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Synergistic Antitumor Effects of Liposomal Honokiol Combined with Adriamycin in Breast Cancer Models

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Honokiol, a novel antitumor agent, could induce apoptosis and inhibit the growth of vascular endothelium in several tumor cell lines and xenograft models. It has been suggested that the antitumor effect of chemotherapy could be increased by combining it with an antiangiogenesis agent in anticancer strategy. The present study explored the potential to increase the antitumor effect of adriamycin by combining it with honokiol in mouse 4T1 breast cancer models, and the underlining mechanism was investigated. Honokiol was encapsulated in liposomes to improve the water insolubility. *In vitro*, liposomal honokiol inhibited the proliferation of 4T1 cells via apoptosis and significantly enhanced the apoptosis of 4T1 cells induced by adriamycin. *In vivo*, the systemic administration of liposomal honokiol and adriamycin significantly decreased tumor growth through increased tumor cell apoptosis compared with either treatment alone. Collectively, these findings suggest that liposomal honokiol may augment the induction of apoptosis in 4T1 cells *in vitro* and *in vivo*, and this combined treatment has shown synergistic suppression in tumor progression according to the analysis of isobologram. The present study may be important in future exploration of the potential application of the combined approach in the treatment of breast cancer. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: synergistic; breast cancer; liposomal honokiol; adriamycin; combined therapy.

INTRODUCTION

Breast cancer accounts for about 1.2 million cases worldwide every year and has become the second leading cause of cancer deaths in Western women (Fioretti *et al.*, 1999; Jemal *et al.*, 2005). Despite aggressive approaches made in the treatment of breast cancer in the past few years, the prognosis remains poor. Therefore, tremendous efforts have to be made to develop new and less toxic therapeutic approaches for the treatment of breast cancer.

Inhibiting tumor angiogenesis is one of the most promising strategies for the treatment of cancer, and antiangiogenesis agents, such as angiostatin, endostatin and antivascular endothelial growth factor (VEGF) antibody, have already been conducted in experimental and clinical trials in recent years (Eichhorn *et al.*, 2007). In most reports, however, the tumors regrow after ceasing treatment with antiangiogenesis agents for their tumoristatic property. Tumor cures have been limited when angiogenesis inhibitors are used as the sole method of treatment (Gao and Xu, 2006). Hence, it has been suggested that chemotherapy could favorably be combined with an antiangiogenesis agent in anticancer strategy since the combination treatment enhances the therapeutic ratio of chemotherapy by targeting both tumor cells and tumor vessels. Moreover, these combined treatment modalities are achieved without increased toxicity compared with chemotherapy alone (Qiu *et al.*, 2006; Xu *et al.*, 2007).

Honokiol, an active compound purified from magnolia, has drawn much attention for its antiangiogenesis and apoptosis properties. Previous reports have demonstrated that honokiol enhanced human HL-60 leukemia cell differentiation (Fong *et al.*, 2005), inhibited mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis (Konoshima *et al.*, 1991) and induced apoptosis of human colon cancer cell RKO via p53-independent pathways (Wang *et al.*, 2004). Furthermore, honokiol could inhibit the growth of new vessels by interfering with the phosphorylation of vascular endothelial growth factor 2 ($VEGF_2$) and induce apoptosis of aggressive angiosarcoma in nude mice (Bai *et al.*, 2003; Reimer

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et al., 2002). Since the antitumor effects of honokiol were reported as the sole therapeutic agent, there is little information about the role of honokiol combined with chemotherapy *in vitro* and *in vivo*.

Adriamycin (ADR), a DNA-intercalating agent, is a significant active chemotherapy medicine for the treatment of a variety of human and murine tumors (Feleszko *et al.*, 2002; Safrit and Bonavida, 1992). ADR could induce the apoptosis of tumor cells by inhibiting DNA polymerases and topoisomerases (Cutts *et al.*, 1996; Tanaka and Yoshida, 1980; Tewey *et al.*, 1984). Moreover, ADR could also induce apoptosis of tumor cells by inhibiting RNA synthesis or processing by binding to RNA substrates (Zhu *et al.*, 1999). In most advanced breast cancer, ADR is a main option and can improve both the quantity and quality of the patients' lives. However, its practical therapeutic use has been limited by conventional toxicities and dose-dependent cardiotoxicity which is manifested by congestive cardiomyopathy (Hequet *et al.*, 2004; Zhou, 2000).

Due to differences in their mechanisms, combined treatment with honokiol and ADR might have clinical potential. To investigate whether honokiol synergistically potentiates the antitumor effects of ADR, the growth inhibitory effects of liposomal honokiol alone and in combination with adriamycin in the mouse 4T1 cell line *in vitro* and in BALB/c mice bearing 4T1 cell line xenografts *in vivo* were examined. The tumor volume and survival time were observed. Honokiol was encapsulated with modified liposomes to improve its poor water insolubility.

MATERIALS AND METHODS

Materials. Cholesterol and PEG4000 were purchased from Sigma Chemical Co, Inc. (St Louis, MO). Honokiol was separated and purified by our laboratory and its purity and identification were analysed by high performance liquid chromatography and nuclear magnetic resonance (Chen *et al.*, 2007). Adriamycin (ADR) was purchased from Zhejiang Hisun Pharmaceutical Company (China). BALB/c mice were purchased from Sichuan University Animal Center (Sichuan, Chengdu, China). Rat monoclonal antibodies against mouse CD31 antibodies were purchased from Santa Cruz Biotechnology. Biotinylated goat anti-rat IgG was purchased from Dako. An *In situ* Cell Death Detection kit was purchased from Roche Co. (Promega, Madison, WI).

