In vivo Inhibition of S180 Tumors by the Synergistic Effect of the Chinese Medicinal Herbs Coptis chinensis and Evodia rutaecarpa

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Key words
Coptis chinensis Franch
Evodia rutaecarpa (Juss.) Benth
Zuojinwan
synergy

Abstract
The cost of treatment and prevention of cancer is 100 million dollars per year. Many research departments focus on the exploration of antitumor drugs for just this reason. Many anticancer drugs from the West, which can rapidly inhibit the growth of tumors, have been used, but some serious side effects (e.g., impairment of immune system, hepatotoxicity, nephrotoxicity, cardiotoxicity) [1] are often produced. Another type of side effect, multiple-drug resistance (MDR), [2] can also occur, which can severely limit the effects of anticancer agents [3]. Traditional Chinese medicines (TCMs) have been widely used to treat cancers for thousands of years in China because they are highly efficacious and have low toxicities. TCMs can also reverse MDR [4] to enhance the anticancer effect of synthetic anticancer agents. The actions of TCMs are due to the combined actions of many types of chemical compounds in the complex mixture. Thus, a synergistic effect may be present in the process of cancer treatment by TCMs, an action that is different to that produced by synthetic anticancer agents [5].

A well-known TCM, Zuojinwan (ZJW), comprises two traditional Chinese medicinal herbs, Coptis chinensis Franch (Huanglian HL in Chinese) and Evodia rutaecarpa (Juss.) Benth (Wuzhuyu WZY in Chinese) mixed in the ratio of 6:1 (w/w). It was first recorded in Danxi Xinhuo-Liuja, a famous ancient medical manuscript, and is listed in the Chinese Pharmacopoeia [6]. Coptis chinensis Franch (Fig. 1) is a buttercup, and its active constituents are considered to be alkaloids, primarily palmatine, berberine, epiberberine and coptisine [7]. It has been used for the treatment of inflammation, diabetes, beriberi, epiderberine and coptisine [7]. It has been used for the treatment of gastric mucosal injury, inflammation, and diarrhea [10–12]. Enhanced apoptosis of human hepatocellular carcinoma SMMC-7721 cells caused by the synergis-
Evodia rutei gistic effects of its components proliferation, apoptosis, tumor necrosis factor-α, body weight, tumor growth inhibition, spleen index, lymphocyte markers (TMs), and expression of Bax and P53 genes were tested and discussed. Our data indicated that ZJW is more powerful than Coptis chinensis Franch or Evodia rutaecarpa (Juss.) Benth used singly in the growth inhibition of S180 tumor in vivo, and a synergistic anticancer effect between the two herbs was clearly present. This is the first report of the synergistic effect of antitumor actions due to the combined use of HL and WZY.

Materials and Methods

Plant material

Cyclophosphamide (CTX) with a purity of 99% was purchased from Jiangsu Hengrui Medicine Company Limited (Jiangsu, China). HL and WZY were purchased from Yunnan Qiancaoyuan Pharmaceuticals Limited (Yunnan, China). The voucher specimens (DLMU-HL0701 and DLMU-WZY0701), identified by Dr. Yunpeng Diao, College of Pharmacy, Dalian Medical University, are deposited in the Herbarium of the School of Pharmacy, Dalian Medical University, Dalian, China. 21 g ZJW (HL 18 g + 3 g WZY), 18 g HL and 3 g WZY were individually weighed and extracted by reflux with 210, 180 and 30 mL water (solvent-sample = 10 : 1, v/w) for 2 h in a round-bottomed flask (500 mL) equipped with a reflux condenser, respectively. The extraction solvent was filtered and evaporated to dryness under reduced pressure at 50° C. Light-yellow powders were obtained and redissolved in water to afford the sample solutions at the concentrations of 42, 36 and 6.0 mg raw material/mL, respectively. These solutions were used for subsequent experiments.

Animals and cell lines

KM mice, weighing 20.0 ± 2.0 g, purchased from the Laboratory Animal Center of Dalian Medical University (Dalian, China). Quality certificated Number: SCXK (Liao) 2004–0017, were randomly divided into five groups (n = 14). The animals were housed five per plastic cage with wood chip bedding in an animal room with a 12 h light and 12 h dark cycle at room temperature (24 ± 2°C) and allowed free access to standard laboratory diet (purchased from the Laboratory Animal Center of Dalian Medical University, Dalian, China). S180 tumor cells were obtained from the Histology and Embryology Department of Dalian Medical University (Dalian, China), and 0.2 mL of the cells (2 × 10^5 cells/mL) were implanted (s.c.) in the right hind groin of the mice on the eighth day. The animal experiment was approved by the School of Animal Science of Shenyang Agricultural College.

