Dihydromyricetin Induces Apoptosis and Reverses Drug Resistance in Ovarian Cancer Cells by p53-mediated Downregulation of Survivin

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Ovarian cancer is one of the leading causes of death in gynecological malignancies, and the resistance to chemotherapeutic agents remains a major challenge to successful ovarian cancer chemotherapy. Dihydromyricetin (DHM), a natural flavonoid derived from Ampeopsis Grossdentata, has been widely applied in food industry and medicine for a long time. However, little is known about the effects of DHM on ovarian cancer and the underlying mechanisms. In this study, we demonstrated that DHM could effectively inhibit the proliferation of ovarian cancer cells and induce cell apoptosis. Survivin, an inhibitor of apoptosis (IAPs) family member, exhibited a decreased expression level after DHM treatment, which may be attributed to the activation of p53. Moreover, DHM markedly sensitized paclitaxel (PTX) and doxorubicin (DOX) resistant ovarian cancer cells to PTX and DOX by inhibiting survivin expression. Collectively, our findings highlight a previously undiscovered effect of DHM, which induces apoptosis and reverses multi-drug resistance against ovarian cancer cells through downregulation of survivin.

Ovarian cancer is one of the most lethal gynecological malignancies in the USA and an estimated 14,180 deaths are expected in 2015¹. Ovarian cancer is typically asymptomatic in its early stages, which decreases the chances of early diagnosis². An ovarian cancer patient who is diagnosed at early stages has a survival rate of 99% throughout his/her lifetime. However, 75% of ovarian cancer patients who are suffering from peritoneal metastasis at the time of diagnosis have a five-year survival rate of less than 30%³. Treatments generally consist of conventional cytotoxic chemotherapy and surgical therapy⁴. Despite successful initial treatment, as many as 70% of ovarian cancer patients would develop advanced-staged disease and resistance to treatment, depriving long-term benefit from treatment⁵. This observation highlights the importance of better understanding the mechanism of therapy and devising more effective strategies to conquer therapeutic resistance.

Upregulation of inhibitors of apoptosis proteins (IAPs) expression has been associated with drug resistance in various cancers⁶. Survivin, a nodal protein of IAPs family, is abundantly expressed in 60 cancer cell lines and in most common human neoplasms⁷,⁸. Survivin is a potential molecular target in cancer therapy because of its important roles in inhibiting apoptosis, enhancing proliferation and promoting angiogenesis. Overexpression of survivin protects cells from various apoptotic stimuli such as chemotherapeutic agents, whereas reducing survivin expression or function can result in enhanced sensitivity to exposure to apoptotic stimuli or the spontaneous apoptosis of cancer cells⁹. Although the regulation of survivin is not completely understood, it has been reported that the retinoblastoma protein and p53 are required for survivin gene repression¹⁰. Furthermore, some transcription factors, such as nuclear factor kappa B (NF-κB), can promote survivin gene transcription¹¹.

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Moreover, survivin is overexpressed approximately 40-fold in tumor tissues and renders cancer cells resistant to radiotherapy and chemotherapy. Therefore, identifying survivin inhibitors represents an important step of effective cancer treatment.

Dihydromyricetin (DHM) (Fig. 1A) is a flavonoid that can be isolated from the stems and leaves of *Ampelopsis grossedentata* ([1](#)). It is associated with multiple pharmacological benefits by performing anti-inflammatory, antioxidant, antibacterial, antihypertensive and antithrombotic activities ([2–5](#)). Moreover, previous studies have demonstrated its potent antitumor activity against a broad range of cancers, including breast cancer, liver cancer, colon cancer, and lung cancer ([6–10](#)). DHM can inhibit cancer cell proliferation and can induce cell cycle arrest and

**Figure 1. The effects of DHM on ovarian cancer cell proliferation.** (A) Chemical structure of DHM. (B) A2780 cells, SKOV3 cells and IOSE80 cells were exposed to different concentrations of DHM for 24 and 48 h. Cell viability was determined by a MTT assay. (C) Colony formation assays of A2780 cells after treatment with different concentrations of DHM (25, 50, 100 μM). (D) The effects of different concentrations of DHM (25, 50, 100 μM) on the cell cycle distribution of A2780 cells. Data are expressed as mean ± SD for three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
apoptosis. It is also known to sensitize cancer cells to chemotherapeutic drugs\textsuperscript{21–24}. However, little is known about its effects on ovarian cancer, and the underlying mechanisms of its anticancer effects still require further investigation. In this study, we aimed to investigate the therapeutic potential of DHM on ovarian cancer and explore its underlying mechanisms of action. Furthermore, the effects of DHM combined with chemotherapeutic agents against resistant ovarian cancer cells were evaluated.

