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Journal of Cellular Biochemistry 115:721–730 (2014)

Journal of Cellular Biochemistry

The Combinational Effect of Vincristine and Berberine on Growth Inhibition and Apoptosis Induction in Hepatoma Cells

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ABSTRACT

The use of vincristine, a known antitumor agent, in hepatoma therapy is limited particularly because of its toxic effect. Meanwhile, berberine has drawn increasing attention to its antineoplastic effect in recent years. In view of the advantages of combinational drug treatment reported in anti-cancer chemotherapy, we evaluated the effects of co-treatment of vincristine and berberine on hepatic carcinoma cell lines in this study. We find that combinational usage of these two drugs can significantly induce cell growth inhibition and apoptosis even under a concentration of vincristine barely showing cytotoxicity in the same cells when used alone. The underlying mechanism about this combinational effect was addressed in this study by monitoring the signals related to mitochondrial function, apoptotic pathway and endoplasmic reticulum stress. Our results suggest a new value of berberine as a potential adjuvant agent in cancer chemotherapy and provide a hopeful approach for developing hepatoma therapy by utilizing the combinational effect of vincristine and berberine. J. Cell. Biochem. 115: 721–730, 2014.

KEY WORDS: VINCRISTINE; BERBERINE; APOPTOSIS; MITOCHONDRIA; HEPATIC CARCINOMA CELLS

Global cancer statistics in 2011 indicates that hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer around the world [Jemal et al., 2011]. However, it is still lack of well established anticancer chemotherapeutic approach against HCC.

Vincristine is a broadly used chemotherapeutic drug for various malignant tumors [Jordan, 2002; Silverman et al., 2013]. It kills cells by disrupting microtubule polymerization and therefore blocking cell division [Mollinedo and Gajate, 2003]. Because it also damages non-neoplastic cells, the toxic side effects of vincristine are obvious in clinic [Skobel and Kammermeier, 1997; Marinina et al., 2000; Dhingra et al., 2013; Smith et al., 2013]. Actually, vincristine does not used alone but commonly administered in combination with other agents for tumor treatment in clinic [Song et al., 2010; Kim et al., 2013; Venkatramani et al., 2013].

Despite of other pharmacological and biological activities, the anticancer effect of berberine, an isoquinoline plant alkaloid isolated from *Coptidis rhizome*, has attracted many attentions [Amin et al., 1969; Ghosh et al., 1985; Averbuch et al., 1988; Huang, 1992; Galle et al., 1994; Chinese Herbals Editor Board tSABoCMoC, 1999; Upadhyay and Yadav, 1999; Ji et al., 2000; El-Serag, 2002a,b; Grycová et al., 2007; Yuen et al., 2009; Singh and Kakkar, 2009; Wu et al., 2010; Tan et al., 2011]. Recent research focused on berberine's anti-tumor effect has shown that berberine can induce growth inhibition or apoptosis in broad tumor cell types derived from leukocytes, liver, lung, gastrointestinal tract, oral, skin, brain, bone, bladder, breast, cervix, and prostate [Mantena et al., 2006; Hsu et al., 2007; Choi et al., 2008; Meeran et al., 2008; Patil et al., 2010]. Animal studies also showed that berberine can suppress chemical-induced carcinogenesis tumor invasion and the growth of HCC xenograft.

[#]Ling Wang and Dandan Wei contributed equally to this work.

Grant sponsor: National Natural Science Foundation of China; Grant number: 81273224; Grant sponsor: Huaxi Hospital of Sichuan University.

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Manuscript Received: 31 October 2013; Manuscript Accepted: 6 November 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 14 November 2013

DOI 10.1002/jcb.24715 • © 2013 Wiley Periodicals, Inc.

Therefore, berberine is elementarily recognized as an anticancer herbal medicine.

Recent years, researchers and tumor therapeutists have taken an increasing interest in the combination therapy by associating anticancer herbal medicine with chemotherapeutic agent, and gained notable successes [Tyagi et al., 2002; Notarbartolo et al., 2005; Zhao et al., 2010]. In these studies, the doses of chemo-drugs efficient for killing cells were apparently cut down and the toxic effects of these drugs were reduced when combinational treatment applied.

Considering the toxicity of vincristine is high to hepatic cells, we investigated the combined effect of vincristine and berberine in two hepatic carcinoma cell lines in present study, by detecting the drug-induced growth inhibition and apoptosis, and exploring underlying mechanisms.

MATERIALS AND METHODS

MATERIALS

Berberine hydrochloride (berberine) was purchased from SCMUST (Chengdu, China) and vincristine sulfate (vincristine) from MingXing Pharmaceutical Co. Ltd (Guangzhou, China). Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Trizol reagent was purchased from Invitrogen (Carlsbad, CA) and the reagents used for quantitative real time RT-PCR (qRT-PCR) were from Transgen (Beijing, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO). Antibody against β-actin was purchased from Zhongshan (Beijing, China), and antibody against poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP) from Boisynthesis (Beijing, China).

CELL CULTURE

Human hepatocellular carcinoma cell lines (HepG2 and SMMC7721) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (v/v), and penicillin (100 U/ml)/streptomycin (100 μg/ml).

CELL VIABILITY ASSAY

Vincristine and berberine were dissolved in phosphate buffered saline (PBS). HepG2 and SMMC-7721 cells were plated in 96-well plate with complete medium at 5×10^3, 3×10^3 and 2×10^3/well. After 24 h, the cells were incubated with increasing concentrations of vincristine and berberine for 24, 48, and 72 h. Thereafter, the cells were incubated with MTT at 37°C for 4 h, and DMSO was added to dissolve the formazan crystals. Absorbance at 570 nm was then measured with a spectrophotometer (BIO-RAD Model 680).

COLONY FORMATION ASSAY

Cells were plated in six-well plates at 800 cells per well. After 2 days of growth, cells were treated by vincristine and berberine alone and together for 24 h. Then the medium was changed to blank complete medium and the cells were cultured for another 10 days. Cell colonies were stained with crystal violet as described previously [Li et al., 2011].

JC-1 STAINING ASSAY

Mitochondrial membrane potential (MMP) was determined with JC-1 assay kit (Beyotime, Shanghai, China). Cells were seeded at 3×10^3 cells/well in a 96-well plate. After incubation with vincristine and berberine for 48 h, the change of fluorescence in cells was visualized and photographed by converted fluorescence microscope (Nikon, ECLIPSE TI-U).

DCFH-DA STAINING ASSAY

Reactive oxygen species (ROS) assay kit (Applygen, Beijing, China) was used for the detection of ROS generation. Cells were treated by vincristine and berberine for 24 h, then washed with PBS and incubated with 10 μM DCFH-DA fluorescent probe for 40 min at 37°C. After two times washing with PBS, cells were digested and collected to quantify the level of ROS. The mean fluorescence intensity was analyzed by flow cytometry as described [Mo et al., 2013]. Cells were also observed and photographed by converted fluorescence microscope.