Measurement of body weight, tumors and spleens

After implantation for 24 h, 0.2 mL of HL, WZY and ZJW, sample solutions were orally administered individually for 10 days according to the body weight of the animal and the references [16, 17], and the S180 model group was fed 0.2 mL of sodium chloride solution (0.9%, w/v). The positive drug CTX was dissolved in water to afford a concentration of 20 mg/kg, and was administered (i.p.) on alternate days for 10 days. Mice were killed on the eleventh day; tumors and spleens were isolated and weighed. The inhibition ratio of tumor growth was calculated according to the following formula:

\[
\text{inhibition ratio (\%)} = \left(\frac{|C - T|}{C}\right) \times 100
\]

where C denotes the mean weight of S180 tumor in the model group, and T is the mean weight of S180 tumor in the experimental groups. The spleen index was also calculated.

Assay of ILS

Mice were inoculated with 0.2 mL of a suspension of S180 cells. They were treated with HL, WZY, and ZJW, individually with the doses of the samples described above after incubation for 24 h. After 10 days, drugs were stopped and the survival time of each animal was recorded. ILS was calculated according to following formula:

\[
\text{ILS (\%)} = \left(\frac{A/B - 1}{1}\right) \times 100
\]

where A is the mean survival time of the mice in the test groups, and B is the mean survival time of the animals in the model group.

Lymphocyte proliferation assay

Mice spleens were extirpated on a very clean bench. Under germfree conditions, spleens were cut into pieces and sieved through a 200-gauge mesh. 2.0 mL of cell-suspension were used and 1.0 mL of a separated medium of lymphocyte cells was slowly added. The mixture was centrifuged for 20 min. The suspension was washed twice with phosphate-buffered saline (PBS). Spleen cells were resuspended in RPMI1640 containing 10% bovine serum albumin (Giboc, USA) and seeded into 96-well flat-bottomed plates (about 1 × 10^5 cells/mL) in concanavalin A (ConA; 10 µg/mL). Cells
were incubated in 5% CO₂.air mixture at 37°C for 44 h. Ten microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) were added into each well. After another incubation of 4 h, 100 µL of sodium dodecyl sulfate (SDS) were added. Cells were subjected to MTT cellular viability assay another incubation of 4 h, 100 µL of sodium dodecyl sulfate (SDS) were added. Cells were subjected to MTT cellular viability assay according to a method described elsewhere [18], and values at the detection wavelength of 570 nm were recorded.

Cell apoptosis
S180 tumor cells were obtained by the following method. S180 tumors were carefully removed from mice and ground in trypsin at 37°C. Tumors were cut into pieces through 200-gauge mesh and the suspension was collected. Tumor cells were washed with PBS and centrifuged at 800 rpm for 10 min. The cell suspension (95 µL) was removed and 5 µL acridine orange: ethidium bromide (AO: EB; 100 µg/mL: 100 µg/mL) mixed liquor were added. A fluorescence microscope (B × 51 TF Olympus, Tokyo, Japan) was used to image the cells after a 15-min reaction in a darkened room.

Assay of TNF-α
Serum from transplanted S180 tumor mice on the eleventh day was collected. The TNF-α concentration was detected using a TNF-α radioimmunoassay (RIA) kit purchased from Dongya Immunity Technology Institute (China) according to manufacturer’s instructions. Data were analyzed by a ZC-2010 γ-counter (USTC Chuangxin Company Limited, China).

Histopathological and morphological observations
Spleens and tumors of mice treated with ZJW, HL and WZY were obtained and put into 10% buffered neutral formalin. They were cut into small pieces and stained with hematoxylin and eosin (HE) for histopathological and morphological analyses.

Assay of TMs
Activities of acid phosphatase (ACP), alkaline phosphatase (AKP), creatine kinase (CK), fructose 1,6-diphosphate aldolase (ALD) and lactate dehydrogenase (LDH) in serum of the transplanted S180 tumor mice were detected using kits purchased from the Najing Jiancheng Bioengineering Institute (China) according to the manufacturer’s instructions.

Immunohistochemistry assay
S180 tumor tissue was fixed with 10% buffered neutral formalin, and non-stained sections (4 µm) were cut from the tissue. After blocking endogenous peroxidases, incubations were carried out using primary antibodies of Bax and P53 (Santa Cruz Biotechnology, USA) at 4°C overnight, and further enhanced by non-biotin-labeled secondary antibodies for 30 min at 37°C (Zhongshan Goldenbrighde Biotechnology Company Limited, China). Samples were stained with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin [19]. Positive rates of Bax and P53 expressions were calculated according to the criteria of an immunohistochemistry reaction [20]. Integrated optical density (IOD) was analyzed by IPP 6.0 software.