Results
DHM inhibits proliferation and induced G0/G1 and S phase arrest in ovarian cancer cells. The \textit{in vitro} anti-proliferation effect of DHM was assessed in A2780 and SKOV3 ovarian cancer cells and IOSE80 human ovarian epithelial cells. The cells were seeded in 96-well plates at a density of $5 \times 10^3$ cells/well 24 h prior to DHM exposure. The cells were treated with various DHM concentrations (12.5, 25, 50, 100, 200 and 400 $\mu$M). MTT assay was conducted to detect the cell viability after treatment with different concentrations of DHM for 24 and 48 h. As shown in Fig. 1B, DHM inhibited cell proliferation in A2780 and SKOV3 ovarian cancer cells in a concentration- and time-dependent manner. In p53 positive A2780 cells, the IC$_{50}$ value was 336.0 $\mu$M after DHM treatment for 24 h. However, in p53 null SKOV3 cells, the IC$_{50}$ value was 845.9 $\mu$M, which was 2.5-fold higher than that in A2780 cells. We also tested whether DHM was cytotoxic to normal ovarian cells. Interestingly, no significant cytotoxicity was observed in human ovarian surface epithelial IOSE80 cells after DHM treatment. Next, to confirm the suppressive effects of DHM on ovarian cancer cell proliferation, we performed colony formation assay on A2780 cells. The cells were exposed to 25, 50 and 100 $\mu$M of DHM for 48 h, and were continued to be cultured for 2 weeks in fresh medium until colonies formed. Consistent with the results of the MTT assay, the colony formation capacity was observably reduced with increasing concentrations of DHM, demonstrating that cell proliferation was suppressed by DHM (Fig. 1C).

Previous studies have shown that DHM can induce cell cycle arrest in various types of cancer cells\textsuperscript{24,25}. In this study, the cell cycle progression was examined on A2780 cells by flow cytometry. The cells were treated with different concentrations of DHM (0, 25, 50, 100 $\mu$M) for 24 h after starvation. As shown in Fig. 1D, DHM specifically arrested A2780 cells at the G0/G1 and S phase in a concentration-dependent manner. Specifically, after exposure to 100 $\mu$M of DHM for 24 h, the number of cells in G0/G1 increased from 56.18% to 63.44%, similar to the number observed in the S phase, whereas the percentage of cells in the G2/M phase decreased from 19.25% to 7.67%. The data displayed the significant cell cycle arrest effects of DHM on ovarian cancer cells at G0/G1 and S phase in a concentration-dependent manner.

DHM induces cell apoptosis and activates the apoptosis-related signaling pathway. To explore whether the deregulation of the cell cycle was correlated with the induction of apoptosis, cell morphology was observed and Annexin V-FITC/PI staining was performed after DHM treatment for 48 h. As shown in Fig. 2A, while the untreated cells were rounded, cells became condensed and cell population showed dramatic depletions after DHM treatment. Moreover, A2780 cells treated with different concentrations of DHM for 48 h displayed significant levels of apoptosis in a concentration-dependent manner (Fig. 2B). The apoptotic rates of the A2780 cells in the presence of 25, 50 and 100 $\mu$M of DHM for 48 h were 12.1, 21.1, and 26.9%, respectively (Fig. 2C). The results confirmed that DHM specifically targeted p53 positive A2780 cells and promoted cell apoptosis, which were consistent with the results of MTT assay and cell cycle study.

We further confirmed this result by evaluating the caspase 3/7 activity using the Caspase3/7-Glo Assay Kit (Promega, Madison, USA). As expected, the apoptotic rates of the DHM-treated were 34.0% (25 $\mu$M), 61.9% (50 $\mu$M) and 150.7% (100 $\mu$M), respectively, which were higher than that of the control group (Fig. 2D), suggesting that an enhanced and concentration-dependent caspase 3/7 activity was induced by DHM. Next, we detected the expression of apoptosis-related protein poly ADR-ribose polymerase (PARP), caspase 8 and caspase 9 after DHM stimulation using a Western blotting detection kit. Of note, DHM treatment increased the expression of cleaved PARP and decreased the expression of caspase 3, 8 and 9 in a concentration-dependent fashion (Fig. 2E), demonstrating that PARP function was impaired via enhanced splicing, which caused cancer cell apoptosis.

