

## Molecular mechanisms of denbinobin-induced anti-tumorigenesis effect in colon cancer cells

Kuo-Ching Yang, Yih-Huei Uen, Fat-Moon Suk, Yu-Chih Liang, Ying-Jan Wang, Yuan-Soon Ho, I-Hsuan Li, Shyr-Yi Lin

Kuo-Ching Yang, Department of Internal Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan; Department of Internal Medicine, Taipei Medical University, Taipei, Taiwan, China  
Yih-Huei Uen, Department of Surgery, Chi-Mei Foundational Medical Center, Yung-Kang City, Tainan, Taiwan, China  
Fat-Moon Suk, Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan, China  
Yu-Chih Liang, School of Medical Technology, Taipei Medical University, Taipei, Taiwan, China  
Ying-Jan Wang, Department of Environmental and Occupational Health, National Cheng Kung University Medical College, Tainan, Taiwan, China  
Yuan-Soon Ho, School of Medical Technology, Taipei Medical University, Taipei, Taiwan, China  
I-Hsuan Li, Department of Internal Medicine, School of Medicine, Taipei Medical University, Taipei, Taiwan, China  
Shyr-Yi Lin, Department of Internal Medicine, School of Medicine and Department of Internal Medicine, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan, China  
Supported by the Shin Kong Wu Ho-Su Memorial Hospital (SKH-TMU-93-16) and the Chi Mei Medical Center (93CM-TMU-09)  
Correspondence to: Dr. Shyr-Yi Lin, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, China. sylin@tmu.edu.tw  
Telephone: +886-2-27361661-3210 Fax: +886-2-27393447  
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### Abstract

**AIM:** To explore both the *in vitro* and *in vivo* effects of denbinobin against colon cancer cells and clarify its underlying signal pathways.

**METHODS:** We used COLO 205 cancer cell lines and nude mice xenograft model to study the *in vitro* and *in vivo* anti-cancer effects of denbinobin.

**RESULTS:** Denbinobin at concentration of 10-20  $\mu\text{mol/L}$  dose-dependently suppressed COLO 205 cell proliferation by MTT test. Flow cytometry analysis and DNA fragmentation assay revealed that 10-20  $\mu\text{mol/L}$  denbinobin treatment induced COLO 205 cells apoptosis. Western blot analysis showed that caspases 3, 8, 9 and Bid protein were activated by denbinobin treatment to COLO 205 cells accompanied with cytochrome *c* and apoptosis-inducing factor (AIF) translocation. Pretreatment of MEK 1 inhibitor (U10126), but not p38 inhibitor (SB203580) and JNK inhibitor (SP600125), reversed denbinobin-induced caspase 8, 9 and Bid activation in COLO 205 cells suggesting that extracellular signal-regulated kinase were involved in the denbinobin-induced apoptosis in COLO 205 cells. Significant regression of tumor up to 68% was further demonstrated *in vivo* by treating nude mice bearing COLO 205 tumor

xenografts with denbinobin 50 mg/kg intraperitoneally.

**CONCLUSION:** Our findings suggest that denbinobin could inhibit colon cancer growth both *in vitro* and *in vivo*. Activation of extrinsic and intrinsic apoptotic pathways and AIF were involved in the denbinobin-induced COLO 205 cell apoptosis.

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**Key words:** Denbinobin; Colon cancer; Nude mice; Apoptosis; ERK pathway

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### INTRODUCTION

Colon cancer is one of the most prevalent malignances in the world. It ranked as the second cause of cancer death in the Western societies<sup>[1]</sup>. In Taiwan, colon cancer is the third most common form of lethal cancer<sup>[2]</sup>. Although surgical treatment of early stage colon cancer showed promising effect, the prognosis for those advanced staging patients is still not satisfactory, mostly owing to high local recurrent rate and metastasis. Therefore, finding new therapeutic strategies to improve survival rate in colon cancer patients are very important issues.

