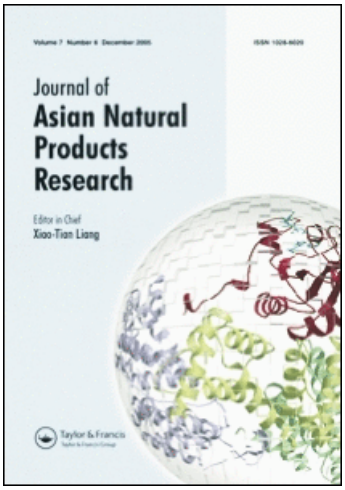

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Berbamine, a natural compound from the plant *Berberis amurensis*, is a traditional Chinese medicine mainly used in stimulating normal hematopoiesis in clinic. Our previous studies demonstrated that berbamine has anti-leukemia activity. In this study, we investigated the anticancer activity of berbamine against human hepatocellular carcinoma (HCC) HepG2 cells *in vitro* and *in vivo*. Berbamine treatment decreased the cell growth in a dose-dependent manner with an IC_{50} value of $34.5 \pm 0.5\,\mu M$. Flow cytometric analysis of apoptosis using Annexin V/propidium iodide staining showed that the percentage of apoptotic cells was increased in a time-dependent manner. Berbamine treatment increased the expression level of Fas and P53, caused depolarization of mitochondrial membrane and decrease of membrane potential, and activated caspase-3, -8, and -9 in HepG2 cells. Berbamine-induced apoptosis could be blocked by the broad caspase inhibitor z-VAD-fmk. HepG2 human HCC xenograft mice treated with berbamine showed a significant reduction in tumor growth rates compared to saline-treated mice. These studies suggest that berbamine exerts anticancer effects on human HCC HepG2 cells *in vivo* and *in vitro*, the induction of p53 and the activity of the Fas apoptotic system may participate in the anticancer activity of berbamine in HepG2 cells.

Keywords: berbamine; apoptosis; Fas; P53; caspase; hepatocellular carcinoma

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently occurring cancers worldwide and the third most common cause of cancer-related death [1]. HCC affects more than 500,000 people globally with 110,000 deaths annually. More than one-half of them were Chinese [2]. The prognosis for patients with hepatoma is poor because there is no effective treatment of metastatic disease. However, the effective chemotherapeutic agents for this disease have not been developed. Therefore, there is a critical need to develop more effective strategies for the chemotherapy of HCC.

Berbamine (bisbenzylisoquinoline) is a natural compound from the plant *Berberis amurensis* used in Chinese traditional medicine. The formula of berbamine is presented in Figure 1, and its molecular weight is 753.80. In recent years, pharmacological studies have demonstrated that berbamine possesses leukogenic, anti-hypertensive, anti-arrhythmic activities, and immunosuppressive [3–6]. Both animal and clinical studies have shown that berbamine could stimulate

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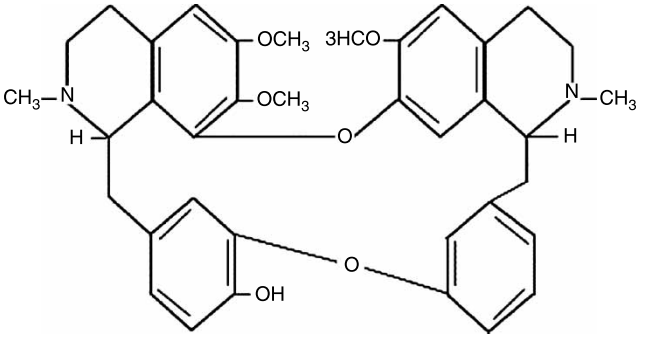

Figure 1. The structural formula of berbamine.

normal hematopoiesis of cancer patients undergoing chemotherapy or radiotherapy and has been used to prevent tumor patients from cytotoxic effects of chemotherapeutic agents on bone marrow in clinic.

In previous study, we found that berbamine had anti-leukemia activity, induced apoptosis of Bcr/Abl-positive K562 leukemia cells by suppressing the expression of Bcr/Abl fusion gene, and activation of caspase-3 [7]; berbamine could also reverse multidrug resistance by reducing P-glycoprotein expression [8]. However, its effect on other kinds of cancers and the mechanism are poorly understood; few studies have reported anticancer activity of berbamine *in vivo* [6,9]. In this study, we evaluated the antitumor effect of berbamine on human HCC HepG2 cells *in vitro* and *in vivo*.