Cell lines and cell culture. The breast carcinoma cell lines 4T1, Bcap-37 and Mcf-7 were obtained from American Type Culture Collection, ATCC. These cells were grown as monolayers in DMEM or RPMI-1640 medium (Gibco), containing 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 units/mL streptomycin, at 37 °C, 95% relative humidity, under 5% CO_2.

Preparation of liposomal honokiol. Liposomal honokiol (LH) was prepared in our laboratory and described briefly as follows: cholesterol, PEG4000 and honokiol in weight ratios of 0.15:0.24:0.22 were mixed and dissolved in 15 mL chloroform/methanol at a ratio of 1:4 [v/v]. The mixture was gently warmed to 40 °C in a round-bottomed flask, and evaporated under vacuum in a rotary evaporator until a thin lipid film was formed. The dried lipid films were left overnight and sonicated in ddwater followed by concentration and lyophilized. The preparation of empty liposomes (LH) was the same as for liposomal honokiol without the addition of honokiol. The final liposomal honokiol and empty liposomes were small multilamellar liposomes in a size range of 150 ± 20 nm and 100 ± 20 nm, respectively. Lyophilized liposomal honokiol and empty liposomes were dissolved in 5% glucose for *in vitro* and *in vivo* studies.

Cell proliferation assay. Cell viability was evaluated by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded in a 96-well plate at a plating density of 2×10^5/mL, and cultured for 24 h to allow them to adhere to the plate. The cells were grown for 24 h and treated with LH or ADR at various doses in fresh DMEM, respectively. Four wells for each treatment schedule were performed. After 24, 48 or 72 h incubation, the drug-containing medium was replaced by 200 μL fresh medium containing MTT for 4 to 6 h. After incubation, the supernatant was discarded and 150 μL DMSO was added to each well. The optical density (OD) of each culture was determined at 570 nm by the M5 (Molecular Corporation, USA). The absorbance of untreated cells and those treated by empty liposomes containing the equivalent dose polyethylene glycol liposomes of LH were both considered 100%. The IC_{50} was defined by the concentration that caused a 50% absorbance decrease of drug-treated cells compared with the control culture cells.

Isobologram analysis. Interactions of the treatment of combination were analysed by isobologram (Chou and Talalay, 1984). The dose-dependent effects were determined for each compound and for one compound with fixed concentrations of another, and the combination index (CI) was calculated according to the following formula:

$$CI = (d1/Dx1) + (d2/Dx2)$$

where $Dx1$ is the concentration of drug 1 (LH) required to produce x percentage effect alone, and $d1$ is the concentration of drug 1 required to produce the same x percentage effect in combination with $d2$. $Dx2$ is similarly the concentration of drug 2 (ADR) required to produce x percentage effect alone, and $d2$ is the concentration of drug 2 required to produce the same x percentage effect in combination with d1. The CI values were defined as follows: <1 synergism, = 1 additive, and >1 antagonism.

Flow cytometry. To determine tumor apoptosis and analyse the specificity of the cell cycle, flow cytometric analysis was done to identify sub-G_1 cells/apoptotic cells and to measure the percentage of sub-G_1 cells after propidium iodide staining in hypotonic buffer and briefly described as follows: the cells were suspended in 1 mL hypotonic fluorochrome solution which contains 50 mg propidium iodide/mL in 0.1% sodium citrate plus 0.1% Triton X-100. The cells were treated with LH plus ADR analysed by flow cytometer (ESP Elite, Beckman-Coulter, Miami, FL). A suitable dose of LH plus ADR was chosen according to the lowest CI value. Apoptotic

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cells appearing in the cell cycle distribution were estimated with Listmode software.

***In vivo* antitumor activity.** In the first experiment, the 4T1 bearing-tumor model was established in BALB/c mice (female; 18–20 g body weight; 8 weeks old). Mouse 4T1 breast cancer cells (1.0×10^5) were suspended in 0.1 mL of normal saline (NS) and injected s.c. in the right axillary fossa of BALB/c mice (Baliga *et al.*, 2005; Demaria *et al.*, 2005; Huang *et al.*, 2002; Laginha *et al.*, 2005; Li *et al.*, 1999; Torrero *et al.*, 2006). Once tumors were palpable, the mice were randomly divided into five groups (n = 8 animals/group). Treatments were given i.p. with normal saline (NS, 0.2 mL each), empty liposomes (EL) (30 mg/kg), LH at different doses of 5 mg/kg, 20 mg/kg and 50 mg/kg every day for 14 days, respectively (Chen *et al.*, 2004; He *et al.*, 2002; To *et al.*, 2004).

In the second study, 4T1 tumor-bearing BALB/c mice were established as described above. When the tumor size reached around 65 mm^3 after 11 days, the mice were pooled and randomly assigned to five groups of eight animals each: NS (0.2 mL each), EL (30 mg/kg), LH (20 mg/kg), ADR (5 mg/kg, in 0.2 mL NS), LH (20 mg/kg) plus ADR 5 mg/kg. Each group was given treatment every day for 2 weeks, except the ADR was given every week for 2 weeks. Two perpendicular diameters of tumors were measured every 2 days with a caliper square, by the same investigator. The mice were killed when they became moribund, and these dates were recorded to calculate the survival time. To detect the microvessel density and apoptosis, the excised tumor tissues were fixed in 10% formalin and frozen at −80 °C. Possible side effects such as weight loss, spirit, appetite, behavior were also observed during the treatment.