There are growing evidences that compounds of plant origin have the ability to treat or prevent cancer. For example, the Chinese herbal preparation called PC-SPEs, a mixture consisting of extracts from eight herbs, has been used increasingly in prostate cancer<sup>[3]</sup> and demonstrated activity against androgen independent prostate cancer in a prospective, multicenter, randomized phase II study<sup>[4]</sup>. Another example was taxol, which was purified from the stem bark of *Taxus brevifolia* and showed anti-tumorigenesis effect on breast and ovarian cancers<sup>[5]</sup>. Other pure compounds isolated from herb could provide novel therapeutic advantage in the treatment of cancers. For example, solamargine, a pure compound isolated from *Solanum incanum* herb, could be a potential drug for cisplatin-resistant human lung cancer cells<sup>[6]</sup> and beta-elemene, a wide spectrum anticancer drug derived from the Chinese herb *Curcuma chaocaulis*, could reverse adriamycin-induced resistance in human breast cancer cell line<sup>[7]</sup>. Therefore, it is reasonable for us to search

useful anti-cancer drugs from the traditional herbal medicine.

Previous study had demonstrated that denbinobin isolated from *Dendrobium nobile*, *Ephemerantha lonchophylla*, and *E. fimbriata* exerted potential anti-inflammatory, antioxidant, and anti-tumorigenesis effect<sup>[8-10]</sup>. Denbinobin was found to be cytotoxic against A549 (human lung carcinoma), SK-OV-3 (human ovary adenocarcinoma), and HL-60 (human promyelocytic leukemia) cell lines<sup>[9]</sup>. However, the anti-cancer effect of denbinobin and its underlying molecular mechanisms remain obscured. The purpose of this study is to explore the *in vitro* and *in vivo* anti-tumorigenesis effects of denbinobin against colon cancer and clarify its underlying molecular mechanisms.

## MATERIALS AND METHODS

### Cell lines and cell culture

The COLO 205 cell line was isolated from human colon adenocarcinoma (American Type Culture Collection CCL-222). The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 mg/mL), and 0.3 mg/mL glutamine in a humidified incubator (37 °C, 50 mL/L CO<sub>2</sub>). Denbinobin (Pharmaceutical Industry, Technology and Development Center, Taiwan) was added at the indicated doses in 0.1% dimethylsulfoxide (DMSO). For control specimens, the same volume of DMSO was added in a final concentration of 1 mL/L without denbinobin.

### MTT assay

COLO 205 cells were seeded in a 96-well plate at a density of  $1 \times 10^4$  cells/well and allowed to adhere overnight. After removing the medium, 200  $\mu$ L of fresh medium per well, containing 10 mmol/L HEPES (pH 7.4) was then added. Then, 50  $\mu$ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the wells and the plate was incubated for 2-4 h at 37 °C in the dark. The medium was removed and 200  $\mu$ L DMSO and 25  $\mu$ L Sorensen's glycine buffer was added to the wells. Absorbance was measured using an ELISA plate reader at 570 nm.

### Drug treatment, and flow cytometry analysis

At 24 h after plating of cells, cells were washed thrice with phosphate-buffered saline (PBS) and then added with medium containing 10% FCS with various concentrations of denbinobin in a final concentration of 1 mL/L DMSO. The cell cycle stages in the denbinobin and DMSO-treated groups were measured by fluorescence-activated cell sorter (FACS) analysis. Cells were harvested and stained with propidium iodide (50  $\mu$ g/mL) (Sigma Chemical Co., St. Louis, MO), and DNA content was measured using a FACScan laser flow cytometer analysis system (Becton-Dickinson, San Jose, CA); and 15 000 events were analyzed for each sample.

### Analysis of DNA fragmentation

Analysis of DNA fragmentation was performed as previously described<sup>[11]</sup>. Briefly, the denbinobin and DMSO-treated cells were seeded on 100-mm dishes. The DNA was extracted twice with equal volumes of phenol and once with

chloroform-isoamyl alcohol (24:1 v:v), then precipitated with 0.1 volume of sodium acetate, pH 4.8, and 2.5 volumes of ethanol at -20 °C overnight, and finally centrifuged at 13 000 *g* for 1 h. Genomic DNA was quantitated, and equal amounts of DNA sample in each lane were electrophoresed in a 2% agarose gel. The DNA was visualized by ethidium bromide staining.