2. Results and discussion

2.1 Berbamine inhibits the growth of HepG2 cells

To determine whether berbamine has growth inhibitory effects on HCC cells, HepG2 cells were exposed to different concentrations of berbamine for 24 h. The cell viability was evaluated by MTT assay. As presented in Figure 2, HepG2 cells were susceptible to the inhibitory effect of berbamine. An obvious growth inhibition of berbamine in a dose-dependent manner was observed. The IC_{50} (50% inhibitory concentration) value was 34.5 ± 0.5 μmol/l in HepG2 cells.

2.2 Berbamine induces apoptosis in HepG2 cells

We used Hoechest 33258 to investigate the changes in the nucleus of cells. Normal cells showed homogeneous staining of their nuclei. By contrast, when HepG2 cells were treated with berbamine at 30 μM for 24 h, apoptotic cells showed irregular staining of their nuclei as a result of chromatin condensation and nuclear fragmentation; some cells appeared as typical apoptotic bleb phenomenon (Figure 3(a)). We further investigated the percentage of cells undergoing apoptosis. Simultaneous staining with Annexin V-FITC and propidium iodide (PI) distinguished between intact cells, early apoptosis, late apoptosis, and cell death. As shown in Figure 3(b) and (c), $HepG_2$ cells were exposed to berbamine at 30 μM at

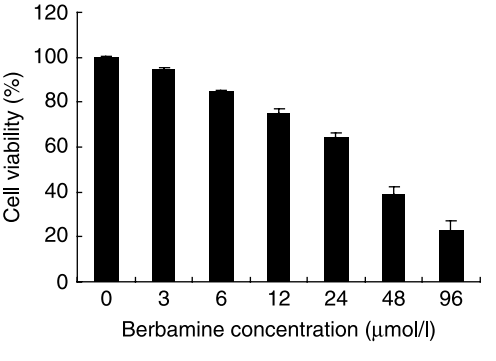

Figure 2. Cytotoxic effects of various concentration of berbamine on HepG2 cells for 24 h. The activity was compared to the control well of the same cells and the results were presented as mean ± SD for triplicate.

