Cancer Chemother Pharmacol (2010) 65:481–489
DOI 10.1007/s00280-009-1053-2

ORIGINAL ARTICLE

Synergistic effect of 5-fluorouracil and the flavanoid oroxylin A on HepG2 human hepatocellular carcinoma and on H_{22} transplanted mice

Li Zhao · Zhen Chen · Jun Wang · Li Yang · Qing Zhao · Jia Wang · Qi Qi · Rong Mu · Qi-Dong You · Qing-Long Guo

Received: 28 April 2009 / Accepted: 12 June 2009 / Published online: 8 July 2009

Abstract
Aim To investigate the synergistic inhibitory effects of the combination of 5-fluorouracil (5-FU) with the natural flavanoid oroxylin A on human hepatocellular carcinoma cells HepG2 in vitro and on transplanted murine hepatoma 22 (H_{22}) tumors in vivo and the preliminary mechanisms.
Methods The inhibitory effects of 5-FU combined with the natural flavanoid oroxylin A in vitro were detected by MTT assay and the effects in vivo were investigated by transplanted H_{22} mice model. DAPI staining and Annexin V/propidium iodide (PI) double staining were used to detect the cell morphological changes and apoptosis. The mRNA levels of thymidine synthetase (TS) and dihydropyrimidine dehydrogenase (DPD) in HepG2 cells after oroxylin A and 5-FU combination treatment were observed by quantitative real-time PCR. Western blotting assay was used to reveal the expressions of apoptotic-inducing proteins P53, cleaved PARP, COX-2, Bcl-2, and pro-caspase3.
Results Oroxylin A in combination with 5-FU presented synergistic effect (CI < 1) on HepG2 cells in vitro when the inhibitory rate was higher than 7.5%. The inhibitory rate on H_{22} murine solid tumor in vivo in the combination group was higher than monotherapy. 5-FU combined with oroxylin A exerted stronger apoptotic induction in HepG2 cells than either single drug treatment. Quantitative real-time PCR discovered the downregulation of TS mRNA and DPD mRNA in HepG2 cells after combination treatment. Western blotting assay revealed oroxylin A enhanced 5-FU-induced apoptosis in HepG2 cells by elevating the expressions of apoptotic-inducing proteins P53 and cleaved PARP and decreasing the expression of apoptotic-inhibitory proteins COX-2, Bcl-2, and pro-caspase3.
Conclusion The anti-hepatocellular carcinoma effects in vitro and in vivo of 5-FU and oroxylin A combinations were synergistic and oroxylin A increased the sensitivity of HepG2 cells to 5-FU by modulating the metabolic enzymes of 5-FU and apoptotic-related proteins.

Keywords 5-Fluorouracil · Oroxylin A · Hepatocellular carcinoma · Synergistic effect

Li Zhao, Zhen Chen contributed equally to this article.

L. Zhao · J. Wang · L. Yang · Q. Zhao · J. Wang · Q. Qi · R. Mu · Q.-L. Guo (✉)
Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University, 24 Tongjiaxiang, 210009 Nanjing, China
e-mail: anticancer_drug@yahoo.com.cn

Q.-D. You (✉)
Department of Medicinal Chemistry, China Pharmaceutical University, 24 Tongjiaxiang, 210009 Nanjing, China
e-mail: youqidong@gmail.com

Z. Chen
Department of Pharmacology, China Pharmaceutical University, 24 Tongjiaxiang, 210009 Nanjing, China

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. Although surgical managements or non-surgical therapeutic modalities have been employed, HCC is rarely curative. Chemotherapy is used in a variety of cancer treatments. Single-agent responses are usually partial and relatively short. Moreover, the toxicity of the single agent to normal tissues has been one of the major obstacles to successful cancer treatment. Therefore,

combined treatments with several chemotherapy drugs are often used to enhance the therapeutic effect.

Oroxylin A (Fig. 1), a bioactive flavonoid, is extracted from the root of a traditional Chinese medicine—*Scutellaria baicalensis* Georgi [16]. It has been shown to be a promising candidate for selective and effective management of inflammation [22]. Chen [2] reported that oroxylin A inhibited lipopolysaccharide (LPS)-induced expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in a concentration-dependent manner. Huang [10] also reported oroxylin A exhibited significant antioxidative and free-radical scavenging activities. The effects of oroxylin A reversing scopolamine and Abeta (25–35) peptide-induced memory and congnitire impairment via the GABAergic neurotransmitter system were reported by Kim [12, 13]. Besides, oroxylin A could inhibit diclofenac 4-hydroxylation (CYP2C9) activity in human liver microsomes to protect liver [14] and be a potent anti-respiratory syncytial virus component [18]. Recently, our studies found oroxylin A induced human hepatocellular carcinoma cell line HepG2 apoptosis by modulating the concerted expression of Bcl-2, Bax, and pro-caspase-3 proteins [9] and inhibited the growth of human gastric carcinoma BGC-823 cells by inducing G2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 [30].

The antimetabolite 5-fluorouracil (5-FU) is widely used in many types of tumors for over 20 years, but responses are obtained in only 10–30% of patients with advanced disease. It exerts antitumor effects by disturbing RNA processing and inhibiting DNA synthesis. It may be converted to 5-fluorouridine-5′-monophosphate (FUMP) either directly by orotate phosphoribosyltransferase or by uridine phosphorylase and uridine kinase. FUMP may then be converted to 5-fluorouridine-5′-triphosphate (FUTP) which, after incorporation into RNA, can interfere with the synthesis and function of all classes of RNA. FUMP may be converted [after reduction of 5-fluorouridine-5′-diphosphate (FUDP) by ribonucleotide reducase] to 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and hence DNA synthesis. Thus, activation of 5-FU may give rise to inhibition of DNA and/or RNA metabolism and DNA synthesis [19, 21].

