**Potentiation of resveratrol-induced apoptosis by matrine in human hepatoma HepG2 cells**

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**Abstract.** Resveratrol, a natural polyphenolic phytochemical, has received considerable attention due to its potential chemopreventive and chemotherapeutic properties. In the present study, we first evaluated the growth-inhibitory effect of resveratrol on HepG2 cells and explored the underlying molecular mechanisms. Resveratrol inhibited proliferation and induced apoptosis in HepG2 cells via activation of caspase-9 and caspase-3, upregulation of the Bax/Bcl-2 ratio and induction of p53 expression. Cell cycle analysis demonstrated that resveratrol arrested cell cycle progression in the G1 and S phase. We further focused on the combination of matrine, a natural component extracted from the traditional Chinese medical herb *Sophora flavescens* Ait., as a mechanism to potentiate the growth-inhibitory effect of resveratrol on HepG2 cells. Both MTT and colony formation assay results indicated that the combined treatment of resveratrol and matrine exhibited a synergistic antiproliferative effect. In addition, resveratrol-induced apoptosis was significantly enhanced by matrine, which could be attributed to activation of caspase-3 and caspase-9, downregulation of survivin, induction of reactive oxygen species (ROS) generation and disruption of mitochondria membrane potential ($\Delta\Psi_m$). Our findings suggest that the combination treatment of resveratrol and matrine is a promising novel anticancer strategy for liver cancer; it also provides new insights into the mechanisms of combined therapy.

**Introduction**

Liver cancer is the fifth most common cancer in men and the seventh in women; it accounts for 9% of all cancer-related deaths worldwide and 12% in developing countries (1). Epidemiologic studies have shown that the main risk factors for hepatocellular carcinoma (HCC) are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections (2,3). Among primary live cancers, HCC accounts for almost 70-85% of the total liver cancer burden. Until recently, there is still a lack of systemic chemotherapy in treating HCC efficaciously. Although sorafenib has been approved by FDA for the treatment of advanced HCC, it causes several adverse effects including diarrhea, hand-foot skin reaction, hypophosphatemia and the risk of bleeding (4,5). Therefore, it is urgent to develop alternative therapeutic strategies for liver cancer.

Resveratrol, a naturally occurring polyphenolic compound, is commonly present in the skin of grapes and in red wine (6). Due to its high toxicity toward tumor cells, resveratrol appears to be a good candidate drug for cancer therapy. Resveratrol can delay or prevent all stages of carcinogenesis in vitro and in vivo, including initiation, promotion and progression (6,7). When resveratrol is combined with other anticancer agents, such as 5-fluorouracil and curcumin, they display synergistic anticancer properties (8-10). Previous studies have also demonstrated that resveratrol inhibits cell proliferation and induces apoptotic cell death in HCC cells in vivo and in vitro (7).

Matrine is an important ingredient of a traditional Chinese herb *Sophora flavescens* Ait., which has widely been used in China for the treatment of viral hepatitis, liver cirrhosis, cardiac arrhythmia and skin inflammations without any obvious side-effects (11). Recently, its anticancer activity has been extensively investigated; it inhibits proliferation and metastasis and induces apoptosis in a variety of malignant cells (12-15). It also reduces the occurrence of multidrug-resistant tumor cells induced by chemotherapy and displays synergistic activities with other anticancer agents (16,17). Matrine inhibits the growth of HCC cells by inducing apoptosis via upregulation of the ratio of Bax/Bcl-2 (13). However, matrine alone weakly inhibits proliferation of cancer cell lines with an IC$_{50}$ value of 2-16 mM (17).

In the present study, we first evaluated the antiproliferative effect of resveratrol on HepG2 cells and then explored the underlying molecular mechanisms. Since both resveratrol and matrine independently exert anticancer activities against liver cancer cells, it led us to hypothesize that an increased benefit would result when using the combination of these two agents.

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when compared with each single agent alone. Thus, the anti-cancer effect of the combination treatment of resveratrol and matrine was also evaluated in HepG2 cells.

Materials and methods

Cell culture and reagents. Matrine (purity ≥98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Resveratrol (purity ≥99%), 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V/PI double staining kit was purchased from Nanjing KeyGen Biotech., Co., Ltd. (Nanjing, China). The HepG2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin at 37˚C in a humidified atmosphere with 5% CO₂.

