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Full Length Research Paper

Honokiol augments the anti-cancer effects of oxaliplatin on colon cancer cell: Apoptosis and analysis of the molecular mechanisms

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The drug oxaliplatin is important in the chemotherapy of colorectal carcinoma, but its toxicity, especially dose-related neurosensory toxicity, is not well tolerated. We investigated if honokiol could augment the anti-tumor effect of oxaliplatin in colon cancer HT-29 cells *in vitro* and determined if honokiol could be used with oxaliplatin to decrease its dose. Cell proliferation, apoptosis, and prostaglandin E2 (PGE_2) and vascular endothelial growth factor (VEGF) levels were investigated. Expression 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 was examined. Honokiol or oxaliplatin alone suppressed the proliferation of HT-29 cells in a concentration-dependent manner. HT-29 cells were more sensitive to oxaliplatin treatment in the presence of honokiol. Oxaliplatin combined with honokiol improved the rate of HT-29 cell apoptosis and reduced PGE_2 and VEGF secretion levels. Expression of COX-2 and VEGF protein and phosphorylation of AKT, ERK1/2, NF-κB and P65 were also inhibited, caspase-3 levels were upregulated after honokiol treatment. Therefore, honokiol can be combined with oxaliplatin in the chemotherapy of colorectal carcinoma, this combination allows a reduction in oxaliplatin dose, and thereby reduces its adverse effects, and may also enhance the chemotherapeutic effect of oxaliplatin for this disease.

Key words: Honokiol, colorectal cancer, apoptosis, oxaliplatin, nuclear factor-kappa B (NF-κB).

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 very important role in the chemotherapy of colorectal and ovarian cancer (Kweekel et al., 2005). The chemotherapy regimens, folinic acid/fluorouracil/oxaliplatin (FOLFOX) or capecitabine/oxaliplatin (XeLOX) are a first-line treatment 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 (Cavalettia et al., 2001; Pasetto et al., 2006), is not well tolerated by most patients. Drug resistance to oxaliplatin is also a problem in chemotherapy. Therefore, finding the right dosing scheme and strategy for each individual patient that minimizes the side effects remains a challenge for individual-based chemotherapy management. Meanwhile, the discovery of new drugs that can augment the anti-tumor effect of oxaliplatin efficiently

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and also enable a reduction in its dose is needed urgently.

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 (Han et al., 2009), which include leukemia (Hibasami et al., 1998; Battle et al., 2005), human breast cancer cells (Liu et al., 2008; Park et al., 2009), human hepatocellular (Han et al., 2009), human multiple myeloma (Ishitsuka et al., 2005), human prostate cancer cells (Hahm and Singh, 2007), and human squamous lung cancer (Yang et al., 2002). In this study, we analyzed 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 of several downstream molecular mechanisms to analyse the way in which honokiol may induce cell apoptosis.

MATERIALS AND METHODS

Reagents

Oxaliplatin was obtained from Sanofi-Aventis Pharmaceutical Co. Ltd. Honokiol (purity, 98.7%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China. The colon cancer cell HT-29 AQueous Cell Viability Assay kit was procured from Promega (WI, USA). Anti-vascular endothelial growth factor (VEGF) and anti-cyclo-oxygenase-2 (COX-2) antibodies used in western blotting analysis were obtained from Santa Cruz Pharmaceuticals (CA, 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-Signaling Technology (MA, USA). All other chemicals were of reagent grade and were obtained from Sigma (St. Louis, MO, USA).