FACS ASSAY

Cell cycle distribution was analyzed by flow cytometry. Cells were plated in six-well plates at 2×10^5 cells per well and treated with vincristine (1 nM) and berberine (30 μM) for 24 h. Cells were harvested, washed with PBS and fixed in 70% ethanol at −20°C for at least 2 h. Fixed cells were washed with PBS again and incubated with PBS containing 50 μg/ml propidium iodide (PI), 100 μg/ml RNase A and 0.1% Triton X-100 for 30 min at 4°C in dark. The stained cells were analyzed by flow cytometry.

TUNEL ASSAY

Apoptotic cell death was evaluated by in situ cell death detection kit (Roche Applied Science, Indianapolis, IN), which is based on labeling of DNA strand breaks (Tunel technology). Cells were seeded on glass cover slips placed in six-well plates at 2×10^5 cells per well and treated by vincristin and berberine for 48 h. The detection of DNA strand breaks was performed according to the manufacturer's instruction and the incorporated fluorescein was visualized under a converted fluorescence microscope. The nucleolus of all cells in the assay was stained with DAPI.

QUANTITATIVE REAL TIME RT-PCR

Total RNA was extracted from about 2×10^5 cells by using Trizol reagent. Five hundred nanograms total RNA was used for reverse transcriptase-mediated cDNA synthesis and qRT-PCR was performed following the manufacturer's instruction. Sequences of the primers used for following human genes detection are as below: p21, 5′-CCT gTC ACT gTC TTg TAC CCT-3′and 5′-gCg TTT ggA gTg gTA gAA ATC T-3; BAX, 5′-gAT gCg TCC ACC AAg AAg CT-3′and 5′-Cgg CCC CAg TTg AAg TTg-3′; Caspase3, 5′-ACT CTg gAA TAT CCC Tgg ACA ACA-3′ and 5′-gAC TTC TAC AAC gAT CCC CTC TgA-3′; GADD153, 5′-TgA TgC TCC CAA TTg TTC ATg-3′ and 5′-TCg CCg AgC TCT gAT TgA C-3′; MDR1, 5′-CCC ATC ATT gCA ATA gCA gg-3′ and 5′-gTT CAA ACT TCT gCT CCT gA-3′ and GAPDH (internal reference gene), 5′-TgA Agg TCg gAg TCA ACg gA-3′ and 5′-CCT ggA AgA Tgg TgA Tgg gAT-3′.

WESTERN BLOT

Whole cell lysates were prepared as before [Yang et al., 2013]. About 100 μg of protein extracts were separated on SDS–polyacrylamide gel. Semi-dry transfer method was adopted for transferring proteins to PVDF membranes. Then the membranes were blocked with

5% skim milk in TBST (Tris-buffered saline containing 0.1% Tween), and probed with anti-PARP (1:500), and anti-β-actin (1:2,000) antibodies at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG (1:2,000) for 1 h at room temperature. The immunoreactive bands were visualized by an enhanced chemiluminescence system.

STATISTICAL ANALYSIS

Results are expressed as the means ± SEM. Statistical significances among groups were analyzed by one-way analysis of variance (ANOVA) followed by a Turkey comparisons test. A value with $P < 0.05$ was considered to be statistically significant.

RESULTS

CYTOTOXICITY OF VINCRISTINE AND BERBERINE ON HEPATIC TUMOR CELLS

At first, Cytotoxicity of vincristine and berberine was evaluated separately in two human hepatocellular carcinoma cell lines, HepG2 and SMMC-7721, and one non-neoplastic hepatic cell line, LO2. Following the cells were treated with serially increasing concentrations of vincristine and berberine for 24, 48, and 72 h, respectively, the viability of these cells were measured via MTT assay. The results showed that either vincristine- or berberine-treatment decreased the viability of cells (Fig. 1). For vincristine, IC50 in HepG2 cells was 4 nM for 72 h, while no IC50 could be figured out in SMMC-7721 cells

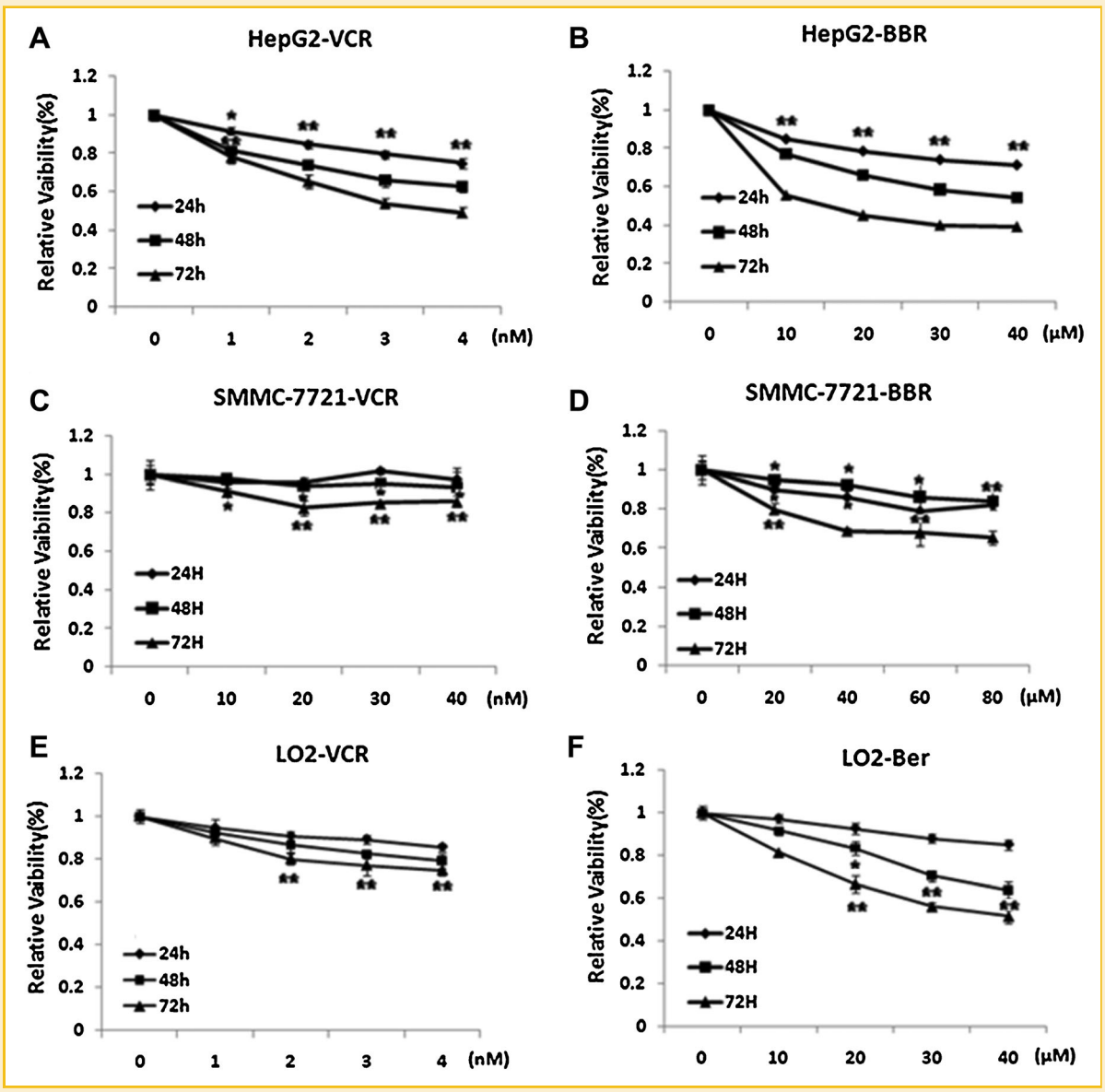

Fig. 1. Time- and dose-dependent viability curves of vincristine and berberine in hepatic cells. HepG2 cells (A,B), SMMC-7721 cells (C,D) and LO2 cells (E,F) were treated with serially concentrations of vincristine and berberine for 24, 48, and 72 h, respectively. MTT assay was performed. Data were analyzed as means ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control.