### Western analysis

Cells were washed with cold PBS, lysed in Golden lysis buffer, and performed Western blotting as described previously<sup>[12]</sup>. Briefly, cell lysates were prepared, electrotransferred, immunoblotted with anti-bax, bcl-2, cytochrome *c* (Cyto *c*), and apoptosis-inducing factor (AIF) monoclonal antibody or anti-caspases 9, 3 and Bid, total Akt polyclonal antibody (1:1 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); or with anti-caspase 8 and phosphorylated Akt monoclonal antibody (1:1 000 dilution, Cell Signaling Technology, Beverly, MA); or with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:2 000 dilution, Biogenesis, Kingston, NH), and then with anti-mouse or anti-rabbit or anti-goat IgG antibody conjugated to horseradish peroxidase (1:5 000 dilution, Santa Cruz Biotechnology) and visualized bands using enhanced chemiluminescence kits (2 min, ECL, Amersham). The expression of GAPDH was used as the control for equal protein loading.

### Subcellular fractionation

As previously described<sup>[13]</sup>, COLO 205 cells were harvested in isotonic mitochondrial buffer (210 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EDTA and 10 mmol/L HEPES pH 7.5), supplemented with protease inhibitor cocktail Complete (Boehringer Mannheim), and homogenized for 40 strokes with a Dounce homogenizer. Samples were centrifuged at 500 r/min for 5 min at 4 °C to eliminate nuclei and unbroken cells. The resulting supernatant was centrifuged at 10 000 *g* for 30 min at 4 °C to separate the heavy membrane pellet, and the resulting supernatant was stored as the cytosolic fraction.

### Treatment of COLO 205-derived xenografts in vivo

As previously described<sup>[14]</sup>, COLO 205 cells were grown in RPMI 1640 supplemented with 10% FCS as described above. Cells were harvested through two consecutive trypsinizations, centrifuged at 300 r/min for 5 min, washed twice, and resuspended in sterile PBS. Cells ( $5 \times 10^6$ ) in 0.2 mL were injected subcutaneously between the scapulae of each nude mouse (purchased from National Science Council Animal Center, Taipei, Taiwan). After transplantation, tumor size was measured using calipers and the tumor volume was estimated according to the formula tumor volume ( $\text{mm}^3$ ) =  $L \times W^2 / 2$ , where *L* is the length and *W* is the width<sup>[15]</sup>. Once tumors reached a mean size of 200  $\text{mm}^3$ , animals received intraperitoneal injections of either 25  $\mu$ L DMSO or 50 mg/kg denbinobin thrice per week for 4 wk.

### Statistical analysis

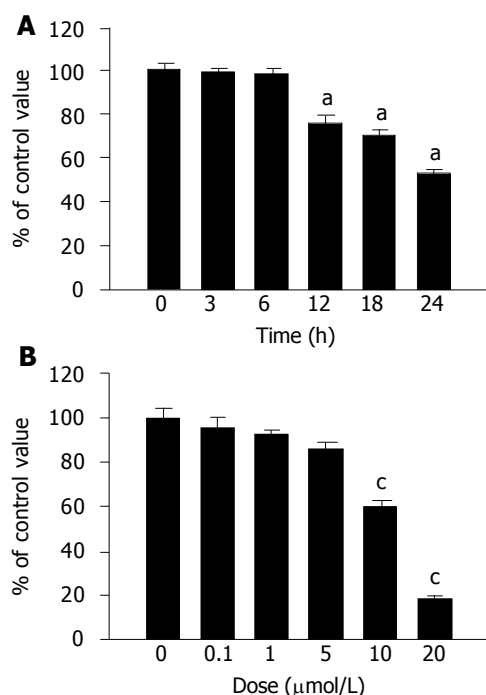
Results were expressed as mean  $\pm$  SE for each study. Data were analyzed by Student's *t*-test or linear regression method;

a *P* value of 0.05 or less was considered statistically significant.