Thymidine synthetase (TS) is a rate-limiting enzyme in pyrimidine de novo deoxynucleotide biosynthesis and is therefore an excellent target for chemotherapeutic strategies. TS plays a central role in DNA synthesis in the reductive methylation of deoxyuridine-5′-monophosphate (dUMP) to deoxythymidine-5′-monophosphate (dTMP). The cosubstrate for TS is the reduced-folate cofactor, 5,10-methylenetetrahydrofolate (CH2THF), which forms a ternary complex with dUMP and TS. FdUMP, the active metabolite of 5-FU competes with dUMP, and can form a ternary complex which does not dissociate readily because the methyl group cannot be transferred [27]. Cancer cells with high levels of FdUMP and low levels of TS are thus known to be sensitive to 5-FU [20]. Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme involved in the degradation of 5-FU and inactivates over 80% of administered 5-FU in vivo. Inactivation of 5-FU by DPD seemed to be a mechanism of clinical resistance to 5-FU, vigorous efforts have been made to design inhibitors of DPD [20]. The activity of DPD in tumor cells is critical to therapeutic effects of 5-FU, thus its inhibition improves the antitumor efficacy and the therapeutic index of 5-FU [8].

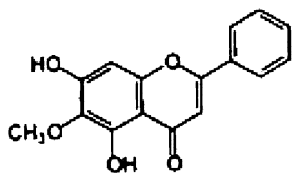

Fig. 1 Molecular structure of oroxylin A. The molecular formula of oroxylin A is $C_{16}H_{12}O_5$ and the molecular weight is 284

In this study, we tested the effects of 5-FU and oroxylin A alone and in combination regarding their activities against hepatocellular carcinoma in vitro and in vivo, and investigated the possible mechanisms involved.

Materials and methods

Chemicals

Oroxylin A was isolated from *S. radix* according to the protocols reported previously [16] with slight modifications. Samples containing 99% or higher oroxylin A were used in all experiments unless otherwise indicated. Oroxylin A was dissolved at a concentration of 100 mM in 100% DMSO (Amresco, Amresco Inc., Solon, Ohio) as a stock solution, stored at −20°C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. 5-FU (25 mg/ml) was purchased from Tianjin Amino Acids Company (Tianjin, China) and diluted to various concentrations with culture medium. Primary antibodies for pro-caspase3, P53, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., CA). Primary antibodies for Bcl-2 and PARP were purchased from Cell Signaling Technology (Beverly, MA). Primary antibody for COX-2 was the product of Bioworld Technology Inc. (China). IRDye™ 800 conjugated secondary antibodies were obtained from Rockland Inc. (Bedford, PA).

Cell culture

Human hepatocellular carcinoma cell line HepG2 was purchased from Cell Bank of Shanghai Institute of

Biochemistry & Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium (GIBCO, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Zhejiang, China), 100 U/ml benzyl penicillin, and 100 U/ml streptomycin in a humidified environment with 5% CO_2 at 37°C.

Murine hepatoma 22 (H_{22}) cells, provided by Jiangsu Institute of Antitumor Pharmaceuticals, were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin G, and 100 U/ml streptomycin, pH 7.4 in a water-jacketed CO_2 incubator (Thermo Forma, USA) with a humidified atmosphere of 5% CO_2 at 37°C.

Animals

Male kunming mice with body weight ranging from 18 to 22 g were provided by the Animals Facility at China Pharmaceutical University [No. SCXK (Su) 2002-0011]. Animals were maintained in a pathogen-free environment (23 ± 2°C, 55 ± 5% humidity) on a 12-h light/12-h dark cycle with food and water supplied adlibitum throughout the experimental period.

Cell growth inhibition assay

HepG2 cells were cultured in the presence of oroxylin A or 5-FU or both drugs for 48 h, the growth inhibitory effect was determined by measuring MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The inhibitory ratio was calculated by the following formula: inhibitory ratio (%) = 1 − average absorbance of treated group/average absorbance of control group × 100%.

Combined effect evaluation

Drug interaction between oroxylin A and 5-FU was assessed at a fixed concentration ratio of 1:1. The combination index (CI) was calculated as follows:

$$CI = \frac{(D)_1}{(D_X)_1} + \frac{(D)_2}{(D_X)_2}$$

where $(D_X)_1$ and $(D_X)_2$ are the doses for oroxylin A and 5-FU in a combination that inhibits 50% cell growth, and $(D)_1$ and $(D)_2$ are the doses for each drug alone that inhibit 50% cell growth. CI < 1, CI = 1, and CI > 1 indicated synergistic, additive, and antagonistic effects, respectively [4, 6]. Data analysis was performed by the Calcusyn software (Biosoft, Oxford, UK).

DAPI staining

Human hepatocellular carcinoma HepG2 cells were cultured in RPMI-1640 till mid-log phase. Then 50-μM oroxylin A, 750-μM 5-FU alone and the both were exposed to cells for 48 h. The morphology of the cells was monitored under an inverted light microscope. All floating and attached cells were harvested with 0.02% (w/v) EDTA and 0.25% (w/v) trypsinase. The cells were fixed with ice-cold 4% paraform for 20 min and washed with ice-cold PBS, then permeabilized with 0.3% Triton X-100 and washed with ice-cold PBS, stained with fluorochrome dye DAPI (Santa Cruz, USA) and observed under a fluorescence microscope (OlympusIX51, Japan) with a peak-excitation wave length of 340 nm.

Annexin V/PI double staining

Cells were incubated for 48 h with either oroxylin A or 5-FU or in combination. Apoptotic cells were identified by the Annexin V-FITC Apoptosis Detection kit (Biovision, Moutain View, CA) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software (Franklin Lakes, NJ, USA).

