotherapeutic function for anticancer drugs. Apoptosis is a physiological mode of cell death, which can be selectively triggered by cells in response to the stimuli[1]. Therefore, the induction of apoptosis is a key factor for anticancer drugs.

Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxole[5,6-a]quinolizinium), a kind of alkaloid, was initially isolated from a Chinese herbal medicine and used as an antibiotic long ago; and it has effects against many bacterial species[2,3]. In the past years, berberine has subsequently been examined for anticancer activity following evidence of antineoplastic properties[3-5]. It has also been shown that berberine interacts with nucleic acids especially DNA[6] *in vitro*. Berberine exhibits the ability to induce apoptosis in human cancer cells[5,7] and promyelocytic leukemia HL-60 cells can form berberine complexes with DNA[8].

Cell cycle studies showed that berberine induces rapid apoptosis in a subpopulation (S phase) of the cells[8]. It is

also reported that berberine has dose-dependent effects of berberine on G_2/M phase and apoptosis in Balb/c 3T3 cells[9]. However, the effects of berberine on human gastric cells are still unclear. Therefore, the purpose of the present study was to find out the molecular mechanism of berberine underlying human gastric cancer cell line (SNU-5).

MATERIALS AND METHODS

Materials

Berberine, propidium iodide (PI), Tris-HCl, trypan blue, ribonuclease-A and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO), and TE buffer were purchased from Merck Co. (Darmstadt, Germany). Iscove's modified Dulbecco's medium, glutamine, fetal bovine serum (FBS), and penicillin-streptomycin, trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human gastric carcinoma cell line (SNU-5)

SNU-5 cell line (human gastric carcinoma; 33 years, female) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were immediately placed into 75 cm×75 cm×75 cm tissue culture flasks and grown at 37 ℃ under a humidified 50 mL/L CO_2 and 950 mL/L air in 800 mL/L Iscove's modified Dulbecco's medium supplemented with 200 mL/L FBS, 10 g/L penicillin-streptomycin (1 MU/L penicillin and 10 g/L streptomycin) and 10 g/L glutamine as described previously[10].

Measurement of cell viability after cells were co-treated with berberine determined by trypan blue exclusion and flow cytometry

SNU-5 cells were plated in 12-well plates at a density of 5×10^5 cells/well and grown for 24 h. Various concentrations of berberine were added to the cells for final concentrations of 0, 25, 50, 100, and 200 μmol/L, while only DMSO (solvent) was added for the control regivnen and grown for a different period of time at 37 ℃, was added 50 mL/L CO_2 and 950 mL/L. The trypan blue exclusion and flow cytometry protocols were used as previously described for determining cell viability[7].

Flow cytometry analysis of DNA content for cell cycle and apoptosis analysis in SNU-5 cells co-treated with different concentrations of berberine

About 5×10^5 SNU-5 cells/well in 12-well plates were incubated with berberine (0, 25, 50, 100, and 200 μmol/L) for different time periods before the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) in 700 mL/L ethanol (in PBS) in ice overnight at -20 ℃ and then re-suspended in PBS containing 40 g/L PI, 0.1 g/L RNase (Sigma) and 0/10 g/L Triton X-100. After 30 min at 37 ℃ in the dark, the cells were transferred to the tube, analyzed with flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. Then cell cycle and apoptosis were determined and analyzed[7,10].

Inhibition of berberine-induced apoptosis by caspase inhibitor z-VAD-fmk in SNU-5 cells

In order to further examine whether caspase-3 activation was involved in apoptosis triggered by berberine, SNU-5 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h prior to the treatment with 100 μmol/L berberine. Apoptosis and caspase-3 activity were then determined as described above.

Detection of reactive oxygen species (ROS) in SNU-5 cells co-treated with berberine by flow cytometry

The level of ROS in the SNU-5 cells was examined and quantitated by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). SNU-5 cells were treated with or without berberine (100 mmol/L) for 0, 0.5, 1, 1.5, 2, 4, 6, and 12 h to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 mL of 2,7-dichlorodihydrofluorescein diacetate (10 μmol/L) and incubated at 37 ℃ for 30 min and analyzed by flow cytometry as described previously[11].