## RESULTS

### Denbinobin suppressed colon cancer cell growth

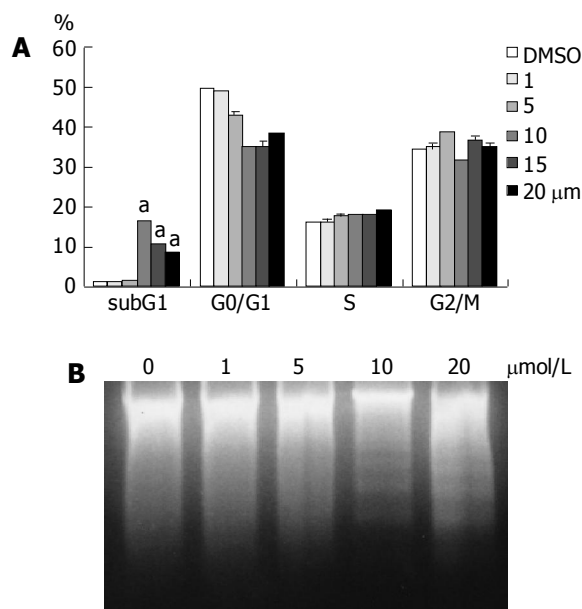
We first examined the antiproliferative effect of denbinobin on the colon cancer cells. Treatment of the COLO 205 cells with 10  $\mu\text{mol/L}$  denbinobin in a time course study showed significant inhibition of COLO 205 cell growth starting from 12 to 24 h by MTT test (Figure 1A). Dose-dependent study by MTT test further demonstrated that treatment of denbinobin (10-20  $\mu\text{mol/L}$ ) to COLO 205 for 24 h suppressed cancer cell growth (Figure 1B).



**Figure 1** Effect of denbinobin on COLO 205 cell growth. COLO 205 cells were seeded in a 96-well plate until subconfluence and viable cells were determined by MTT assay. **A:** Time course study with 10  $\mu\text{mol/L}$  denbinobin in 0.1% DMSO. Two independent experiments were performed in triplicate.  $^aP < 0.05$  vs time 0. **B:** Dose-dependent study after treatment of denbinobin for 24 h. Two independent experiments were performed in triplicate.  $^cP < 0.05$  vs DMSO control.

### Denbinobin induced apoptosis

To further investigate the cellular mechanism of the denbinobin-induced growth inhibition, FACS analyses of DNA content in both DMSO- and denbinobin-treated COLO 205 cells were conducted. Figure 2A showed that 10-20  $\mu\text{mol/L}$  denbinobin treatment to COLO 205 cells for 24 h induced a significant accumulation of cells at the sub-G<sub>1</sub> phase of the cell cycle, suggesting that the observed growth inhibitory effect of denbinobin was due to induction of cell death. Further study by using DNA fragmentation assay also demonstrated that, at concentrations of 0-5  $\mu\text{mol/L}$  denbinobin, apoptosis was not observed. However, when denbinobin concentration was increased to 10-20  $\mu\text{mol/L}$ , apoptosis was observed in COLO 205 cells (Figure 2B).



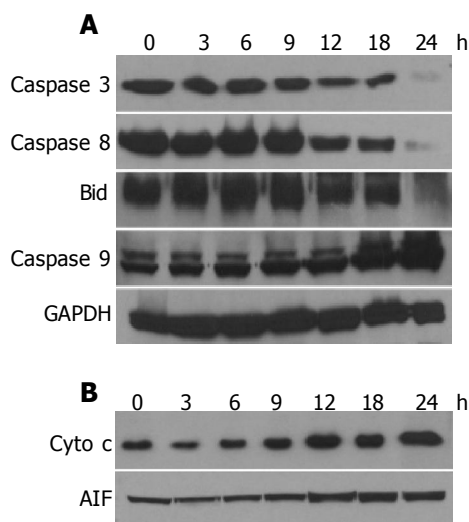
**Figure 2** Treatment of COLO 205 cells with denbinobin (10-20  $\mu\text{mol/L}$ ) for 24 h induced apoptosis. **A:** FACS analysis of DNA content after 24-h incubation in culture medium supplemented with 10% FCS and 0.1% DMSO or 0-20  $\mu\text{mol/L}$  denbinobin in 0.1% DMSO. Percentages of cells in sub-G<sub>1</sub>, G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases of the cell cycle determined using established CellFIT DNA analysis software were shown. Increased sub-G<sub>1</sub> percentage of COLO 205 cells was shown after treatment with 10-20  $\mu\text{mol/L}$  denbinobin. Two independent experiments were performed in triplicate.  $^aP < 0.05$  vs DMSO control. **B:** Electrophoresis of genomic DNA from COLO 205 treated with denbinobin. A typical DNA ladder pattern associated with apoptosis was seen at 10-20  $\mu\text{mol/L}$  denbinobin.