DHM induces apoptosis by downregulating the expression of survivin. Survivin, as an anti-apoptotic gene, was shown to be overexpressed in ovarian cancer\textsuperscript{26}. Intrigued by the apoptotic effect of DHM in A2780 cells, we therefore sought to explore the effects of DHM on the expression of survivin. Cells were treated with DHM for 48 h, and the expression of survivin was examined by an immunoblot assay. As shown in Fig. 3A, compared with control cells, DHM downregulated the expression of survivin in A2780 cells in a concentration-dependent manner. This finding was further evidenced in the immunofluorescence images (Fig. 3B).

Overexpression of survivin attenuates DHM-mediated apoptosis. To confirm whether DHM triggered apoptosis by decreasing the survivin expression, we performed survivin transfection taking advantage of the plasmid pIRES-survivin we constructed based on the previously reported method\textsuperscript{27}. pIRES vector was adopted as the control. The results of transfection were confirmed with Western blotting analysis and flow cytometry. As shown in Fig. 3C, cells transfected with pIRES-survivin showed a higher expression level of survivin with or without DHM treatment. In contrast, in the empty vector groups, survivin was present at lower levels under the same condition. The results of Annexin V-FITC/PI dual staining showed that cells transfected with pIRES-survivin plasmid had a decreased apoptotic rate compared to that with empty plasmid when treated with 100 $\mu$M of DHM (Fig. 3D). On the contrary, the apoptotic rate of cells transfected with survivin siRNA reached 45.3% after DHM treatment for 48 h, 13.5% higher than that with scrambled siRNA due to survivin was knocked down by survivin siRNA (Fig. 3E).
Figure 2. Effects of DHM on cell apoptosis. (A) Cell morphologies of A2780 cells treated with DHM (25, 50, 100 μM) for 48 h. (B) A2780 cells were treated with different concentrations of DHM (25, 50, 100 μM) for 48 h and analyzed with flow cytometry after Annexin V-FITC/PI staining. Annexin-V-FITC−/PI− populations in Quadrant 3 were non-apoptotic cells, while Annexin-V-FITC+/PI− cells in Quadrant 4 and Annexin-V-FITC+/PI+ cells in Quadrant 2 were considered late apoptotic cells, respectively. (C) The apoptotic ratio of A2780 cells was calculated. (D) The Caspase 3/7 activity was determined after exposure to DHM for 24 h. (E) Expression of activated PARP, caspase 8 and caspase 9 were determined by Western blotting. Results were obtained based on two or three separate experiments. Data were expressed as mean ± SD. *p < 0.05, ***p < 0.001.
Activation of p53 was critical for DHM-induced apoptosis. It was documented that p53 and survivin could together modulate cell apoptosis. As the above data indicated, DHM triggered increased cell apoptosis using Western blotting. Accordingly, we sought to verify the effects of p53 on DHM-mediated apoptosis using Western blotting. As shown in Fig. 4A, expressions of both p53 and p53 phosphorylation sites, including p-p53 (ser15) and p-p53 (ser37), were upregulated in A2780 cells after exposure to different concentrations of DHM for 48 h. However, DHM treatment at all tested concentrations did not increase the expression of p-p53 (ser20) and p-p53 (ser46) in A2780 cells and p53 in SKOV3 cells. We then conducted immunofluorescence assay to measure the expression of p53 in the A2780
cells. The fluorescence intensity of p53 was prominently enhanced in the DHM-treated cells (100 μM), while no obvious green fluorescence was observed in the untreated cells (Fig. 4B). Next, we determined the effect of p53 downregulation on the apoptosis of A2780 cells. Notably, knockdown of p53 using p53 siRNA inhibited the expression of p53, leading to a significantly decreased apoptotic rate in response to DHM (Fig. 4C and D).