Quantitative real-time reverse transcription-PCR analysis

Cells were incubated for 48 h with either oroxylin A or 5-FU or in combination. Then total cellular RNA was extracted using the TriPure Solution (Takara, Takara Bio Inc., Otsu, Shiga, Japan) following the manufacturer's instructions. cDNA of 1 μg total RNA was synthesized using random primers and Primescript reverse transcriptase (Takara, Takara Bio Inc., Otsu, Shiga, Japan). Quantitative PCRs for indicated genes were carried out using SYBR green qPCR kit (Takara, Takara Bio Inc., Otsu, Shiga, Japan) by a fluorescent temperature cycler (Real-Time PCR System, Eppendorf, Hamburg, Germany). The primers used in the reactions are as follows: TS 5′-CAC CCT GTC GGT ATT CG-3′ and 5′-CCA CTG GAA GCC ATA AAC T-3′; DPD 5′-AAA GAA GCC TTG AGC T-3′ and 5′-TAG CCA AAC CAA CGA C-3′. Thermocycling was done in a final volume of 20 μl containing 2-μl cDNA sample (1 μg/μl), 0.4 μl of each primer (10 nM), 10-μl SYBR green I, 7.2-μl H2O. After an initial denaturation at 94°C for 10 s, 40 cycles at 94°C for 30 s, annealing at 55°C for 30 s, and 72°C for 30 s were carried out. All cDNA samples were synthesized in parallel, and PCR reactions were run in triplicate. mRNA levels were derived from standard curves and expressed as relative changes after normalization versus GAPDH mRNA levels [28].

Western blot analysis

Cells were treated with oroxylin A or 5-FU single or in combination. Then they were lysed in lysis buffer (100 mM Tris–HCl, pH 6.8, 4% (m) SDS, 20% (v) glycerol, 200 mM β-mercaptoethanol, 1 mM PMSF, and 1 g/ml aprotinin) for 1 h on the ice. The lysates were clarified by centrifugation (12,000 rpm) at 4°C for 15 min. The protein concentration in the supernatants was detected by a Varioskan multimode microplate spectrophotometer (Thermo, Waltham, MA, USA). Then equal amount of protein was separated by SDS-PAGE [34]. Proteins were detected using specific antibodies of Bcl-2, P53, COX-2, PARP, pro-caspase3, and β-actin followed by IRDye™800-conjugated secondary antibodies for 1 h at 37°C. Detection was performed by the Odyssey Infrared Imaging System (LI-COR Inc., Superior St. Lincoln, NE, USA). All blots were stripped and probed with polyclonal anti-β-actin antibody to ascertain equal loading of the proteins.

Inhibitory effects on H_{22} murine solid tumor

Murine hepatoma 22 (H_{22}) cells were diluted with icy 0.9% saline and inoculated subcutaneously at right axilla of mice (5×10^6 viable cells/each ml) [29]. Twenty-four hours after inoculation, mice were divided randomly into four groups (with 10 mice/group): saline tumor control group; oroxylin A 1,000 mg/(kg/day) orally group; 5-FU 10 mg/(kg/day) group; and oroxylin A + 5-FU combination group. Oroxylin A was administered orally (po) and 5-FU was given intravenously (iv). Both were given once every day. From the third day after treatment, the tumors were measured continuously. Tumor volume (TV) was calculated using the following formula:

$$\mathrm{TV}\left(\mathrm{mm}^3\right) = \mathrm{d}^2 \times \mathrm{D}/2$$

where d and D were the shortest and the longest diameter, respectively.

At the seventh day after treatment, the mice were killed and tumors were ablated carefully and weighed after washing off any remaining blood with PBS. The dose of oroxylin A was 1,000 mg/(kg/day), which was about one-fifth of LD50 value. The 5-FU 10 mg/(kg/day) was determined as a half of its effective dose 20 mg/(kg/day). This study was approved in SPF Animal Laboratory of China Pharmaceutical University.

Statistical analysis

All results shown represent means ± SEM from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using one-way ANOVA by SPSS 11.5 software.

Results

Effects of 5-FU or oroxylin A on the inhibition of cell viability and combination index of two drugs mixture

The inhibitionary effect of oroxylin A or 5-FU on cell viability in HepG2 cells was assessed after 48 h exposure, following a 24 h cultivation in drug-free medium. Results indicated that oroxylin A and 5-FU showed inhibitory effect by a concentration-dependent manner. As shown in Fig. 2a, b, the IC_{50} of oroxylin A and 5-FU was 39.79 ± 2.84 and 792.61 ± 14.65 μM, respectively. To detect the inhibitory effect of the combination treatment, cells were exposed to oroxylin A and 5-FU concurrently for 48 h at a fixed ratio (oroxylin A IC_{50}:5-FU IC_{50} ratios were 1:1) [4]. Data showed the CI values were <1 (Fig. 2c) when the values of fraction affects (Fa) were higher than 0.075 (at the point, the concentration of oroxylin A and 5-FU was about 0.6 and 1.4 μM, respectively), which indicated that the combination of oroxylin A and 5-FU exerted a synergistic inhibitory effect on the proliferation of HepG2 cells at concentrations higher than the threshold values (drug concentrations when CI = 1). Moreover, when the value of Fa is 0.5 (the inhibitory rate was 50%), the $C_{5\text{-FU}}$ is 157.57 μM, which is only about 20% of the IC_{50} value of 5-FU single treatment.

Cell morphological assessment by DAPI staining

After oroxylin A (50 μM) or 5-FU (750 μM) or the combination treatments for 48 h, the morphological changes of the cells were examined by light microscopy. As shown in Fig. 3a, untreated HepG2 cells stained with equal intensity of DAPI and demonstrated homogeneously distributed chromatin within their nuclei, while treated cells displayed chromatin condensation and nucleolus condensation (pyknosis), which indicated an early apoptotic event (arrow designating) [31]. Moreover, we observed that the apoptosis events in the combination group were more distinguished than that of either single drug treatment group. These results suggested that oroxylin A increase the cytotoxic effect of 5-FU on HepG2 cells.

Apoptosis induced by 5-FU or oroxylin A or in combination of them by Annexin V/PI staining

Apoptosis induced by oroxylin A and 5-FU in HepG2 cells was analyzed using Annexin V/PI staining. Compared with control group, the proportion of apoptotic cells increased after cells were exposed to 50 μM of oroxylin A or 750 μM of 5-FU or both drugs for 48 h with the early apoptosis rate 17.5%, 21.0%, and 26.3%, respectively. Among which the apoptotic induction of the combination was the strongest.