even the concentration of vincristine up to 40 nM (Fig. 1A,C). For berberine, the IC50 in HepG2 cells was around 40 μM for 48 h and 10 μM for 72 h (Fig. 1B) but the largest viability inhibition in SMMC-7721 cells was less than 30% even at 72 h when 40 μM berberine was applied (Fig. 1D). The toxicity of these two drugs in LO2 cells was lower than that in HepG2 cells but higher than that in SMMC-7721 cells. These results reveal the cytotoxicities of vincristine and berberine are diverse in different hepatic cells and demonstrate that HepG2 cells were more sensitive to these toxicities than SMMC-7721 cells.

CO-TREATMENT OF VINCRISTINE AND BERBERINE PROMOTED THE DECREASE OF CELL VIABILITY IN HEPATIC TUMOR CELLS

To test the combined effect of vincristine and berberine on cell viability, co-treatment experiments were conducted. Based on above experiment, we adopted the concentrations of vincristine and berberine used for co-treatment as following: 1 nM vincristine and 30 μM berberine for HepG2 cells and 20 nM vincristine and 40 μM berberine for SMMC-7721 cells. In these experiments, cells were incubated with vincristine and berberine alone or together, followed by detecting the cell viability via MTT assay. The results were summarized in Figure 2. As shown, the co-treatment of vincristine and berberine clearly promoted the decline of cell viability, This promotion was mostly significant in comparison the co-treatment group with either vincristine alone group or berberine alone group (V vs. VB or B vs. VB). Notably, the combinational effect was more apparently come into play in vincristine sensitive HepG2 cells.

CO-TREATMENT OF VINCRISTINE AND BERBERINE ENHANCED THE GROWTH INHIBITION IN HEPATIC TUMOR CELLS

With 48 h incubation, neither 1 nM of vincristine nor 30 μM of berberine triggered visible cell death in HepG2 cells, whereas the co-treatment induced obvious cell floating and rounding (Fig. 3A). To measure the effect of co-treatment on growth inhibition in HepG2 cells, colony-forming assay, cell cycle analysis and qRT-PCR were performed. From colony-forming assay, we observed that even though single drug treatment decreased visible cell colonies, co-treatment resulted in much severe colony reduction (Fig. 3B). By cell cycle analysis, we found that vincristine alone caused G2/M arrest, but berberine alone or co-treatment induced evident G1 arrest (Fig. 3C). Consistent with above measurements, the results based on quantitative real time RT-PCR assay showed that the transcription of the p21 gene, a regulator of cell cycle progression at G_1 and S phase, was obviously elevated by co-treatment, although berberine alone did the same (Fig. 3D). These results show that the combinational effect of vincristine and berberine can enhance the growth inhibition of HepG2 cells.

CO-TREATMENT OF VINCRISTINE AND BERBERINE INDUCED APOPTOTIC CELL DEATH IN HEPG2 CELLS

To assess whether co-treatment can promote apoptosis in HepG2 cells, we examined the DNA brand break by Tunel technology. Apparent numbers of Tunel-positive cells were observed in co-treatment group, whereas only a few in berberine group and vincristine group (Fig. 4A,B). Similar results were obtained by DAPI staining, where the numbers of condensed nucleolus in co-treatment group were more than those in other groups (Fig. 4A). In addition, qRT-PCR assay was conducted for detecting the transcriptional activity of apoptosis-marker genes, BAX and Caspase 3. In berberine and co-treatment groups, the expressions of both genes were increased significantly, with higher increase in co-treatment group (Fig. 4C). To get the evidence about the situation of drug-induced apoptotic cell death from the view of protein level, the cleavage of poly (ADP-ribose) polymerase (PARP), another known apoptosis-

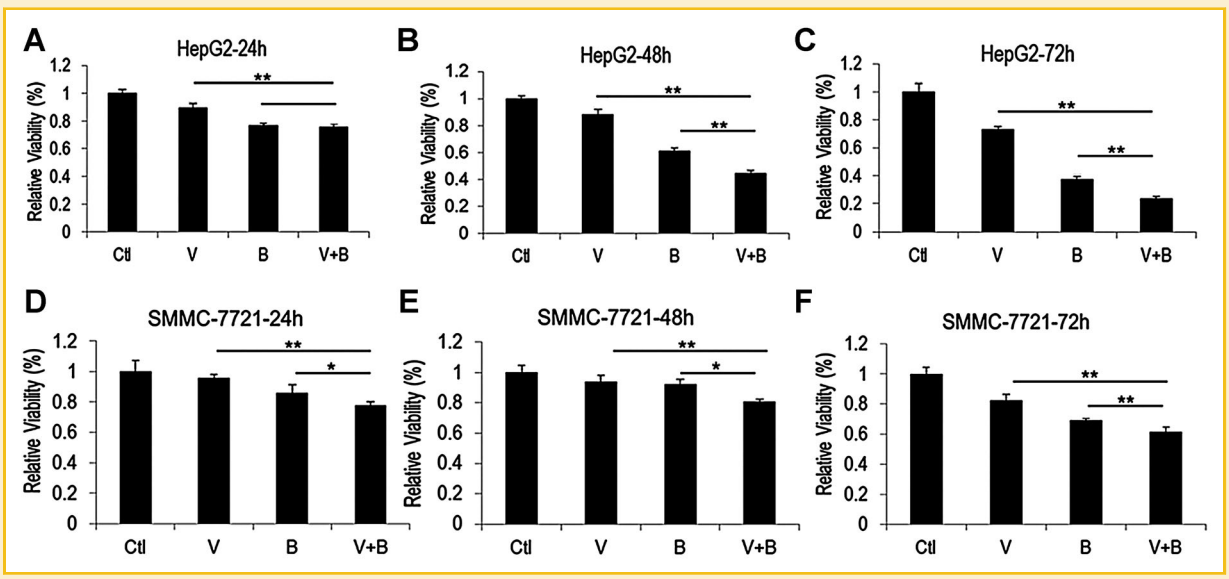

Fig. 2. The combinational effect of vincristine and berberine on cell viability. HepG2 cells (A–C) were left untreated (Ctl) or treated with vincristine (V, 1 nM), berberine (B, 30 μM) and both (V + B) for 24, 48, and 72 h, respectively. SMMC-7721 cells (D–F) were treated with vincristine (20 nM), berberine (40 μM) and both for 24, 48, and 72 h, respectively. Cell viability was detected by MTT assay. Data were analyzed as means ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control.