Analysis of the effects of combinations of drugs *in vivo*. The tumor volume was calculated according to the following formula: $V\ (mm^3) = d^2\ (mm^2) \times D\ (mm)/2$, where d and D are, respectively, the smallest and the largest tumor diameters. Treated animals were monitored and killed if any signs of death were observed. Mice in all groups were killed 60 days after tumor establishment. At the endpoint, tumor regression was calculated using the formula: Tumor suppression index = $(V_O - V)/V_O \times 100\%$, where V represents the mean tumor volume of the treated group, and V_O represents mean tumor volume of the control group.

In each group, the relative tumor volume was expressed as Vt/V_0 ratio where Vt is the mean tumor volume on a given day during the treatment and V_0 is the mean tumor volume at the beginning of the treatment. The expected reduced percentage of tumor volume (Rptv) of the combined treatment was calculated according to the following modified Jin's formula (Jin, 1980):

$$\text{Expected reduced percentage of tumor volume} = \text{drug A} + (1 - \text{drug A}) \times \text{drug B or drug B} + (1 - \text{drug B}) \times \text{drug A}$$

The combination index (CI) was assessed by the ratio of the actual Rptv divided by that of expected Rptv. If the CI was <0.85, the combination was antagonistic; >1.15 the combination was synergistic; and $0.85 \geq CI \leq 1.15$, the combination was additive.

Immunohistochemistry. The antiangiogenesis effects of LH plus ADR were determined by CD31 immunostaining. Tissue samples were cut into 4 mm slices and immersed in icy acetone. The frozen sections were probed with a monoclonal rat anti-mouse CD31 antibody (1:400) at 4 °C overnight, followed by incubation with biotinylated polyclonal goat anti-rat antibody (1:200) in a humidified chamber for 1 h. Positive reaction was visualized using 3,3-diaminobenzidine as chromagen (DAB substrate kit). Sections were counterstained with hematoxylin and mounted with glass coverslips. Then tissue sections were visualized in an Olympus microscope at 20 magnifications to determine the microvessel density (MVD) (Vermeulen *et al.*, 1996; Wei *et al.*, 2001).

TUNEL assay. Apoptosis cells were identified by the fluorescent *in situ* terminal deoxynucleotidyltransferase-mediated nick end-labeling (TUNEL) assay (*In Situ* Cell Death Detection Kit; Roche) according to the manufacturer's recommendations (Xiao *et al.*, 2002). Images were captured by fluorescence microscope at an excitation wavelength of 488 nm. The apoptosis cells were counted from five areas in each section in a blinded manner to determine the apoptosis rate. The apoptosis index = the number of apoptotic cells/total cells × 100%.

Statistical analysis. All statistical tests were performed using the SPSS 13.0 software. Statistical comparisons were made with one-factor analysis of variance (ANOVA) with the Student–Newmann–Keuls (SNK) test used for post hoc comparisons. For the survival time of animals, Kaplan–Meier curves were established for each group, and the survivals were compared by means of the log rank test. Values of $p < 0.05$ were considered significant. The results are presented as mean ± SD. Experiments were performed at least in duplicate.

RESULTS

Combined effects of liposomal honokiol and adriamycin on the growth of 4T1 cells *in vitro*

To evaluate the most sensitive cell line towards LH, dose 12 μg/mL was chosen to treat with breast cancer cell lines 4T1, MCF-7 and Bcap-37, respectively. 4T1 cell line was found more sensitive towards LH treatment.

Liposomal honokiol increased the effectiveness of ADR in a dose and time-dependent manner (Fig. 1A, 1B). After the cells were treated for 48 h, either ADR or LH produced moderate cytotoxicity in 4T1 cells. The concentrations leading to a 50% decrease after 48 h in cell number (IC_{50}) were about 17.65 ± 0.32 μg/mL for LH and 0.78 ± 0.08 μg/mL for ADR. However, this combination of LH and ADR was significantly enhanced and caused 0.5-fold, 3-fold, 15-fold decreases in the IC_{50} of ADR after 10 μg/mL, 12 μg/mL and 14 μg/mL LH was added. The data indicated that LH had synergistic effects (CI < 1) on antiproliferation of ADR in the mouse breast cancer cell line 4T1 by isobologram analysis. The CI had the lowest value when ADR

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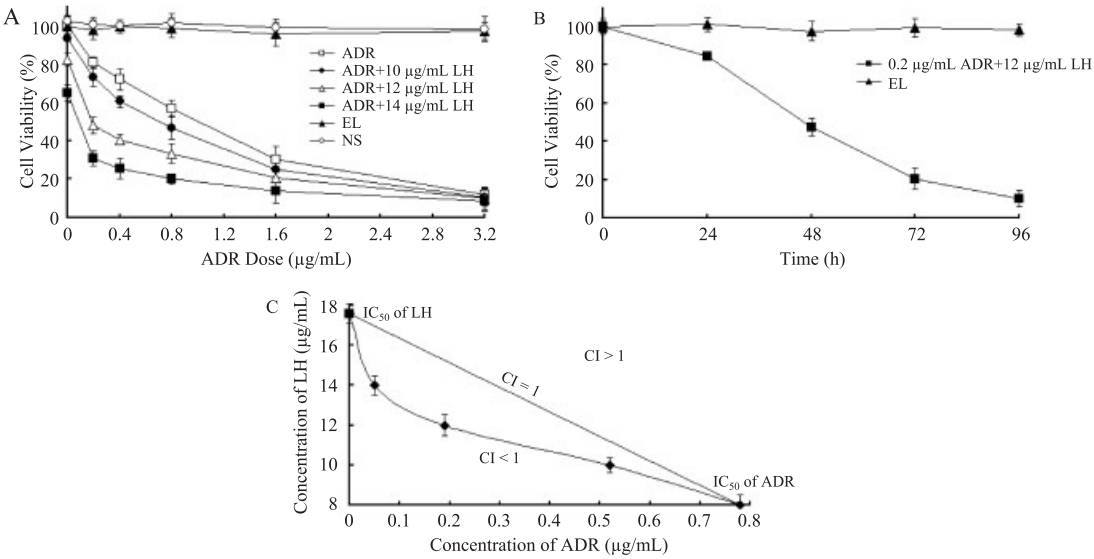