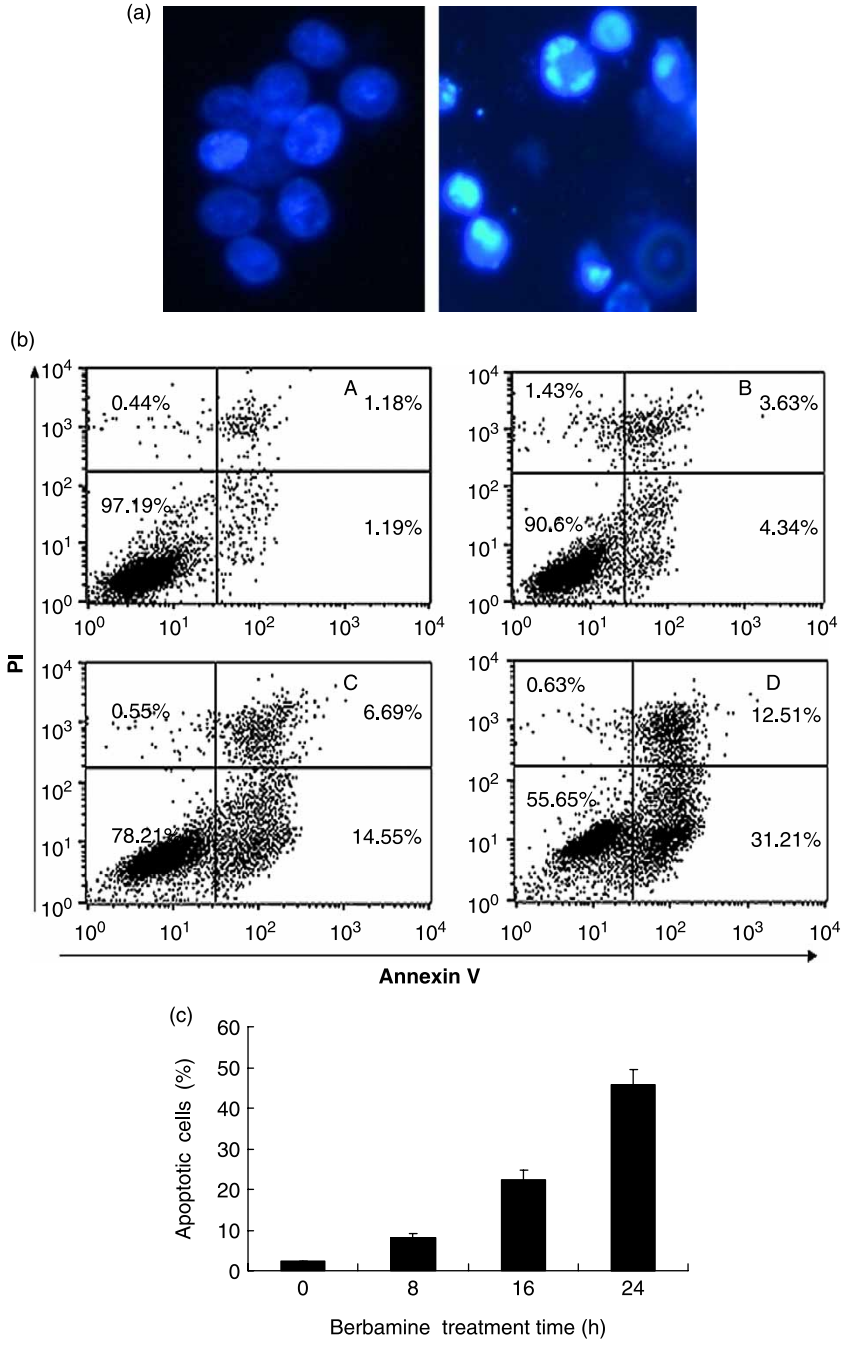

Figure 3. (a) Fluorescence photomicrographs of HepG2 cells stained with Hoechst 33258 (×400). (left) Untreated cells showing diffusely stained intact nuclei; (right) after cells treated with berbamine at 30 μmol/l for 24 h. (b) Fluorescence-activated sorting analysis of Annexin V-FITC and PI for quantification of berbamine-induced apoptosis in HepG2 cells at 30 μmol/l. (A) Untreated, (B) treated for 8 h, (C) treated for 16 h, and (D) treated for 24 h. (c) Time-dependent alteration of apoptosis induced by berbamine at 30 μmol/l. The results were presented as mean ± SD for triplicate.

different time (0, 8, 16, and 24 h); 1.56, 10.62, 25.97, and 55.4% cells were undergoing apoptosis, which indicated that berbamine induced apoptosis in HepG2 cells in a time-dependent manner. It showed that the growth inhibition of berbamine for HepG2 cells was caused by apoptosis.

2.3 *Berbamine upregulates Fas expression in HepG2 cells*

We further investigated whether Fas pathway, which is a key signaling transduction pathway of apoptosis in cells, was involved in berbamine-mediated apoptosis in Fas-positive HepG2 cells. We observed that berbamine exhibited a significant enhancement on the expression of Fas. As shown in Figure 4, when $HepG_2$ cells were exposed to berbamine for different times (0, 8, 16, and 24 h), the percentage of Fas-positive cells increased accordingly. It is suggested that Fas pathway was involved in berbamine-induced apoptosis in HepG2 cells.

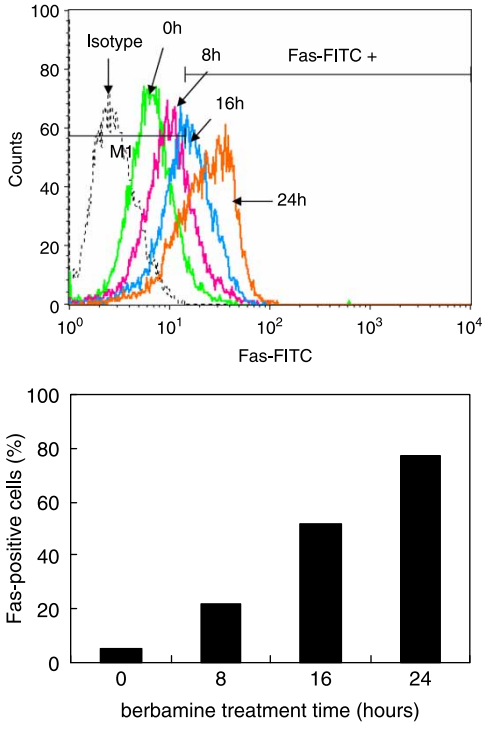

Figure 4. FCM analysis of Fas expression in HepG2 cells treated by berbamine at 30 μmol/l for different times.