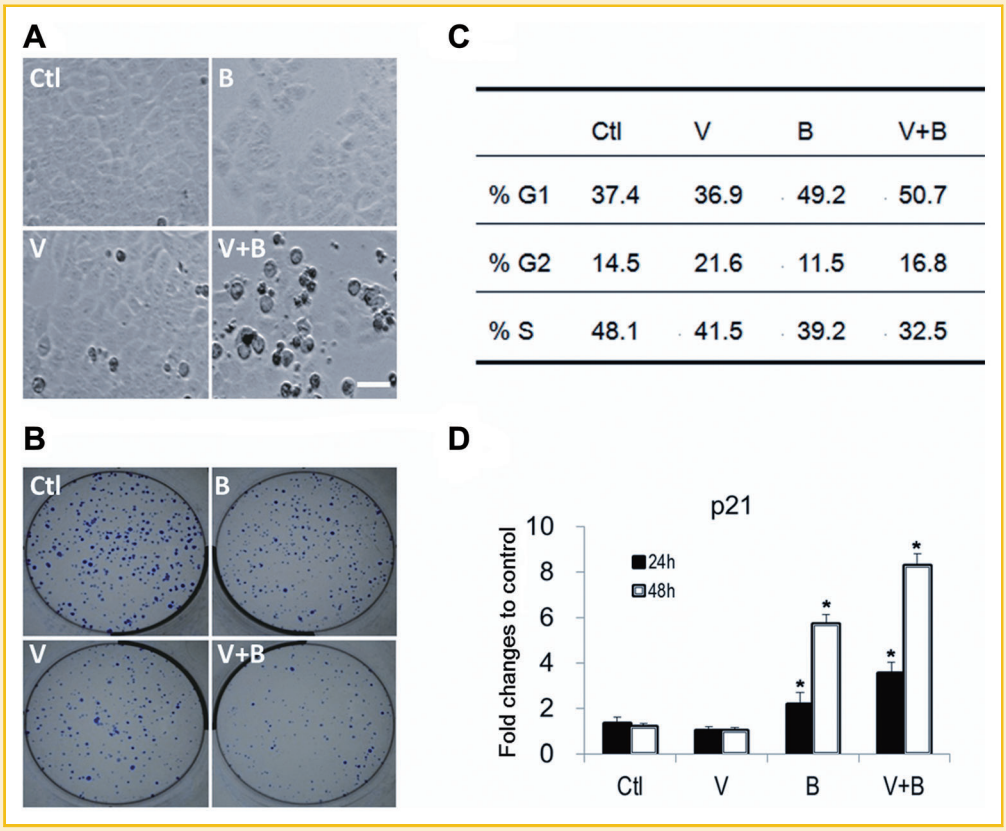

Fig. 3. Co-treatment of vincristine and berberine induced growth inhibition in HepG2 cells. A: Microscopic images of cells after 48 h treatments as indicated, 1 nM of vincristine and 30 μM of berberine were applied. magnification, 200×. B: Cells were treated with vincristine or/and berberine for 24 h, the colonies of cells growing for another 10 days were shown by crystal violet staining assay. C: After 24 h treatment, the phase distributions of cell cycles were detected by flow cytometry. D: The mRNA level of the p21 gene was analyzed by qRT-PCR. Fold changes relative to control were displayed. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control.

marker, was checked by Western blot. The results showed that cleaved PARP protein markedly increased in berberine and co-treatment groups (Fig. 4D). These results consistently indicate that the co-treatment of vincristine and berberine enhanced the apoptosis in HepG2 cells.

CO-TREATMENT OF VINCRISTINE AND BERBERINE CAUSED THE DAMAGES OF MITOCHONDRIAL FUNCTION

We next addressed intracellular reactive oxygen species (ROS) level and mitochondrial membrane potential (MMP), as they are wildly accepted parameters of mitochondria function. To measure intracellular ROS, the cells with 24 h-treatment were labeled by DCFH-DA probe. Figure 5A,B demonstrates the results obtained by flow cytometry, showing that berberine alone and co-treatment markedly increased ROS generation in cells. Figure 5C presents a set of fluorescent microimages displaying that the visible ROS positive cells were more in co-treatment group than those in other groups. To evaluate MMP status, JC-1 staining was conducted, in which the JC-1 aggregates (red) and JC-1 monomers (green) reflect higher or lower level of mitochondrial membrane polarization, respectively. As shown in Figure 5D, the overall intensity of red fluorescence in berberine groups markedly decreased, indicating a reduction of MMP. Coherently, the green fluorescence positive cells were the most in co-treated group.

THE EXPRESSION ALTERATIONS OF ER-STRESS AND DRUG-RESISTANT RELATED GENES

Endoplasmic reticulum (ER)-stress is another general incentive of apoptosis, so we investigated the expression of GADD153 gene, one of the components of the ER stress-mediated apoptosis pathway [Liu et al., 2013]. As shown in Figure 6A, the transcription of the GADD153 gene do increased when the cells were exposed to berberine for 24 and 48 h. Clearly, the increased transcription of this gene was particularly higher in co-treatment cells.

The expression of the MDR1 gene, a well known drug efflux transporter, generally influences the intracellular concentration and the cytotoxicity of vincritine [Woodahl et al., 2009; Zhou et al., 2010]. For this reason, the transcriptional expression of the MDR1 gene was measured by quantitative real time RT-PCR in our system. The result indicated that nevertheless vincristine alone slightly elevated the transcription of the MDR1 gene, berberine and co-treatment obviously reduced the transcription of this gene (Fig. 6B). These results show that berberine as well as co-treatment can elevate the cytotoxicity of these drugs to hepatic carcinoma seemed through a