Figure 1. Combined effect of LH and ADR on the proliferation of mouse breast cancer cell line 4T1. (A) Dose-dependent inhibition of cell growth: 4T1 cells were treated with ADR at different concentrations (□), LH (10 μg/mL ●, 12 μg/mL, 14 μg/mL ■) plus ADR at different concentrations, EL (▲) and NS (□). Cell viability experiments were performed after 48 h of exposure to the compounds. (B) Time-dependent inhibition of cell growth: 4T1 cells were treated with 0.2 μg/mL ADR + 12 μg/mL LH (■) and EL (▲) for various time intervals. (C) Interaction of the combination treatment: Isoboles for the combination of LH with ADR in 4T1 cells that were isoeffective (IC_{50}) for inhibition of the growth of 4T1 cells. The dashed line indicates the zero interaction of the isobole. Cells were treated with LH and ADR for 48 h. During the antitumor assays, the EL concentration was always the same as LH. Results are expressed as mean ± SD for four separate experiments.

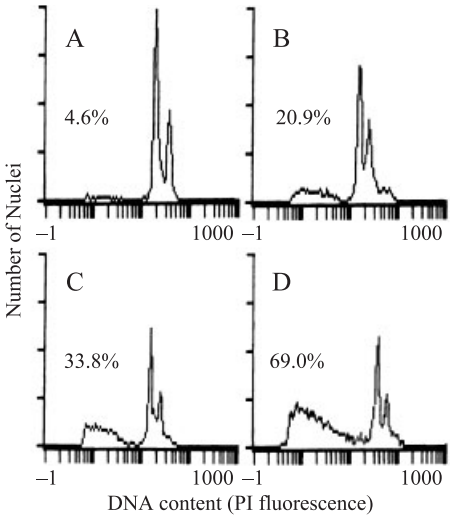

Figure 2. Flow cytometry histograms for 4T1 cancer cells exposed to control (A), 0.2 μg/mL ADR (B), 14 μg/mL LH (C) and 14 μg/mL LH plus 0.8 μg/mL ADR (D) for 48 h. The cells in sub-G_1 phase were considered as apoptotic cells. The apoptosis rates in nontreated and drug-treated cells were 4.6% (A), 20.9% (B), 33.8% (C), 69.0% (D) as assessed by flow cytometry.

(0.2 μg/mL) was combined with LH (14 μg/mL). This might suggest that a low dose of ADR combined with LH could generate more synergistic effects than a high dose.

The rates of cell apoptosis were assessed by flow cytometry. The 4T1 cells were treated in the control group (Fig. 2A), ADR group (Fig. 2B), LH group (Fig. 2C) and ADR plus LH group (Fig. 2D) for 48 h, respectively. The combination treatment resulted in 69% apoptosis which is more than 3-fold that of ADR and 2-fold that of LH alone. This apoptosis with the combination of the two agents is consistent with the definition of synergy.

Effect of the combined treatment with liposomal honokiol plus adriamycin on tumor growth *in vivo*

In the first test, 4T1-bearing BABL/c mice were treated with LH at different doses every day for 2 weeks. The 5 mg/kg doses of LH had almost no significant inhibition response on the tumor ($p > 0.05$). Both the 25 and 50 mg/kg doses of LH could significantly suppress the tumor growth, and they almost have no difference in the effect on tumor growth ($p > 0.05$). There was no difference between the NS and EL control ($p > 0.05$). Therefore, a dose of 20 mg/kg was selected as an effective dose for combination treatment.

In the second test, the tumor volume was measured every 3 days. Figure 3A indicates that the combination group had more effective suppression of tumor growth while either LH or ADR exhibited moderate antitumor efficiency. After 26 days, the average tumor volumes in mice treated with NS, EL, LH, ADR and combined therapy were respectively 1165.82 ± 383.17 mm^3, 1116.57 ± 256.64 mm^3, 605.98 ± 121.51 mm^3, 539.99 ± 128.29 mm^3 and 150.62 ± 36.42 mm^3 ($p < 0.05$). The antitumor effects of this combined therapy of LH and ADR showed that LH could synergistically (CI > 1.15) enhance the antitumor efficacy of ADR from day 26 to day 32 (Fig. 3B). On day 29, the CI reached a maximal value of 1.32, indicating that these enhancements are more effective at a moderate treatment period.

The results illustrated that the combination treatment resulted in a 9 day delay of tumor growth to reach a volume of 1000 mm^3 compared with the NS and EL control in 4T1 tumor-bearing mice. The life span of mice showed that the control animals receiving NS, EL, LH or ADR treatment survived 41, 41, 43, 43 days on average, respectively ($p < 0.05$). In contrast, the combination group survived 51 days. At 60 days after treatment, the survival of LH plus ADR was still 75% (Fig. 3C).

