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G0/G1 Cell Cycle Arrest and Activation of Caspases in Honokiol-mediated Growth Inhibition of Human Gastric Cancer Cells

You-Jin Kang[1], Hwa-Jin Chung[2], Hye-Young Min[1], Jayoung Song[2], Hyen Joo Park[2], Ui Joung Youn[3], KiHwan Bae[3], Yeong Shik Kim[2], and Sang Kook Lee[2,*]

[1]*College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea*
[2]*College of Pharmacy, Seoul National University, Seoul 151-742, Korea*
[3]*College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea*

Abstract – Honokiol, a naturally occurring neolignan mainly found in *Magnolia* species, has been shown to have the anti-angiogenic, anti-invasive and cancer chemopreventive activities, but the molecular mechanism of actions has not been fully elucidated yet. In the present study, we investigated the effect of honokiol on the growth inhibitory activity in cultured SNU-638 human gastric cancer cells. We found that honokiol exerted potent anti-proliferative activity against SNU-638 cells. Honokiol also arrested the cell cycle progression at the G0/G1 phase and induced the apoptotic cell death in a concentration-dependent manner. The cell cycle arrest was well correlated with the downregulation of Rb, cyclin D1, cyclin A, cyclin E, and CDK4 expression, and the induction of cyclin-dependent kinase inhibitor p27. The increase of sub-G1 peak by honokiol was closely related to the induction of apoptosis, which was evidenced by the induction of DNA fragmentation, the cleavage of poly(ADP-ribose) polymerase, and the sequential activation of caspase cascade. These findings suggest the cell cycle arrest and induction of apoptosis might be one possible mechanism of actions for the anti-proliferative activity of honokiol in human gastric cancer cell.
Keywords – Honokiol, Growth inhibition, Cell cycle arrest, Apoptosis, Human gastric cancer cells

Introduction

Although the incidence and mortality of gastric cancer has been declined in Western countries, particularly in USA and Europe, gastric cancer is still the second-ranked most common cancer worldwide. Especially, gastric cancer is the most prevalent malignant neoplasm and the leading cause of cancer-related human death in Asian countries including Korea, China and Japan (Whelan *et al*., 1993; Roukos, 2006). The excessive intake of salt and *Helicobacter pyroli* infection have been considered one of major etiologic factors to induce the gastric carcinogenesis (Miller *et al*., 1994; Tsugane *et al*., 1994). The strategy for prevention and treatment of gastric cancer with vegetables and dietary antioxidants is also proposed for reducing the risk of gastric carcinogenesis (Wu *et al*., 1999). Therefore, there is still needed to identify and procure promising chemotherapeutic agents for better treatment of advanced malignancy of gastric cancer.

Honokiol is a neolignan type phytochemical found in the bark, leaf, and seed cones of the magnolia tree, and has been shown to exhibit a variety of important biological effects such as antithrombosis, antimicrobial and anxiolytic activity (Chang *et al*., 1998; Kuribara *et al*., 2000; Zhang *et al*., 2007). Many studies have also demonstrated the potential of natural honokiol to mediate the strong antioxidant, anti-inflammatory, or potent cancer chemopreventive effects in carcinogenesis (Lin *et al*., 2006; Munroe *et al*., 2007). In addition, honokiol inhibited the growth of several human cancer cell lines, including human lung squamous carcinoma, promyelocytic leukemia, breast, and colon cancer cells (Hirano *et al*., 1994; Yang *et al*., 2002; Chen *et al*., 2004; Wolf *et al*., 2007). In our recent study we also reported that the growth inhibition of breast cancer cells by honokiol was associated with the down-regulation of c-Src/EGFR-mediated cell signaling activation (Park *et al*., 2009). In view of the growth inhibitory potential of honokiol against cancer cells, we further designed to investigate the cellular mechanisms of honokiol on the growth of human gastric cancer cells. Here, we report for the first time that honokiol inhibited the growth of human gastric cancer