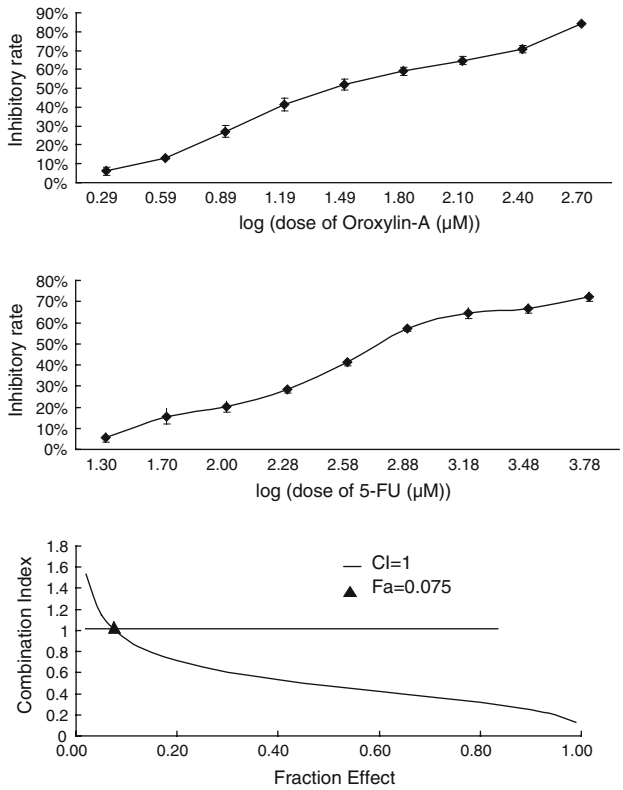

Fig. 2 Inhibitory effect of oroxylin A, 5-FU and the mean-effect of these two drugs on HepG2 cells. Effects of two single drugs on HepG2 cells and the combination index of the two drugs. HepG2 cells were treated with various concentration of oroxylin A (**a**) or 5-FU (**b**) alone for 48 h. Cell viability was determined using MTT assay

The condition of the late apoptosis and dead cells was similar with that of the early apoptotic cells (Fig. 3b). These results indicated that oroxylin A could increase the apoptotic-inducing effect and cytotoxic effect of 5-FU in HepG2 cells.

Changes of the mRNA levels of the TS and DPD metabolic enzymes after treatment with 5-FU or oroxylin A or the combination in HepG2 cells

To further elucidate the synergistic mechanism of 5-FU and oroxylin A, we examined the effect of them on the mRNA levels of TS and DPD on HepG2 cells. As shown in Fig. 4, 5-FU treatment alone caused a 5.18 ± 0.39-fold increase at mRNA level of TS in HepG2 cells compared with the control group ($P < 0.01$). In contrast, the dual treatment decreased it nearly to one quarter at 1.43 ± 0.13-fold of control. And, the DPD mRNA level in 5-FU treatment group was 9.31 ± 0.17-fold increase compared with the control group ($P < 0.01$), while the level in combined group was down-regulated to about one-third of 5-FU group at 2.92 ± 0.15 (compared with control, $P < 0.01$).

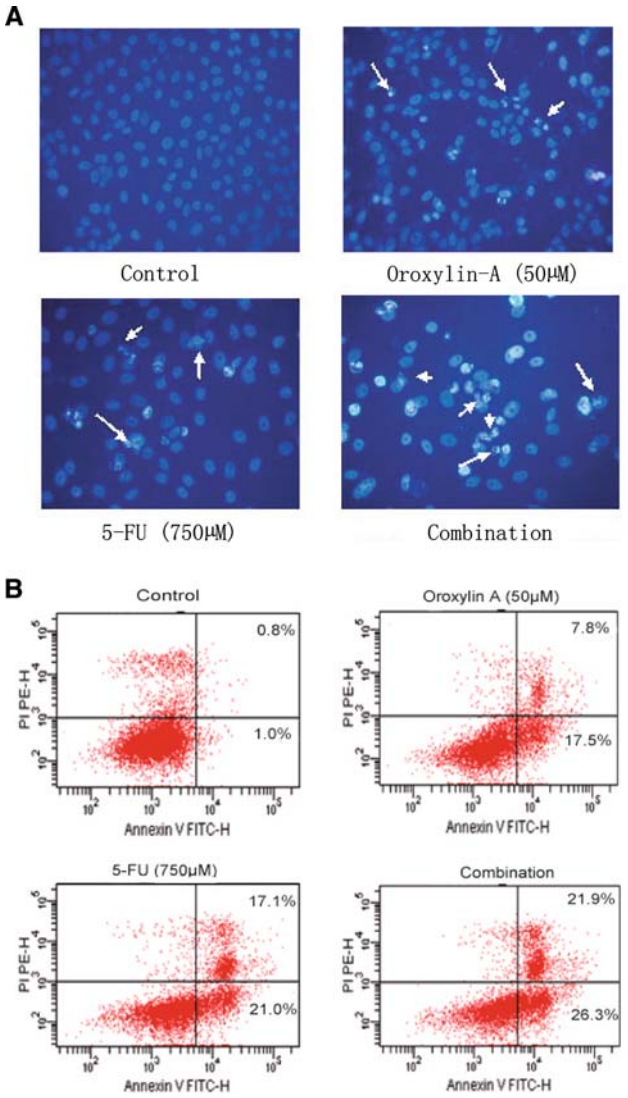

Fig. 3 Apoptosis assessment in HepG2 cells. **a** Nucleolus morphological changes observed by fluorescence microscope (200×). HepG2 cells were incubated with 50 μM oroxylin A, 750 μM 5-FU alone and the combination of them for 48 h. DAPI staining was used to visualize the cell nucleus. The nucleolus changes of HepG2 cells were observed under the fluorescent microscope. **b** Apoptosis assessment by Annexin V/PI staining in HepG2 cells. Apoptosis in HepG2 cells treated with oroxylin A (50 μM), 5-FU (750 μM) either in combination or alone for 48 h. Early apoptotic cells were Annexin V-positive, PI-negative cells (*low right*), and late apoptosis and death cells were defined as Annexin V-positive, PI-positive cells (*up right*)