**Activation of p53 by DHM is responsible for DHM-induced survivin downregulation.** To investigate the relationship of p53 and survivin in the regulation of DHM-induced cell apoptosis, we further knocked down the p53 protein with p53 siRNA. The cells were seeded in dishes and transfected with 10 μg/mL siRNA (p53 or scrambled) in 10 μL/dish Lipofectamine® diluted in fresh medium. After 24 h, the cells were exposed to various concentrations of DHM, and the expression of survivin expression was evaluated by Western blotting and apoptosis assays. As expected, the cells transfected with p53 siRNA showed a higher level of survivin than those transfected with scrambled siRNA, indicating the opposite role of p53 and survivin in modulating DHM-induced apoptosis (Fig. 4C). Taken together, our data demonstrated that DHM might downregulate survivin expression via p53 activation, leading to A2780 cell apoptosis.

**DHM sensitizes resistant ovarian cancer cells to paclitaxel and doxorubicin through suppressing survivin expression.** In retrospective studies, patients with high expression level of survivin show a resistance to chemotherapy and an increased recurrence rate. In tumor tissues, overexpression of survivin for 40-fold renders to cancer cells resistant to chemotherapy. DHM, capable of downregulating the expression of survivin, might serve as a survivin inhibitor and reverse tumor resistance. Currently, paclitaxel (PTX) and doxorubicin (DOX) are two major chemotherapeutic drugs for treatment of ovarian cancer clinically. Thus, we evaluated whether low dose of DHM in combination with PTX and DOX was able to sensitize cancer cells to
Chemotherapeutic agents. To this end, PTX-resistant A2780/PTX cells were treated with 50 μM of DHM combined with PTX (ranging from 0.01 to 1 μM), or cotreated with DHM combined with different concentrations of PTX for 48 h. Cell viability was determined by MTT assay. A2780/PTX cells were treated with 50 μM of DHM, 0.1 μM of PTX, or their combination for 48 h and cell apoptosis was detected by flow cytometry. DOX-resistant A2780/DOX cells were exposed to 25 μM of DHM combined with DOX (ranging from 1 to 4 μM). Cell viability was determined by MTT assay. As shown in Figs 5A and 6A, the cell viability was decreased after treatment of the combination of DHM with either PTX or DOX. Annexin V assay was performed to evaluate their anticancer effects. Figure 5B showed that the apoptotic rate of the PTX group was 17.16%, whereas the combination of DHM and PTX increased the apoptotic rate to 29.25%. Meanwhile, the apoptotic rate in DHM and DOX combination group was 41.27%, 2.4-fold higher than that of DOX group (17.20%) (Fig. 6B). These observations suggested that DHM was able to sensitize A2780 resistant cancer cells to both PTX and DOX.

The expression levels of cleaved PARP, p53 and survivin were visualized by Western blotting. As shown in Figs 5C and 6C, low concentrations of PTX and DOX resulted in low expression levels of cleaved PARP and p53 in A2780 resistant cancer cells, indicating that the resistant cancer cells were relatively unresponsive to these chemotherapeutical agents. However, combination of DHM and PTX or DOX significantly increased the expression of cleaved PARP and p53, suggesting that the combination therapy enhanced the apoptotic effects to some extent. On the contrary, a decreased expression level of survivin was witnessed after combination treatment of DHM and PTX or DOX. This result consistently confirmed that DHM played an important role as a survivin inhibitor and contributed to reducing the expression level of survivin, which was probably modulated by the upregulation of p53, leading to an enhanced pro-apoptotic effects.

Discussion

Ovarian cancer is the sixth most common cancer among women worldwide. The successful treatment of ovarian cancer has faced several impediments, including a symptomless early state, a high incidence of recurrence and the development of chemoresistance. The discovery of promising therapeutic agents for ovarian cancer therapy, particularly, to improve the current responses to chemotherapy, remains a key goal for achieving a better outcome. DHM, which is a natural flavonoid that induces negligible side effects in mice, has received wide interest as a potential candidate for cancer therapy. Previous studies have revealed the effects of DHM on cell proliferation, colony formation, cell cycle distribution and cell apoptosis in a plethora of cancer cell lines, such as hepatocellular carcinoma, osteosarcoma, and melanoma.
Based on the results of our MTT assay, DHM treatment significantly inhibited the proliferation of both A2780 and SKOV3 cells in a concentration- and time-dependent manner but induced no significant cytotoxicity in IOSE80 cells. Obviously, A2780 cells (wild-type p53) were more vulnerable than SKOV3 cells (p53 null) at higher concentrations of DHM (Fig. 1B). For instance, treatment with 200 μM DHM for 24 h decreased the viability of A2780 cells to 70.0% which was significantly lower than that of the SKOV3 cells (84.7%). It was previously documented that the antitumor activity of DHM was at least partially due to the activation of the p53-dependent apoptosis pathway. Our results demonstrated that the p53 status might be an important determinant of the sensitivity of ovarian cancer cells to DHM.