Detection of Ca^{2+} concentrations in SNU-5 cells co-treated with berberine by flow cytometry

The level of Ca^{2+} in the SNU-5 cells was determined and quantitated by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem; La Jolla, CA, USA). Cells were treated with or without berberine (100 mmol/L) for 0, 0.5, 1, 1.5, 2, 4, 6, and 12 h to detect the changes of Ca^{2+} concentrations. The cells were harvested and washed twice, and re-suspended in Indo 1/AM (3 mg/L) and incubated at 37 ℃ for 30 min and analyzed by flow cytometry as described previously[12].

Detection of mitochondrial membrane potential in SNU-5 cells co-treated with berberine by flow cytometry

The level of mitochondrial membrane potential in the SNU-5 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using $DiOC_6$ (4 mol/L) (Calbiochem, Inc., La Jolla, CA, USA). Cells were treated with or without various concentrations (0, 25, 50, 100, and 200 mmol/L) of berberine for 1, 2, 4, 6, 12, 24 h to detect the changes of mitochondrial membrane potential. The cells were harvested and washed twice, re-suspended in 500 mL of $DiOC_6$ (4 mol/L) and incubated at 37 ℃ for 30 min and analyzed by flow cytometry[11].

Detection of caspase-3 activity and apoptosis in SNU-5 cells co-treated with berberine by flow cytometry

The caspase-3 activity and apoptosis in the SNU-5 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using PhiPhiLux-G_2D_2 (4×10^{-4} mmol/L) (OncoImmunin, Inc., MD, USA). Cells were treated with or without various concentrations (0, 25, 50, 100, and 200 mmol/L) of berberine and co-treated with or without caspase-3 inhibitor (z-VAD-fmk) for 24 h to detect the changes of caspase-3 activity and apoptosis. The cells were harvested and washed twice, re-suspended in 50 mL PhiPhiLux-G_2D_2 of (4×10^{-4} mmol/L) and incubated at

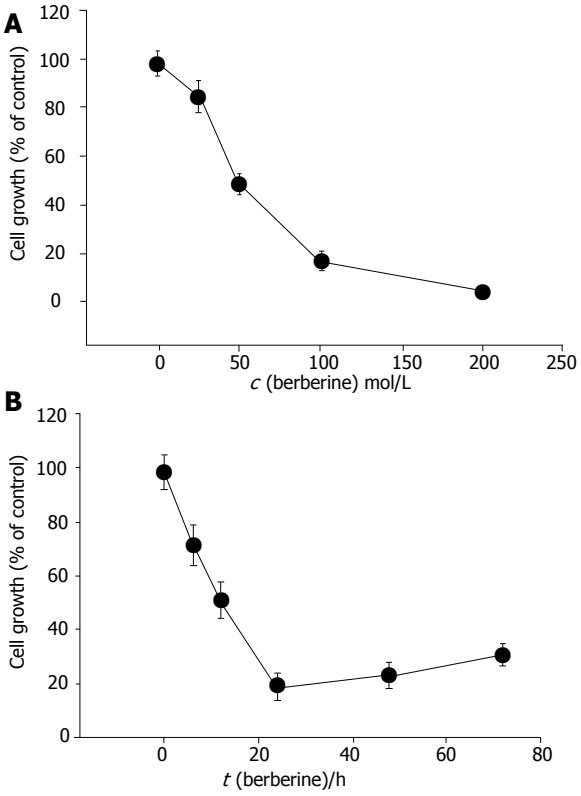

Figure 1 Percentage of viable SNU-5 cells treated with berberine with 24-h incubation. The SNU-5 cells (2×10^5 cells/well; 12-well plate) were plated in 80% Iscove's modified Dulbecco's medium+20% FBS with different concentrations of berberine for 24 h (Panel **A**) or 100 μmol/L berberine for 6, 12, 24, 48, and 72 h (Panel **B**). Then the cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry as described in Materials and Methods. Each point is mean ± SD of three experiments.