2.4 *Berbamine induces loss of mitochondrial membrane potential*

Human HCC HepG2 cell is Fas positive. There are two Fas signaling pathways: type I cells undergo apoptosis independently of mitochondrial signal amplification and type II cells rely on mitochondria for cell death [10,11]. We measured the membrane potential using flow cytometry. Berbamine (30 μmol/l) treatment to HepG2 cells resulted in a rapid dissipation of $\Delta\psi_m$ in a time-dependent manner (Figure 5). Flow cytometric results revealed high level of rhodamine 123 binding to the mitochondrial of untreated HepG2 cells. However, a significant decrease in the fluorescence was observed starting from 8 h after berbamine treatment. The result showed that HepG2 cells were type II cells, which rely on mitochondria in Fas-induced apoptosis.

2.5 *Berbamine-activated caspases expression*

We assessed the activated caspase-3, -8, and -9 levels of HepG2 cells before and after treatment with berbamine using colorimetric assay kit. As shown in Figure 6(a), when HepG2 cells were exposed to 30 μmol/l berbamine for different times (0, 8, 16, and 24 h), the activated caspase-3/8/9 levels increased. Caspase-3 and -9 activities show higher enhancement ratios relative to caspase-8. For further definition on the role of caspase in berbamine-induced apoptosis, cells were pretreated with the broad-spectrum caspase inhibitor, z-VAD-fmk. Cells were treated with 100 μmol/l of z-VAD-fmk 2 h prior to berbamine treatment. The apoptosis was detected 24 h after treatment. As shown in Figure 6(b), z-VAD-fmk attenuated berbamine-induced apoptosis. These findings suggest that the mechanism of HepG2 cell apoptosis induced by berbamine is involved in the activation of caspases.

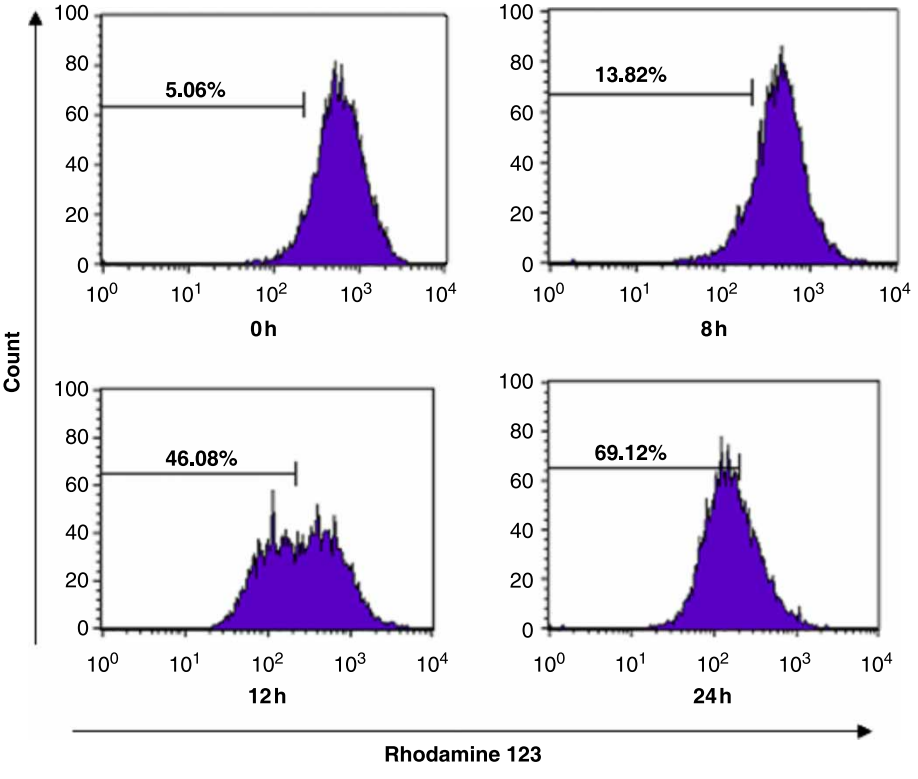

Figure 5. Effect of berbamine on the changes in mitochondrial membrane potential. Loss in mitochondrial membrane potential was analyzed by FCM at a single-cell level using rhodamine 123 as a fluorescent probe. After 30 μmol/l berbamine treatment at 8, 12, and 24 h, cells were stained with rhodamine 123, analyzed on a flow cytometer and histogram display of Rh123 (*x*-axis) vs. counts (*y*-axis) has been shown in logarithmic fluorescence intensity. Data were representative of three similar experiments.

Type II cells produce very little active caspase-8 at the DISC, which is sufficient to cleave the pro-apoptotic BH3 domain containing only Bcl-2 family member Bid [12,13], causing its translocation to the mitochondria, where it induces the release of mitochondrial factors, which ultimately enhance the apoptotic signal. Our study showed that berbamine upregulated Fas expression in HepG2 cells, activated little caspase-8, caused depolarization of mitochondrial membrane, and decreases mitochondrial transmembrane potential, then activated caspase-9 and -3, resulting in the disassembly of HepG2 cell. The results suggest that berbamine induces apoptosis in HepG2 cells through type II Fas signaling pathway.