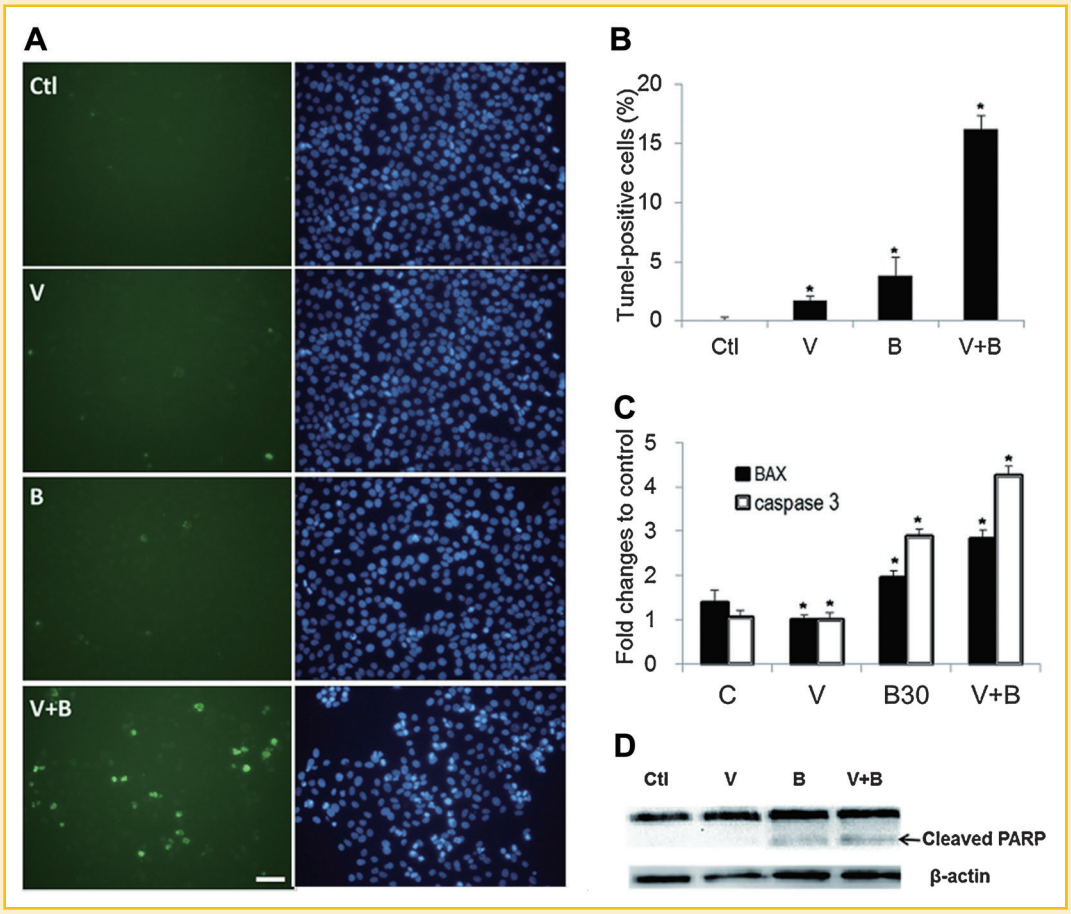

Fig. 4. Co-treatment of vincristine and berberine induced apoptosis in HepG2 cells. A: Cells were treated with vincristine (1 nM) or/and berberine (30 μM) for 48 h and the apoptotic cells were detected by Tunel technology. Photographs were taken by converted fluorescence microscope, magnification, 100×. B: The ratios of Tunel positive cells were counted from the results of Tunel assay. C: Cells were treated as above for 48 h, and the mRNA levels of the BAX gene and the caspase 3 gene were analyzed by qRT-PCR. Fold changes relative to control were displayed. D: Cells were treated for 24 h (upper) and 48 h (lower), and cleaved poly (ADP-ribose) polymerase (PARP) was estimated by Western blot analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control.

way involved in reducing the expression of the MDR1 gene and sequential drug efflux.

DISCUSSION

In the present study, the combinational effects of vincristine and berberine on growth inhibition and apoptotic cell death of hepatoma cells is evidenced. This finding is based on the facts that co-treatment with vincristine and berberine significantly reduced the viability of hepatocellular carcinoma cell lines when comparing to the drugs were applied separately; and that co-treatment also increased apoptotic cell death in our system. These results demonstrate that berberine should be a powerful adjuvant for anti-cancer therapy, particularly when it combines with vincristine for hepatoma treatment. Our finding provides a new evidence for the importance and the effectiveness of combinational drug treatment in anti-cancer chemotherapy.

Despite of being a powerful anti-cancer drug in experiments, the clinical usage of vincristine is largely limited because its therapeutic dosages usually cause several toxic effects [Jackson and Bender, 1979; McCune and Lindley, 1997; Skobel and Kammermeier, 1997; Marinina et al., 2000]. It has been wildly accepted that the high toxicity of vincristine comes from its action on inhibiting microtubule polymerization, which can damage both malignant and nonmalignant cells [Himes et al., 1976; Owellen et al., 1976]. Even many efforts have been paid for lowering the toxic effect of vincristine by combinational using it with other anti-cancer drugs, the balance between the therapeutic efficiency and tolerable side effect has not been well established [Evans et al., 1982; Allerton, 1996; Gillies et al., 1998]. Importantly, this problem not only exists in vincristine utilization but also in the use of other approved drugs directed for cancer therapy [Tange and Vuzevski, 1984; Löw-Friedrich et al., 1990; Sasson et al., 1994; Koutinos et al., 2002]. Given this fact, it is a common view to develop new anti-cancer therapy by expanding our attention from the combinational usage of traditional anti-cancer drugs to broader resources.

Berberine has been studied recently for its antineoplastic nature [Mantena et al., 2006; Hsu et al., 2007; CHOI et al., 2008; Meeran