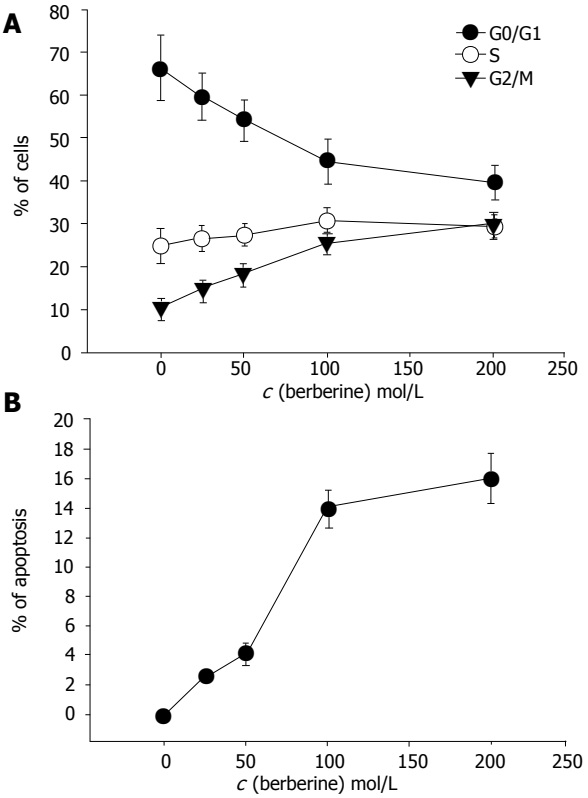

Figure 2 Flow cytometric analysis of the effects of berberine on SNU-5 cell cycle and sub-G_1 group. The SNU-5 cells were exposed to various concentrations of berberine for 48 h, and the cells were harvested and analyzed for cell cycle (Panel **A:** the percent of cells in phase) and sub-G_1 group (Panel **B:** the percent of cells in apoptosis) were analyzed by flow cytometry as described in Materials and methods. Data represents mean ± SD of three experiments.

37 ℃ for 30 min and analyzed by flow cytometry as described previously[11].

Effect of berberine on CDK1, Wee1, Cdc25, p53, JNK, Bcl-2, Bax, and cytochrome C of SNU-5 cells

The total protein was collected from SNU-5 cells treated with or without various concentrations of berberine for 48 h before CDK1, Wee1, Cdc25, p53, JNK, Bcl-2, Bax, and cytochrome C were measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously[12,13].

Statistical analysis

Student's *t*-test was used in statistical analysis between berberine-treated and control groups. $P<0.05$ was considered statisticahy significant

RESULTS

Effects of berberine on growth of SNU-5 cells

Percentage of cell growth was significantly different between the berberine-treated group and control groap. The effects of berberine on SNU-5 cells were dose-dependent (Figure 1A). Increasing the time of incubation led to the decrease of percentage of cell growth (Figure 1B). Apparently the effects of berberine on SNU-5 cells also were time dependent.

Cell cycle arrest and apoptosis induced by berberine in SNU-5 cells

First, we studied the cell cycle and occurrence of apoptosis induced by berberine. Cell cycle and apoptosis were detected by PI staining and annexin V method after 48 h of continuous exposure to berberine before analyzed by flow cytometry (Figures 2A and 2B). As shown in Figure 2, berberine induced G_2/M arrest and apoptosis in a concentration- and time-dependent manner.

Effect of berberine on cyclin B, CDK1, Wee1, and CDC25C of SNU-5 cells

Berberine increased the expression of Wee1 and CDC25C (Figures 3A and 3B) but decreased the expression of cyclin B and CDK1 (Figures 3C and 3D) as detected by western blotting.

Effects of berberine on the production of reactive oxygen species (ROS) in SNU-5 cell line

Percentage of ROS was significantly different between the berberine-treated group and control group. The effects of berberine on taking up of DCFH-DA dye by SNU-5 cells were dose-dependent (Table 1) and time dependent (Figure 4A).

