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Original Article

Honokiol augments the anti-cancer effects of oxaliplatin in colon cancer cells

Hanju Hua[1], Wenbin Chen[1], Ling Shen[2], Qinsong Sheng[1], and Lisong Teng[3]*

[1]Department of Colorectal Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China
[2]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China
[3]Department of Oncological Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China
*Correspondence address. Tel: +86-571-87236878; Fax: +86-571-87236734; E-mail: tomorrow97@163.com

Oxaliplatin is an important drug in the chemotherapy of colorectal carcinoma, but its toxicity, especially dose-related neurosensory toxicity, is not well tolerated. In this study, we investigated whether honokiol could augment the anti-tumor effect of oxaliplatin in colon cancer HT-29 cells *in vitro* and whether honokiol could be used with oxaliplatin to decrease oxaliplatin dose. We used the normal colon cells, human colonic epithelial cells (HCoEpiCs) as control cells. Cell proliferation, apoptosis, prostaglandin E2 (PGE_2) and vascular endothelial growth factor (VEGF) levels were also investigated. Expression levels of cyclo-oxygenase 2 (COX-2), VEGF, AKT/p-AKT, extracellular signal-related kinase (ERK)1/2/p-ERK1/2, nuclear factor kappa B (NF-κB) P65/p-P65, and caspase-3 were measured. Honokiol or oxaliplatin suppressed the proliferation of HT-29 cells in a concentration-dependent manner, but only high concentrations of honokiol would suppress the proliferation of HCoEpiCs. HT-29 cells were more sensitive to oxaliplatin treatment in the presence of honokiol. Oxaliplatin combined with honokiol improved the apoptosis rate of HT-29 cell and reduced PGE_2 and VEGF secretion levels. Expression levels of COX-2 and VEGF protein and phosphorylation of AKT, ERK1/2, and NF-κB P65 were also inhibited. Caspase-3 levels were upregulated after honokiol treatment. Therefore, honokiol can be used in combination with oxaliplatin in the chemotherapy of colon cancer. This combination allows a reduction in oxaliplatin dose, and thereby reduces its adverse effects. It may also enhance the chemotherapeutic effect of oxaliplatin for this disease.

Keywords honokiol; colorectal cancer; apoptosis; oxaliplatin; nuclear factor-κB

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Introduction

Oxaliplatin is a platinum-based chemotherapeutic agent with a 1,2-diaminocyclohexane carrier ligand that produces bulkier DNA conjugates due to the restricted freedom of motion of the platinum atom. Oxaliplatin plays a very important role in the chemotherapy of colorectal and ovarian cancer [1]. FOLFOX (folinic acid/fluorouracil/oxaliplatin) or XELOX (capecitabine/oxaliplatin) is used as the first-line chemotherapy regimen in advanced colorectal cancer. Oxaliplatin combined with fluorouracil (5-Fu) can markedly improve the 5-year survival rates of colorectal cancer patients, but oxaliplatin toxicity, especially its dose-related neurotoxicity [2,3], is not well tolerated by most patients. Drug resistance to oxaliplatin is also a problem in chemotherapy. Therefore, finding the right dosage scheme and strategy for each individual patient that minimizes side effects remains a chemotherapeutic challenge. Meanwhile, there is an urgent need for new drugs that can efficiently augment the anti-tumor effect of oxaliplatin and enable a reduction in its dose.

Honokiol is an active component that has been isolated and purified from the Chinese traditional herb magnolia. It has been shown to have anti-angiogenic, anti-invasive, and anti-proliferative activities in several types of human cancer cells [4], which include leukemia [5,6], human breast cancer cells [7,8], human hepatic cells [4], human multiple myeloma [9], human prostate cancer cells [10], and human squamous lung cancer cells [11]. In this study, we investigated the effect of honokiol, either alone or in combination with oxaliplatin, on the proliferation and apoptosis of the human colon cancer cell line HT-29. We also investigated the expression levels of several downstream molecules to explore the mechanism by which honokiol may induce cell apoptosis.