*Author for correspondence
Tel: +82-2-880-2475; E-mail: sklee61@snu.ac.kr

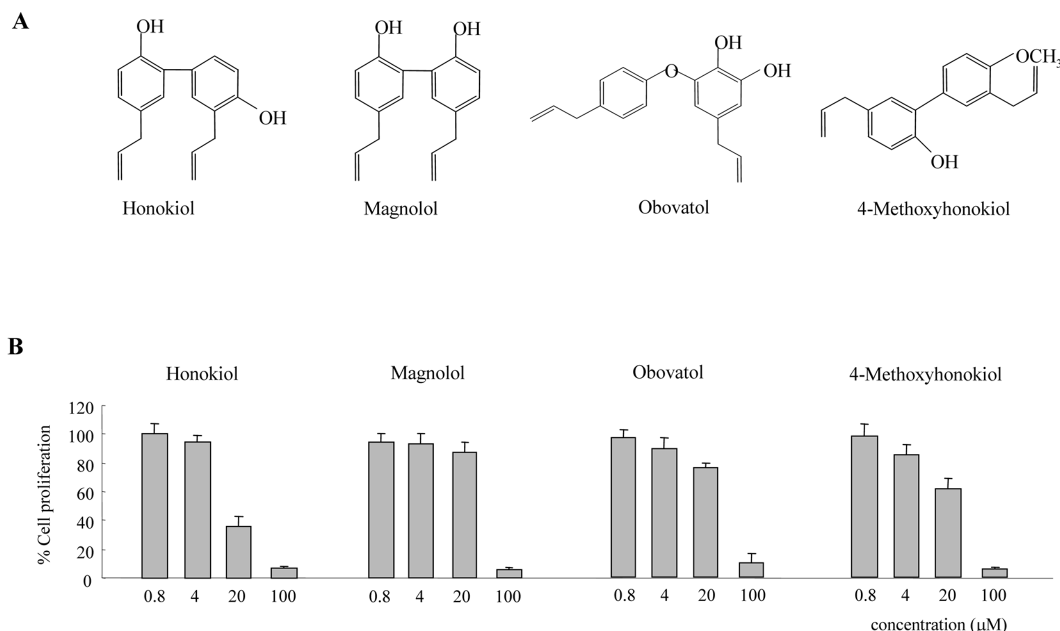

Fig. 1. Chemical structures of honokiol and neolignans isolated from the bark of *M. obovata* (A), and the growth inhibitory effects of isolates neolignans on SNU-638 human gastric cancer cells (B). Human gastric cancer (SNU-638) cells were plated at 10,000 cells in 96-well plate in RPMI supplemented with 10% FBS, and incubated with the test compound as the indicated concentrations for 3 days. The values of % cell growth are calculated by the mean absorbance of samples/absorbance of vehicle-treated control. Data are represented as the means ± S.E. (n = 3).

cells and remarkably induced arrest at the G0/G1 of the cell cycle, and also induced apoptosis in human gastric (SNU-638) cancer cells.

Experimenal

Chemicals – Trichloroacetic acid (TCA), sulforhodamine B, propidium iodide, trypsin inhibitor, RNase A, and anti-β-actin primary antibody were purchased from Sigma (St. Louis, MO, USA). Rosewell Park Memorial Institute medium 1640 (RPMI 1640), fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100X), trypsin-EDTA solution (1X) and antibiotic-antimycotic solution (PSF) were from GIBCO-BRL (Grand Island, NY, USA). Rabbit polyclonal anti-CDK4, anti-cyclin A, anti-caspase-3, anti-p27 antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-Rb, anti-caspase-8, rabbit anti-caspase-9, and anti-phospho-Rb (Ser 807/811) primary antibody were obtained from Cell Signaling (Danvers, MA, USA). Mouse monoclonal anti-PARP, and anti-cyclin E was from BD Biosciences (San Diego, CA, USA).

Honokiol, magnolol, obovatol, and 4-methoxyhonokiol (Fig. 1A) isolated from the bark of *Magnolia obavata* were provided from Dr. KiHwan Bae (Chungnam National University, Korea).