Effects of berberine on the production of Ca^{2+} in SNU-5 cells

Percentage of Ca^{2+} concentrations was significantly

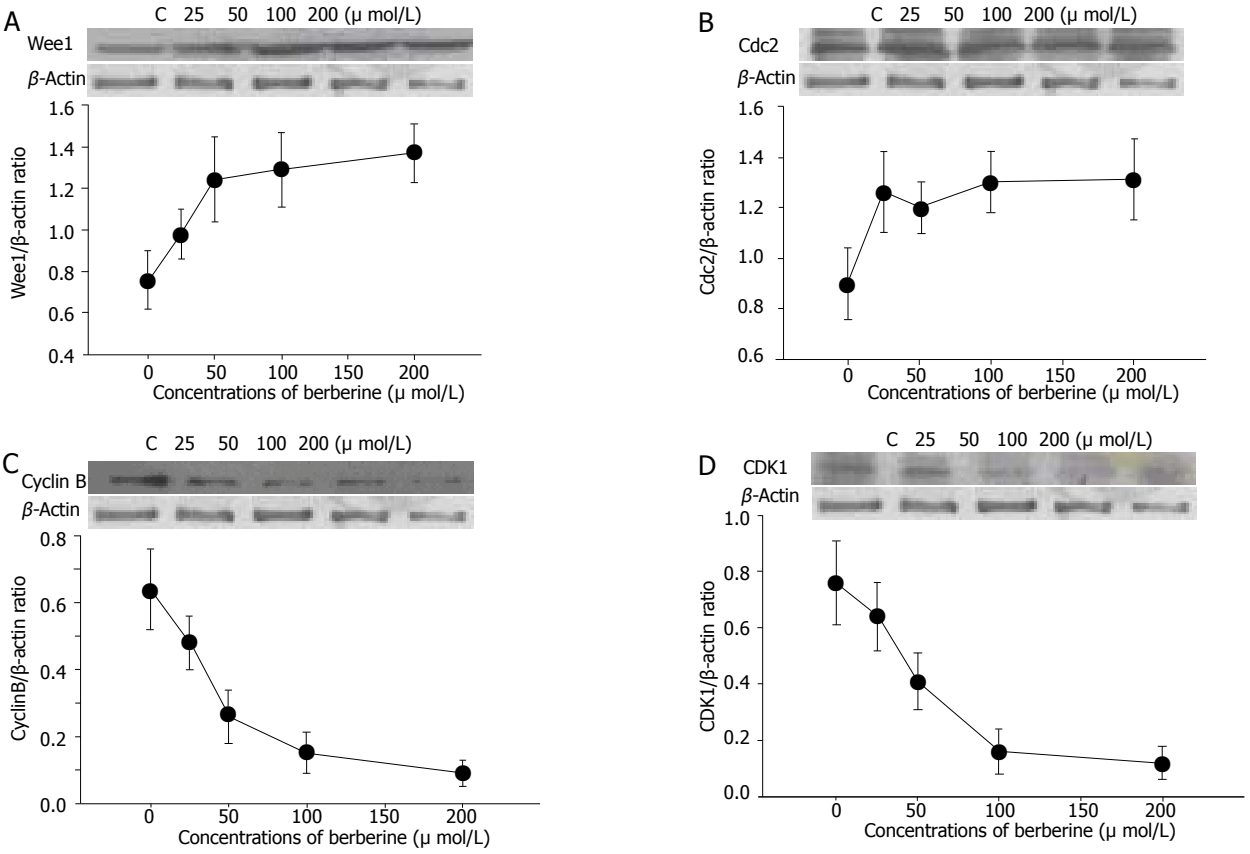

Figure 3 Changes of levels of CDK1 (A), cyclin B1 (B), Wee1 (C), and Cdc2 (D) in SNU-5 cells after exposure to berberine. SNU-5 cells (5×1 0 ^{9} / L) were treated with 0, 25, 50, 100, and, 200 μmol/L berberine for 24 h, then cytosolic fraction and total protein were determined as described in Materials and Methods.

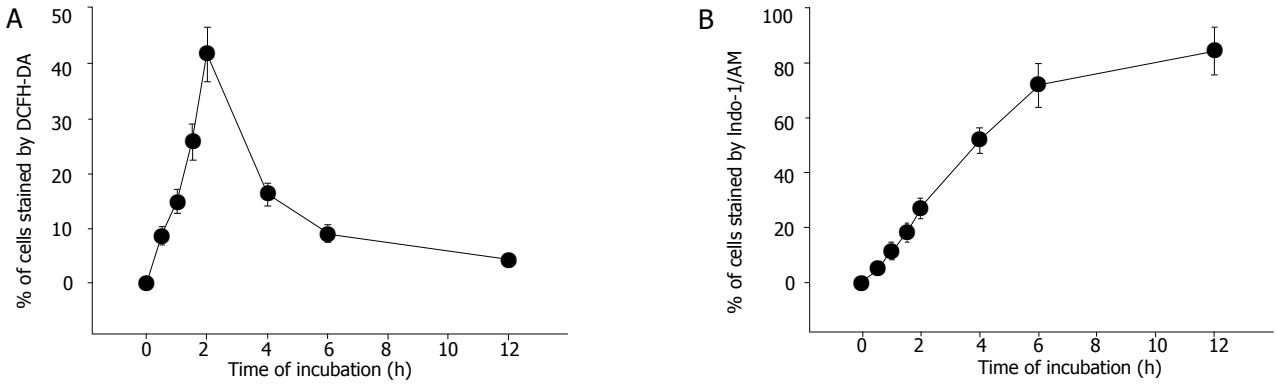

Figure 4 Flow cytometric analysis of reactive oxygen species (A) and Ca^{2+} concentration (B) in human gastric carcinoma SNU-5 cells with 100 μmol/L berberine for various time periods. The SNU-5 cells (5×10^{5} cells/mL) were treated with 100 μmol/L berberine for 0, 0.5, 1, 1.5, 2, 4, 6, and 12 h to detect the changes of ROS and Ca^{2+} cmcentration. The zero cancentration was defined as control. The percentage of cells stained with DCFH-DA dye was determined by flow cytometry as described in the Materials and Methods section.