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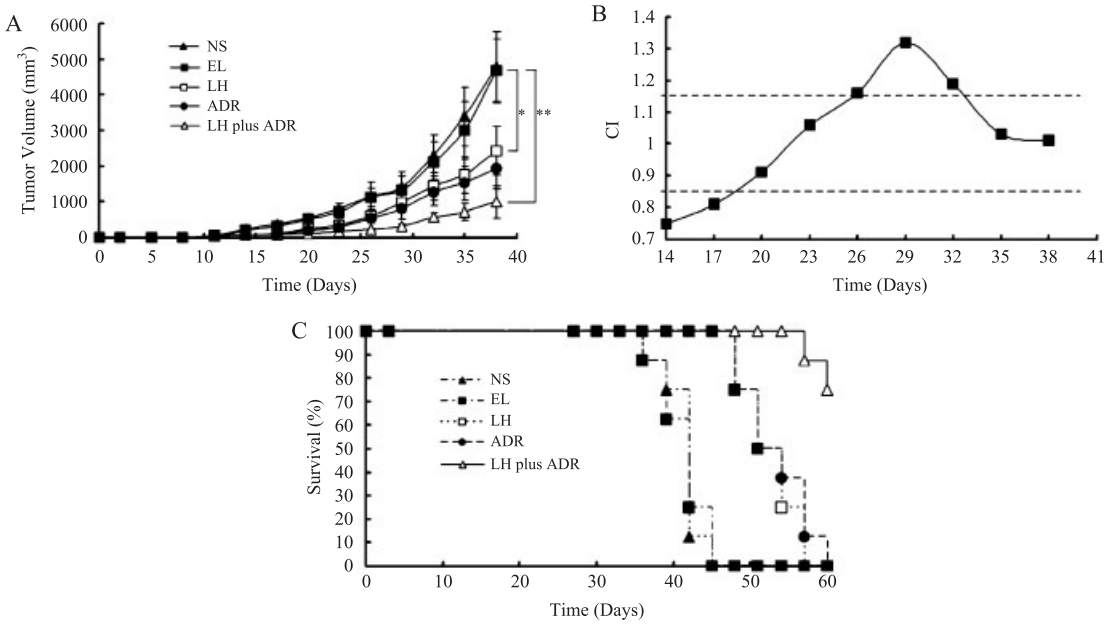

Figure 3. Antitumor effects of LH plus ADR in BALB/c mice bearing 4T1 models. Mice were i.p. injected with respectively 0.2 mL NS (▲), 30 mg/kg EL (■), 20 mg/kg LH (□), 5 mg/kg ADR (●) and 5 mg/kg ADR plus 20 mg/mL LH (□) every 3 days. The results are expressed as the mean ± SD of eight mice. (A) Reduction of tumor volume in mice: No difference was discovered between NS and EL control ($p > 0.05$). * $p < 0.05$; ** $p < 0.001$. (B) The interaction of combined therapy: From 26 day to 30 day, this treatment showed a synergistic effect. (C) A delay of the survival time: Combination therapy also significantly increased the survival time compared with the control ($p < 0.05$).

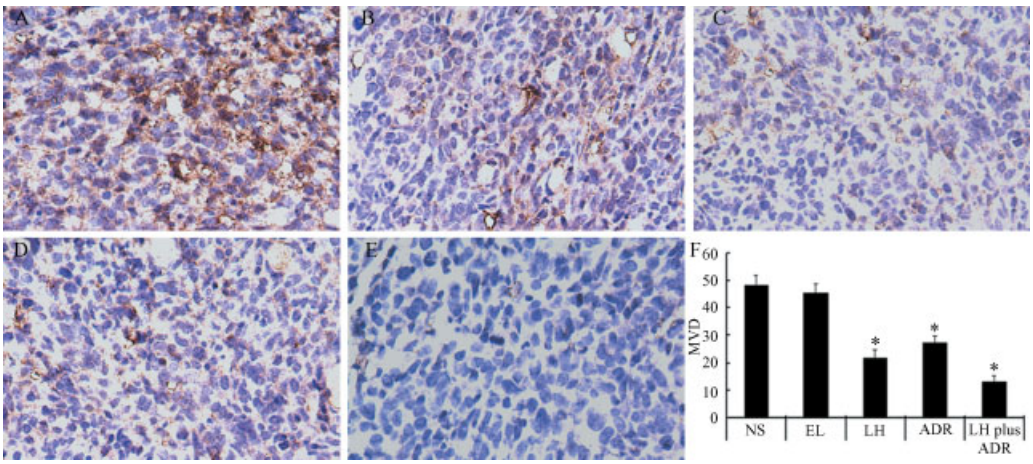

Figure 4. Tumor angiogenesis was assessed by immunohistochemical staining with anti-CD31 antibody on frozen tumor tissue sections. Microvessel counting was performed at 200 ×. Significantly reduced numbers of blood vessels in tumors treated with combination (E) in comparison with ADR (D) or LH alone (C). No significant difference between NS (A) and EL (B) groups ($p < 0.05$) was observed. (F) shows the influence of combination therapy on MVD. Data represent the mean ± SD of microvessels per high-power field. * $p < 0.05$ vs control (NS or EL). ADR or LH were i.p. administered every day for 2 weeks. The extent of inducing apoptosis was more severe than that of any other treatment ($p < 0.05$).