### Denbinobin activated caspases 3, 8 and 9

Since it has been suggested<sup>[16]</sup> that the occurrence of apoptosis requires the activation of caspases, we investigated the involvement of caspase activation in the denbinobin-induced apoptosis in COLO 205 cells by Western blot analyses. Treatment of COLO 205 cells with 20  $\mu\text{mol/L}$  denbinobin for 24 h induced caspase 3 activation evidenced by degradation of procaspase 3 bands (Figure 3A). To elucidate the apoptotic pathways involved in the activation of caspase 3, we examined the changes of caspases 8 and 9 protein levels in the denbinobin-treated COLO 205 cells. After treatment of COLO 205 cells with 20  $\mu\text{mol/L}$  denbinobin for 12 h, activations of caspases 8 and Bid were evidenced by the degradation of the proenzymes of caspases 8 and cleavage of Bid proteins. Furthermore, increased expression of activation form of caspase 9 was noted after 18 h of 20  $\mu\text{mol/L}$  denbinobin treatment to COLO 205 cells (Figure 3A).

### Denbinobin induced Cyto c and AIF release from mitochondria

It has been demonstrated that activation of caspase 9 occurred during the release of Cyto *c* from mitochondria<sup>[17]</sup>. To examine whether this occurs in the denbinobin-induced apoptosis in COLO 205 cells, Cyto *c* release was monitored at various times after 10  $\mu\text{mol/L}$  denbinobin treatment. Figure 3B showed that denbinobin treatment resulted in a significant accumulation of Cyto *c* as well as AIF in the cytosol fraction of cell extracts (Figure 3B). This denbinobin-induced elevation of cytosolic Cyto *c* was observed at 12 h and peaked at 24 h after denbinobin treatment. Under the same



**Figure 3** Effect of denbinobin on caspases, Bid protein levels or Cyto *c* and AIF translocation from mitochondria to cytosol. The whole cell proteins (A) or cytosolic proteins for Cyto *c*, AIF (B) were extracted from the cultured COLO 205 cells, which had been grown in 10% FCS and incubated for the indicated times with 0.1% DMSO or 20  $\mu\text{mol/L}$  denbinobin in 0.1% DMSO. After electrophoresis, proteins were transferred onto Immobilon-P membranes, and then probed with proper dilutions of specific antibodies. Membranes were also probed with anti-GAPDH antibody to correct for difference in protein loading. Denbinobin time-dependently induced the activation of caspases and Bid (A), translocation of Cyto *c* and AIF (B).

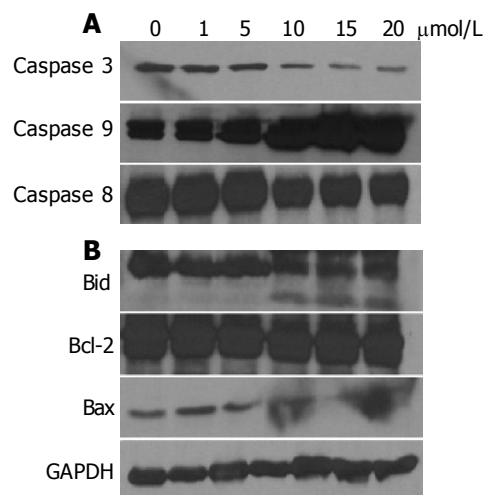
conditions, apoptosis was not observed until 12 h after denbinobin treatment (Figure 1A) suggesting that translocation of Cyto *c* and AIF occurred in the denbinobin-treated COLO 205 cells beforehand, and then caspases 9 and 3 activation, and apoptosis followed thereafter.