different between the berberine-treated group and control groap. The effects of berberine on taking up of Indo-1/AM dye by SNU-5 cells were dose and time-dependent (Table 1, Figure 4B).

Effects of berberine on the mitochondrial membrane potential in SNU-5 cells

Percentage of mitochondrial membrane potential (MMP) was significantly different between the berberine-treated group and control group. Apparently the effects of berberine on the levels of MMP determined by the take up of the DiOL6 dye in SNU-5 cells were dose and time-dependent (Table 1, Figure 5).

Inhibition of berberine-induced caspase-3 activity and apoptosis by z-VAD-fmk in SNU-5 cells

The results indicate the caspase inhibitor that berberine increased caspase-3 activity in a dose- and time-dependent manner (Figure 6A). The SNU-5 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (z-VAD-fmk) 3 h prior to the treatment with berberine. The z-VAD-fmk decreased the caspase-3 activity. After

Table 1 Flow cytometric analysis of reactive oxygen species Ca^{2+} concentration and mitochondrial membrane potential in gastric carcinoma SNU-5 cells with treated various concentrations of berberine (mean ± SD)

Berberine (μ mol/L)	cells taking up DCFH-DA (%)	cells taking up Indo-1/AM (%)	cells taking up $DiOC_6$
0	0.24 ± 0.06	1.69 ± 0.22	94.20 ± 9.29
25	10.47 ± 2.02[a]	28.78 ± 2.49[a]	74.14 ± 8.28[a]
50	28.18 ± 3.14[a]	42.47 ± 3.96[a]	40.21 ± 5.08[a]
100	62.28 ± 5.46[a]	74.62 ± 6.48[a]	17.40 ± 1.87[a]
200	79.49 ± 7.08[a]	91.38 ± 8.41[a]	8.16 ± 1.04[a]

The SNU-5 cells (5×10^5 cells/mL) were treated with 0, 25, 50, 100, and 200 μmol/L of berberine. The zero concentration was defined as control. The percentage of cells taking up DCFH-DA dye, was determined by flow cytometry as described in the Materials and Methods. [a]$P < 0.05$. *vs* control.

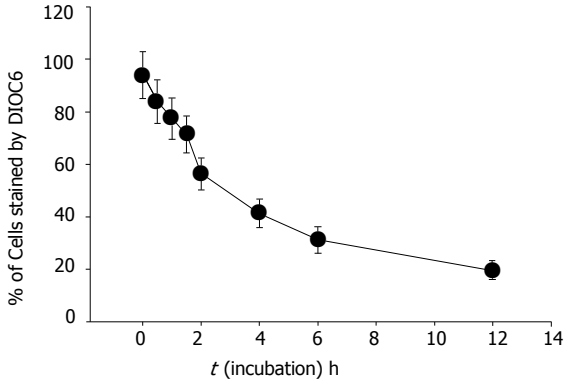

Figure 5 Flow cytometric analysis of mitochondrial membrane potential in human SNU-5 cells with 100 μmol/L berberine for various time periods. The SNU-5 cells (5×10^5 cells/L) were treated with various concentrations of berberine. The zero concentration was defined as control. The percentage of cells stained with $DiOL_6$ dye, was determined by flow cytometry as described in the Materials and Methods.

treated with berberine and z-VAD-fmk in SNU-5 cells, inhibition of berberine-mediated caspase-3 activation was accompanied with the marked attenuation of berberine-induced apoptotic cell death (Figure 6B).