Materials and Methods

Reagents

Oxaliplatin was obtained from the Sanofi-Aventis Pharmaceutical Co. Ltd (Paris, France). Honokiol (purity, 98.7%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing,

China). The CellTiter 96 AQueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, USA). Anti-VEGF and anti-cyclo-oxygenase-2 (COX-2) antibodies used in western blot analysis were obtained from Santa Cruz (Santa Cruz, USA). Anti-AKT/phospho-AKT, extracellular signal-related kinase (ERK)1/2/phospho-ERK1/2, nuclear factor (NF)-κB P65/p-P65, caspase-3, and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibodies were obtained from Cell Signalling (Beverly, USA). All other chemicals were of reagent grade and obtained from Sigma (St Louis, USA).

Cell culture and treatment

HT-29 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). Human colonic epithelial cells (HCoEpiCs) were purchased from ScienCell Research Laboratories (Carlsbad, USA). Cells were passaged three to five times and used throughout the study. HT-29 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, USA), 5.6 mM glucose, glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C under 5% CO_2 in air. HCoEpiCs were cultured in colonic epithelial cell medium. HT-29 cells were plated at 1×10^4 cells/well in 96-well plate. Cells were treated with either honokiol at various concentrations (0, 0.25, 0.5, 1, 2, 5, 10, 20, 50, or 100 μM) or oxaliplatin (0, 0.07, 0.15, 0.3, 0.6, 1.25, 2.5, 5, or 10 μM) alone, or with oxaliplatin (0.6 μM) plus honokiol (0.2, 1, 5, or 20 μM) for 48 h respectively. HCoEpiCs were treated with honokiol at various concentrations as controls. Cell viability was evaluated by the 3,4-(5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay. MTS and an electron coupling reagent, phenazine methosulfate, were used in the MTS assay. The reduction of MTS to formazan, measured as absorbance at 490 nm using a spectrophotometer, was used to estimate the number of viable cells. Three duplicate experiments were performed for each experimental condition.

Annexin V/propidium iodide apoptosis assay

Cell apoptosis was measured using an annexin V-FITC apoptosis detection kit (BD Pharmingen, Franklin Lakes, USA). Briefly, HT-29 cells and HCoEpiCs were removed from the culture dish, stained with annexin V-FITC and propidium iodide (PI), and then analysed by flow cytometry (FACSCalibur; BD Pharmingen) after treatment. Cells that were annexin V-FITC and PI double-negative were considered to be non-apoptotic. The cells stained with Annexin V were considered apoptotic.

Analysis for PGE_2 and VEGF production

The concentrations of PGE2 and VEGF in culture supernatants were determined using a competitive enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA). In brief, 100 μl of culture supernatants or standards were added to each well coated with the specific antibodies (Abs) followed by incubation with biotinylated detection Abs. Streptavidin-HRP binding to biotinylated detection Abs was visualized with TMB substrate. Absorption was measured at 450 nm by a Model 680 microplate reader (Bio-Rad, Hercules, USA). The sample concentrations of VEGF or PGE_2 were calculated according to the standard curve.

Western blot analysis

Cells were treated with honokiol or oxaliplatin alone or combination of both, then harvested and washed three times with ice-cold phosphate-buffered saline (PBS). Cell lysates were prepared for western blot analysis of VEGF, COX-2, and GAPDH using whole cellular protein extraction kits (Active Motif, Carlsbad, CA, USA). A DC protein assay kit was used (Bio-Rad) to examine the protein concentration in each cell lysate; 40 μg protein was mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis sample loading buffer and denatured for 10 min at 95°C. Proteins were separated on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane (Bio-Rad). Nitrocellulose membranes were blocked with 5% bovine serum albumin (Sigma) in Tris-buffered saline (25 mM Tris-HCl, 150 mM sodium chloride, pH 7.2) for 2 h at room temperature. Membranes were incubated with rabbit polyclonal immunoglobulin G (IgG) primary antibody overnight at 4°C. Then membranes were washed three times with washing buffer (PBS with 0.1% Tween-20) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1 : 2000 dilution) for 2 h at room temperature. The blot was developed using enhanced chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, UK) and analyzed using a VersaDoc MP5000 imaging system (Bio-Rad).