2.6 Berbermine upregulates P53 expression

Fas expression on the cell's surface increased upon drug treatment, in particular, in cells harboring wild-type p53 [14,15]. We measured the expression level of the tumor suppressor factor P53 in P53-positive HepG2 cells. As shown in Figure 7, HepG2 cells were exposed to 30 μmol/l berbamine for different time (0, 8, 16, and 24 h), and P53 expression increased accordingly. Rapid transport of pre-synthesized Fas receptor molecules stored in vesicles to the cell membrane upon activation of p53 may contribute to the upregulation of Fas surface expression in response to chemotherapy.

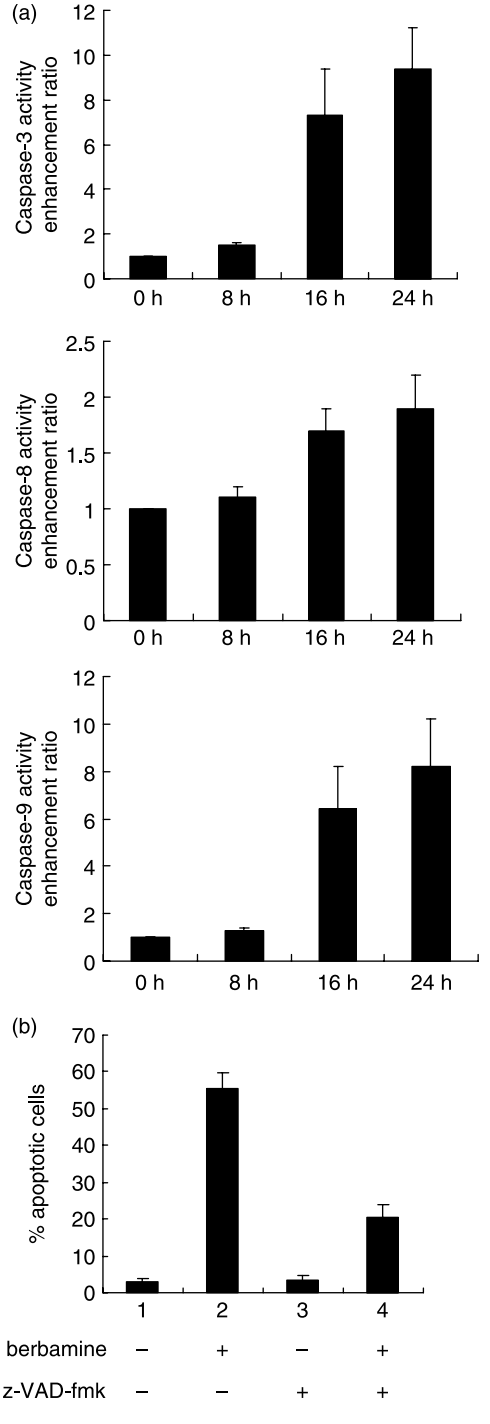

Figure 6. (a) Time-dependent alteration of active caspase-3/8/9 induced by berbamine 30 μmol/l. To investigate whether the caspases are activated in HepG2 cells, a colorimetric assay based on cleavage of caspase-associated substrates were used. Results show that berbamine treatment results in the increase of caspase-3/8/9. (b) Effects of caspase inhibitor, z-VAD-fmk, on berbamine-induced apoptosis detected by Annexin V-staining. Apoptotic cells were determined 24 h after treatment with berbamine (30 μmol/l) in the presence or absence of z-VAD-fmk. Each value is the mean ± SD of three determinations.

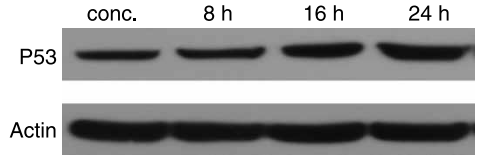

Figure 7. Effect of berbamine on P53 expression in HepG2 cells treated with berbamine at 30 μmol/l for different times.