Western blot analysis for apoptosis-related proteins in HepG2 cells

To further illuminate the mechanism of co-treatment with oroxylin A which may enhance the apoptotic effect induced by 5-FU in HepG2 cells, the expressions of some apoptotic related proteins were investigated. Expressions of COX-2, Bcl-2, and P53 were detected after cells were incubated with either oroxylin A or 5-FU or in combination

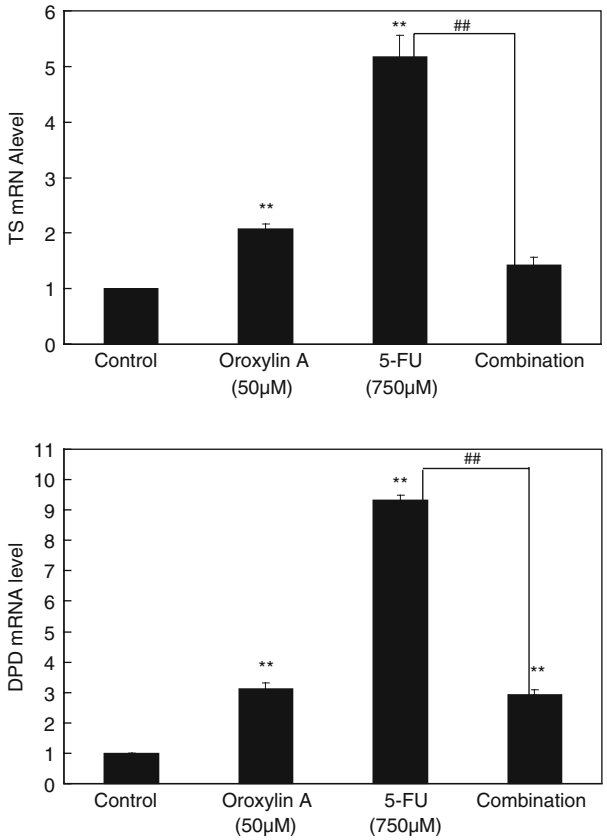

Fig. 4 Changes of mRNA levels of TS and DPD in HepG2 cells. The mRNA expression of TS and DPD was detected by real-time PCR. HepG2 cells were treated with oroxylin A and 5-FU either in combination or alone for 48 h. Results represent mean values of three experiments ± SEM, $^{*}P < 0.05$ compared with control, $^{**}P < 0.01$ compared with control, $^{\#\#}P < 0.01$ combination compared with 5-FU group

for 24 h, and the expressions of PARP and pro-caspase3 were observed after cells were treated for 48 h. As shown in Fig. 5, 5-FU combined with oroxylin A significantly decreased the protein level of Bcl-2, whereas COX-2 and P53 protein levels were increased compared with 5-FU single group. PARP, a 112 kDa protein, is a substrate for caspases and cleaved during apoptosis to an 89 kDa fragment. Oroxylin A or 5-FU alone activated caspase-3 and induced PARP cleavage and the combined effect was more significant than single treatment.

Inhibitory effect of oroxylin A or 5-FU or the combination on the growth of H_{22} murine solid tumor

We further examined the effects of 5-FU combined with oroxylin A on the growth of H_{22} murine solid tumor in vivo. As shown in Fig. 6, combination group presented strongest inhibitory effect on the growth of H_{22} murine solid tumor. As shown in the curves of tumor growth volumes (Fig. 6a), all treated groups showed inhibitory effects starting from the fifth day. At the seventh day, the mean volume of the tumors in control group reached 1,588 mm^3, while oroxylin A group and 5-FU group were at 1,311 mm^3 and 1,096 mm^3 respectively, while that in the drug-combination group was smallest with 720 mm^3, which was about one half of the data of the control group ($P < 0.05$ compared with control). The same trend is shown in the tumor weight figure (Fig. 6b). In the drug-combined group, the average tumor weight was the smallest (0.66 g) and the inhibitory rate reached 59.67% ($P < 0.01$ compared with control), whereas those of mice treated with 5-FU or oroxylin A alone were 34.71% ($P < 0.05$ compared with control) and 14.72% ($P > 0.05$ compared with control), respectively. These results showed that the anti-tumor effect of oroxylin A combined with 5-FU was superior to that of either single drug treatment.

Discussion

At present, the most effective treatment for patients with HCC is the surgical resection of hepatic lesions. Local therapeutic approaches, such as transcatheter arterial embolization [3], percutaneous transhepatic ethanol injection [7], microwave coagulation [11], and radiofrequency ablation [25], are also effective. However, these therapies are not sufficient for patients with advanced HCC, for whom surgery is often not suitable and whose 5-year survival rate is extremely low [17]. For patients with advanced HCC, the clinical responses of most anticancer drugs were insufficient, and several combination chemotherapies have been tried. In clinic, 5-FU combined with cisplatin, FAM (5-FU + adriamycin + mitomycin) and BAF (5-FU + adriamycin + bleomycetin) have been used, but the effect is not satisfied. Therefore, to find more effective combined therapeutic schedule becomes eager. In recent years, the interest in exploiting traditional medicines for prevention or treatment of tumors has increased.

In our study, the inhibitory effects of 5-FU combined with oroxylin A on HCC cells in vitro and in vivo were investigated. We found that oroxylin A could increase the sensitivity of HCC cells to 5-FU. In vitro (Fig. 2), by the method of Chou and Talalay [5], when $Fa > 0.075$, $CI < 1$, oroxylin A in combination with 5-FU presented synergistic effect on HepG2 cells when the inhibitory rate was higher than 7.5%. The IC50 of 5-FU was reduced to about 20% when combined with oroxylin A. The inhibitory rate on H_{22} murine solid tumor in vivo was 59.67% in the drug-combination group, which was higher than either monotherapy (Fig. 6). While the dosing of oroxylin A in mice is 1,000 mg/kg which indicates a low bioavailability. In our next work, we would make the drug to injectable