Cell culture and treatment

Human colon cancer HT-29 cells were purchased from the American Type Culture Collection (ATCC, VA, USA). Cells were passaged three to five times and were used throughout the study. HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 5.6 mM glucose, glutamine, and antibiotics (penicillin and streptomycin) at 37°C under 5% CO_2 in air. HT-29 cells were plated out at 1×10^4 cells/well in 96-well plate; cells were treated at a range of concentrations with either honokiol (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) in combination for 48 h, respectively, and cell viability was evaluated through 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 (PMS), were used in the MTS assay. The reduction 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 (PI) apoptosis assay

Cell apoptosis was measured using an annexin V-FITC apoptosis detection kit (BD PharMingen, CA, USA). Briefly, HT-29 cells were removed from the culture dish and stained with annexin V-FITC and PI and were analyzed by flow cytometry (FACSCalibur, BD PharMingen) after treatment. Cells that were annexin V-FITC and PI double-negative were considered to be non-apoptotic for statistical analysis.

Analysis for prostaglandin E2 (PGE_2) and VEGF production

The concentrations of PGE_2 and VEGF in culture supernatants were determined using a competitive enzyme-linked immuno-sorbent assay (ELISA) kit (R&D Systems, MN, USA).

Western blotting

Cells were treated with honokiol or oxaliplatin alone or combined, 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, GAPDH using whole cellular protein extraction kits (Active Motif, California, USA). A DC protein assay kit was used (Bio-Rad, Richmond, CA, USA) to examine the protein concentration in each cell lysate; 40 µg protein was mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and denatured for 10 min at 95°C. Proteins were separated on a 10% polyacrylamide gel and blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes were blocked with 5% bovine serum albumin (BSA; Sigma) in Tris-buffered saline (TBS; 25 mM Tris–HCl, 150 mM sodium chloride and pH 7.2) for 2 h at room temperature. Blots were incubated with rabbit polyclonal immunoglobulin G (IgG) primary antibody overnight at 4°C. Blots were washed three times with washing buffer (PBS with 0.1% Tween-20) and then incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit second antibody (1:2000 dilution) for 2 h at room temperature. After thorough washing, the blots were incubated with HRP-conjugated secondary antibody. The reaction was developed using enhanced chemiluminescence (ECL) reagents (Amersham, NJ, USA) and analyzed using a VersaDoc MP5000 imaging system (Bio-Rad, CA, USA).

Statistical analysis

The results were expressed as the mean value and standard error of the mean. Statistical significance was analyzed by one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

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

We used the 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to detect HT-29 cells viability after treatment with different concentrations of honokiol or oxaliplatin. The addition of either honokiol or oxaliplatin had a concentration-dependent inhibitory effect (Figure 1). A honokiol concentration of 20 µM was the maximum concentration that did not affect HT-29 cell