Cell culture – Human gastric carcinoma SNU-638 cells, obtained from the Korean Cell Line Bank (Seoul, Korea), were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2.

Evaluation of growth inhibitory potential – SNU-638 cells (5×10^4 cells/mL) were treated with various concentrations of test compound for 3 days. After treatment, cells were fixed with 10% TCA solution, and cell proliferation was determined with sulforhodamine B (SRB) protein staining method (Lee *et al.*, 1998a). The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

DNA fragmentation assay – SNU-638 cells were plated in 100-mm culture dish at a density of 1×10^6 cells/dish. Twenty-four hours later, fresh media containing test sample were added to cultured dishes. After 24 h, the cells were washed with PBS and lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 1.0% NP-40. After centrifugation, 1% SDS and RNase A

(5 μg/mL) were added to the supernatants, and then incubated at 56 ºC for 2 h. Subsequently, proteinase K (2.5 μg/mL) was added and then incubated at 37 ºC for 2 h. DNA was precipitated with 0.5 volume of 10 M ammonium acetate and 2.5 volumes of cold ethanol at –20 ºC overnight. Precipitated DNA was dissolved in 50 μL of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA. DNA samples (4 μg) were resolved by electrophoresis on a 2% agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, Oregon, USA), and visualized under Alpha Imager^{TM} (Alpha Innotech Co., USA) (Lee *et al*., 1998b).

Analysis of cell cycle dynamics by flow cytometry – Cell cycle analysis by flow cytometry was performed as previously described (Lee *et al*., 1998b). Briefly, SNU-638 cells were plated at a density of 1×10^6 cells per 100-mm culture dish and incubated for 24 h. Fresh media containing test samples were added to culture dishes. After 24 h, the cells were harvested (trypsinization and centrifugation), fixed with 70% ethanol, and incubated with a staining solution containing 0.2% NP-40, RNase A (30 μg/mL), and propidium iodide (50 μg/mL) in phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 20,000 cells were used for each analysis, and results were displayed as histograms of DNA content. The distribution of cells in each phase of cell cycle was calculated using ModFit LT2.0 program.

Evaluation of the protein expression by Western blot – SNU-638 human gastric cancer cells were exposed with various concentrations of honokiol for 24 h. After incubation, cells were lysed and protein concentrations were determined by BCA method. Each protein (30 μg) was subjected to 10% SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were treated for 1 h with blocking buffer [5% non-fat dry milk in phosphate-buffered saline-0.1% Tween 20 (PBST)]. Membranes were then incubated with indicated antibodies (rabbit anti-p27, diluted 1 : 750; mouse anti-β-actin, diluted 1 : 1,500; other antibodies, diluted 1 : 1,000 in PBST) overnight at 4 ºC, washed three times for 5 min with PBST. After washing, membranes were incubated with HRP-conjugated anti-mouse IgG diluted 1 : 1,500 in PBST for 3 h at room temperature, washed three times for 5 min with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature and visualized by HRP-chemiluminescent detection kit (Lab Frontier, Seoul, Korea) using LAS-3000 Imager (Fuji Film Corp., Japan) (Lee *et al*., 1998b).

Statistical analysis – Data were presented as means ± SE for the indicated number of independently performed experiments. Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with Dunnett's *t*-tests.

Results

Effects of honokiol on the proliferation of SNU-638 human gastric cancer cells – The growth inhibitory potential of honokiol was determined in cultured SNU-638 human gastric cancer cells by a colorimetric SRB protein dye staining method. As shown in Fig. 1B, honokiol exhibited a remarkable growth inhibitory effect against SNU-638 human gastric cancer cells, and the IC_{50} value of honokiol was 14.7 μM. In the same experimental condition, biphenolic compounds which are isolated and similar chemical structures with honokiol from the bark of *Magnolia obavata* showed a relatively less potent than honokiol (magnolol, $IC_{50} = 67.6$ μM; obovatol, $IC_{50} = 50.1$ μM; 4-methoxyhonokiol, $IC_{50} = 30.9$ μM). In addition, the treatment of high concentration of honokiol (100 μM) exerted a significant cytotoxic effect with the observation of dead cells.