Cell viability assay. MTT assay was used to assess cell viability. HepG2 cells were seeded into 96-well plates at a cell density of 5,000/well and allowed to adhere for 24 h, followed by resveratrol and/or matrine treatment for 48 h. Then 5 µl of 5 mg/ml MTT was added to the medium and incubated for 2 h at 37˚C. After removing the culture medium, 100 µl of DMSO was added. The plates were read using an enzyme-linked immunosorbent assay plate reader at 570 nm. All experiments were performed in triplicate, and the cell viability of HepG2 cells was calculated as the ratio of each experimental condition to the control. The IC₅₀ value was calculated from the nonlinear regression analysis.

Cell cycle analysis. To determine cell cycle distribution, HepG2 cells were treated with resveratrol for 24 h. The cells were trypsinized and fixed with cold 70% ethanol overnight at 4˚C. Then the fixed cells were washed twice with phosphate-buffered saline (PBS) and incubated with 100 µg/ml of ribonuclease A at 37˚C for 30 min and then stained with 50 µg/ml PI for 1 h. The fluorescence intensity was detected using BD FACSCalibur cytometer, and the cell cycle distribution was assayed using ModFit LT software (both from BD Biosciences, San Jose, CA, USA).

Colony formation assay. HepG2 cells were seeded into 6-well plates at a density of 400 cells/well. After overnight incubation, the cells were exposed to resveratrol and/or matrine for 48 h. Thereafter, the drugs were removed by replacing the medium with fresh medium, and the cells were then maintained in culture for another 10 days and the medium was replaced every three days, during which time the surviving cells produced colonies. The colonies were visualized by staining for 4 h with 1% methylene blue (in 100% methanol), and the colonies that contained >50 cells were counted. The colony formation efficacy was calculated according to the following formula: Colony formation efficacy = colony counts/seeded cells x 100%. All experiments were performed in triplicate.

Synergy between the resveratrol and matrine combination. The nature of the combined effect of resveratrol and matrine was determined using the method described by George et al (9), based on the principles described by Chou and Talalay (18). In brief, the expected value of the combined effect between agent 1 and 2 is calculated as: [(observed agent 1 value)/(control value)] x [(observed agent 2 value)/(control value)] x (control value); and the ratio is calculated as (expected value)/(observed value). A ratio of >1 indicates a synergistic effect, and a ratio of <1 indicates a less than additive effect.

Quantification of apoptosis. HepG2 cells were seeded in 6-well plates at a density of 3x10⁵ cells/well and exposed to resveratrol and/or matrine treatment for 48 h. The cells were harvested and washed twice in PBS, and then resuspended in 500 µl binding buffer at a density of 1x10⁶ cells/ml. The cell suspension was incubated with 5 µl Annexin V and 5 µl PI in the dark for 15 min at room temperature. Finally, apoptotic cells were detected by flow cytometry. The amount of apoptosis was evaluated as the percentage of Annexin V+/PI⁻ and Annexin V+/PI⁺ cells.

Western blot analysis. Western blot analysis was performed as previously described (19). In brief, after treatment with resveratrol and/or matrine, HepG2 cells were collected, lysed and subjected to 7.5-12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% non-fat milk in the blocking buffer (PBS containing 0.1% Tween-20, pH 7.5), the membrane was probed with designated first and second antibodies. The immunoreactive bands were visualized using the ECL Plus Western Blotting Detection System (Piscataway, NJ, USA). The level of β-actin was used as a loading control. The antibody against caspase-3 and caspase-9, poly(ADP-ribose) polymerase-1 (PARP-1), p53, survivin, Bax, Bcl-2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies against β-actin were purchased from Sigma-Aldrich.

Reactive oxygen species (ROS) and mitochondrial membrane potential (Δψₘ) assays. HepG2 cells were seeded into 6-well plates at a density of 3x10⁵ cells/well. After overnight incubation, the cells were treated with resveratrol and/or matrine for 24 h. Then the HepG2 cells for the detection of ROS were incubated with 10 µM H₂DCFDA at 37˚C for 30 min in the dark. For the Δψₘ assay, the HepG2 cells were incubated with 0.5 mM Rhodamine 123 at 37˚C for 30 min in the dark. The intracellular fluorescence intensity was measured with a BD FACSCalibur cytometer.

Statistical analysis. All experiments were repeated as least three times. One-way analysis of variance (ANOVA) was used to analyze the variance for the means of multiple groups. Statistical analysis was performed using SPSS, and significant differences were considered at values of P<0.05.

Results

Resveratrol inhibits HepG2 cell proliferation. The effect of resveratrol on the cell proliferation of HepG2 cells was evaluated by the MTT assay. The viability of HepG2 cells was decreased significantly when resveratrol was used at
concentrations >10 µM (P<0.05) (Fig. 1). The cell viabilities at 10, 20, 50 and 100 µM concentrations of resveratrol were recorded as 91, 83, 70 and 46%, respectively. The results showed that resveratrol inhibited the growth of HepG2 cells in a dose-dependent manner. The IC\textsubscript{50} value was 70 µM after incubation for 48 h.