Effect of berberine on p53, Bax, Bcl-2, and cytochrome C in SNU-5 cells

The results indicated that DADS increased the expression of p53 (Figure 3A), JNK phosphorylation (Figure 3B) and cytochrome C (Figure 3D) release but decreased the expression of Bcl-2 (Figure 3C) as detected by western blotting

DISCUSSION

Berberine (25-200 μmol/L) was cytotoxic to SNU-5 cells in a dose- and time-dependent manner. The IC_{50} for SNU-5 cells was 48 μmol/L (Figure 7). It is slightly different in HL-60 cells[8]. But the sensitivity of murine leukemia L1210 cells to the berberine is higher than that of HeLa cells[14]. Our results also showed that berberine induced ROS in a dose-dependent manner. It may be due to the cell death induced by ROS. Although it was demonstrated that berberine can decrease the intracellular ROS[5], such variable effects are not uncommon. However, cells after treated with berberine for 48 and 72 h slightly increased their viability. They therefore may lead to the resistance to berberine due to the expression of multidrug-resistant transporters (mdr) because berberine can modulate expression of mdr1 gene product (pgp-170) that leads to reduced response to paclitaxel in digestive track cancer cells[15].

Berberine arrests cells in S- and G_2/M-phase of the cell cycle, but the former effect is transient. However, G_2/M arrest is obvious. Apparently this effect is dose-dependent. Although it was reported that berberine could induce G_0/G_1 cell cycle arrest in murine L1210 cells[14], it was also reported that berberine can induce G_2/M cell cycle arrest in Balb/c 3T3 cells[4,16], suggesting that berberine induces cell cycle arrest depending on cell types. Therefore, the mechanism of berberine is not the same in all cell types. Western blot results from the present studies also demonstrated that berberine inhibits the levels of cyclin B and CDK1 but increases the levels of Wee1 and Cdc2, which may be the factors for G_2/M arrest in SNU-5 cells. It has been reported that increases of Cdc2 activity are in response to drug-induced G_2/M arrest[16,17]. Formation of Cdc2-cyclin B complex is necessary for G_2/M transition and cells to enter mitosis[16]. Our result demonstrated that berberine declined cyclin b levels in SNU-5 cells, and that G_2/M arrest could be controlled by cyclin B rather than by Cdc2 activation. Cyclin-dependent kinases (Cdks) are the central regulators of cell division cycle. Inhibitors of Cdks ensure proper coordination of cell cycle events and regulate cell proliferation in tissues and organs. Wee1 homologs phosphorylate a conserved tyrosine to inhibit the mitotic cyclin-dependent kinase Cdk1[18]. It was also reported that the induction of Cdc2 phosphorylation due to the increase of Wee1 and Myt1 as well as the reduction of Cdc2 and cyclin B1, is involved in 1,25 [OH] 2VD3-induced G_2/M arrest of keratinocytes[16,19]. It has been shown that multisite phosphorylation of either CDK, Cdc2, Wee1, or CDK-activating kinase is sufficient to generate dynamical behaviors including bistability and limited cycles[20,21]. Experimental depletion of Wee1 by a small interfering RNA directed to Wee1 mRNA could alleviate Vpr-induced G(2) arrest and allow normal progression from M into G phase[16].

Cell cycle analysis revealed the presence of apoptotic cell death (sub-G_1 group) following treatment with berberine. We also did morphological examination which showed cell shrinkage, loss of cell-to-cell contact, membrane blebbing and chromatin condensation elicited by increasing berberine concentration and length of exposure. These results were also confirmed by fluorescence microscopy and flow cytometry. So far, many signals and stimuli have been reported to join the induction of apoptosis, therefore the survival of specific cells is under the control of a wide complex of signals. Especially the caspase activities have been demonstrated to be the regulators of apoptosis[22,23]. Most apoptosis models are involved in caspase activation and two main pathways: caspases-8 and -3 or activation of caspases-9 and -3 activation[24]. The caspases-9 and -3 are involved in the