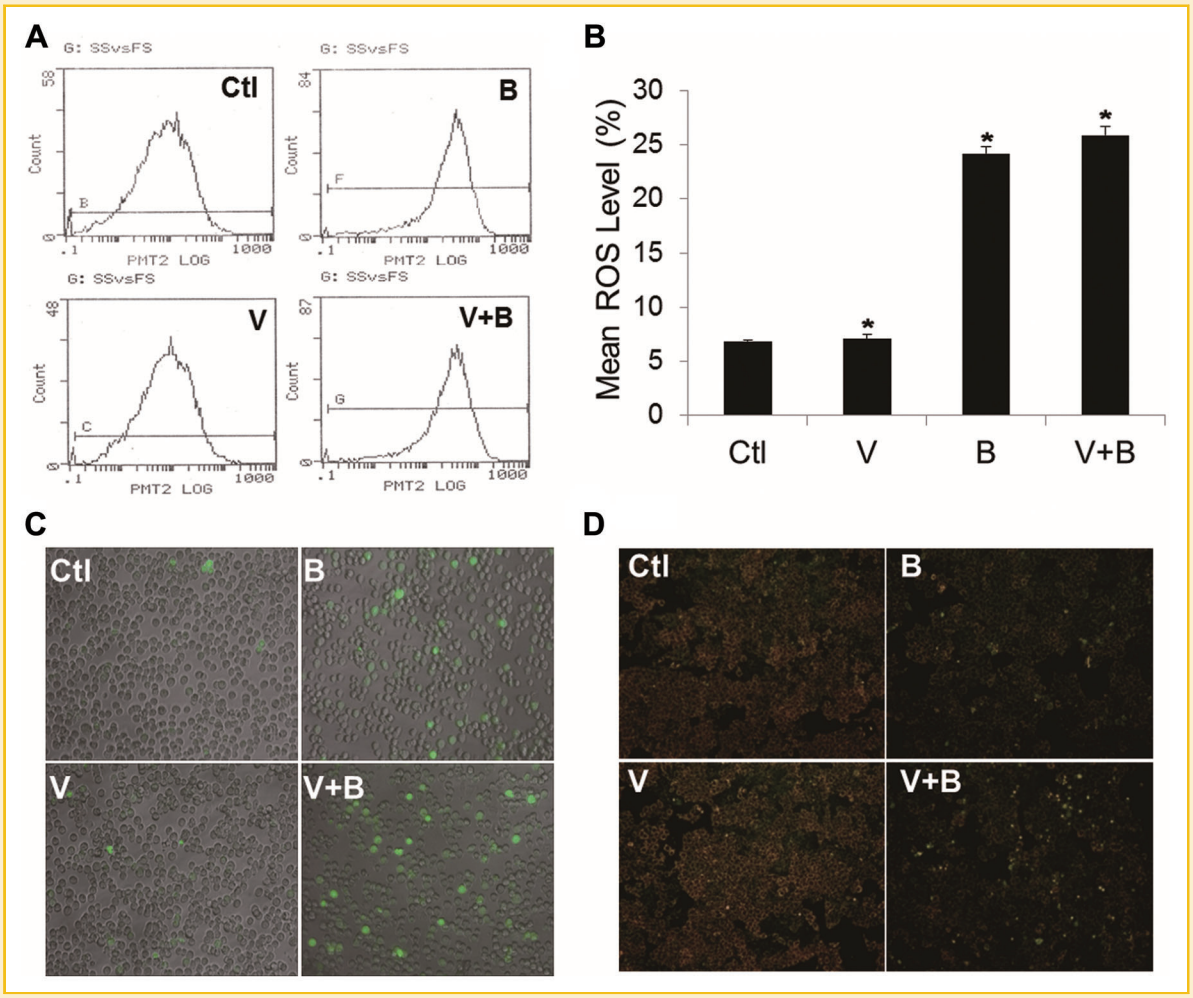

Fig. 5. The combinational effect of vincristine and berberine on cell mitochondrial functions. A: Cells were treated as indicated for 24 h and the generation of reactive oxygen species (ROS) was assayed after DCFH-DA staining and by FACS analysis. B: Mean ROS level calculated from FACS analysis. C: DCFH-DA stained cell populations were visualized by converted fluorescence microscope, and randomly selected images are shown. magnification, 100×. D: After 48 h treatment, mitochondrial membrane potential (MMP) in the cells was displayed by JC-1 staining and observed under converted fluorescence microscope, magnification, 100×.

et al., 2008; Patil et al., 2010]. However, the mechanisms of this nature remain unclear. Someone reported that berberine induced mitochondrial-dependent apoptosis in breast cancer cells (MCF-7) [Patil et al., 2010]. It is also known that berberine can directly damage DNA in high concentration and induce apoptosis [Kuo et al., 1995]. To know whether berberine can be used for combinational therapy against cancer, we focused on assessing the effect of berberine in hepatoma cell system when conbinationally used with vincristine, because of the well known hepatocyte toxicity of vincristine [Mattos et al., 2001]. Our results clearly displayed not only the antiproliferative effect of vincristine and berberine alone, but also the strengthened antitumor effect when these two drugs were used together. These results are accordant to the combinational effect of berberine and carmustine [Zhang et al., 1990] and of berberine and cisplatin [Youn et al., 2008], suggesting the wide usage value of berberine in anti-cancer therapy. It is worth to mention here that, based on statistic analysis, the combinational effect of vincristine and berberine in our experimental system is additive for growth inhibition (Fig. 2), but synergistic for apoptosis induction (Fig. 4B).

In this study, the combinational effect of vincristine and berberine includes not only cell growth inhibition, but also apoptotic cell death induction. As shown in results, the signs of apoptotic cell death were faint when vincristine and berberine was used alone, but became stronger when they were used together. We noticed from previous reports, that the IC50 of vincristine in HepG2 cells are inconsistent, ranging from 4.4 to 200 nM [Jongsma et al., 2000; Gasparotto et al., 2006; Kim et al., 2009], which is similar to or higher than what we observed. The difference in the IC50 of vincristine in this cells may be explained by the discrepancy of culture conditions and cell passages, or the activity variation among different lots of drugs.

To understand the mechanisms for the combinational effect observed, we investigated mitochondrial function of the cells. Berberine is a molecule predominantly distributed in mitochondria.

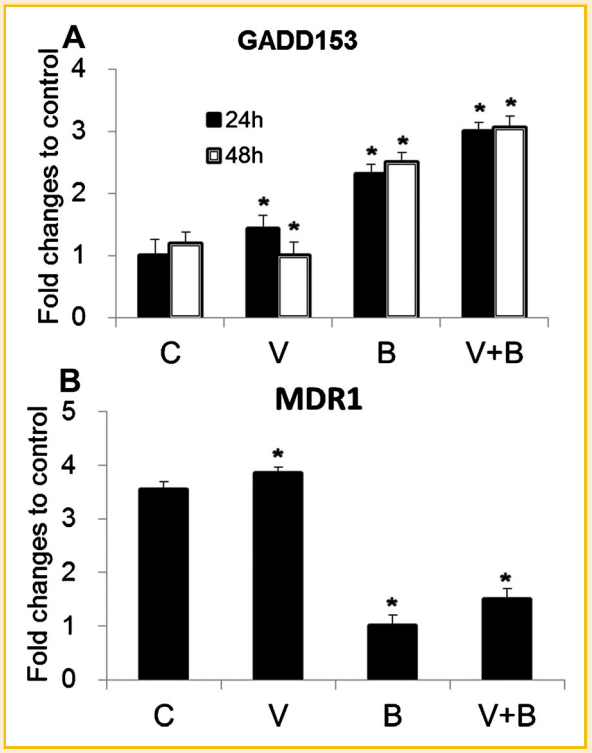

Fig. 6. The influence of co-treatment in the expression of the GADD153 gene and the MDR1 gene. A: Relative folds of GADD153 mRNA in the cells accepted indicated treatments for 24 and 48 h. B: Relative folds of MDR1 mRNA in the cells accepted indicated treatments for 24 h. All data were obtained by qRT-PCR assay. Fold changes relative to control were displayed. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control.

By checking intracellular distribution of berberine under fluorescent microscopy (berberine is an auto-fluorescence), we found that lower than 50 μM of berberine was mostly distributed in mitochondrial (data not shown). The same result has been reported previously [Serafim et al., 2008]. In our experiments, 20–30 μM of berberine alone showed slight influence for mitochondrial functions, but the co-treatment with vincristine strengthened mitochondrial damage, showing by increased ROS generation and decreased MMP. Then, we investigated the involvement of ER stress-mediated apoptosis in the combinational effect, as it was reported that berberine can induce GADD153-mediated, caspase-dependent apoptosis in human cervical cancer Ca ski cells [McCullough et al., 2001; Oyadomari and Mori, 2003; Lin et al., 2007]. Coincide with their studies, the treatment by berberine alone or with vincristine induced the elevation of GADD153 expression in our study. Together with other signs of apoptosis we observed, such as elevated expression of the BAX gene, increased tunel positive cells, condensed DAPI-staining nucleus, and boosted the cleavage of PRAP, we think the apoptosis promoted by co-treatment is related to ER stress.

Considering the expression of drug resistance genes is important for the therapeutic efficiency of vincristine, we also preliminarily checked the influence of this combinational treatment in the expression of a major drug-resistant gene, MDR1 (P-glycoprotein [P-gp, ABCB1]) in HepG2 cells. Vincristine did not significantly affect the expression of the MDR1 gene in our system. However, berberine, either alone or together with vincristine, could suppress the transcription of the MDR1 gene. This result needs to be further confirmed in future study at protein level.