Statistical analysis

The results were expressed as the mean value ± standard error of the mean. All experiments were performed in triplicates. Statistical significance was analysed by one-way analysis of variance. A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of honokiol and oxaliplatin on inhibition of HT-29 cell proliferation

MTS assay was used to detect HT-29 cell viability after treatment with different concentrations of honokiol or

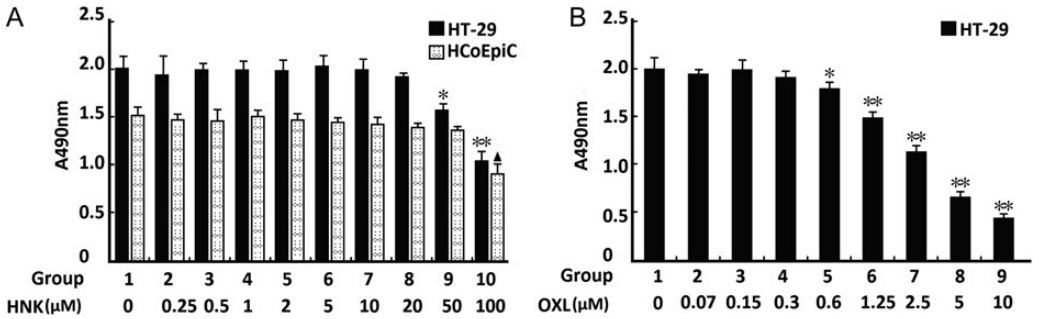

Figure 1 MTS assay to detect HT-29 cell and HCoEpiC viability Absorbance at 490 nm of cell cultures treated with different concentrations of (A) honokiol (HNK) or (B) oxaliplatin (OXL) alone. *$P < 0.05$ vs normal control (0 μM) in HT-29 cells, **$P < 0.01$ vs normal control (0 μM) in HT-29 cells. ▲$P < 0.05$ vs normal control (0 μM) in HCoEpiCs. Data were from three independent experiments (mean ± SEM).

oxaliplatin. The inhibitory effect of either honokiol or oxaliplatin on cell viability is in a concentration-dependent manner (**Fig. 1**). A concentration of 20 μM for honokiol was the maximum concentration that did not affect HT-29 cell proliferation and for oxaliplatin, 0.6 μM was the minimum effective concentration. The LC_{10} of honokiol was about 20 μM. Only a high concentration (100 μM) of honokiol could suppress the HCoEpiC proliferation. The anti-proliferation capability of oxaliplatin was significantly enhanced when honokiol was also added (**Fig. 2**). Results showed that HT-29 cells were more sensitive to the combined treatment than treatment with single reagent alone. The addition of 20 μM honokiol increased markedly the anti-proliferation effect of low concentrations of oxaliplatin, while having no effect on HCoEpiCs.

Effect of honokiol and oxaliplatin on induction of HT-29 cell apoptosis *in vitro*

A range of concentrations of honokiol (0, 0.2, 1, 5, 20 μM) combined with 0.6 μM oxaliplatin were used. The percentage of apoptotic HT-29 cells increased significantly when honokiol was added (**Fig. 3**). For example, the addition of 20 μM honokiol increased the rate of apoptotic cells from 11.46% to 50.43%, when compared with the control. Therefore, there was a significant synergistic effect following honokiol and oxaliplatin treatment. The induction of cell apoptosis was more effective at a lower concentration of oxaliplatin in the presence of honokiol. However, 20 μM honokiol had no effect on the apoptosis of HCoEpiCs.