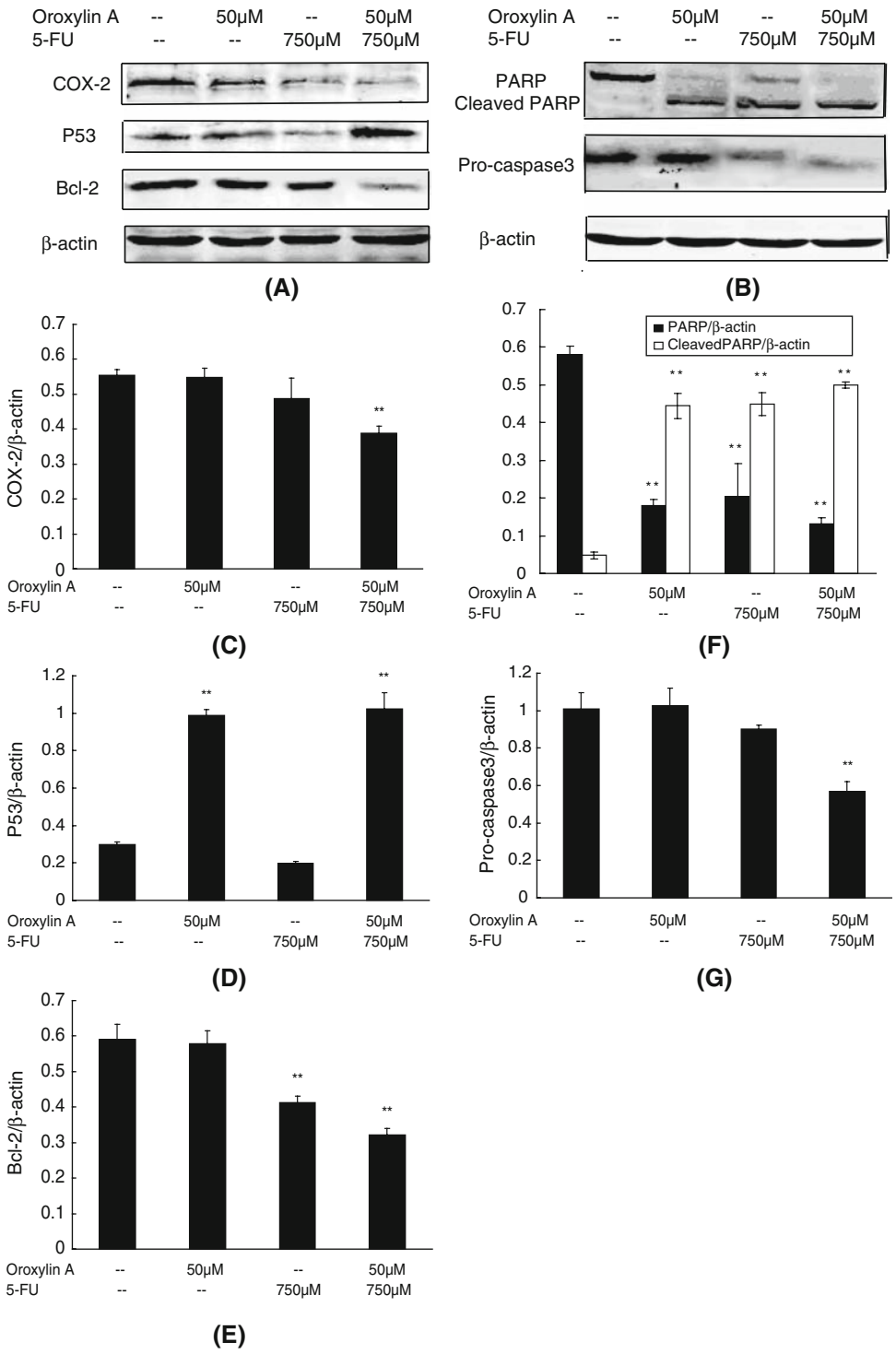

Fig. 5 Effects of oroxylin A and 5-FU combination on the expression of apoptotic-related proteins. HepG2 cells were incubated with either oroxylin A (50 μM) or 5-FU (750 μM) alone or dual treatment. Western blot assay was used to examine COX-2, Bcl-2, and P53 expression after cells incubated for 24 h, and PARP and pro-caspase3 expression after cells were treated for 48 h. $^{*}P < 0.05$ compared with control, $^{**}P < 0.01$ compared with control

preparation, which would possess higher bioavailability in vivo for future clinical application.

Furthermore, the apoptosis induced by oroxyloin-A (50 μM) and 5-FU (750 μM) was examined by DAPI staining and flow cytometry analysis. The concentrations were selected based on determination of the IC50 for both compounds. By DAPI staining (Fig. 3a), more apoptosis events were observed in the combination group after HepG2 cells were incubated with drugs for 48 h. By Annexin V/PI staining (Fig. 3b), the highest early apoptotic rate appeared in the combination group at 26.3%, which was higher than 17.5% in oroxylin A group and 21.0% in 5-FU group.

The above results encouraged further research on the mechanism of this synergistic effect. 5-FU is converted in