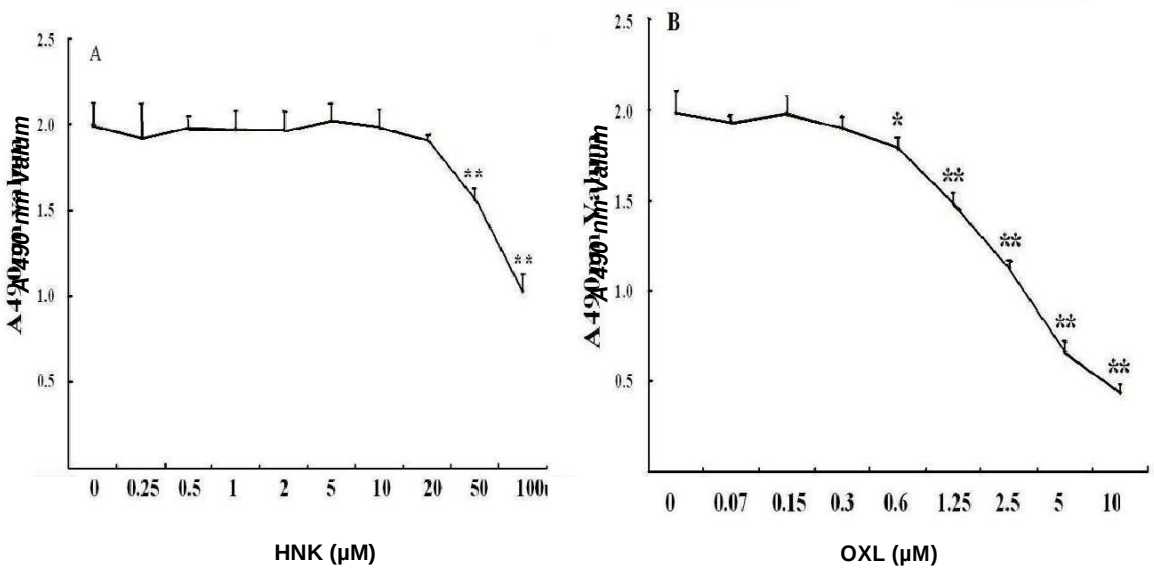


Figure 1. MTT assay to detect HT-29 cells viability after treatment. Absorbance at 490 nm of cell cultures treated with different concentrations of (A) oxaliplatin (OXL) or (B) honokiol (HNK) alone. *P < 0.05 versus normal control (0 μM), **P < 0.01 versus normal control (0 μM).

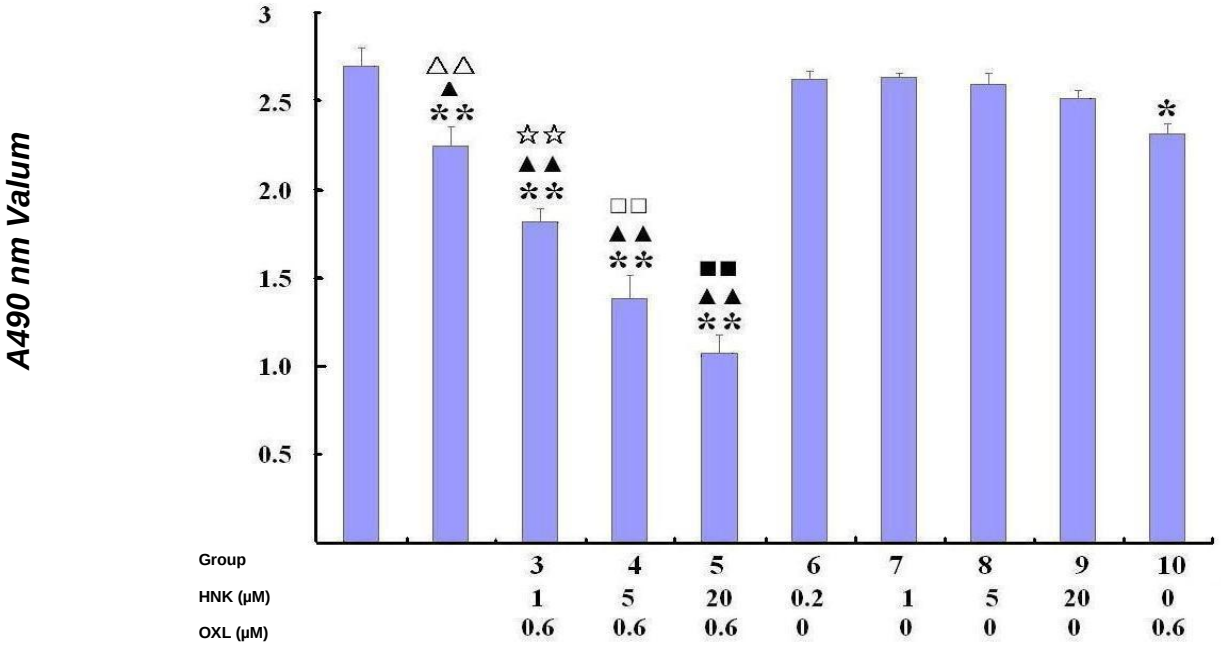


Figure 2. MTT assay to detect HT-29 cells viability after treatment. Absorbance at 490 nm of HT-29 cell cultures treated honokiol (HNK) combined with oxaliplatin (OXL). HNK combined with OXL can improve significantly the suppression of cell proliferation compared with the control group and with the OXL alone treated group. **P < 0.01 versus group 1, *P < 0.05 versus group 1. ▲▲P < 0.01 versus group 10. ▲P < 0.05 versus group 10, P < 0.01 versus group 6, ☆☆P < 0.01 versus group 7, □□P < 0.01 versus group 8, ■■P < 0.01 versus group 9.