Resveratrol arrests HepG2 cells in the G\textsubscript{1} and S phase in HepG2 cells. To explore the growth-inhibitory mechanisms of resveratrol, the effect of resveratrol on cell cycle perturbations was examined. The cell cycle profile was assessed in HepG2 cells after exposure to 0, 20, 50 and 100 µM resveratrol for 24 h. A clear dose-dependent cell cycle arrest was observed in the HepG2 cells (Fig. 2). At lower concentrations of resveratrol (20 and 50 µM), the number of HepG2 cells increased in the S phase. However, when resveratrol was used at a higher concentration (100 µM), there was a considerable accumulation of cells in the G\textsubscript{1} phase. These results indicate that resveratrol arrests the cell cycle in the G\textsubscript{1} and S phase in a concentration-dependent manner.

Resveratrol triggers apoptosis in HepG2 cells. In order to determine whether apoptosis participates in resveratrol-induced cell death, the expression levels of PARP-1, p53, Bax, Bcl-2, caspase-3 and -9 were measured by western blotting. Resveratrol increased PARP-1 cleavage and caspase-3 and caspase-9 activation, which are hallmarks of an increase in apoptosis, in a dose- and time-dependent manner (Fig. 3). Resveratrol also inhibited anti-apoptotic protein Bcl-2 expression and upregulated expression of pro-apoptotic protein Bax and tumor suppressor protein p53. Therefore, resveratrol induced apoptotic cell death via a caspase- and p53-dependent pathway.

Resveratrol and matrine synergistically inhibit the growth of HepG2 cells. The effect of matrine on the growth of HepG2 cells was evaluated by the MTT assay. Matrine inhibited the growth of HepG2 cells in a dose-dependent manner (Fig. 4A). However, matrine treatment at concentrations <2 mM only resulted in a slight decrease in cell survival rate. To assess whether matrine can enhance the anticancer activity of...
resveratrol, we treated HepG2 cells with increasing concentrations of resveratrol (25, 50, 100 and 200 µM) in the presence or absence of matrine (1 or 2 mM) for 48 h. The MTT assay results showed that the combination treatment was more effective in inhibiting the proliferation of HepG2 cells when compared with either agent alone (Fig. 4B).

In order to confirm the growth-inhibitory effect of the combined treatment of resveratrol and matrine, a colony formation assay was used to further study the combined treatment of resveratrol and matrine. HepG2 cells were treated with increasing concentrations of resveratrol (50 and 100 µM) with or without 2 mM matrine. When resveratrol was combined with matrine, the colony formation efficacy of the HepG2 cells was significantly reduced when compared with either agent alone (P<0.05) (Fig. 4C). The precise nature of this combination was further analyzed by the method described by George et al (9). The expected effect of the combination treatment on the cell proliferation was greater than the observed combination, suggesting a synergistic effect between resveratrol and matrine on HepG2 cells (Table I). Based on these results, we selected 50 µM resveratrol and 100 µM resveratrol in the presence or absence of matrine (2 mM) to carry out the subsequent studies.

Matrine enhances resveratrol-induced apoptosis in HepG2 cells. To understand the molecular basis of the growth-inhibitory mechanism caused by resveratrol and matrine, Annexin V/PI double staining was used to quantify the extent of apoptosis after 48 h treatment with resveratrol and/or matrine. When matrine was combined with resveratrol, matrine significantly enhanced resveratrol-induced apoptosis of HepG2 cells (P<0.05) (Fig. 5A). We further detected the effects of resveratrol and/or matrine on apoptosis-related proteins. The combination treatment of resveratrol and matrine significantly enhanced the cleavage of PARP-1, activation of caspase-3 and caspase-9 when compared to either agent alone (Fig. 5B). In addition, the combined treatment significantly inhibited survivin expression in HepG2 cells compared to the expression following treatment with either agent alone.
Combined treatment of resveratrol and matrine induces ROS generation and decreases Δψ\textsubscript{m} in HepG2 cells. In order to study the mechanisms of the apoptosis induced by the combined treatment, we further examined the combined effect of resveratrol and matrine on ROS production in HepG2 cells after 24 h treatment. The combined treatment significantly induced ROS generation in the HepG2 cells when compared with either agent alone (\textit{P}<0.05) (Fig. 6A). To better characterize the apoptotic cell death induced by resveratrol and matrine in HepG2, the role of mitochondria was also evalu-
ated after the treatment of resveratrol and/or matrine for 24 h. The combined treatment of resveratrol and matrine caused a marked loss of Δψm in the HepG2 cells in respect to each agent alone (P<0.05) (Fig. 6B).