Survivin, a nodal protein that belongs to the IAP family, is ubiquitously expressed in various types of cancers. Importantly, it is scarcely detectable in normal cells and tissues, which distinguishes it from other potential targets. The downregulation of survivin was reported to be responsible for suppressing the viability and colony formation ability of cancer cells. Considering its significance in cancer development, survivin has become an important anticancer target. YM155, a small-molecule survivin suppressant, has shown therapeutic potential against a range of cancers in clinical trials. Our results showed that DHM could downregulate the expression of survivin in a concentration-dependent manner. This result was further confirmed by transfecting A2780 cells with pIRES-survivin plasmid, which led to the overexpression of survivin and a lower apoptosis rate induced by DHM compared with the empty vector groups (Fig. 3). These findings supported the fact that the downregulation of survivin is one of the mechanisms involved in DHM-mediated apoptosis.

It was reported that silence of survivin could sensitize ovarian cancer cells to chemotherapeutical agents. Inhibition of survivin by DHM might facilitate the sensitization to chemotherapeutical agents. Thus, we further evaluated the influence of DHM on conquering drug resistance. Apoptosis assay showed that combination of DHM with PTX or DOX increased the apoptotic rate for 1.7-fold and 2.4-fold on PTX or DOX resistant ovarian cancer cells, respectively, compared to the treatment of PTX or DOX alone. Furthermore, while the treatment of PTX or DOX had little influence on survivin expression levels, and the addition of DHM significantly downregulated the expression of survivin, suggesting that DHM was able to sensitize A2780 resistant cancer cells to both PTX and DOX.

Figure 6. DHM reversed drug resistance in DOX-resistant A2780/DOX cells. (A) A2780/DOX cells were exposed to 25 μM of DHM, different concentrations of DOX (1, 2, 4 μM), or cotreated with DHM combined with different concentrations of DOX for 48 h. Cell viability was determined by a MTT assay. (B) A2780/DOX cells were treated with 25 μM of DHM, 4 μM of DOX, or cotreated with DHM (25 μM) and DOX (4 μM) for 48 h and cell apoptosis was detected by flow cytometry. (C) A2780/DOX cells were treated as indicated for 48 h and the expression of cleaved PARP, p53, and survivin was evaluated using Western blot analysis. *p < 0.05, **p < 0.01.
Previous studies have shown that wild-type p53 repressed survivin gene expression transcriptionally by direct binding to survivin promoter and activating p21, and DHM was reported to induce cell apoptosis by activating p53. We found that DHM increased the expression of p53 and phosphorylated p53 (ser15) in a concentration-dependent fashion, demonstrating that p53, the "guardian of the genome", was involved in DHM-triggered apoptosis in A2780 cells. In light of this finding, the association between p53 and survivin has subsequently been investigated. Furthermore, the present study showed changes in the survivin levels after knockdown of p53 (Fig. 4). In addition, while the combination of DHM with PTX or DOX increased the expression level of survivin, the expression level of p53 tended to be lower (Figs 5 and 6). Taken together, it was not surprising that the expression level of survivin showed an increasing trend after DHM treatment, which revealed the biphasic role of p53 and survivin in mediating DHM-triggered apoptosis.

In conclusion, this study demonstrated that DHM may be a promising chemotherapeutic agent for the treatment of ovarian cancer. The suppression of cell proliferation was stimulated by the activation of p53 and the downregulation of survivin. This study also showed a decreased expression level of survivin after DHM exposure, suggesting that DHM may trigger apoptosis though the p53-mediated survivin inhibition. Downregulation of survivin by DHM was also witnessed to sensitize A2780 resistant cancer cells to both PTX and DOX. This finding may shed new light on the direction of an effective strategy for ovarian cancer therapy.