proliferation and oxaliplatin at 0.6 μM was the minimum effective concentration. The anti-proliferation capability of oxaliplatin was enhanced significantly when honokiol was also added to the cells (Figure 2). The data showed that HT-29 cells were more sensitive to the combined treatment than treatment with single reagents alone. The addition of honokiol markedly increased the anti-proliferation effect of low concentrations of oxaliplatin.

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

A range of concentrations of honokiol (0, 0.2, 1, 5, and 20 µM) combined with 0.6 µM oxaliplatin was used. The percentage of apoptotic HT-29 cells increased significantly when honokiol was added (Figure 3); for example, the addition of honokiol (20 µM) increased the number of cells that were undergoing apoptosis from 9.61 to 44.22%. 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.

PGE_2 and VEGF production in culture supernatants

The levels of PGE_2 and VEGF in HT-29 cells culture supernatants were examined by competitive ELISA after treatment with honokiol or oxaliplatin alone or combined. Addition of honokiol at concentrations greater than 1 µM reduced the production of PGE_2 and VEGF (Figure 4) in a concentration-dependent manner. Honokiol added at concentrations above 5 µM had a significant suppressive effect when compared with the control group, independent of the addition of oxaliplatin ($P < 0.01$). There was a synergetic suppressive effect between oxaliplatin and honokiol when honokiol was at added at concentrations between 1 to 5 µM ($P < 0.05$).

Possible mechanisms of honokiol induction of HT-29 cell apoptosis

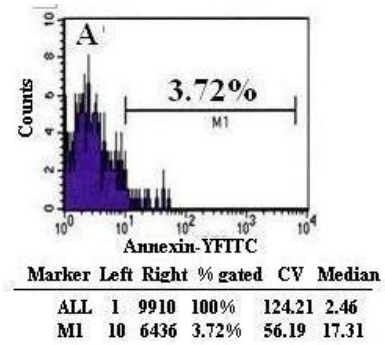
We found that honokiol combined with low concentrations of oxaliplatin (0.6 µmol/L) suppressed HT-29 cell proliferation and induced apoptosis markedly; therefore, we investigated the possible mechanisms of honokiol-induced HT-29 cell apoptosis using western blotting. Honokiol at a concentration of 20 µM reduced the production of VEGF and COX-2 proteins significantly, inhibited the phosphorylation of AKT, ERK1/2 and NF-κB P65, and caspase-3 expression was upregulated (Figure 5). This effect was increased if oxaliplatin and honokiol were given together. However, there was no effect of addition of oxaliplatin alone when compared with the control group.

DISCUSSION

Oxaliplatin therapy has been considered to be a first-line therapy strategy in the chemotherapy of advanced colorectal cancer. But oxaliplatin toxicity, especially its neurotoxicity, is not well tolerated by most patients. After long-term administration of oxaliplatin, patients may present with deep sensory loss, sensory ataxia and functional impairment. This type of neurotoxicity usually has late onset and is correlated with the cumulative dose of oxaliplatin. Resistance to oxaliplatin is also a problem after long-term therapy. Severe toxic reactions and high resistance to this drug after long-term treatment limit its clinical application, therefore, there is an urgent need to minimize the adverse effects and improve the anti-cancer functions.