#### Denbinobin activated Bid protein

Proteins of the bcl-2 family are also believed to be involved in the control of apoptosis<sup>[18]</sup>. Activation of Bid by caspase 8 links the extrinsic to the intrinsic apoptotic pathway through mitochondrial damages<sup>[19]</sup> by releasing Cyto *c* from mitochondria, and thereby initiate apoptosis. Bcl-2 directly or indirectly operates to prevent the release of Cyto *c* from mitochondria. On the other hand, bax can trigger mitochondria to release Cyto *c* from mitochondria, and thereby initiate apoptosis. Accordingly, we examined the changes of bcl-2 protein levels in denbinobin-treated COLO 205 cells. Treatment of COLO 205 cells with 10-20  $\mu\text{mol/L}$  denbinobin caused a dose-dependent activation of caspases 3, 8, and 9 (Figure 4A) as well as Bid activation evidenced by decreased Bid protein and appearance of its cleavage product (Figure 4B). In contrast, bcl-2 protein levels were not changed significantly (Figure 4B). Surprisingly, proapoptotic bax protein showed decreased expression (Figure 4B). These findings suggest that Bid but not bcl-2 or bax participated in the denbinobin-induced COLO 205 cells apoptosis.

#### Denbinobin-induced apoptosis involved extracellular signal-regulated kinase (ERK) pathway

To further study the signaling pathways involved in the denbinobin-induced COLO 205 cell apoptosis, the following experiments were performed. Since the extracellular signal-regulated kinase (ERK), p38 and JNK pathways have been suggested to be involved in the regulation of apoptotic



**Figure 4** Effect of denbinobin on caspases (A) or Bid, bcl-2 and bax protein levels (B). The whole cell proteins were extracted from the cultured COLO 205 cells, which had been grown in 10% FCS and incubated for the indicated times with 0.1% DMSO or 20  $\mu\text{mol/L}$  denbinobin in 0.1% DMSO. After electrophoresis, proteins were transferred onto Immobilon-P membranes, and then probed with proper dilutions of specific antibodies. Membranes were also probed with anti-GAPDH antibody to correct for difference in protein loading. Denbinobin dose-dependently induced the activation of caspases (A), Bid protein activation and decreased expression of bax (B).

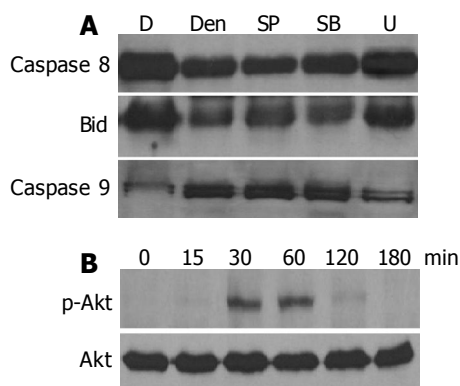
processes, we applied an MEK 1 inhibitor (U10126), a p38 MAP kinase (p38 MAPK) inhibitor (SB203580) and a JNK inhibitor (SP600125) to examine whether they also participated in the denbinobin-induced COLO 205 cell apoptosis. As illustrated in Figure 5A, pretreatment of COLO 205 cells with 100 nmol/L U10126, but not SB20358 (20  $\mu\text{mol/L}$ ) and SP600125 (100 nmol/L), blocked the denbinobin-induced caspases 8, 9 and Bid protein activation in COLO 205 cell, suggesting that the ERK, but not p38 and JNK pathways, was involved in this process.

The serine/threonine kinase Akt is important for anti-apoptotic signaling pathways for normal cells. Accordingly, we tested whether Akt activity was regulated by denbinobin treatment to COLO 205 cells. Time course study in Figure 5B demonstrated that treatment of denbinobin to COLO 205 cells induced activation of Akt evidenced by increased phosphorylated form of Akt peaked from 30 to 60 min after treatment. Pretreatment of PI3K-Akt pathway inhibitor (Wortmannin, 100 nmol/L) failed to affect denbinobin-induced caspases activation (data not shown). These findings suggested that Akt pathway might not be directly involved in the denbinobin-induced apoptosis in COLO 205 cells.