Analysis of the cell cycle distribution – SNU-638

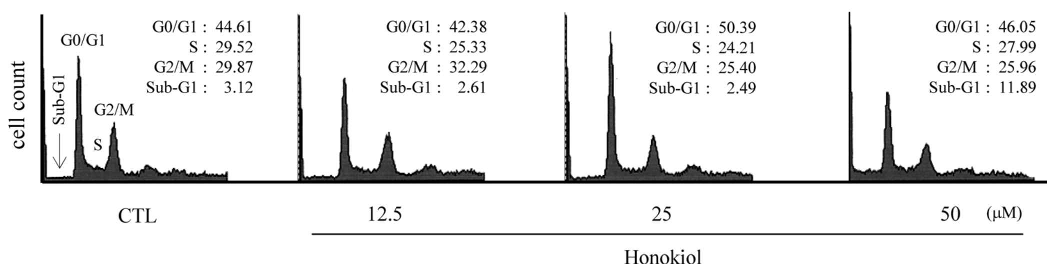

Fig. 2. Effect of honokiol on cell cycle distribution in cultured SNU-638 cells. Cells were seeded at 1×10^6 cells in 100 mm dish in RPMI supplemented with 10% FBS, and then treated with various concentration of honokiol for 24 h and analyzed for cell cycle distribution by flow cytometric analysis of the DNA content.

cells were treated with honokiol (up to 50 μM) for 24 h, and the distribution of cells in various compartments of the cell cycle was analyzed by flow cytometry. When treated with honokiol of up to 25 μM, cells were gradually increased the accumulation of the G0/G1 phase. However, the treatment of 50 μM of honokiol subsequently increased in the sub-G1 phase, indicative of apoptotic peaks, during incubation time (Fig. 2).

Effect of honokiol on DNA fragmentation of SNU-638 cells – To further evaluate whether the cytotoxic effect of honokiol was associated with apoptosis, DNA was extracted from SNU-638 cells at 24 h after treatment of various concentrations of honokiol (0 - 50 μM) and analyzed on a 2% agarose gel. Compared to the non-degraded high molecular weight DNA in vehicle-treated control cells, the treatment of honokiol (50 μM) induced the fragmented DNA (DNA ladder), indicative of an apoptotic cell death (Fig. 3).

Effect of honokiol on the expressions of cell cycle checkpoints and apoptosis biomarker proteins – Based on the data of cell cycle arrest and induction of apoptosis by honokiol we further analyzed whether honokiol modulates the expression of regulatory proteins. Cells were treated with various concentrations of honokiol for 24 h, and the regulatory protein expressions were determined by Western blot analysis. As indicated in Fig. 4A, honokiol suppressed the expressions of Rb phosphorylation, cyclin D1, cyclin A, cyclin E, and CDK4, which are correlated with the induction of the arrest of cell cycle progression at the G0/G1 phase. The cell cycle arrest in the phase of G0/G1 was also evidenced by the induction of CDK inhibitor p27, which is related with the retardation of cell cycle progression in the G1. In

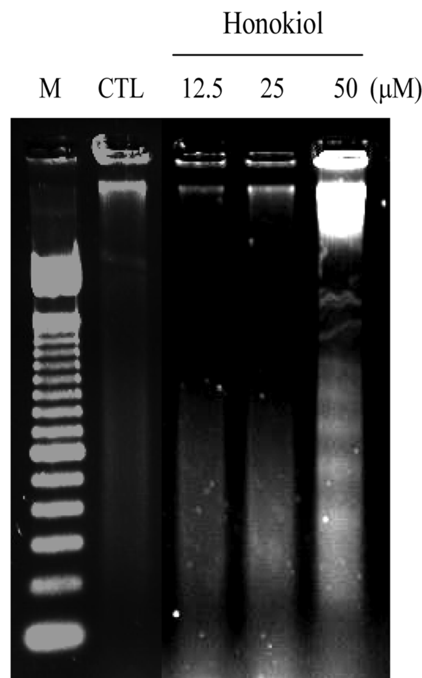

Fig. 3. Effects of honokiol on DNA fragmentation in cultured SNU-638 cells. Cells were seeded at 1×10^6 cells in 100 mm dish in RPMI supplemented with 10% FBS and incubated for 24 h in the presence of honokiol (0- 50 mM). DNA was extracted and separated using 2% agarose gel electrophoresis.