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 *Magnoliae* cortex, it has been shown to be effective in the therapy of several types of human cancer cells, such as breast cancer, human hepatocellular carcinoma, leukemia, human prostate cancer cells, human squamous lung cancer, and human multiple myeloma. Honokiol can traverse the blood-brain barrier and induce apoptosis of neuroblastoma (Lin et al., 2012). Honokiol was also observed to have antimetastatic activity in osteosarcoma (Steinmann et al., 2012). In this study, we evaluated the anti-cancer value of honokiol in colon cancer HT-29 cells. We found that low concentrations 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 human colon HT-29 cells than either oxaliplatin or honokiol alone. We also investigated the molecule mechanisms of honokiol induction of 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.

AKT has been recognized as a key mediator of cell proliferation, differentiation, and survival. AKT is phosphorylated in response to variety of stimuli (hormones, growth factors, cytokines) (Mora et al., 2004; Yoeli-Lerner and Toker, 2006). A large variety of proteins can then be activated by phosphorylated AKT, these protein 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 (Brazil et al., 2004; Fresno Vara et al., 2004; Martelli et al., 2005, 2006). AKT is recognized, therefore, as a key mediator of cell proliferation, differentiation, and survival. Moreover, increased evidence points to the likelihood that AKT plays an important role in tumorigenesis and resistance to chemotherapeutic drugs (Fresno Vara et al., 2004; Martelli et al., 2005), 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 respond to anti-cancer drugs (McCubrey et al., 2006).



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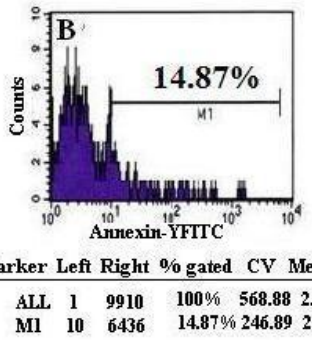

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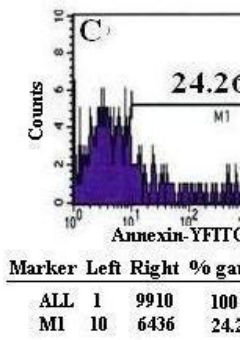
M1	10	6436	14.87%	246.89	23.50



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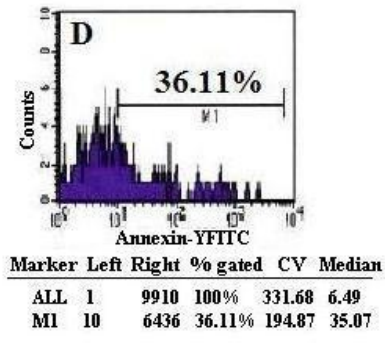
ALL	1	9910	100
M1	10	6436	24.2



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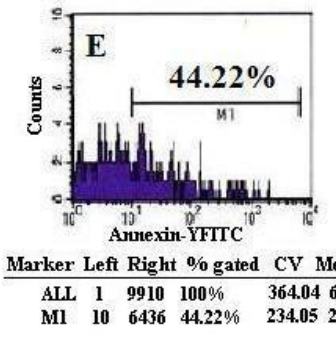
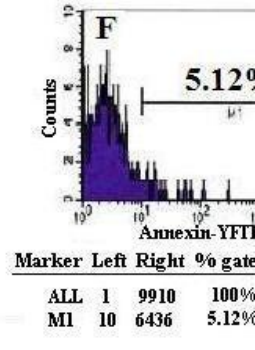
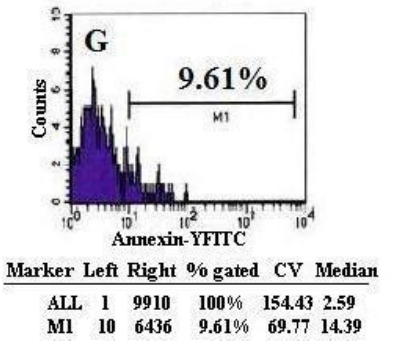
M1	10	6436	36.11%	194.87	35.07