Statistical analysis
Statistical analysis was done using the statistical software SPSS11.5. Data were expressed as mean ± S.D. Groups were compared using Student’s rank sum and ANOVA methods. P < 0.05 was considered significant.

Results

Inhibition values of the drugs and the ILS of S180 tumor-transplanted mice are shown in Table 1. A significantly enhanced inhibition of tumor growth was produced by ZJW (50.54%), which was much higher than those by HL (25.87%) and WZY (15.53%) used singly (p < 0.001). The ILS of mice treated with ZJW, HL and WZY were 64.91%, 26.31% and 21.93%, respectively. The efficient inhibition and prolonged ILS of S180-bearing mice were observed when HL and WZY were used together. ZJW could significantly enlarge the spleen index and body weight compared with the mice treated with CTX (Fig. 2). The immunological function of S180 tumor-transplanted mice was increased in groups treated with HL, WZY and ZJW (Fig. 3) and higher than that seen in the CTX-treated group. Compared with HL and WZY, the ZJW-treated groups produced the best actions (p < 0.001), indicating that an obvious synergistic effect was produced when HL and WZY were used together to influence lymphocyte proliferation in S180 tumor-transplanted mice.

Table 1 Effect of ILS and inhibition in S180-bearing mice treated with ZJW, HL and WZY (means ± S.D.).

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug dose (mg/mL)</th>
<th>ILS (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S180 model</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CTX</td>
<td>2.0</td>
<td>45.61</td>
<td>32.53</td>
</tr>
<tr>
<td>ZJW</td>
<td>10.0</td>
<td>64.91*</td>
<td>50.54**</td>
</tr>
<tr>
<td>HE</td>
<td>8.5</td>
<td>26.31</td>
<td>25.87</td>
</tr>
<tr>
<td>WZY</td>
<td>2.0</td>
<td>21.93</td>
<td>15.53</td>
</tr>
</tbody>
</table>

*ILS (%) = (A/B - 1) × 100, in which A is the mean survival time of the S180-bearing mice in test groups; B is the mean survival time of the S180-bearing mice in model group. *Inhibition (%) = [(C - T)/C] × 100, in which C is the mean body weight of S180-bearing mice in model group, and T denotes the average body weight of the S180-bearing mice in experimental group. The ILS of the mice treated with ZJW was significantly higher than HL and WZY single-use, *p < 0.05. Inhibition of tumor growth in ZJW-treated group was significantly higher than that in the HL-, WZY- and CTX-treated groups, **p < 0.001

Fig. 2 Effect of drug treatment on body weight, spleen index, tumor weight, and survival time in S180-transplanted tumor mice.

Fig. 3 Effect of drug treatment on lymphocyte proliferation in S180 tumor-transplanted mice.
The normal cells emitted a green fluorescence (Fig. 4A). The orange apoptotic cells of the group treated with ZJW (Fig. 4C) were significantly increased compared with HL (Fig. 4D) and WZY (Fig. 4E) as shown by AO: EB staining.

The effects of ZJW, HL and WZY on TNF-α levels in the serum of S180 tumor-transplanted mice were determined by RIA (Fig. 5). The TNF-α concentration was 1.04 ng/mL in the serum of S180-bearing mice treated with ZJW, which was significantly higher than those of the HL (0.66 ng/mL) and WZY (0.56 ng/mL)–treated groups (p < 0.001). The histopathology of S180 tumors was analyzed. Tumor cells of the S180 model group were not only significantly enlarged, but also had many irregular karyons and polikaryocytes. The morphology of tumor cells treated with ZJW was more regular, and karyon pyknosis was more significant than in the CTX-treated group. HL and WZY had weaker effects on the necrosis of tumor cells than ZJW.

The effects on the spleen in S180-bearing mice were also investigated. The white pulp of the spleen was markedly more decreased in the CTX-treated group than in the S180 model group. The white pulp in ZJW-treated group was conspicuously increased compared with that in the CTX group, which was also larger than those in HL and WZY singly-used groups.

S180 tumor-transplanted animals were treated with or without ZJW, HL and WZY, and the activities of the tumor markers in serum were detected. Activities of ACP, AKP, CK, ALD and LDH were 126.72 U/L, 78.05 U/100 mL and 67.27 U/100 mL after treatment with ZJW, respectively, which were much higher than after HL- and WZY-treated groups (Fig. 6). Activities of ACP, AKP, CK, ALD and LDH in serum of ZJW-treated mice were considerably reduced compared with those in the HL (7.88 U/L) and WZY (5.67 U/L)–treated groups.