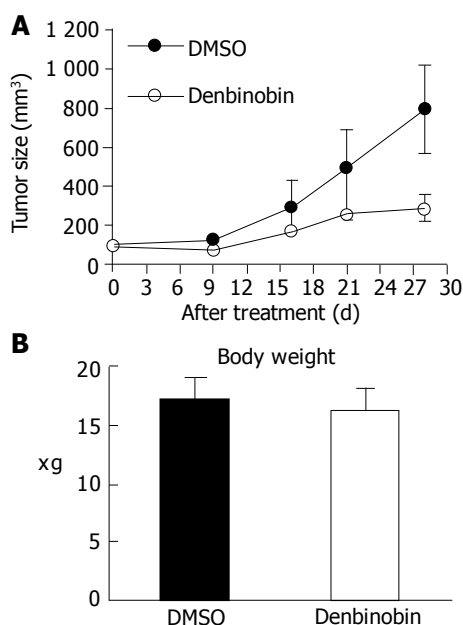
#### Denbinobin inhibited colon tumor growth in vivo

We further examined the anti-tumor effect of denbinobin *in vivo* by treating athymic mice bearing COLO 205 tumor xenografts. After establishment of palpable tumors (mean tumor volume, 200  $\text{mm}^3$ ), animals received denbinobin at dosage of 50 mg/kg or DMSO (control) thrice a week. A reduction in tumor volume between denbinobin-treated *vs* the DMSO-treated control group was detected by d 15. This difference became progressively more conspicuous, with the average tumor volume in the denbinobin-treated mice 32% that of the DMSO-treated mice after 4 wk of

treatment (Figure 6A). In mice receiving these treatment regimens, no significant difference of body weight was noted (Figure 6B) and no gross signs of toxicity were observed (visible inspection of general appearance, and microscopic examination of individual organs) (data not shown). Our results indicated that denbinobin might be a potential useful chemotherapeutic agent in treating colon tumor.



**Figure 5** Effect of MAPK and Akt pathways in the denbinobin-induced COLO 205 cells apoptosis. **A:** Cells were pretreated with various kinds of MAPKs inhibitors; U10126 (U, 100 nmol/L), SB203580 (SB, 20  $\mu$ mol/L), or SP600125 (SP, 100 nmol/L) for 1 h. The whole cell proteins were extracted from the cultured COLO 205 cells, which had been grown in 10% FCS and incubated for the indicated times with 0.1% DMSO or 20  $\mu$ mol/L denbinobin in 0.1% DMSO for 24 h. After electrophoresis, proteins were transferred onto Immobilon-P membranes, and then probed with proper dilutions of specific antibodies; **B:** Effect of Akt pathway on the denbinobin treatment to COLO 205 cells. Time course study of 20  $\mu$ mol/L denbinobin-treated COLO 205 cells was used to detect the total Akt proteins and the phosphorylated Akt by Western blot.



**Figure 6** Growth of COLO 205 tumor xenografts in nude mice was suppressed by denbinobin treatment. Athymic nude mice injected with COLO 205 cells into subcutaneous tissue of inter-scapular area. Once tumor volume reached approximately 200 mm<sup>3</sup>, the animal received treatment of 50 mg/kg denbinobin or DMSO intraperitoneally thrice per week for 4 wk. **(A)** Average tumor volume **(B)** body weight of DMSO-treated ( $n = 5$ , filled circles) vs denbinobin-treated ( $n = 4$ , open circles) nude mice. Samples were analyzed in each group, and values represent the mean  $\pm$  SE. Comparisons were subjected to linear regression method in **(A)** and showed significant difference between DMSO vs denbinobin treatment group.

## DISCUSSION

Denbinobin is one of the pure compounds isolated from the stems of *D. moniliforme*, known as Shi-Hu in Chinese medicine, which has been used for a long time to treat respiratory tract infection, fever and heat stroke<sup>[10]</sup>. Denbinobin has been shown to suppress the lipopolysaccharide-induced TNF- $\alpha$  formation in mouse macrophage cells suggesting that it exerts potent anti-inflammatory effect<sup>[10]</sup>. Chemical compounds with anti-inflammatory activity has been shown to protect against chemical toxicity and cancer<sup>[20,21]</sup>. In our *in vitro* study, we demonstrated that denbinobin at concentrations of 10-20  $\mu$ mol/L inhibited the growth of COLO 205 cells in a dose-dependent manner (Figure 1). These results were not due to inhibition of cell division and indicated that there was an induction of apoptosis by denbinobin in the subcultured cancer cells (Figure 2). *In vivo* studies showed that intraperitoneal administration of denbinobin caused a striking and substantial regression of the COLO 205 tumor in nude mice (Figure 6). To our knowledge, this is the first demonstration that denbinobin inhibits the growth of colon cancer cells both *in vitro* and *in vivo*.