In summary, our study firstly demonstrate that berberine in combination with vincristine has markedly combinational effect on growth inhibition and apoptosis induction in human hepatoma cells. The mechanisms underlying these effects may relevant to the dysfunction of mitochondria and the induction of endoplasmic reticulum stress. Besides, berberine seems play a leading role in these combinational effect. These results indicate that berberine might be an optional adjuvant for cancer therapy, especially for HCC. However, in vivo study and further mechanistic analysis are necessary to confirm the effect of this combination.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China to H. Xiao (Grant Number 81273224) and the grant from Huaxi Hospital of Sichuan University to H. Xiao. The authors thank Dr. Ping Lin, Xiujie Wang and Yi Chen for all-around supports.

REFERENCES

Allerton R. 1996. Acute mesenteric ischaemia associated with 5-FU, cisplatin and vincristine chemotherapy. Clin Oncol 8:116–117.

Amin A, Subbaiah T, Abbasi K. 1969. Berberine sulfate: Antimicrobial activity, bioassay, and mode of action. Can J Microbiol 15:1067–1076.

Averbuch SD, Steakley CS, Young RC, Gelmann EP, Goldstein DS, Stull R, Keiser HR. 1988. Malignant pheochromocytoma: Effective treatment with a combination of cyclophosphamide, vincristine, and dacarbazine. Ann Intern Med 109:267.

Chinese Herbals Editor Board tSABoCMoC. 1999. Chinese Herbals (Zhong Hua Ben Cao)editor^editors. Shanghai: Shanghai Science and Technology Press. p 11.

Choi MS, Yuk DY, Oh JH, Jung HY, Han SB, Moon DC, Hong JT. 2008. Berberine inhibits human neuroblastoma cell growth through induction of p53-dependent apoptosis. Anticancer Res 28:3777–3784.

Dhingra D, Sethi GR, Mantan M. 2013. Unilateral phrenic nerve palsy: A rare manifestation of vincristine neurotoxicity. Indian J Pediatr [Epub ahead of print].

El-Serag HB. 2002a. Hepatocellular carcinoma: An epidemiologic view. J Clin Gastroenterol 35:S72.

El-Serag HB. 2002b. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 36:s74–s83.

Evans AE, Land VJ, Newton WA, Randolph JG, Sather HN, Tefft M. 1982. Combination chemotherapy (vincristine, adriamycin, cyclophosphamide, and 5-fluorouracil) in the treatment of children with malignant hepatoma. Cancer 50:821–826.

Galle K, Blodt S, Wagner H. 1994. TLC and HPLC detection of alkaloids in Mahonia aquifolium and related plants. Dtsch Apoth Ztg 134:35.

Gasparotto V, Castagliuolo I, Chiarelotto G, Pezzi V, Montanaro D, Brun P, Palù G, Viola G, Ferlin MG. 2006. Synthesis and biological activity of 7-phenyl-6, 9-dihydro-3 H-pyrrolo [3,2-f] quinolin-9-ones: A new class of antimitotic agents devoid of aromatase activity. J Med Chem 49:1910–1915.

Ghosh AK, Bhattacharyya FK, Ghosh DK. 1985. Leishmania donovani: Amastigote inhibition and mode of actior of berberine. Exp Parasitol 60: 404–413.

Gillies J, Hung K, Fitzsimons E, Soutar R. 1998. Severe vincristine toxicity in combination with itraconazole. Clin Lab Haematol 20:123–124.

Grycová L, Dostal J, Marek R. 2007. Quaternary protoberberine alkaloids. Phytochemistry 68:150–175.

Himes RH, Kersey RN, Heller-Bettinger I, Samson FE. 1976. Action of the vinca alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules in vitro. Cancer Res 36:3798.

Hsu WH, Hsieh YS, Kuo HC, Teng CY, Huang HI, Wang CJ, Yang SF, Liou YS, Kuo WH. 2007. Berberine induces apoptosis in SW620 human colonic carcinoma cells through generation of reactive oxygen species and activation of JNK/p38 MAPK and FasL. Arch Toxicol 81:719–7728.

Huang W. 1992. A study of the antiarrhythmic mechanism of berberine on delayed activation potassium current by voltage clamp. Zhonghua Xin Xue Guan Bing Za Zhi 20:310.

Jackson DV, Bender RA. 1979. Cytotoxic thresholds of vincristine in a murine and a human leukemia cell line in vitro. Cancer Res 39:4346.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. CA Cancer J Clin 61:69–90.

Ji X, Li Y, Liu H, Yan Y, Li J. 2000. Determination of the alkaloid content in different parts of some Mahonia plants by HPCE. Pharm Acta Helv 74: 387–391.

Jongsma A, Riethorst A, Lankelma J, Dekker H, Westerhoff H. 2000. Evaluating limited specificity of drug pumps. Eur J Biochem 267:5369–5377.

Jordan M. 2002. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. Curr Med Chem Anticancer Agents 2:1–17.

Kim SN, Kim NH, Park YS, Kim H, Lee S, Wang Q, Kim YK. 2009. 7-Diethylamino-3 (2′-benzoxazolyl)-coumarin is a novel microtubule inhibitor with antimitotic activity in multidrug resistant cancer cells. Biochem Pharmacol 77:1773–1779.

Kim H, Kang HJ, Lee JW, Park JD, Park KD, Shin HY, Ahn HS. 2013. Irinotecan, vincristine, cisplatin, cyclophosphamide, and etoposide for refractory or relapsed medulloblastoma/PNET in pediatric patients. Childs Nerv Syst 29:1851–1858.

Koutinos G, Stathopoulos GP, Dontas I, Perrea-Kotsarelis D, Couris E, Karayannacos PE, Deliconstantinos G. 2002. The effect of doxorubicin and its analogue mitoxantrone on cardiac muscle and on serum lipids: An experimental study. Anticancer Res 22:815–820.

Kuo CL, Chou CC, Yung BYM. 1995. Berberine complexes with DNA in the berberine-induced apoptosis in human leukemic HL-60 cells. Cancer Lett 93:193–200.

Lin JP, Yang JS, Chang NW, Chiu TH, Su CC, Lu KW, Ho YT, Yeh CC, Mei-Dueyang, Lin HJ, Chung HG. 2007. GADD153 mediates berberine-induced apoptosis in human cervical cancer Ca ski cells. Anticancer Res 27:3379.

Li N, He Y, Wang L, Mo C, Zhang J, Zhang W, Li J, Liao Z, Tang X, Xiao H. 2011. D-galactose induces necroptotic cell death in neuroblastoma cell lines. J Cell Biochem 112(12):3834–3844.

Liu WT, Huang CY, Lu IC, Gean PW. 2013. Inhibition of glioma growth by minocycline is mediated through endoplasmic reticulum stress-induced apoptosis and autophagic cell death. Neuro Oncol 15:1127–1141.

Löw-Friedrich I, Von Bredow F, Schoeppe W. 1990. In vitro studies on the cardiotoxicity of chemotherapeutics. Chemotherapy 36:416–421.