Methods

Reagents. A powdered form of DHM, PTX, and DOX were purchased from Dalian Meilun Biology Technology Co., Ltd. (Liaoning, China). DMEM culture medium, fetal bovine serum (FBS), penicillin-streptomycin, 0.25% (w/v) trypsin/EDTA, phosphate-buffered saline (PBS), propidium iodide (PI) and Hoechst 33342 were obtained from Life Technologies (Grand Island, USA). Paraformaldehyde (PFA) and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Crystal purple was purchased from Beyotime Biotechnology (Jiangsu, China). Primary antibodies against PARP, cleaved PARP, p53, survivin, GADPH and second antibodies were supplied by Cell Signaling Technology (Danvers, MA, USA). All reagent water used was pretreated with a Milli-Q apparatus (Millipore Corporation, Darmstadt, Germany). All other chemicals were of the highest purity commercially available.

Cell lines and cell culture. Human ovarian cancer A2780 and SKOV3 cells were obtained from Boster Biotech (Wuhan, Hubei, China) and the American type culture collection (ATCC), respectively. Human ovarian surface epithelial IOSE80 cells were obtained from Shanghai Huiying Biotech (Shanghai, China). PTX- and DOX-resistant A2780 cells (A2780/PTX and A2780/DOX) were selected in stepwise increasing concentrations of PTX or DOX as previously described, respectively. Cells were cultured in DMEM medium with 10% (v/v) heat-inactivated FBS and antibiotics (100 U/ml penicillin, 100 μg/mL streptomycin) and maintained at 37°C in a 5% CO₂ atmosphere. All cells were passaged using trypsin/EDTA, and the medium was changed every other day.

Cell viability assay. Exponentially growing A2780 and SKOV3 cells were seeded in 96-well plates at a density of 5,000 cells per well in 100 μL of medium and treated with a concentration series of DHM for 24 and 48 h. For drug combination experiments, A2780/PTX were treated with 50 μM of DHM, 0.01 to 1 μM of PTX or cotreated with different concentrations of PTX and DHM for 48 h. A2780/DOX cells were treated with 25 μM of DHM, 1 to 4 μM of PTX or cotreated with different concentrations of PTX and DHM for 48 h. Cell viability was determined with incubation with medium containing 1 mg/mL of MTT for 4 h, followed by dissolving formazan crystals in 100 μL of DMSO. Cell viability was evaluated based on the absorbance at 570 nm by a microplate reader (SpectraMax M5, Molecular Devices, USA). The results were analyzed according to three independent biological replicates.

Colony formation assay. A2780 cells were seeded in 6-well plates at a density of 50 cells per well. After exposure to 25, 50 and 100 μM DHM for 48 h, the cells were cultured for 2 weeks until colonies formed. Cell colonies were visualized by staining with crystal violet, and colonies with cell counts more than 50 were considered to be surviving colonies.

Cell cycle analysis. A2780 cells (2.0 × 10⁵ cells) were seeded in 6-well plates following 24 h of starvation. Cells were exposed to 25, 50, and 100 μM of DHM for 24 h, respectively. After trypsinization, cells were harvested and washed twice with PBS, followed by fixation in 70% ethanol at −20°C overnight. The collected cells were stained with 100 μL of PI staining solution containing 10 μg/mL PI plus 8 μg/mL RNase for 30 min in dark conditions. Sample acquisition was performed using a flow cytometer (BD FACSCantoTM, BD Biosciences, San Jose, USA). The cell distributions in phases of SubG1, G0/G1, S, and G2/M were analyzed using ModFit LT software (version 3.0, Verity, USA). The results were analyzed based on three independent replicates.

Assessment of apoptosis. Cells were exposed to 25, 50, and 100 μM of DHM for 48 h. After treatment, cell morphology was observed and captured using a microscope (Olympus MVX10, Japan) equipped with a digital camera (ColorView II, So Imaging System, Olympus). Moreover, an Annexin V-FITC/PI apoptosis detection kit was used to detect cell apoptosis. After being treated with various concentrations of DHM for 48 h, A2780 cells were collected by centrifugation, washed twice with cold PBS and suspended in binding buffer. The cells were stained with 5 μL of Annexin-V-FITC and 5 μL of PI while being protected from light, after which they were analyzed using a flow cytometer (BD Biosciences). For drug combination experiments, A2780/PTX were cotreated with 50 μM of DHM and 0.1 μM of PTX, while A2780/DOX cells were cotreated with 25 μM of DHM and 4 μM of PTX for 48 h, and the same procedures were performed as mentioned above. At least three independent experiments were conducted.
Caspase 3/7 activity assay. Caspase 3/7 assay was performed using the Caspase3/7-Glo Assay Kit (Promega, Madison, USA). A2780 cells were seeded in white 96-well plates and treated with different concentrations of DHM (25, 50, 100μM) for 24 h. Then, 100μL of Caspase 3/7 reagent was added to each well and mixed using a plate shaker. The Caspase 3/7 activity was then determined using a microplate reader (SpectraMax M5). The Caspase 3/7 activity was expressed as a fold of the untreated control treatment. At least three independent experiments were performed.