Discussion

Previous studies have demonstrated that resveratrol inhibits the proliferation of various types of tumor cells in vivo and in vitro (20-22). In the present study, we showed that resveratrol decreased the viability of HepG2 cells in a dose-dependent manner. In addition, we further dissected the mechanisms underlying the antitumor effect of resveratrol. Resveratrol arrested HepG2 cells in the G1 and S phase which is consistent with reported findings (23), suggesting that retardation of cell cycle progression may be one of the mechanisms underlying the antitumor effect of resveratrol.

Apoptosis, or programed cell death, is a well-documented phenomenon in many cellular systems, which has been recognized as a major anticancer therapeutic response (24). Our findings are consistent with previous reports that resveratrol induces apoptosis in cancer cells (25-27). In the present study, we confirmed that resveratrol induced apoptosis in HepG2 cells, as shown by the cleavage of PARP-1, the hallmark of the degradation process of apoptosis, such as cell shrinkage, membrane blebbing, DNA fragmentation and finally the breakdown of the cell into smaller units (apoptotic bodies) (29). The Bcl-2 family members Bax and Bcl-2 serve as critical regulators of the mitochondrial-dependent apoptotic pathway. Bcl-2 that negatively regulates apoptosis promotes cell survival, whereas Bax that positively regulates apoptosis stimulates mitochondrial damage (30). Consistent with the ability of resveratrol to kill HepG2 cells via apoptotic processes, resveratrol upregulated the ratio of Bax/Bcl-2, indicating that the increased ratio of Bax/Bcl-2 may trigger resveratrol-induced apoptosis in HepG2 cells.

Matrine has been approved as an adjuvant drug for the treatment of various malignant cancers in China (11). However, matrine alone weakly inhibits proliferation of cancer cell lines with an IC50 value of 2-16 mM (17). Combination of anticancer agents for cancer therapy and prevention has been extensively studied in numerous in vivo and in vitro models (31,32). Since each agent may have its own targets and also share common targets, the combination of two anticancer agents may exert a synergistic effect. Thus, the effect of the combined treatment of resveratrol and matrine on HepG2 cells was also evaluated here. The combined treatment significantly enhanced the antiproliferative effect when compared with either agent alone. The ratio of expected value/observed value was >1 for the concentrations tested, indicating that the combined treatment of resveratrol and matrine exhibited a synergistic antiproliferative effect. To our knowledge, the present study was the first to investigate the effect of the combination treatment of resveratrol and matrine on cancer cells. The combination treatment significantly induced apoptotic cell death in HepG2 cells as compared to either agent alone, indicating that induction of apoptosis is an important mechanism of enhancing the anticancer effects of resveratrol by matrine.

Survivin is a member of the inhibitors of apoptosis-related proteins, which has been found to be frequently overexpressed in most types of cancer cells, including HCC cells. Therefore, survivin has emerged as a potential therapeutic target for natural anticancer compounds (33,34). Our results showed that the combination treatment of resveratrol and matrine significantly reduced the expression of survivin in HepG2 cells compared with the control or either drug alone. These data suggest that downregulation of survivin expression is also involved in the antiproliferative effects of the combined treatment.

ROS are known to disrupt Δψm, and therefore trigger a series of mitochondrial-associated events (35). A high level of ROS leads to oxidative stress, loss of cell function and ultimately apoptosis or necrosis (36). Natural products may exert anticancer effects by inducing ROS-mediated apoptosis. Both resveratrol and matrine were found to induce ROS production in cancer cell lines (37-39). However, there is no evidence of the combined effect of resveratrol and matrine on ROS generation. In the present study, we demonstrated that the combination treatment significantly enhanced the generation of ROS in HepG2 cells when compared with either agent alone. Furthermore, the combined treatment also resulted in loss of Δψm. Therefore, induction of ROS generation and disruption of Δψm are involved in potentiating resveratrol-induced apoptosis by matrine.

Taken together, resveratrol exhibits multiple anticancer effects by inducing cell growth inhibition, cell cycle arrest and apoptosis in HepG2 cells. Moreover, the present study is the first to demonstrate that the combination treatment of resveratrol and matrine exhibits a synergistic antiapoprotic effect on HepG2 cells. Therefore, the combined treatment of resveratrol and matrine is an effective and promising strategy for the prevention and treatment of liver cancer.

Acknowledgements

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References