Liposomal honokiol inhibited angiogenesis and induced apoptosis by combination with adriamycin

Microvessel density (MVD) was quantified to measure angiogenesis by immunolabeling of CD31 in frozen tumor tissue sections. The microvessel density in mice treated with ADR, LH, or LH plus ADR was, respectively, 21.60 ± 3.05 (Fig. 4C), 27.60 ± 2.19 (Fig. 4D), 13.20 ± 2.22 (Fig. 4E) ($p < 0.05$) while the NS (Fig. 4A) group and EL (Fig. 4B) group were relatively higher ($p > 0.05$). The microvessel density was significantly reduced in tumors treated with the combination in comparison with LH alone or ADR alone ($p < 0.05$). The observations suggest that the combination LH with ADR may inhibit tumor angiogenesis to a significant extent (Fig. 4H).

Increase of apoptosis (TUNEL)

Histological sections of tumors from each group were stained with a TUNEL reagent (Fig. 5) kit and detected by immunofluorescence microscopy to determine the apoptosis rate. The data showed no significant differences in the apoptosis count between 0.2 mL NS and 30 mg/kg EL groups, while more apoptotic cells were observed in those from the 5 mg/kg ADR or 20 mg/kg dose LH administered alone. Furthermore, tumors from

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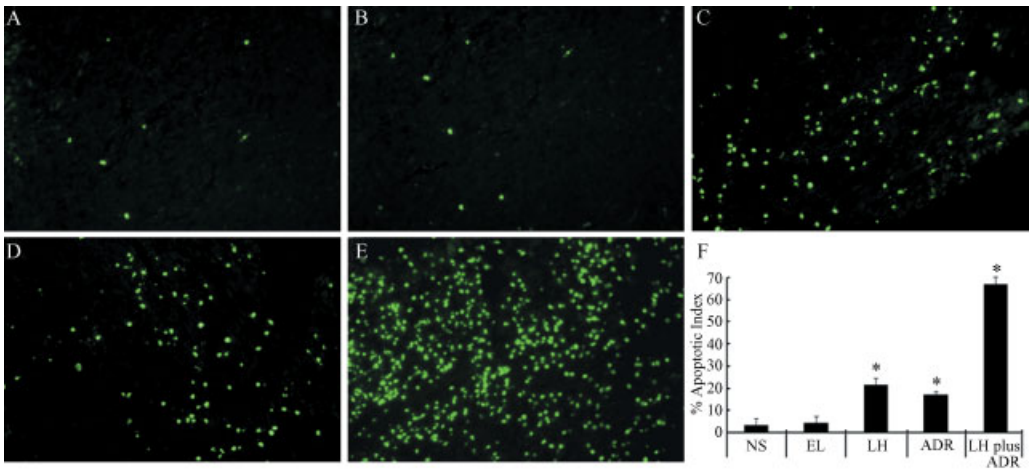

Figure 5. TUNEL assay for apoptotic cells. Single LH (C) or ADR (D) treatment sections revealed a little necrosis, which was much less than the combination of ADR plus LH treatment section (E). There was no obvious difference between NS (A) and EL (B) groups which had little necrosis ($p > 0.05$). (F) shows apoptotic index within tissue from 4T1.

animals receiving 20 mg/kg LH plus 5 mg/kg ADR showed the highest apoptotic rate compared with the control group. Figure 5 shows the synergistic tumor apoptosis effect of LH combined with ADR.

Toxicity assays

The classic toxic side effects of ADR therapy are cardiovascular, gastrointestinal, hematologic and neurological (Hogberg *et al.*, 2001; Kimby *et al.*, 2001; Zhou, 2000). All groups' mice maintained normal activity, without gross signs of cumulative adverse results, such as weight loss, ruffling of fur, behavioral and postural changes. Furthermore, no pathological changes in heart, lung, liver, spleen or kidney were found by microscopic examination.

DISCUSSION

Cytotoxic chemotherapy has been one of the mainstays in the treatment of solid cancers. Although ADR is the first line drug in combating the growth and spread of tumors, the dose-dependent toxicity and the development of resistance during treatment have become obstacles to the cure of most solid tumors, thus the cancer mortality rate remains staggering (Hvizdala *et al.*, 1976; Nguyen *et al.*, 1992). Antiangiogenesis, for instance, is a promising approach for the treatment of breast cancer. Folkman and others thoroughly demonstrated that the rationale of why tumor expansion is stalled in a quiescent state, is that the tumor growth and metastasis are inhibited by reducing angiogenesis which is caused by decreasing the essential nutrients and oxygen (Ellis *et al.*, 2001; Folkman, 1990; Nguyen *et al.*, 1992, 1994). As the most abundant and active component of magnoliae cortex, honokiol has also been shown to have antiangiogenic property. Recent studies have shown that treatment with honokiol could exhibit an inhibitory effect on several cancer cells *in vitro* and a variety of murine and human tumors *in vivo* (Bai *et al.*, 2003; Battle *et al.*, 2005; Fong *et al.*, 2005; Hirano *et al.*, 1994; Nagase *et al.*, 2001; Wang *et al.*, 2004; Yang *et al.*, 2002). It has also been demonstrated that honokiol could enhance ADR-cytotoxicity by the down-regulation of P-gp in the MCF-7/ADR cell line (Xu *et al.*, 2006). Therefore, the objectives of the present study were to determine whether LH could enhance apoptosis induced by ADR and to evaluate the therapeutic efficacy of a combination with LH and ADR on tumor growth in breast cancer models.