PGE_2 and VEGF production in culture supernatants

The levels of PGE_2 and VEGF in cell culture supernatants were examined by competitive ELISA after treatment with honokiol or oxaliplatin alone or combination of both. When the honokiol concentration was higher than 1 μM, the production of PGE_2 and VEGF (**Fig. 4**) was reduced in a concentration-dependent manner. At concentrations above 5 μM, honokiol had a significant suppressive effect compared with the control group, which was independent of the

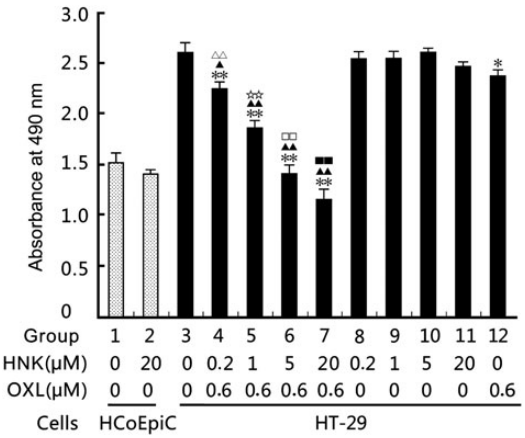

Figure 2 MTS assay to detect the combination effect of honokiol and oxaliplatin Absorbance at 490 nm of HT-29 cell cultures treated with honokiol (HNK) in combination with oxaliplatin (OXL) and HCoEpiC cultures treated with HNK. HNK combined with OXL can significantly improve the suppression of cell proliferation compared with the control group and with the OXL alone treated group. Low concentrations of HNK had no effect on HCoEpiCs. **$P < 0.01$ vs group 3, *$P < 0.05$ vs group 3. ▲▲$P < 0.01$ vs group 12. ▲$P < 0.05$ vs group 12, △△$P < 0.01$ vs group 8, ☆☆$P < 0.01$ vs group 9, □□$P < 0.01$ vs group 10, ■■$P < 0.01$ vs group 11. Data are from three independent experiments (mean ± SEM).

addition of oxaliplatin ($P < 0.01$). There was a synergistic suppressive effect between oxaliplatin and honokiol when honokiol concentrations were between 1 and 5 μM ($P < 0.05$). Honokiol (20 μM) could also reduce the production of PGE_2 in HCoEpiCs ($P < 0.05$).

Possible mechanisms of honokiol to induce HT-29 cell apoptosis

We found that honokiol, in combination with low concentrations of oxaliplatin (0.6 μM), suppressed HT-29 cell proliferation and induced apoptosis markedly. Therefore, we further investigated the possible mechanisms of honokiol-induced HT-29 cell apoptosis using western blot analysis. Honokiol, at a concentration of 20 μM, reduced the production of VEGF and COX-2 proteins significantly, inhibited the phosphorylation of AKT, ERK1/2, and NF-κB P65, and upregulated