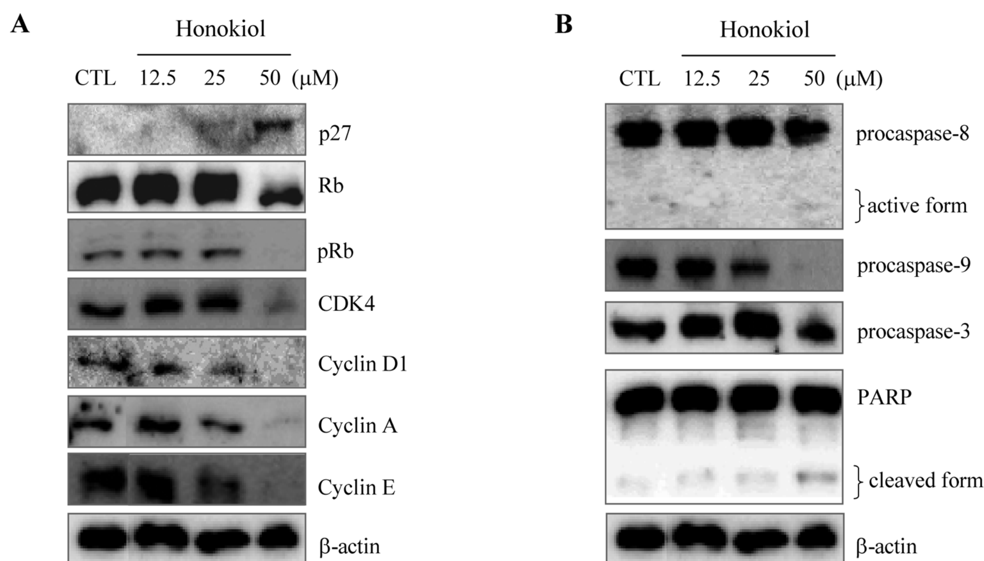

Fig. 4. Effects on the expressions of biomarkers of cell cycle regulation (A) and apoptosis (B). SNU-638 cells were plated at 1×10^6 cells in 100 mm dish in RPMI supplemented with 10% FBS and treated with various concentrations of honokiol for 24 h. The protein expressions were anaylzed by Western blot.

addition, honokiol induced the apoptotic cell death at a high concentration of honokiol (50 μM). The expressions of apoptosis biomarker proteins by honokiol were manifested in the activation of caspase cascade. As shown in Fig. 4B, the sequential downregulation of procaspase-8, -9,- and -3 was investigated at 50 μM honokiol, and subsequently PARP cleavage, a target of caspase-3, was induced, indicating that honokiol evoked apoptotic cell death by the sequential activation of caspase cascade.