The present study, to our best knowledge, for the first time demonstrated that LH potentiated the therapeutic index of ADR in the treatment of the 4T1 tumor bearing mice model. The combination treatment of LH plus ADR resulted in synergistic and significant antitumor activity *in vitro* and *in vivo*. This conclusion is based on the following observations. First, combination therapy displayed a dose and time-dependent inhibition of 4T1 cell proliferation, and significantly decreased the IC_{50} of ADR when combined with LH. The isobologram analysis indicated that this combination therapy was synergistic even at a low dose of ADR (Fig. 1C). The breast 4T1 cancer cells treated with LH plus ADR obviously showed growth arrest of the cells at the G1 phase of propidium iodide (PI)-stained nuclei analysed by flow cytometry. Second, the combination therapy substantially reduced tumor growth and caused prolonged tumor survival in a synergistic manner from the period of day 26 to day 32 *in vivo* (Fig. 3B). This result might suggest that this is the most effective therapeutic time, while the effects during other periods showed antagonism or were additive. Even when the combined treatment was continued for 14 days and then stopped, 75% mice were survived to day 60 compared with none surviving in the other treatment group. Furthermore, the synergistic effect of combination treatment resulted in reduction of tumor vascular (Fig. 4) which might contribute to enhanced tumor regression, and enhancement of apoptosis of 4T1 cells (Fig. 5) compared with either treatment alone. The continuous administration of LH inhibits the process of reparation of tumor vessels and enhances antitumor efficacy during combination therapy. Throughout the study, it was observed that combination treatment was well tolerated, and no obvious undesired toxicity such as loss of weight was observed throughout the study. Taken together, all these results suggest that the combination therapy of LH plus

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ADR has a synergistic and significant antitumor effect in the breast cancer model, and may have therapeutic value for the treatment of advanced breast cancer.

CONCLUSION

In summary, the present data indicate that the combination of LH with ADR could increase the therapeutic effectiveness of each agent. The study showed that tumor growth was inhibited to a greater extent by combined treatment with LH plus ADR, resulting in a synergistic interaction for all concentrations tested *in vitro* and a moderate treatment time *in vivo*. Moreover, the combined treatment with LH and ADR significantly inhibited the growth of 4T1 cells as xenografts without apparent adverse effects, providing a promising approach in breast cancer therapy.

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REFERENCES

Bai XH, Cerimele F, Ushio-Fukai M, Waqas M, Campbell PM, Govindarajan B. 2003. Honokiol, a small molecular weight natural product, inhibits angiogenesis *in vitro* and tumor growth *in vivo*. *J Biol Chem* **278**: 35501–35507.

Baliga MS, Meleth S, Katiyar SK. 2005. Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells *in vitro* and *in vivo* systems. *Clin Cancer Res* **11**: 1918–1927.

Battle TE, Arbiser J, Frank DA. 2005. The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells. *Blood* **106**: 690–697.

Chen F, Wang T, Wu YF *et al.* 2004. Honokiol, a potent chemotherapy candidate for human colorectal carcinoma. *World J Gastroenterol* **10**: 3459–3463.

Chen LJ, Zhang Q, Yang GL *et al.* 2007. Rapid purification and scale-up of honokiol and magnolol using high performance countercurrent chromatography (HPCCC). *J Chromatogr A* **1142**: 115–122.

Chou TC, Talalay P. 1984. Quantitative analysis of dose-effect relationship: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**: 27–55.

Cutts SM, Parsons PG, Sturm RA, Phillips DR. 1996. Adriamycin-induced DNA adducts inhibit the DNA interactions of transcription factors and RNA polymerase. *J Biol Chem* **271**: 5422–5429.

Demaria S, Kawashima N, Yang AM *et al.* 2005. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. *Clin Cancer Res* **11**: 728–734.

Eichhorn ME, Kleespies A, Angele MK, Jauch KW, Bruns CJ. 2007. Angiogenesis in cancer: molecular mechanisms, clinical impact. *Langenbecks Arch Surg* **392**: 371–379.

Ellis LM, Liu W, Ahmad SA, Fan F, Jung YD, Shaheen RM. 2001. Overview of angiogenesis: biologic implications for antiangiogenic therapy. *Semin Oncol* **28**: 94–104.

Feleszko W, Mlynarczuk I, Balkowiec-Iskra EZ *et al.* 2002. Lovastatin potentiates antitumor activity of doxorubicin in murine melanoma via an apoptosis-dependent mechanism. *Int J Cancer* **100**: 111–118.

Fioretti F, Tavani A, Bosetti C *et al.* 1999. Risk factors for breast cancer in nulliparous women. *Br J Cancer* **79**: 1923–1928.

Folkman J. 1990. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Institute* **82**: 4.

Fong WF, Tse AK, Poon KH, Wang C. 2005. Magnolol and honokiol enhance HL-60 human leukemia cell differentiation induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Int J Biochem Cell Biol* **37**: 427–441.

Gao BB, Xu ZF. 2006. Advances in anti-tumor angiogenesis and combination with radiotherapy or chemotherapy. *Chin J Cancer Prevent Treat* **13**: 235–238.

He W, Qin XJ, Hai CX *et al.* 2002. Adriamycin's acute toxicity in mice through celiac injection and its effect on the peripheral blood. *J Fourth Mil Med Univ* **23**: 667–669.

Hequet O, Le QH, Moullet I *et al.* 2004. Subclinical late cardiomyopathy after doxorubicin therapy for lymphoma in adults. *J Clin Oncol* **22**: 1864–1871.

Hirano T, Gotoh M, Oka K. 1994. Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. *Life Sci* **55**: 1061–1069.