To explore the relationship between the antitumor action and the level of protein expression, Bax and wild-type P53 protein expression were analyzed by immunohistochemistry (Fig. 7 and Fig. 8). A mean score for the IOD of staining was determined, and the percentage of positive cases was detected for each group using an individual score of > 0 as a positive result. According to results in Table 2, Bax protein appears to be predominantly localized within the cytoplasm of the tumour cells, and the expression was significantly higher in the ZJW-treated group (mean IOD, 932.66 ± 180.72; positive rate, 90%) than in the HL- and WZY- and CTX-treated groups (mean IOD, 272.52 ± 73.43, 186.35 ± 55.50 and 213.46 ± 64.04; positive rate, 50%, 32% and 42.86%, respectively). Expression of wild-type P53 protein was the highest (main IOD, 28.03 ± 91.78; positive rate, 86.67%) compared with HL-, WZY- and CTX–treated groups (main IOD, 219.40 ± 43.58, 268.09 ± 26.38 and 367.52 ± 87.70; positive rate, 32%, 35% and 40%, respectively) treated groups.

Discussion

ZJW could produce significant antitumor activity compared with HL and WZY used singly. ZJW could not only inhibit the S180 tumor growth, but also significantly enhance the ILS of tumor-bearing mice.

CanA, a lectin protein originally extracted from the jackbean Canavalia ensiformis, has been utilized in lymphokine production, mitogenic assays, characterization of normal and malignant cells, and glycoprotein purification [21]. In a study by Das et al. [21], the lymphocyte proliferation in HL-, WZY- and ZJW-treated ani-
TNF-α is a cytokine released from activated macrophages that can inhibit the proliferation of tumor cells. Not only can normal cells be detected, but also early apoptotic cells, late apoptotic cells, and necrotic cells can be distinguished by this method. Early apoptotic cells (densely green-stained or displaying green-yellow fragments), late apoptotic cells (densely cardinal red-stained or displaying cardinal red fragments) and cardinal red swollen necrotic cells can be observed. Significant apoptosis was induced by ZJW, and apoptotic cells detected by AO: EB staining were markedly increased compared with the other groups.

TNF-α is a cell factor released from activated macrophages that can be combined with the receptors of tumor cell membranes to kill tumor cells specifically without injuring healthy cells. It can regulate many genes associated with inflammation, infection and malignancy. TNF-α can inhibit the proliferation of tumor cells [23] by cytotoxicity and regulate immunomodulation. TNF-α is an important factor in the detection and study of tumors. The TNF-α concentration in the serum of mice treated with ZJW was much higher than in the HL- and WZY-treated groups, and more tumor cells were killed by ZJW compared with the two herbs used singly.

Histopathological and morphological analyses revealed that ZJW could significantly increase antitumor activity, and promote immune function by regulation of the white pulp of the spleen, which was markedly higher than in the HL- and WZY-treated groups. Abnormal genes are expressed after protein synthesis within the cell and are changed due to cancer. Tumor markers are displayed in the form of enzymes in blood, and have an important role in tumor determination [24]. ACP activity directly affects the apoptosis and acidification of cytolympth [25]. AKP can participate in the information transfer of cells. Activities of ACP and AKP were increased significantly, which indicated that ZJW could increase the activities of ACP and AKP to regulate the DNA expression, phosphorylation and dephosphorylation of related proteins to inhibit the growth of tumor cells. ALD is a lyase in glycolysis, and its activity is increased significantly in serum when cancer is present. The endocellular enzyme CK [26] can enter the blood when cells are damaged in cancer. LDH [27], an important oxidoreductase in glycolysis, can catalyze the reversible production of lactic acid. Activities of ALD, CK and LDH were reduced in the serum of ZJW-treated mice, which would benefit the inhibition of tumor growth.

The P53 gene family contains wild-type (wtp 53) and mutant-type (mut p53) genes. The wtp 53 genes are more likely to suppress tumor cell expression than the mut P53 genes [28]. Wtp53 genes are tumor suppressors, and are expressed in karyon genes when introduced into cells [29]. Expression of high levels of wild-type (but not mutant) P53 can induce cell cycle arrest or apoptosis. Wtp53 protein was expressed at the highest level in ZJW-treated mice compared with the HL and WZY counterparts. P53 protein also reached a higher level in CTX compared with the control group. Bax, a pro-apoptotic member of the Bcl-2 protein family, is responsible for apoptotic cell death. It can induce growth arrest, apoptosis and cell senescence. HL could promote higher Bax protein expression than WZY, and highest expression of the protein was produced in the mice treated with ZJW. Expressions of Bax and wild-type P53 proteins could be significantly increased by ZJW compared with HL and WZY used singly, and there was a synergistic effect in the expression of the two proteins when HL and WZY were used together.
The anticancer activity produced by Z/W was much better than those of HL and WZY singly used, and an obvious synergistic effect was also shown by the combination use of the two herbs. The mechanism of enhanced antitumor activity due to the synergy of HL and WZY at the gene and protein level deserves further investigation.

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