Mantena SK, Sharma SD, Katiyar SK. 2006. Berberine inhibits growth, induces G1 arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating Cdki–Cdk-cyclin cascade, disruption of mitochondrial membrane potential and cleavage of caspase 3 and PARP. Carcinogenesis 27:2018.

Marinina J, Shenderova A, Mallery SR, Schwendeman SP. 2000. Stabilization of vinca alkaloids encapsulated in poly (lactide-co-glycolide) microspheres. Pharm Res 17:677–683.

Mattos DMM, Gomes ML, Freitas RS, Bernardo-Filho M. 2001. Model to evaluate the toxic effect of drugs: Vincristine effect in the mass of organs and in the distribution of radiopharmaceuticals in mice. Mut Res 496:137–143.

McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21:1249.

McCune JS, Lindley C. 1997. Appropriateness of maximum-dose guidelines for vincristine. Am J Health Syst Pharm 54:1755.

Meeran SM, Katiyar S, Katiyar SK. 2008. Berberine-induced apoptosis in human prostate cancer cells is initiated by reactive oxygen species generation. Toxicol Appl Pharmacol 229:33–43.

Mo C, Wang L, Zhang J, Numazawa S, Tang H, Tang X, Han X, Li J, Yang M, Wang Z, Wei D, Xiao H. 2013. The Crosstalk Between Nrf2 and AMPK Signal Pathways Is Important for the Anti-Inflammatory Effect of Berberine in LPS-Stimulated Macrophages and Endotoxin-Shocked Mice. Antioxid Redox Signal [Epub ahead of print].

Mollinedo F, Gajate C. 2003. Microtubules, microtubule-interfering agents and apoptosis. Apoptosis 8:413–450.

Notarbartolo M, Poma P, Perri D, Dusonchet L, Cervello M, D′Alessandro N. 2005. Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-kB activation levels and in IAP gene expression. Cancer Lett 224:53–65.

Owellen RJ, Hartke CA, Dickerson RM, Hains FO. 1976. Inhibition of tubulin-microtubule polymerization by drugs of the Vinca alkaloid class. Cancer Res 36:1499.

Oyadomari S, Mori M. 2003. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 11:381–3389.

Patil JB, Kim J, Jayaprakasha G. 2010. Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway. Eur J Pharmacol 645:70–78.

Sasson Z, Morgan CD, Wang B, Thomas G, MacKenzie B, Platts ME. 1994. 5-Fluorouracil related toxic myocarditis: Case reports and pathological confirmation. Can J Cardiol 10:861.

Serafim TL, Oliveira PJ, Sardao VA, Perkins E, Parke D, Holy J. 2008. Different concentrations of berberine result in distinct cellular localization patterns and cell cycle effects in a melanoma cell line. Cancer Chemother Pharmacol 61:1007–1018.

Silverman JA, Reynolds L, Deitcher SR. 2013. Pharmacokinetics and pharmacodynamics of vincristine sulfate liposome injection (VSLI) in adults with acute lymphoblastic leukemia. J Clin Pharmacol 53:1139–1145.

Singh J, Kakkar P. 2009. Antihyperglycemic and antioxidant effect of Berberis aristata root extract and its role in regulating carbohydrate metabolism in diabetic rats. J Ethnopharmacol 123:22–26.

Skobel E, Kammermeier H. 1997. Relation between enzyme release and irreversible cell injury of the heart under the influence of cytoskeleton modulating agents. Biochim Biophys Acta 1362:128–134.

Smith EM, Li L, Hutchinson RJ, Ho R, Burnette WB, Wells E, Bridges C, Renbarger J. 2013. Measuring vincristine-induced peripheral neuropathy in children with acute lymphoblastic leukemia. Cancer Nurs 36:E49–E60.

Song XR, Zheng Y, He G, Yang L, Luo YF, He ZY, Li SZ, Li JM, Yu S, Luo X, Hou SX, Wei YQ. 2010. Development of PLGA nanoparticles simultaneously loaded with vincristine and verapamil for treatment of hepatocellular carcinoma. J Pharm Sci 99:4874–4879.

Tan W, Li Y, Chen M, Wang Y. 2011. Berberine hydrochloride: Anticancer activity and nanoparticulate delivery system. Int J Nanomedicine 6:1773–1777.

Tange R, Vuzevski V. 1984. Changes in the stria vascularis of the guinea pig due to cis-platinum. Eur Arch Otorhinolaryngol 239:41–47.

Tyagi AK, Singh RP, Agarwal C, Chan DCF, Agarwal R. 2002. Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis. Clin Cancer Res 8:3512–3519.

Upadhyay SN, Yadav RP. 1999. Application of protoberberine alkaloid, berberine, an immunosuppressive agenteditor^editors: Google Patents.

Venkatramani R, Malogolowkin M, Davidson TB, May W, Sposto R, Mascarenhas L. 2013. A phase I study of vincristine, irinotecan, temozolomide and bevacizumab (vitb) in pediatric patients with relapsed solid tumors. PLoS ONE 8:e68416.

Woodahl EL, Crouthamel MH, Bui T, Shen DD, Ho RJ. 2009. MDR1 (ABCB1) G1199A (Ser400Asn) polymorphism alters transepithelial permeability and sensitivity to anticancer agents. Cancer Chemother Pharmacol 64:183–188.

Wu M, Wang J, Liu L. 2010. Advance of studies on anti-atherosclerosis mechanism of berberine. Chin J Integr Med 16:188–192.

Yang M, Wei D, Mo C, Zhang J, Wang X, Han X, Wang Z, Xiao H. 2013. Saturated fatty acid palmitate-induced insulin resistance is accompanied with myotube loss and the impaired expression of health benefit myokine genes in C2C12 myotubes. Lipids Health Dis 12:104.

Youn MJ, So HS, Cho HJ, Kim HJ, Kim Y, Lee JH, Sohn JS, Kim YK, Chung SY, Park R. 2008. Berberine, a natural product, combined with cisplatin enhanced apoptosis through a mitochondria/caspase-mediated pathway in HeLa cells. Biol Pharm Bull 31:789–795.

Yuen MF, Hou JL, Chutaputti A. 2009. Hepatocellular carcinoma in the Asia pacific region. J Gastroenterol Hepatol 24:346–353.

Zhang R, Dougherty D, Rosenblum M. 1990. Laboratory studies of berberine used alone and in combination with 1, 3-bis (2-chloroethyl)-1-nitrosourea to treat malignant brain tumors. Chin Med J 103:658.

Zhao Q, Wang J, Zou MJ, Hu R, Zhao L, Qiang L, Rong JJ, You QD, Guo QL. 2010. Wogonin potentiates the antitumor effects of low dose 5-fluorouracil against gastric cancer through induction of apoptosis by down-regulation of NF-kappaB and regulation of its metabolism. Toxicol Lett 197:201–210.

Zhou Y, Ling XL, Li SW, Li XQ, Yan B. 2010. Establishment of a human hepatoma multidrug resistant cell line in vitro. World J Gastroenterol 16:2291–2297.