2.7 *Berbamine inhibits tumor growth in human HCC HepG2-xenografted nude mice*

To confirm the anticancer activity of berbamine on human HCC cells, an *in vivo* experiment was carried out using HepG2-xenografted animal model. Male nude mice were inoculated with human HCC HepG2 cells and treated with berbamine by i.p. injection at 40 or 80 mg/kg once a day for 10 days. At the end of the experiment, a significant suppression of growth was found on berbamine-treated HepG2 xenografts ($p < 0.01$), but not in that of control group (administration of 100 μl PBS) (Figure 8(a) and (b)). Furthermore, berbamine could also induce regression of HepG2 tumors. Tumor growth inhibitory rate was 34.9% at a dosage of 40 mg/kg ($p < 0.05$) and 56.1% at a dosage of 80 mg/kg ($p < 0.001$).

In vivo toxicity was roughly examined by body weight change; there were no significant body weight drop at a dose of 40 mg/kg berbamine during the berbamine treatment period (Figure 8(c)). But there was more drop in body weight in the 80 mg/kg berbamine treatment group compared with the control. No evidence of drug-related toxicity was

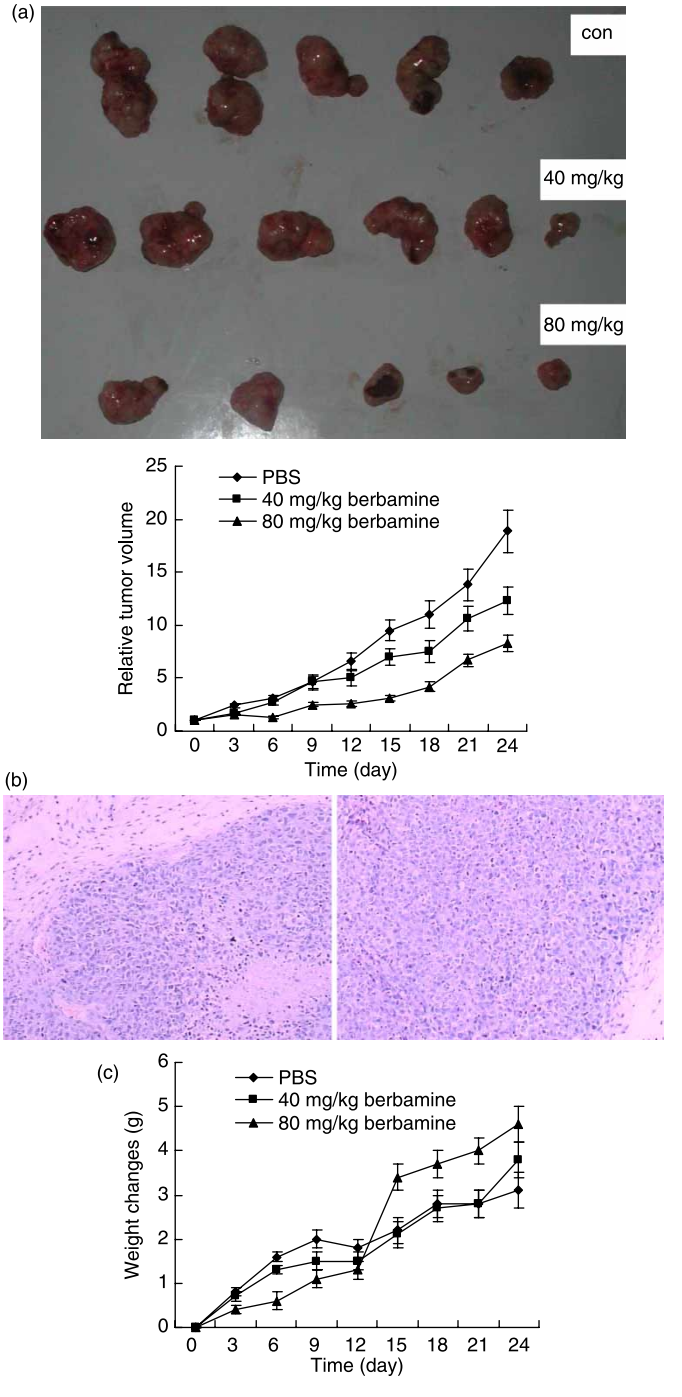

Figure 8. Inhibition of tumor growth in nude mice xenografted with human HCC HepG2 by berbamine. Two weeks after inoculation of human HepG2, mice were randomly divided into three groups, five to six mice each. In treated mice, berbamine was administered i.p. on day 0–10 at a dose of 40 or 80 mg/kg. A significant reduction in tumor volume was observed in treated mice. The figures show the relative tumor volume (a), tumor HE staining (b, left: control; right: 80 mg/kg treatment), and body weight changes (c).

identified in treated animals by histopathologic changes of major organs (the liver, the heart, the lung, and the kidney) of both groups (data not shown). Some researchers have reported that berbamine and other related alkaloids induce gastric lesions [16]. Gastric lesions may affect the mice appetite and induce weight loose. After the berbamine treatment is finished, the 80 mg/kg berbamine treatment group gained more body weight. It may be related to the immunomodulatory properties of berbamine, which could stimulate normal hematopoiesis of cancer patients undergoing chemotherapy or radiotherapy [17–19]. The experimental findings showed that berbamine had antitumor activity in nude mice-bearing human HCC HepG2 xenografts.