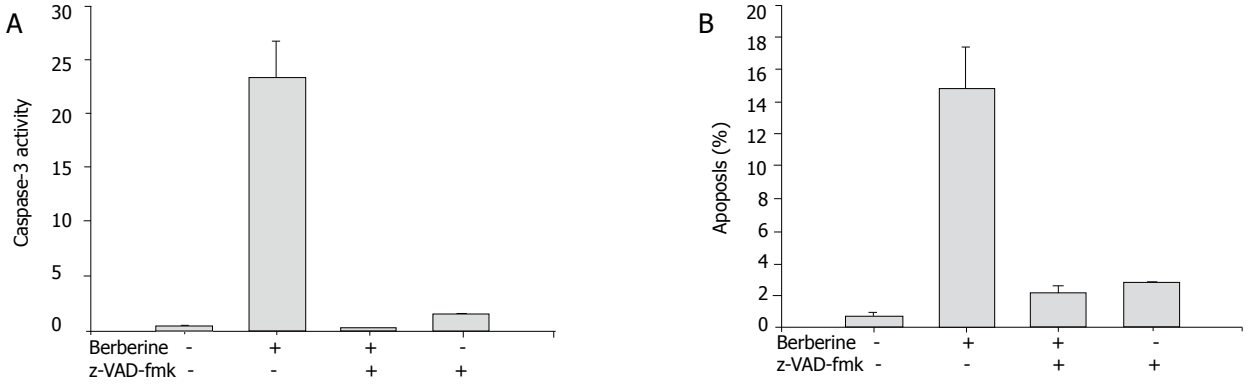

Figure 6 Flow cytometric analysis of the effects of berberine induced caspase-3 activity **(A)** and apoptosis **(B)**. The SNU-5 cells were incubated with 100 μmol/L berberine with or without z-VAD-fmk treatment for determination of caspase-3 activity and apoptosis.

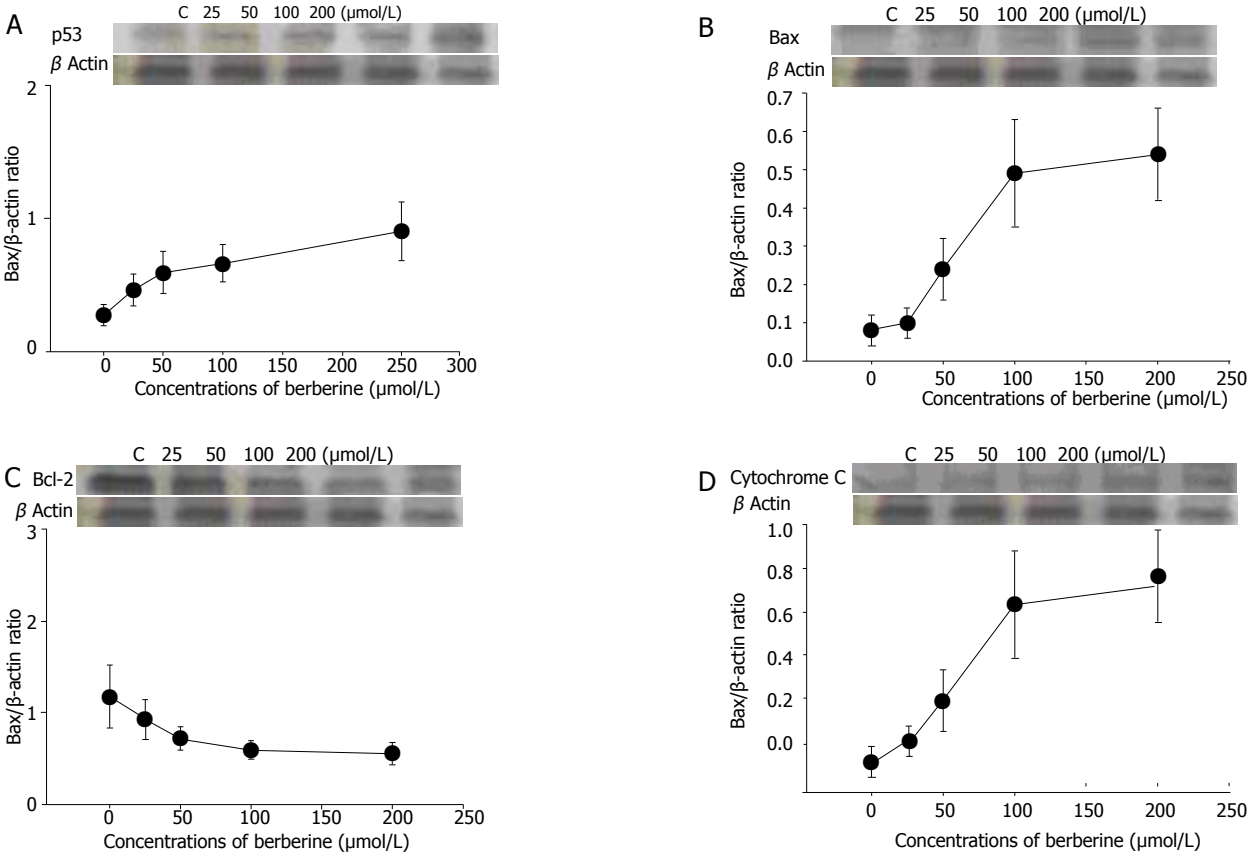

Figure 7 Changes of levels of p53**(A)**, Bcl-2**(B)**, Bax**(C)**, and cytochrome C **(D)** in SNU-5 cells after exposure to berberine. SNU-5 cells (5×10^6/mL) were treated with 0, 25, 50, 100, and 200 μmol/L berberine for 24 h, then cytosolic fraction and total protein were determined as described in Materials and Methods. The levels of p53, p21, Bcl-2, Bax, and cytochrome C were determind by Western blotting as described in Materials and Methods.