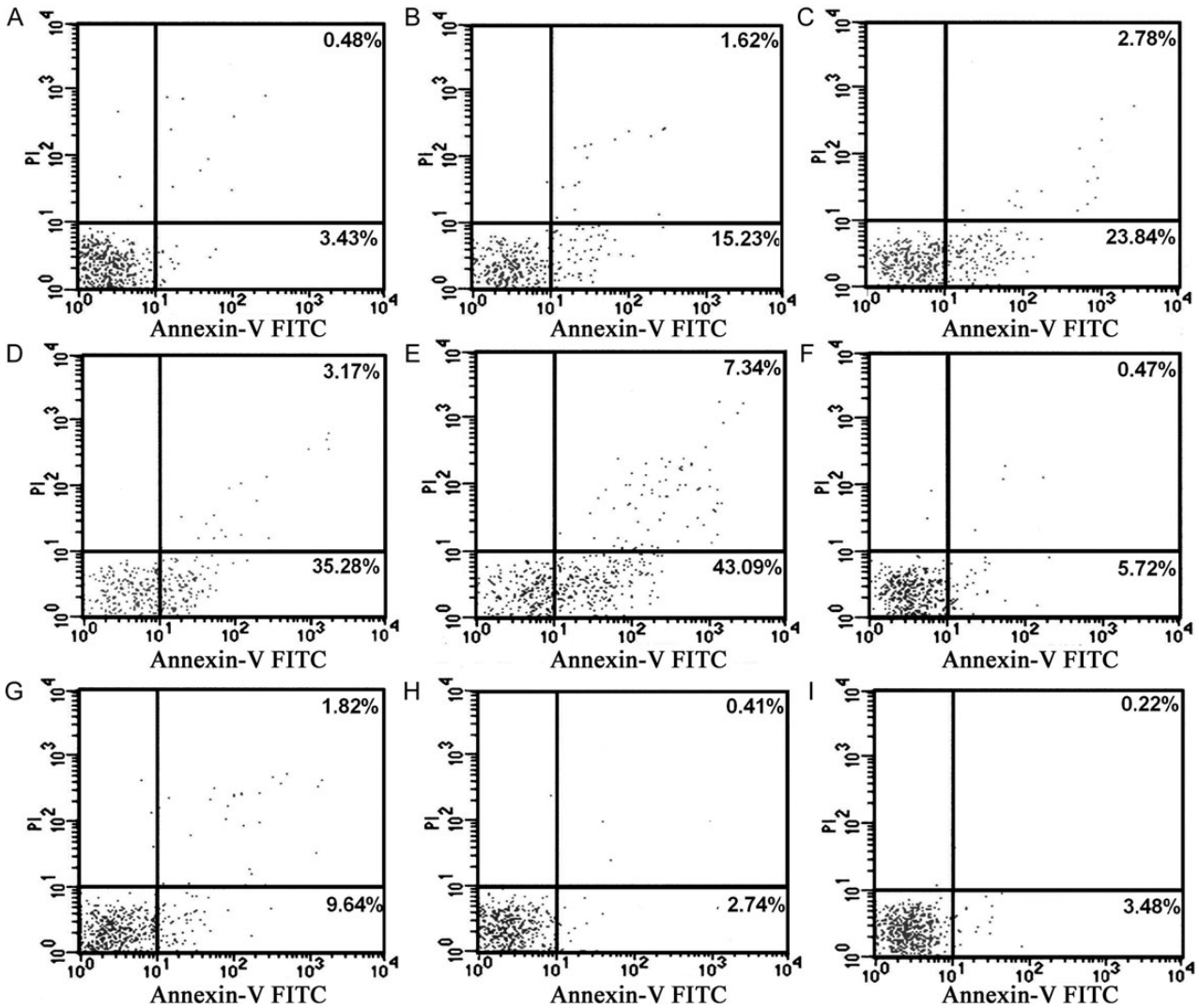

Figure 3 Measurement of cell apoptosis (A–G) HT-29 cells and (H and I) HCoEpiCs treated with honokiol (HNK) and oxaliplatin (OXL) at a series of concentrations. HT-29 cells and HCoEpiCs were removed from the culture dish and stained with annexin V-FITC and PI and analysed by flow cytometry. (A) HT-29 cells Normal control; (B) HNK 0.2 μM + OXL 0.6 μM; (C) HNK 1 μM + OXL 0.6 μM; (D) HNK 5 μM + OXL 0.6 μM; (E) HNK 20 μM + OXL 0.6 μM; (F) HNK 20 μM; (G) OXL 0.6 μM; (H) normal control; (I) HNK 20 μM. Data were from one experiment that was representative of three independent experiments that had similar results.

caspase-3 expression (**Figs. 5** and **6**). These effects were stronger if oxaliplatin and honokiol were added together. However, there was no effect of addition of oxaliplatin alone when compared with the control. Although the inhibitory effect on the COX-2 and VEGF expression was also shown in HCoEpiCs, this effect in HT-29 cells was more significant.