Marker	Left	Right	% gated	CV	Median
ALL	1	9910	100%	364.04	6.15
M1	10	6436	44.22%	234.05	24.80



Marker	Left	Right	% gate
ALL	1	9910	100%
M1	10	6436	5.12%



Marker	Left	Right	% gated	CV	Median
ALL	1	9910	100%	154.43	2.59
M1	10	6436	9.61%	69.77	14.39

Figure 3. Measurement of apoptosis. (A to G) HT-29 cells treated with honokiol (HNK) and oxaliplatin (OXL) at concentrations. HT-29 cells were removed from the culture dish and stained with annexin V-FITC and PI and analy cytometry. A: 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 µM + OXL 0.6 µM, F: HNK 20 µM, G: OXL 0.6 µM.

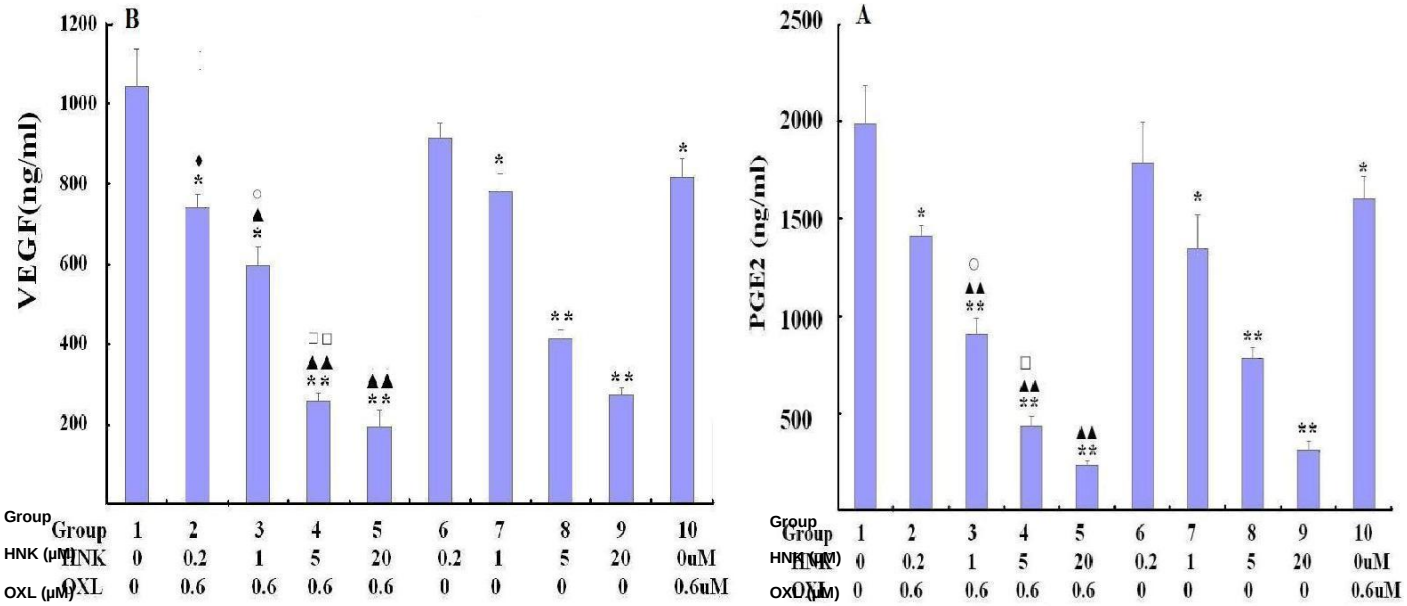


Figure 4. Enzyme-linked immunosorbant assay (ELISA) of levels of (A) prostaglandin E2 (PGE_2) and (B) vascular endothelial growth factor (VEGF) in culture supernatants treated by a range of concentrations of honokiol (HNK) or oxaliplatin (OXL) alone or combined. **P < 0.01 versus group 1, *P < 0.05 versus group 1, ▲▲P < 0.01 versus group 10, °P < 0.05 versus group 7, □P < 0.05 versus group 8 (A). **P < 0.01 versus group 1, *P < 0.05 versus group 1, ▲▲P < 0.01 versus group 10, ▲P < 0.05 versus group 10, ♦P < 0.05 versus group 6, °P < 0.05 versus group 7, □□P < 0.01 versus group 8 (B).