3. Experimental

3.1 Chemicals and reagents

Fetal calf serum, Dulbecco's modified Eagle's medium (DMEM), antibiotic–antimycotic (100×) were purchased from Gibocal BRL (Gaithersburg, MD, USA). A stock solution of berbamine (1 mg/ml) in saline was prepared, from which a series of working dilutions were made. Berbamine, dimethyl sulfoxide, MTT, Hoechst 33258, PI, mouse anti-actin, and ribonuclease were purchased from Sigma Chemical Co. (St Louis, MO, USA). Annexin V-FLUOS Staining kit was purchased from Immunotec (Marseille, France). FITC-Fas, mouse IgG-FITC were purchased from PharMingen (San Diego, CA, USA). Mouse anti-P53 was purchased from Maxim Biotech, Inc. (San Diego, CA, USA). Caspase-3, -8, -9 Colorimetric Assay kit and the broad-spectrum caspase inhibitor z-VAD-fmk were purchased from BioVision (Mountain View, CA, USA).

3.2 Cell lines and culture

Human HCC HepG2 cell line was cultured in DMEM, supplemented with 10% FCS, and antibiotics in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

3.3 Cell growth inhibition assay

Cells (3000/well) were plated in 96-well plates in 200 μl medium containing different doses of berbamine from 0 to 96 μmol/l. After 48 h, IC_{50} value was calculated by MTT method. Cell survival rate was calculated as the percentage of MTT inhibition as follows: %survival = (mean experimental absorbance/mean control absorbance) × 100.

3.4 Staining of apoptotic cells with Hoechst 33258

After drug treatment, cells were washed with 0.1 mol/l PBS (pH 7.2) and resuspended in the same buffer. One hundred microliters of cell suspension (1×10^6/ml) were incubated with 1 μl of Hoechst 33258 (1 mg/ml in ddH_2O) for 10 min. Apoptotic cells were evaluated by fluorescence microscopy.

3.5 Annexin V/PI staining assay

Apoptosis was assessed by measuring membrane redistribution of phosphatidylserine using an Annexin V-FITC apoptosis detection kit. According to the manufacturer's protocol, after drug treatments, cells were collected and washed twice with PBS, followed by being resuspended in 500 μl of staining solution containing FITC-conjugated Annexin V antibody (5 μl) and PI (5 μl of 250 μg/ml stock). After incubation in ice for 30 min, cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA). Basal apoptosis and necrosis were identically determined on untreated cells. The percentage of cells undergoing apoptosis was determined by three independent experiments.

3.6 Flow cytometry assay for Fas

After drug treatment, cells were collected and washed with PBS, then labeled with Fas-FITC. Mouse IgG-FITC was used as isotype control. The cells were analyzed by flow cytometry (FACSCalibur).

3.7 Flow cytometry assay for mitochondrial membrane potential ($\Delta\psi_m$)

After drug treatment, cells were collected and washed twice with PBS, then incubated in the presence of rhodamine 123 (final concentration of 2.5 μm) for 10 min in the dark. Then cells were washed with 0.1 M PBS and resuspended in the same buffer. Analysis was performed on a FACSCalibur flow cytometer.

3.8 Caspase activity assay

Activities of caspase-3, -8, or -9 were detected by using a colorimetric assay kit according to the manufacturer's protocol. In brief, 2×10^6 cells were lysed in 50 μl of chilled cell lysis buffer and incubated in ice for 10 min. Cell lysates of 50 μl were added to 50 μl reaction buffer and 5 μl of colorimetric report substrates specific for caspase-3, -8, or -9 in a 96-well microplate. After incubated at 37°C for 1 h, the samples were read at 405 nm.

3.9 Western blotting

Cell lysates were prepared and quantified according to the established methods. To each well of a 10% SDS–polyacrylamide gel, 30 μg of total protein were applied, electrophoresed, and transferred to PVDF membrane. Membranes were blocked using Tris-buffered saline with 5% nonfat milk. Blots were then probed with the primary antibody mouse anti-P53, in blocking buffer, and subsequently by a secondary anti-mouse IgG antibody. Detection was done using an ECL kit. The expression of actin was used as a control.