Discussion

Natural products have been played an important role in drug discovery and development. Especially, plant derived phytochemicals have been shown to alleviate various human diseases including cancer. Honokiol, a natural neolignan, is found abundantly in *Magnolia* species which are mainly prescribed in Oriental herbal medicines for thrombotic stroke, gastrointestinal complaints, anxiety and nervous disturbance (Kuribara *et al*., 2000; Hu *et al*., 2005; Lin *et al*., 2005; Zhang *et al*., 2005). Previous studies were also reported that honokiol exhibited various pharmacological activities including antioxidant, antiangiogenic and cancer chemopreventive effects (Bai *et al*., 2003). In particular, honokiol inhibits the growth of several human cancer cells including colon, leukemia, and lung carcinoma (Hirano *et al*., 1994; Yang *et al*., 2002; Wolf *et al.,* 2007). Honokiol induced the cell cycle arrest at G0/G1 phase in human prostate cancer cells in association with the suppression of Rb phosphorylation and inhibition of transcriptional activity of E2F1 (Hahm *et al.,* 2007). In our recent study we also reported the potential growth inhibition of honokiol against human breast cancer cells via modulation of c-Src/EGFR signaling pathway. The additional benefit of honokiol is known as a relatively non-toxic to normal cells and good oral absorption potential (Bai *et al*., 2003). Although the anticancer potential of honokiol has been suggested the underlying mechanisms of actions for the growth inhibition of cancer cells are still needed to be clarified. In this study, we further evaluated the anticancer activity of honokiol against human gastric cancer cells because the morbidity and mortality of gastric cancer in Asian countries, especially, Korea and Japan, are top-ranked. Previous studies revealed that honokiol-mediated growth inhibition of cancer cells might be related with different modes of actions depending on cell types. Honokiol induces cancer cell death via either apoptosis or necrotic pathway, and also honokiol-induced apoptosis was suggested in either caspase-dependent or –independent (Ishitsuka *et al*., 2005). In the course of bioassay-guided fractionation from plant extracts aiming to isolate active compounds for development of anticancer agents we recently obtained several neolignan compounds including honokiol from the bark of *Magnolia obovata* and evaluated the growth inhibitory activity against human gastric cancer cells. As a result, compared to other isolated neolignan compounds from the bark of *M. obovata*, honokiol exerted a remarkable growth inhibitory activity in cultured SNU-638 human gastric cancer cells (Fig. 1B). Although honokiol showed a potential growth inhibitory effect against several human cancers the mechanisms of action study with human gastric cancers are not fully elucidated yet. Characterization of growth inhibition by honokiol was determined to evaluate whether honokiol induces the growth arrest or apoptosis using cell cycle distribution and biomarker expression analysis. SNU-638 cells were treated with honokiol for 24 h and then subjected to an analysis of cell cycle distribution. As shown in Fig. 2, cells treated with 25 μM of honokiol significantly accumulated in the G0/G1 phase of the cell cycle, but the higher concentration of honokiol (50 μM) induced the accumulation of the sub-G0 peaks of DNA contents, assuming evidence of apoptotic phenomena. The apoptosis evoked by honokiol was further confirmed by the observation of DNA fragmentation in the treatment of 50 μM honokiol (Fig. 3). The induction of cell cycle arrest in the G0/G1 phase by honokiol was analyzed by the cell cycle checkpoint protein expression, and the cell cycle arrest was well correlated with the dephosphorylation of Rb and the decrease of cyclin D1, cyclin A, cyclin E, and CDK4 expression, and the induction of p27 (Fig. 4A). In addition, the induction of caspase activation and subsequential PARP cleavage (Fig. 4B) suggested that honokiol-mediated apoptosis is evoked by the mitochondrial-dependent casapase activation pathway. Therefore, the accumulation of G0/G1 phase of the cell cycle and induction of apoptosis appear to be probably the growth inhibitory mechanism of honokiol in SNU-638 human gastric cancer cells.

Other studies for the anti-proliferative effects of honokiol suggest that honokiol induced cell cycle arrest in the G0/G1 phase and apoptosis in RKO human colorectal carcinoma cells (Wolf *et al*., 2007), and induced a mitochondrial permeability transition pore-associated necrotic cell death by the regulation of cyclophilin D in human leukemia HL-60 and breast cancer MCF-7 cells (Li *et al*., 2007). Therefore, the anti-proliferative effects of honokiol might be probably dependent on cell-types or the culture conditions.

In summary, this study suggests that honokiol is a potent inhibitor of the growth of human gastric cancer cells, and the growth inhibition is highly related to cell cycle arrest and induction of apoptosis. As currently reported with human gastric cancer cells, the potential growth inhibitory activity of honokiol should be taken into account when considering the further development and prioritization as a cancer chemopreventive or chemotherapeutic agent in human gastric cancer cells.

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