Western blotting. After treatment with DHM for 48 h, A2780 cells were lysed with RIPA lysis buffer containing 1% protease inhibitor and 1% phenylmethylsulfonylfluoride (PMSF). For drug combination experiments, A2780/PTX were cotreated with 50μM of DHM and 0.1μM of PTX, while A2780/DOX cells were cotreated with 25μM of DHM and 4μM of PTX for 48 h, and the same procedures were performed as mentioned above. The samples were incubated in lysis buffer for 20 min and centrifuged at 12,000 g for 20 min, followed by the determination of the protein concentration with a BCA protein assay kit. Equivalent amounts of each sample were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After blocking in a solution of blocking powder for 1 h, the membranes were incubated with primary antibodies (1: 1000) at 4 °C overnight, followed by the corresponding secondary antibodies for 1 h. The membranes were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After blocking in a solution of blocking powder for 1 h, the membranes were incubated with primary antibodies (1: 1000) at 4 °C overnight, followed by the corresponding secondary antibodies for 1 h. The membranes were incubated with primary antibodies (1: 1000) at 4 °C overnight, followed by the corresponding secondary antibodies for 1 h.

siRNA transfection. The cells were seeded in dishes (100 mm²) and the transfection groups were added to transfection reagents containing 20μM of siRNA (final concentration) diluted in fresh media without antibiotics. After incubation overnight, the medium was removed, and the cells were treated with various concentrations of DHM 24 h later. Western blots and apoptosis assays were used to evaluate the efficiency of siRNA transfection.

Plasmid and transfection. An efficient survivin vector was constructed based on the previous method27. Briefly, the total mRNA from A2780 cells was used as templates. The forward and reverse primers targeting survivin were 5′-CTTCAATTTGCCACCACCATGGGTGCACCCGAGCTTGTCGATGG-3′ and 5′-GGGCGGATCCTCAATCCATGGCAGCCAGCTGCTCGATGG-3′, respectively. The purified RT-PCR product was inserted into the pIRES-vector (Clontech, CA, USA) to form the pIRES-survivin plasmid. Using the TurboFect transfection reagent (Thermo Scientific), A2780 cells (2 × 10⁵) were transfected with 3μg pIRES-vector or pIRES-survivin, followed by incubation with 48 h for subsequent experiments.

Immunofluorescence. Cells were seeded in 6-well plates for 24 h before being treated with DHM. After treatment for 48 h, cells were fixed in 4% PFA and hydrated with PBS for 30 min at room temperature to block non-specific antibodies. The samples were incubated in lysis buffer for 20 min and centrifuged at 12,000 g for 20 min, followed by the determination of the protein concentration with a BCA protein assay kit. Equivalent amounts of each sample were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After blocking in a solution of blocking powder for 1 h, the membranes were incubated with primary antibodies (1: 1000) at 4 °C overnight, followed by the corresponding secondary antibodies for 1 h.

Statistical analysis. All of the presented data were obtained based on three independent experiments and were expressed as mean ± SD. The results were analyzed by Graphpad Prism 6. Significant differences were assessed using Student’s t-test. A significance value of p < 0.05 level was considered to be significant in all cases.

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2. Liu, B. et al. Dihydromyricetin induces mouse hepatoma HepA1c cell apoptosis via the transforming growth factor beta pathway. Molecular Medicine Reports 11, 1609–1614 (2015).

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Author Contributions

X.Y.Q. and W.S.P. carried out the experiments, conducted the data analysis and drafted the manuscript. C.H.F., L.H.W., L.Z.Q., and H.C.W. revised the manuscript and discussed the results. C.M.W. coordinated the study, revised, and confirmed the paper. All authors read, revised, and approved the manuscript.

Additional Information

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