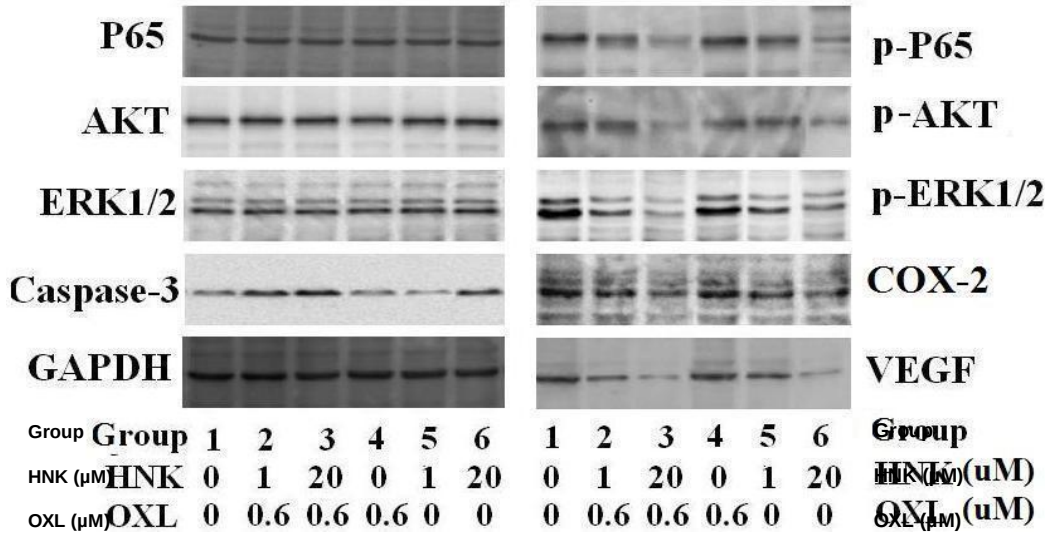


Figure 5. Western blood analysis of protein cell lysates to investigate a possible mechanism of honokiol induction of HT-29 cells apoptosis. GAPDH was used as the positive control.

ERKs are typical members of the mitogen-activated protein kinase (MAPK) family and have been shown to be involved in cell proliferation and survival (Park et al., 2010). ERKs are phosphorylated and activated in cells upon exposure to serum or oncogenes (Troppmair et al., 1994). Upregulated expression of ERKs is observed in cancer cells (Nakayama et al., 2001; Kallergi et al., 2008). Increased activities of ERKs are also suggested to give resistance to cancer cells in response to anti-cancer drugs (McCubrey et al., 2006).

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 studies suggest that honokiol could induce 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 show, 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, and lead to improved efficacy and less drug resistance of oxaliplatin in chemotherapy. Patients who cannot stand the oxaliplatin toxicity or have poor effect will benefit from honokiol.

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ABBREVIATIONS

ANOVA, Analysis of variance; **BSA,** bovine serum albumin; **CREB,** cAMP-response-element binding; **DMEM,** Dulbecco's modified Eagle's medium; **ECL,** enhanced chemiluminesence; **EGFR,** epidermal growth factor receptor; **ELISA,** enzyme-linked immunosorbent assay; **ERK,** extracellular signal-related kinase; **FBS,** fetal bovine serum; **MAPK,** mitogen-activated protein kinase; **PBS,** phosphate-buffered saline; **PI,** propidium iodide; **PMS,** phenazine methosulfate; **VEGF,** vascular endothelial growth factor.

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