Running title: hispolon induces human hepatoma cell apoptosis

Hispolon Induces Apoptosis and Cell Cycle Arrest of Human Hepatocellular Carcinoma Hep3B Cells by Modulating ERK Phosphorylation

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ABSTRACT

Hispolon is an active phenolic compound of *Phellinus igniarius*, a mushroom that has recently been shown to have antioxidant, anti-inflammatory, and anticancer activities. In this study, we investigated the antiproliferative effect of hispolon on human hepatocellular carcinoma Hep3B cells by using the MTT assay, DNA fragmentation, DAPI **(**40, 6-diamidino-2-phenylindole dihydrochloride) staining and flow cytometric analyses. Hispolon inhibited cellular growth of Hep3B cells in a time-dependent and dose-dependent manner, through the induction of cell cycle arrest at S phase measured using flow cytometric analysis and apoptotic cell death, as demonstrated by DNA laddering. Hispolon-induced S-phase arrest was associated with a marked decrease in the protein expression of cyclins A, and E and cyclin-dependent kinases (CDKs) 2, with concomitant induction of p21waf1/Cip1 and p27Kip1. Exposure of Hep3B cells to Hispolon resulted in apoptosis as evidenced by caspase activation, PARP cleavage, and DNA fragmentation. Hispolon treatment also activated JNK, p38 MAPK and ERK expression. Inhibitors of ERK (PB98095), but not those of JNK (SP600125) and p38 MAPK (SB203580), suppressed hispolon-induced S-phase arrest and apoptosis in Hep3B cells. These findings establish a mechanistic link between MAPK pathway, and hispolon-induced cell cycle arrest and apoptosis in Hep3B cells.

KEYWORKS: *Phellinus igniarius*; apoptosis; Hep3B; caspase; mitochondria

INTRODUCTION

Hepatocellular carcinoma (HCC) is a lethal and one of the four most prevalent malignancies in adults in Taiwan, China, and Korea. Several etiologic factors, including exposure to aflatoxin B1, and infection with hepatitis B virus and hepatitis C virus, have been classified as high-risk factors associated with HCC (*1*). Apoptosis is important in the control of cell quantity during development and proliferation. The mechanism of apoptosis is conserved from lower eukaryotes to mammals and exhibits a network of tightly ordered molecular events that finally converge into the enzymatic fragmentation of chromosomal DNA, driving a cell to death (*2*). Apoptosis involves the activation of a family of caspases, which cleave a variety of cellular substrates that contribute to detrimental biochemical and morphological changes (*3*). At least two pathways of caspase activation for apoptosis induction have been characterized. One is mediated by the death receptor, Fas. Activation of Fas by binding with its natural ligand (Fas ligand) induces apoptosis in sensitive cells (*4*). Fas ligand characteristically initiates signaling via receptor oligomerization and recruitment of specialized adaptor proteins followed by proteolysis and activation of procaspase-8. Caspase-8 directly cleaves and activates caspase-3, which in turn cleaves other caspases (e.g., caspase-6 and -7) for activation (*5*). The other pathway, driven by Bcl-2 family proteins, which may be anti-apoptotic (Bcl-2 and Bcl-X_L) or pro-apoptotic

(Bax, Bak, and Bid), regulates cell death by controlling the permeability of mitochondrial membrane during apoptosis (*6*). Upon apoptosis, pro-apoptotic proteins translocate to the mitochondria and accelerate the opening of mitochondrial porin channels, leading to release of cytochrome *c* and thereby triggering the cascade of caspase activation (*7*). The induction of apoptosis by natural products on malignant cells validates a promising strategy for human cancer chemoprevention (*8*).