Discussion

In recent years, anti-cancer agents derived from natural products have been considered to play an important role in the development of cancer therapy. Honokiol is a neolignan isolated from the traditional medicinal herb magnolia cortex. It has been shown to be effective in the therapy of several types of human cancer cells. Honokiol can traverse the blood–brain barrier and induce apoptosis of neuroblastoma [12]. Honokiol was also observed to have antimetastatic activity in osteosarcoma [13]. In this study, we evaluated the anti-cancer activity of honokiol in colon cancer HT-29 cells. We found that low concentrations of oxaliplatin combined with non-toxic concentrations of honokiol had a much more powerful effect on inhibition of cell proliferation, induction of apoptosis, and inhibition on PGE_2 and VEGF expression in HT-29 cells than either oxaliplatin or honokiol alone. Low concentrations of honokiol had no effect on inhibition of HCoEpiC proliferation. We also investigated the possible molecular mechanisms of honokiol to induce cell apoptosis. Honokiol could suppress the expression of VEGF and COX-2, inhibit the phosphorylation of AKT, ERK1/2, and NF-κB P65, and upregulate the expression of caspase-3.

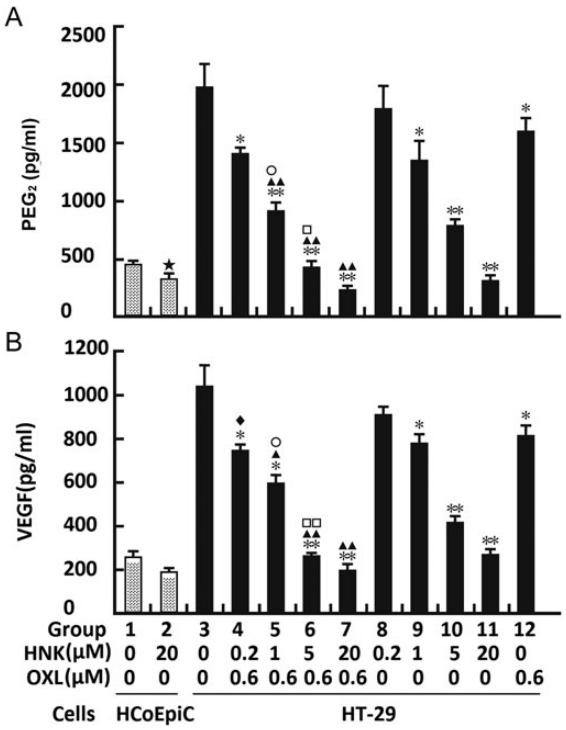

Figure 4 Enzyme-linked immunosorbent assay to determine the expression levels of PGE_2 and VEGF The levels of PGE_2 (A) and VEGF (B) in cell culture supernatants were examined after being treated by a series of concentrations of honokiol (HNK) or oxaliplatin (OXL) alone or combined. (A) $^{\star}P < 0.05$ vs group 1, $^{**}P < 0.01$ vs group 3, $^{*}P < 0.05$ vs group 3, $^{\blacktriangle\blacktriangle}P < 0.01$ vs group 12, $^{\circ}P < 0.05$ vs group 9, $^{\square}P < 0.05$ vs group 10. (B) $^{**}P < 0.01$ vs group 3, $^{*}P < 0.05$ vs group 3, $^{\blacktriangle\blacktriangle}P < 0.01$ vs group 12, $^{\blacktriangle}P < 0.05$ vs group 12, $^{\blacklozenge}P < 0.05$ vs group 8, $^{\circ}P < 0.05$ vs group 9, $^{\square\square}P < 0.01$ vs group 10. Data were from three independent experiments (mean ± SEM).