Apoptosis is a cell suicide mechanism that requires specialized cellular machinery. A central component of this machinery is a proteolytic system involving caspases, a highly conserved family of cysteine proteinases with specific substrates<sup>[16]</sup>. By Western blot analysis, we demonstrated that 10-20  $\mu$ mol/L denbinobin induces activations of caspases 8 and 9 in COLO 205 cells, evidenced by decreases in the stainable procaspases 8, and increases in the active form of caspase 9 enzyme expression (Figures 3A and 4). Activation of caspases 8 and 9 can cleave and activate downstream caspases such as caspase 3 (Figures 3A and 4), which eventually leads to apoptosis<sup>[22]</sup>.

Bcl-2 family proteins have been suggested to be involved in the regulation of apoptosis by controlling the release of Cyto *c* from mitochondria<sup>[18]</sup>. Activation of Bid by caspase 8 links the extrinsic to the intrinsic apoptotic pathway through mitochondrial damages<sup>[19]</sup>. Bcl-2 prevents apoptosis by blocking the release of Cyto *c* from mitochondria<sup>[23]</sup>. Bax, on the other hand, directly induces Cyto *c* release from mitochondria, and thereby triggers caspase 9 activation<sup>[24]</sup>. The results of the present study demonstrate that activation of Bid protein by denbinobin treatment in COLO 205 cells seems to be responsible for stimulating the release of Cyto *c* (Figure 3).

One of the major challenges in the cancer treatment is that many tumor cells carry mutations in key apoptotic genes such as p53, Bcl family proteins or those affecting caspase signaling. These defects resulted in failure of traditional chemotherapeutic treatments. Therefore, it is important to search for caspase-independent cell death mechanism in tumor<sup>[25]</sup>. It has been discovered that, in response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effector such as AIF<sup>[26]</sup>. AIF is a phylogenetically ancient mitochondrial intermembrane flavoprotein endowed with the unique capacity to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation when added to purified nuclei<sup>[27]</sup>. In our study, 20  $\mu$ mol/L denbinobin treatment induced the release of AIF from mitochondria to cytosol. These findings suggest a potential therapeutic advantage of denbinobin in the

treatment of colon cancer.

Mitogen-activated protein (MAP) kinases, including ERK, c-Jun NH<sub>2</sub>-terminal kinases (JNK) and p38 MAPK are important intermediates of the signal-transduction pathway associated with proliferation or apoptosis<sup>[28]</sup>. A report has shown that ursodeoxycholic acid could induce colon cancer cell apoptosis through modulation of ERK pathway. Our results demonstrated that MEK 1 inhibitor but not JNK or p38 inhibitors could reverse the denbinobin-induced caspases 8, 9 and Bid activation (Figure 5A) suggesting that ERK pathway was involved in the denbinobin-mediated apoptosis in COLO 205 cells.

Akt promotes cell survival by inhibiting proapoptotic proteins such as Bad, forkhead, and p53, and activating pro-survival proteins such as NF- $\kappa$ B. It is likely that hyperactivity of the PI3K-Akt pathway is a selected mechanism for tumor cells to overcome apoptotic stimuli. In our results, we demonstrated that Akt was not suppressed but transiently activated after denbinobin treatment to COLO 205 cells (Figure 5B) and pretreatment of Akt inhibitor has no effect on the denbinobin-induced COLO 205 cell apoptosis. These findings suggest that Akt pathway was not primarily regulated by denbinobin, instead its activation might be a secondary effect.

Our findings introduced our basic observations that denbinobin, a pure compound derived from Chinese herb, could induce apoptosis in colon cancer cells and suppress colon cancer growth in tumor bearing nude mice. The results from the present *in vitro* and *in vivo* studies highlight the molecular mechanisms of denbinobin-induced apoptosis in COLO 205 cells, which might have potential applications in the treatment of human colon cancer.

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