3.10 Antitumor activity in vivo

Eighteen 6-week-old male BALB/c nude mice (Experimental Animal Center, Zhejiang Traditional Medicine University) were maintained under specific pathogen-free conditions and provided with sterile food and water. *In vitro* cultured HepG2 cells (1×10^7) were injected s.c. into the right back of mice. When the tumor reached 150–200 mm^3 in volume (one mouse was excluded for no tumor formation), animals were divided randomly into test groups consisting of five to six mice per group. Berbamine was administered intraperitoneally at a dose of 40 or 80 mg/kg once a day for 10 days; the control group was injected with the same volume of saline. Mice were weighed every 3 days. The tumor volumes were also determined at the same time points by caliper measurement using the following equation: tumor volume $=$ length $\times$ $(\text{width})^2/2$ and subsequently transformed into relative values (v) ($v = V_t/V_0$, where V_0 is the tumor volume at initiation of treatment, whereas V_t is the tumor volume at any given day during entire treatment period). Tissue samples from mice were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin, cut in 4 μm sections for histologic study.

3.11 Statistical analysis

Data obtained represented mean values of at least three different experiments and are expressed as mean ± SD. Statistical analysis was determined by t-test. $P < 0.05$ was considered statistically significant.

Acknowledgements

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References

[1] D.M. Parkin, F. Bray, J. Ferlay, and P. Pisani, *Int. J. Cancer* **94**, 153 (2001).
[2] A.J. Hall and C.P. Wild, *Br. Med. J.* **326**, 994 (2003).
[3] K. Yang, X. Zhao, P. Xiao, and G. Liu, *Yao Hsueh Tung Pao* **17**, 21 (1982).
[4] C.Q. Xu, D.L. Dong, Z.M. Du, D.W. Chen, D.M. Gong, and B.F. Yang, *Acta Pharm. Sin.* **39**, 691 (2004).

[5] E. Kupeli, M. Kosar, E. Yasilada, K. Husnu, and C. Baser, *Life Sci.* **72**, 645 (2002).
[6] D. Wu, M.F. Lin, and X.Y. Zhao, *Zhongguo Shi Yan Xue Ye Xue Za Zhi* **13**, 373 (2005).
[7] R.Z. Xu, Q.H. Dong, Y. Yu, X. Zhao, X. Gan, D. Wu, Q. Lu, X. Xu, and X.F. Yu, *Leuk. Res.* **30**, 17 (2006).
[8] Q.H. Dong, S. Zheng, R.Z. Xu, Q.H. Lu, and L. He, *Zhongguo Zhong Xi Yi Jie He Za Zhi* **24**, 820 (2004).
[9] X.W. Zhu and W.Z. Sui, *Zhong Xi Yi Jie He Za Zhi* **6**, 611 (1986).
[10] V. Sharma, R. Tewari, U.H. Sk, C. Joseph, and E. Sen, *Int. J. Cancer* **123**, 2204 (2008).
[11] B.C. Barnhart, E.C. Alappat, and M.E. Peter, *Immunology* **15**, 185 (2003).
[12] H. Li, H. Zhu, C.J. Xu, and J. Yuan, *Cell* **94**, 491 (1998).
[13] M.D. McKenzie, E.M. Carrington, T. Kaufmann, A. Strasser, D.C. Huang, T.W. Kay, J. Allison, and H.E. Thomas, *Diabetes* **57**, 1284 (2008).
[14] J.K. Rho, Y.J. Choi, B.Y. Ryoo, I.I. Na, S.H. Yang, C.H. Kim, and J.C. Lee, *Cancer Res.* **67**, 1163 (2007).
[15] M. Muller, S. Wilder, D. Bannasch, D. Israeli, K. Lehlbach, M. Li-Weber, S.L. Friedman, P.R. Galle, W. Stremmel, M. Oren, and P.H. Krammer, *J. Exp. Med.* **188**, 2033 (1998).
[16] E. Kupeli, M. Kosar, E. Yasilada, K. Husnu, and C. Baser, *Life Sci.* **72**, 645 (2002).
[17] C. Luo, X. Lin, W.K. Li, F. Pu, L.W. Wang, S. Xie, and P. Xiao, *J. Ethnopharmacol.* **59**, 211 (1998).
[18] X. Liu and Z.R. Zhou, *J. Chin. Med. Univ.* **25**, 229 (1996).
[19] M.Z. Ge, X. Liu, and Y. Zhang, *Immunol. J.* **14**, 238 (1998).