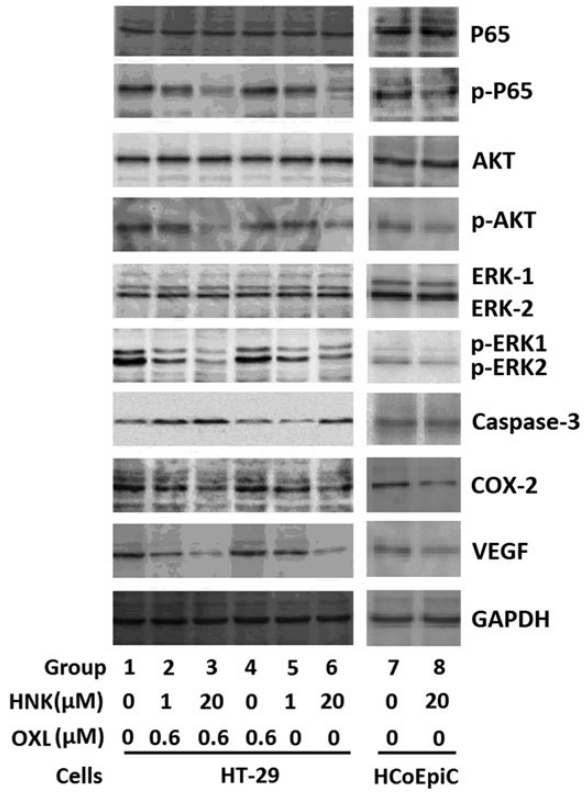

Figure 5 Western blot analysis of proteins from cell lysates It aims to investigate a possible mechanism of honokiol induction of HT-29 cell apoptosis and its effect on HCoEpiCs. GAPDH was used as the internal control. Data were from one representative experiment of three independent experiments.

COX-2 plays an important role in the carcinogenesis of colon cancer. COX-2 enzymes are integral membrane proteins located in the endoplasmic reticulum and nuclear membrane [14]. COX-2 catalyze the rate-limiting step in the metabolism of arachidonic acid, resulting in the production of prostaglandin G2 (cyclooxygenase reaction). Prostaglandin G2 is then converted to prostaglandin H2 (peroxidase reaction), a target of several specific prostanoid synthases, resulting in the production of prostaglandins including PGE_2 [15]. Increased levels of PGE_2 are detected in colon cancer tissue, whereas PGE_2 is only moderately presented in normal mucosa [16]. PGE_2 was quickly secreted from cells and bound locally to membrane-bound prostanoid receptors termed EP. With regard to tumor biology, PGE_2 stimulated the growth and invasion of colon cancer cells and promoted angiogenesis by increasing VEGF production [17–19]. VEGF, one of the best characterized proangiogenic factors, plays a critical role in angiogenesis that is essential for tumor growth [20]. It can be secreted by colon carcinoma cells, *via* its interaction with its receptor VEGFR-2. It mediates many key components of angiogenesis, including endothelial cell proliferation, invasion, migration, and survival, as well as vessel permeability [21,22]. Inhibition of PGE_2 and VEGF expression can increase the apoptosis of colon cancer cells. In this study, we found honokiol inhibited the COX-2 and PGE_2 expression both in HT-29 cells and HCoEpiCs, and the inhibitory effect was more significant in HT-29 cells than in HCoEpiCs.

AKT has been recognized as a key mediator of cell proliferation, differentiation, and survival, which is phosphorylated in response to a variety of stimuli (hormones, growth factors, and cytokines) [23,24]. A large number of proteins can be activated by phosphorylated AKT. These proteins include Bcl-associated death (BAD), cAMP-response-element binding protein (CREB), members of the forkhead box protein O (FoxO) family of transcriptions factors, inhibitory (I)κ-B kinase, procaspase-9, glycogen synthase kinase (GSK3)-α/β, mammalian target of rapamycin (mTOR)/FK506 binding protein 12-rapamycin associated protein 1 (FRAP), and p21 [25–28]. Moreover, increasing evidence points to the likelihood that AKT plays an important role in tumorigenesis and resistance to chemotherapeutic drugs [26,27], as overexpression of phosphoinositide (PI3K)/AKT has been observed in many cancer cells. Furthermore, increased activities of PI3K/AKT are considered to be related to the resistance of cancer cells to anti-cancer drugs [29].