Hogberg T, Glimelius B, Nygren P. 2001. A systematic overview of chemotherapy effects in ovarian cancer. *Acta Oncol* **40**: 340–360.

Huang XJ, Wong MK, Yi HM *et al.* 2002. Combined therapy of local and metastatic 4T1 breast tumor in mice using SU6668, an inhibitor of angiogenic receptor tyrosine kinases, and the immunostimulator B7.2-IgG fusion protein1. *Cancer Res* **62**: 5727–5735.

Hvizdala EV, Komp DM, Arnold JB. 1976. Low-dose adriamycin remission maintenance therapy for pediatric solid tumors. *Cancer* **39**: 2411–2414.

Jemal A, Murray T, Ward E *et al.* 2005. Cancer statistics 2005. *CA Cancer J Clin* **55**: 10–30.

Jin ZJ. 1980. Addition in drug combination. *Acta Pharmacol Sin* **1**: 70.

Kimby E, Brandt L, Nygren P, Glimelius B. 2001. A systematic overview of chemotherapy effects in B-cell chronic lymphocytic leukaemia. *Acta Oncol* **40**: 224–230.

Konoshima T, Kozuka M, Tokuda H *et al.* 1991. Studies on inhibitors of skin tumor promotion, IX: neolignans from *Magnolia officinalis*. *J Nat Prod* **54**: 816–822.

Laginha KM, Verwoert S, Charrois GJ, Allen TM. 2005. Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. *Clin Cancer Res* **11**: 6944–6949.

Li Y, Santiago A, O'Connor W *et al.* 1999. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res* **59**: 5209–5218.

Nagase H, Ikeda K, Sakai Y. 2001. Inhibitory effect of magnolol and honokiol from *Magnolia obovata* on human fibrosarcoma HT-1080. Invasiveness *in vitro*. *Planta Med* **67**: 705–708.

Nguyen HN, Sevin BU, Averette H *et al.* 1992. Comparative evaluation of pirarubicin and adriamycin in gynecologic cancer cell lines. *Gynecol Oncol* **45**: 164–173.

Nguyen M, Shing Y, Folkman J. 1994. Quantitation of angiogenesis and anti-angiogenesis in the chick embryo chorioallantoic membrane. *Microvasc Res* **47**: 31.

Qiu M, Yi C, Hou M. 2006. Combined low-dose chemotherapy inhibiting angiogenesis and growth of Lewis lung carcinoma xenografts in mice. *J Sichuan Univ* **37**: 534–537.

Reimer CL, Agata N, Tammam JG *et al.* 2002. Antineoplastic effects of chemotherapeutic agents are potentiated by NM-3, an inhibitor of angiogenesis. *Cancer Res* **62**: 789–795.

Safrit JT, Bonavida B. 1992. Sensitivity of resistant human tumor cell lines to tumor necrosis factor and adriamycin used in combination: correlation between down-regulation of tumor necrosis factor-messenger RNA induction and overcoming resistance. *Cancer Res* **52**: 6630–6637.

Tanaka M, Yoshisa S. 1980. Mechanism of the inhibition of calf thymus DNA polymerase α and β by daunomycin and adriamycin. *J Biochem* **87**: 911–918.

Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF. 1984. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226**: 466–468.

To H, Shin M, Tabuchi M *et al.* 2004. Influence of dosing schedule on toxicity and antitumor effects of a combination of adriamycin and docetaxel in mice. *Clin Cancer Res* **10**: 762–769.

Torrero MN, Henk WG, Li SL. 2006. Regression of high-grade malignancy in mice by bleomycin and interleukin-12 electrochemo-gene therapy. *Clin Cancer Res* **12**: 257–263.

Vermeulen PB, Gasparini G, Fox SB *et al.* 1996. Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. *Eur J Cancer* **32**: 2474–2484.

Wang T, Chen F, Chen Z *et al.* 2004. Honokiol induces apoptosis through p53-independent pathway in human colorectal cell line RKO. *World J Gastroenterol* **10**: 2205–2208.

Wei YQ, Huang MJ, Yang L *et al.* 2001. Immunogene therapy of tumors with vaccine based on *Xenopus homologous* vascular endothelial growth factor as a model antigen. *Proc Natl Acad Sci USA* **98**: 11545–11550.

Xiao F, Wei YQ, Yang L *et al.* 2002. A gene therapy for cancer based on the angiogenesis inhibitor, vasostatin. *Gene Ther* **9**: 1207–1213.

Xu D, Lu QH, Hu X. 2006. Down-regulation of P-glycoprotein expression in MDR breast cancer cell MCF-7/ADR by honokiol. *Cancer Lett* **243**: 274–280.

Xu TM, Xin Y, Cui MH, Jiang X, Gu LP. 2007. Inhibitory effect of ginsenoside Rg3 combined with cyclophosphamide on growth and angiogenesis of ovarian cancer. *Chin Med J* **120**: 584–588.

Yang SE, Hsieh MT, Tsai TH, Hsu SL. 2002. Down-modulation of Bcl-XL, release of cytochrome c and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells. *Biochem Pharmacol* **63**: 1641–1651.

Zhou JC. 2003. *Practical Tumor Medicine.* 2nd Edition. Beijing People's Medical Publishing House.

Zhu K, Henning D, Iwakuma T, Valdez BC, Busch H. 1999. Adriamycin inhibits human RH II/Gu RNA helicase activity by binding to its substrate. *Biochem Biophys Res Commun* **266**: 361–365.