release of cytochrome C from mitochondria which causes the decrease of mitochondrial membrane potential. Our data demonstrated that berberine was able to induce the increase of Ca^{2+} and mitochondrial membrane potential loss and cytochrome C release in cytosolic fraction of SNU-5 cells, which correlates well with the activation of caspases-9 and -3. Our result also showed that berberine decreased the levels of Bcl-2 which control mitochondrial membrane potential and Bax was increased which promotes the cytochrome C release (Figure 8).

Berberine can decrease apoptosis induced by paclitaxel in human cancer cell lines including gastric cancer cells[25,26], whereas in the present study berberine induced cell cycle arrest and cell death (apoptosis) in SNU-5 cells in a dose- and time-dependent manner. Based on these findings, we suggest that berberine may contribute to the antineoplastic activity of gastric cancer cells. However, the molecular basis of such effects needs for further investigations. Although the exact binding sites or receptors of berbarine on the SNU-5 cells are still unknown, the antineoplastic activity of berberine may provide a basis for further studies.

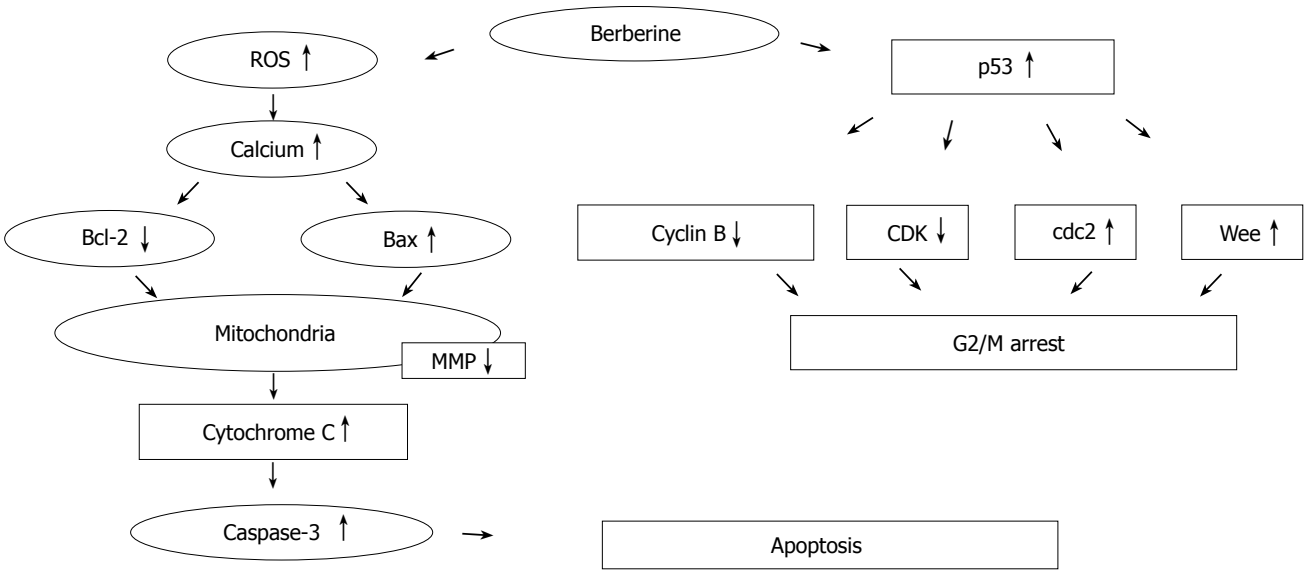

Figure 8 Proposed model of berberine mechanism of action on G2/M arrest and apoptosis in SNU-5 cells. Berberine induced p53 expression that led to the decrease of cyclin B and CDK1 but increase of the expression of cdc25c and Wee1 for G2/M arrest. Berberine induced ROS, Ca^{2+} production and decreased MMP levels led to cytochrome C release and caspase-3 activity, causing apoptosis in SNU-5 cells.

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