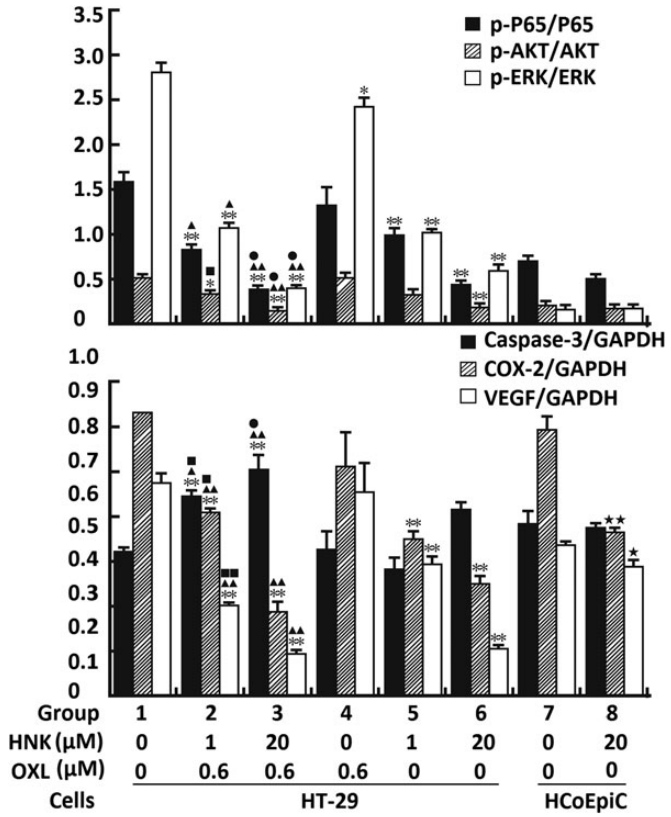

Figure 6 Quantification and analysis of Western blot protein bands $^{**}P < 0.01$ vs group 1, $^{*}P < 0.05$ vs group 1, $^{\blacktriangle\blacktriangle}P < 0.01$ vs group 4, $^{\blacktriangle}P < 0.05$ vs group 4, $^{\blacksquare\blacksquare}P < 0.01$ vs group 5, $^{\blacksquare}P < 0.05$ vs group 5, $^{\bullet}P < 0.05$ vs group 6, $^{\star\star}P < 0.01$ vs group 7, $^{\star}P < 0.07$ vs group 7. Data were from three independent experiments (mean ± SEM).

ERKs are typical members of the mitogen-activated protein kinase family and have been shown to be involved in cell proliferation and survival [30]. ERKs are phosphorylated and activated in cells upon exposure to serum or oncogenes [31]. Upregulated expression of ERKs is observed in cancer cells [32,33]. It has also been suggested that increased activity of ERKs is responsible for the resistance of cancer cells to anti-cancer drugs. [29].

NF-κB plays a major role in the control of apoptosis, cell proliferation, and differentiation, and is activated in response to several pro-apoptotic stimuli, such as tumor necrosis factor (TNF)-α, ionizing radiation, oxidative stress, and cytotoxic. NF-κB phosphorylated by these stimuli then translocates into the nucleus and regulates the expression of anti-apoptotic genes. Therefore, inhibition of NF-κB in cancer cells has become one of the major targets of anti-cancer therapy.

These results suggested that honokiol could induce HT-29 cell apoptosis and inhibit cell proliferation by the suppression of AKT, ERK1/2 and NF-κB P65 phosphorylation, by the suppression of COX-2 and VEGF expression, and by the upregulation of caspase-3 expression. These results showed, as far as we know for the first time, that honokiol can augment the anti-tumor effect of oxaliplatin. This effect may not only enable a reduction of the dose of oxaliplatin given to patients and thereby prevent the associated adverse effects, but may also enhance the chemotherapeutic effect on colon cancer. Honokiol can reduce the toxicity and side effects of oxaliplatin by decreasing the dosage, leading to improved efficacy and less drug resistance of oxaliplatin in chemotherapy. Those patients who can not tolerate the oxaliplatin toxicity or respond poorly will benefit from the use of honokiol.

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