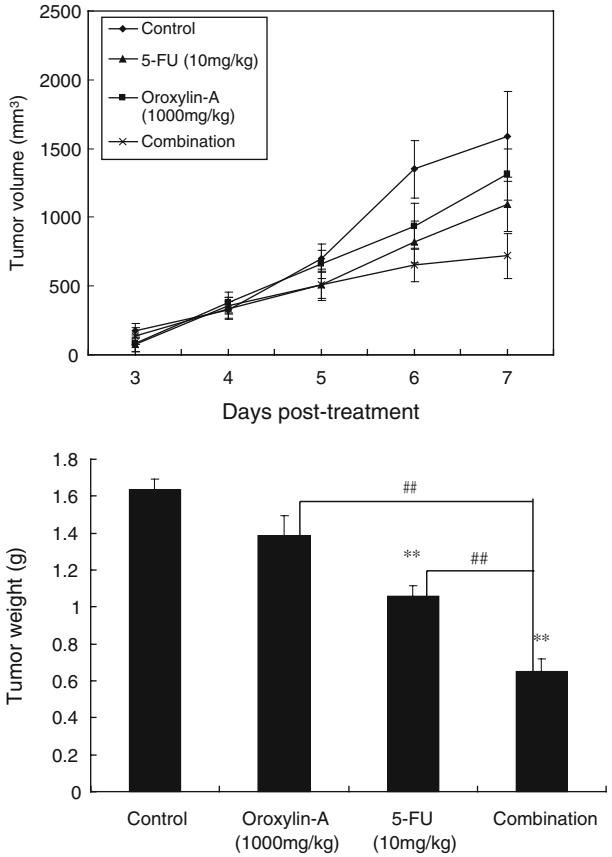

Fig. 6 Inhibitory effect of oroxylin A and 5-FU on the growth of H_{22} murine solid tumor. H_{22} tumor-bearing mice were randomly allocated into four groups after being inoculated for 24 h and administered with oroxylin A 1,000 mg/(kg/day) orally and 5-FU 10 mg/(kg/day) intravenously once every day. From the third day, the tumors were measured every day. On the seventh day, the mice were killed and the tumors were weighed. Data were represented as mean ± SEM, $n = 10$; $^{*}P < 0.05$ compared with control; $^{**}P < 0.01$ compared with control. $^{\#\#}P < 0.01$ combination compared with the monotherapy

tumor cells to FdUMP, which forms a tight covalent complex with TS in the presence of the folate cofactor 5-10-methylene tetrahydrofolate. And a decrease in TS levels in tumor cells blocks DNA synthesis in dividing cells [1]. Thus, levels of TS expression in tumors have been reported to be a critical indicator of chemosensitivity to 5-FU [15, 23]. In our study, the mRNA level of TS in the combination group was 3.62-fold lower than that in 5-FU single group (Fig. 4). DPD converted 5-FU accumulated into inactivated metabolites and high DPD activity in cancer cells is an important determinant of 5-FU response [26]. In our study, the mRNA level of DPD in the combination group was 3.18-fold lower than that in 5-FU alone group (Fig. 4). These results proved strongly that oroxylin A could increase the sensitivity of HepG2 cells to 5-FU though regulating the mRNA levels of 5-FU-related enzymes—TS and DPD.

In addition, the changes of apoptosis-related protein might explain the higher apoptotic rate in the drug-combined group. P53 is known to induce apoptosis by transcriptional repression of Bcl-2 and inhibitors of apoptosis [32]. We detected that the expression of P53 in the combined group was increased dramatically campared with 5-FU group (Fig. 5). At the same time, the expression of Bcl-2 protein was down-regulated significantly (Fig. 5). Moreover, 5-FU combined oroxylin A elevated PARP cleavage and depressed the expression of pro-caspase3. Previous reports have suggested that 5-FU could down-regulate Bcl-2 family proteins and induces caspase family proteins [33]. So we supposed that the combined treatment could increase the apoptotic effects induced by 5-FU. Since P53 and Bcl-2 were the upstream events in apoptotic pathway, we observed the changes at 24 h after HepG2 cells being treated. The changes were not obvious at 48 h (data not shown). Moreover, we found that at 24 h, the expression of COX-2 was decreased in the combined group compared with oroxylin A and 5-FU alone group. Sun et al. [24] have reported that forced COX-2 expression significantly attenuates apoptosis induction by 5-FU through predominant inhibition of the cytochrome *c*-dependent apoptotic pathway. COX-2-mediated up-regulation of Bcl-2 suggests a potential mechanism for reduced-apoptotic susceptibility. Based on these data, we concluded that oroxylin A increase the apoptosis induced by 5-FU by modulating the expressions of apoptotic-related proteins.

Taken together, HCC in vitro and in vivo showed higher sensitivity to 5-FU when combined with oroxylin A. The most likely mechanism was that oroxylin A could regulate the gene expressions of TS and DPD, the key enzymes in 5-FU metabolic procession. Moreover, the combination of 5-FU and oroxylin A exerted stronger apoptosis-inducing effect than either single drug treatment, which involved the regulation of the expression of COX-2, P53, Bcl-2, cleaved PARP and caspase3 in HepG2 cells. All the results suggested the combinational use of 5-FU and oroxylin A might be an effective candidate in clinical treatment of HCC in future.

Acknowledgments This work was supported by the National High Technology Research and Development Program of China (863 program, No. 2003AA2Z3267), National Institutes of Health (SCORE S06GM-008205) and Jiangsu Province Six Talent Pools in Education, Medical care and hygiene, Electronic information, Machinery and motor, Architecture and Agriculture Project.

References

1. Carrico CK, Glazer RI (1979) Effect of 5-fluorouracil on the synthesis and translation of polyadenylic acid-containing RNA from regenerating rat liver. Cancer Res 39:3694–3701