Phellinus linteus (Berk. & M.A. Curt.) (PL) is a mushroom that belongs to the genus *Phellinus* and is commonly called “Sangwhang” in Taiwan. It is popular in oriental countries and has been traditionally used as food and medicine. PL contains many bioactive compounds, and is known to improve health and to prevent and remedy various diseases, such as gastroenteric disorders, lymphatic diseases, and cancer (*9*). Recently, a few pharmacological actions of PL have been elucidated. For instance, PL suppresses cellular proliferation and it induces apoptosis in lung and prostate cancer cells (*10*). The anticancer effects of PL have been demonstrated by the inhibition of invasive melanoma B16-BL6 cells (*11*). PL has been found to inhibit the growth, angiogenesis and invasive behavior of breast cancer cells via the suppression of AKT phosphorylation (*12*). We recently reported that hispolon, a phenol compound isolated from PL, anti-inflammatory (*13*) and antimetastatic effects (*14*). Others have also shown that hispolon has antiproliferative and immunomodulatory activities (*15*).

However, there have been no reports on the antiproliferative effects of hispolon in liver cancer cells. In this study, we investigated the anticancer effects of hispolon on three different hepatoma cell lines, including J5, HepG2, and Hep3B cells. A major difference of these three hepatoma cell lines lies in their invasive activities, i.e., J5 > HepG2 =Hep3B, based on their expression levels of thyroid hormone b1 nuclear receptor and nm23-H1 (*16*); the latter is a tumor metastatic suppressor gene that has been identified in murine and human cancer lines (*17-19*). The purpose of this study was to investigate the anticancer effect of hispolon and to provide scientific rationales for using hispolon as chemopreventive and/or chemotherapeutic agents against liver cancer.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM), 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin–EDTA, fetal bovine serum (FBS) and penicillin/streptomycin were from Gibco Life Technologies, Inc. (Paisley, UK). Cell culture supplies were purchased from Costar (Corning, Inc., Cypress, CA, USA). The antibody against Bax, Bcl-2, Fas, FasL, Bid, caspase 3,

caspase 8, caspase 9, MAPK/extracellular signal–regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK proteins and phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA). Anti-cyclin A, anti-cyclin E, anti-CDK 2, anti-p27, anti-p21, and anti-PARP mouse monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA).

Isolation and Characterization of hispolon from Fruiting Body of PL. The fruiting body of PL (about 1.0 kg, air dry weight) was powdered, and extracted with 95% EtOH 6 L at room temperature (3 times, 72h each). Extracts were filtered and combined together, and then evaporated at 40 ℃ (N-11, Eyela, Japan) to dryness under reduced pressure to give a dark brown residue (40 g). The yield obtained for PL is about 4 %. The crude extract was suspended in H_2O (1 L), and then partitioned with 1 L *n*-hexane (× 2), 1 L EtOAc (× 2) and 1 L *n*-butanol (× 2), successively.

Hispolon (Fig.1A) was purified from the EtOAc soluble portion (8 g) by a bioassay-guid separation. A portion of the active EtOAc fraction was subjected to silica gel chromatography using stepwise $CHCl_3$-MeOH (9:1, 8:2, 1:1 v/v) as eluent. Final purification was achieved by preparative HPLC (Spherisorb ODS-2 RP18, 5 μm

(Promochem), 250×25 mm, acetonitrie-H_2O (83: 17 v/v), at a flow rate of 10 mL/min and UV detection at 375nm). The identification of hispolon was performed by comparing their physical spectral data with literature values (*20*).

Cell Culture. The hepatocarcinoma J5, HepG2, and Hep3B cell was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a CO_2 incubator (5% CO_2 in air) at 37 ℃ and subcultured every 2 days at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).

Assay of Cell Viability. The cells (2 x 10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with hispolon for 24, 48, and 72 h. Then, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37℃ testing for cell viability. The medium was then discarded and 100 μL dimethylsulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570

nm was read by a microplate reader. At least three repeats were done for each sample to determine cell proliferation. Decolorization was plotted against the concentration of the sample extracts, and the amount of test sample necessary to decrease 50% absorbance of MTT (IC_{50}) was calculated.