2. Chen Y, Yang L, Lee TJ (2000) Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kappaB activation. Biochem Pharmacol 59:1445–1457
3. Cheung YC, Ko SF, Ng SH, Chan SC, Cheng YF (2005) Survival outcome of lobar or segmental transcatheter arterial embolization with ethanol–lipiodol mixture in treating hepatocellular carcinoma. World J Gastroenterol 11:2792–2795
4. Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58:621–681
5. Chou TC, Motzer RJ, Tong Y, Bosl GJ (1994) Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst 86:1517–1524
6. Chou TC, Talalay P (1984) Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
7. Ebara M, Okabe S, Kita K, Sugiura N, Fukuda H, Yoshikawa M, Kondo F, Saisho H (2005) Percutaneous ethanol injection for small hepatocellular carcinoma: therapeutic efficacy based on 20-year observation. J Hepatol 43:458–464
8. Fischel JL, Etienne MC, Spector T, Formento P, Renee N, Milano G (1995) Dihydropyrimidine dehydrogenase: a tumoral target for fluorouracil modulation. Clin Cancer Res 1:991–996
9. Hu Y, Yang Y, You QD, Liu W, Gu HY, Zhao L, Zhang K, Wang W, Wang XT, Guo QL (2006) Oroxylin A induced apoptosis of human hepatocellular carcinoma cell line HepG2 was involved in its antitumor activity. Biochem Biophys Res Commun 351:521–527
10. Huang WH, Lee AR, Yang CH (2006) Antioxidative and anti-inflammatory activities of polyhydroxyflavonoids of *Scutellaria baicalensis* GEORGI. Biosci Biotechnol Biochem 70:2371–2380
11. Kawamoto C, Ido K, Isoda N, Hozumi M, Nagamine N, Ono K, Sato Y, Kobayashi Y, Nagae G, Sugano K (2005) Long-term outcomes for patients with solitary hepatocellular carcinoma treated by laparoscopic microwave coagulation. Cancer 103:985–993
12. Kim DH, Jeon SJ, Son KH, Jung JW, Lee S, Yoon BH, Lee JJ, Cho YW, Cheong JH, Ko KH, Ryu JH (2007) The ameliorating effect of oroxylin A on scopolamine-induced memory impairment in mice. Neurobiol Learn Mem 87:536–546
13. Kim DH, Kim S, Jeon SJ, Son KH, Lee S, Yoon BH, Cheong JH, Ko KH, Ryu JH (2008) The effects of acute and repeated oroxylin A treatments on Abeta(25–35)-induced memory impairment in mice. Neuropharmacology 55:639–647
14. Kim JY, Lee S, Kim DH, Kim BR, Park R, Lee BM (2002) Effects of flavonoids isolated from *Scutellariae radix* on cytochrome P-450 activities in human liver microsomes. J Toxicol Environ Health A 65:373–381
15. Kitchens ME, Forsthoefel AM, Barbour KW, Spencer HT, Berger FG (1999) Mechanisms of acquired resistance to thymidylate synthase inhibitors: the role of enzyme stability. Mol Pharmacol 56:1063–1070
16. Li HB, Chen F (2005) Isolation and purification of baicalein, wogonin and oroxylin A from the medicinal plant *Scutellaria baicalensis* by high-speed counter-current chromatography. J Chromatogr A 1074:107–110
17. Llovet JM (2005) Updated treatment approach to hepatocellular carcinoma. J Gastroenterol 40:225–235
18. Ma SC, Du J, But PP, Deng XL, Zhang YW, Ooi VE, Xu HX, Lee SH, Lee SF (2002) Antiviral Chinese medicinal herbs against respiratory syncytial virus. J Ethnopharmacol 79:205–211
19. Piper AA, Fox RM (1982) Biochemical basis for the differential sensitivity of human T- and B-lymphocyte lines to 5-fluorouracil. Cancer Res 42:3753–3760
20. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV (2000) Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 6:1322–1327
21. Sasaki E, Tominaga K, Kuwamura H, Watanabe T, Fujiwara Y, Oshitani N, Higuchi K, Arakawa T (2007) Synergistic antitumor effect of combined 5-fluorouracil (5-FU) with 5-chloro-2, 4-dihydroxypyridine on 5-FU-resistant gastric cancer cells: possible role of a dihydropyrimidine dehydrogenase-independent mechanism. J Gastroenterol 42:816–822
22. Sonoda M, Nishiyama T, Matsukawa Y, Moriyasu M (2004) Cytotoxic activities of flavonoids from two Scutellaria plants in Chinese medicine. J Ethnopharmacol 91:65–68
23. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH (1982) In vivo kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. Cancer Res 42:450–456
24. Sun Y, Tang XM, Half E, Kuo MT, Sinicrope FA (2002) Cyclooxygenase-2 overexpression reduces apoptotic susceptibility by inhibiting the cytochrome c-dependent apoptotic pathway in human colon cancer cells. Cancer Res 62:6323–6328
25. Tateishi R, Shiina S, Teratani T, Obi S, Sato S, Koike Y, Fujishima T, Yoshida H, Kawabe T, Omata M (2005) Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer 103:1201–1209
26. van Kuilenburg AB, Haasjes J, Richel DJ, Zoetekouw L, Van Lenthe H, De Abreu RA, Maring JG, Vreken P, van Gennip AH (2000) Clinical implications of dihydropyrimidine dehydrogenase (DPD) deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DPD gene. Clin Cancer Res 6:4705–4712
27. Van Triest B, Pinedo HM, Giaccone G, Peters GJ (2000) Downstream molecular determinants of response to 5-fluorouracil and antifolate thymidylate synthase inhibitors. Ann Oncol 11:385–391
28. Wada Y, Yoshida K, Suzuki T, Mizuiri H, Konishi K, Ukon K, Tanabe K, Sakata Y, Fukushima M (2006) Synergistic effects of docetaxel and S-1 by modulating the expression of metabolic enzymes of 5-fluorouracil in human gastric cancer cell lines. Int J Cancer 119:783–791
29. Wang X, Zhang J, Xu T (2008) Thioredoxin reductase inactivation as a pivotal mechanism of ifosfamide in cancer therapy. Eur J Pharmacol 579:66–73
30. Yang Y, Hu Y, Gu HY, Lu N, Liu W, Qi Q, Zhao L, Wang XT, You QD, Guo QL (2008) Oroxylin A induces G2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 in human gastric carcinoma BGC-823 cells. J Pharm Pharmacol 60:1459–1463
31. Yang Y, Yang L, You QD, Nie FF, Gu HY, Zhao L, Wang XT, Guo QL (2007) Differential apoptotic induction of gambogic acid, a novel anticancer natural product, on hepatoma cells and normal hepatocytes. Cancer Lett 256:259–266
32. Yee KS, Vousden KH (2005) Complicating the complexity of p53. Carcinogenesis 26:1317–1322
33. Yim EK, Lee KH, Bae JS, Namkoong SE, Um SJ, Park JS (2004) Proteomic analysis of antiproliferative effects by treatment of 5-fluorouracil in cervical cancer cells. DNA Cell Biol 23:769–776
34. Yu J, Guo QL, You QD, Zhao L, Gu HY, Yang Y, Zhang HW, Tan Z, Wang X (2007) Gambogic acid-induced G2/M phase cell-cycle arrest via disturbing CDK7-mediated phosphorylation of CDC2/p34 in human gastric carcinoma BGC-823 cells. Carcinogenesis 28:632–638