Assay of DNA Fragmentation. Apoptosis was determined by the presence of internucleosomal DNA fragmentation (DNA laddering) after cell treated with increasing dose of hispolon for 48 h, or treated with 45 μM for 24, 48, and 72 h. Hep 3B cells were cultured in 24-well microtiter plates at a density of 2 x 10^6 cells/well (1 mL final volume). To extract genomic DNA, cells were harvested, washed with cold 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, and lysed by adding 0.5% SDS. Cell lysates were then incubated at 56°C for 3 h in the presence of 100 μg/mL of proteinase K. DNA was purified by successive phenol/chloroform extractions and the resultant aqueous phase was mixed with 3M sodium acetate (pH 5.2) and absolute ethanol. The mixture was incubated at -20°C overnight and the ethanol-precipitated DNA was washed with 70% ethanol. Purified DNA was resuspended in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and treated with 50 μg/mL DNase-free RNase A for 1 h. Samples were resolved on a 1% agarose gel and stained with 0.5 μg/mL ethidium bromide before DNA was visualized with ultraviolet light (*21*).

DAPI (40, 6-diamidino-2-phenylindole dihydrochloride) Staining. Cells were seeded onto a 12-well plate at a density of 5 x 10^4 cells/well before treating with drugs. Hep3B cells were cultured with vehicle alone or 45 μM hispolon in DMEM medium for 24, 48, and 72 h. After the treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/mL DAPI for 5 min at 37 ℃. The cells were then washed with PBS and examined by fluorescence microscopy (Nikon, Tokyo, Japan).

Flow Cytometric Analysis for Cell Cycle Distribution. Human hepatocellular carcinoma Hep3B cells (1 x 10^6 cells) were suspended in a hypotonic solution (0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, and 0.1 mM EDTA) and stained with 50 μg/mL of propidium iodide (PI). DNA content was analyzed with a FACScan (Becton Dickinson, San Jose, CA). The population of cells in each phase of cell cycle was determined using CellQuest PRO software (Becton Dickinson, San Jose, CA).

Assay of Cell Apoptosis. Quantitative assessment of apoptosis was analyzed by an Annexin V-FITC assay kit (BD Biosciences, San Jose, CA). Briefly, cells grown in 10

cm Petri dishes were harvested with trypsin and washed in PBS. Cells were then resuspended in a binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$) and stained with Annexin V-FITC and PI at room temperature for 15 min in the dark. Cells were analyzed in an EPICS flow cytometer (Coulter Electronics) within 1 h after staining. Data from 10,000 cells were detected for each data file. Early apoptotic cells were defined as Annexin V-FITC-positive and PI-negative cells (Annexin V+/PI- fraction) and late apoptosis or necrotic cells were defined as annexin V+/PI+ cells.

Preparation of Whole-Cell Lysates. Hep3B cells (1×10^5 cells) were plated in a 100-mm Petri dish and were treated with various concentrations of hispolon. Hep3B cells were washed twice with PBS and were scraped into a microcentrifuge tube. The cells were centrifuged at 1,250*g* for 5 min, and the pellet was lysed with iced-cold RIPA (Radio-Immunoprecipitation Assay) buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5), to which was added freshly prepared phenylmethylsulfonyl fluoride (10 mg/mL), leupeptin (17 mg/mL), and sodium orthovanadate (10 mg/mL). After incubation for 5 min on ice, the samples were centrifuged at 10,000*g* for 10 min, and then the supernatants were collected as whole-cell lysates. The lysates were denatured and subjected to SDS-PAGE and

Western blotting. The protein content was determined with Bio-Rad protein assay reagent using BSA as a standard.

Western Blotting Analysis. Whole-cell lysates proteins (30-50 μg of partially purified protein) were mixed with an equal volume of electrophoresis sample buffer, and the mixture was then boiled for 10 min. Then, an equal protein content of total cell lysate from control, 0.2% DMSO, and hispolon-treated sample were resolved on 10-12% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA) by electroblotting using an electroblotting apparatus (Bio-Rad). Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times each for 10 min and then incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 ℃. The membranes were washed with TBST and then incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated, goat antimouse, or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min in TBST, the bands were visualized using ECL reagents (Millipore, Billerica, MA). Band intensity on scanned films was quantified

using Kodak Molecular imaging (MI) software and expressed as relative intensity compared with control.

Statistical Analysis. Values are expressed as means ± SD and analyzed using one-way ANOVA followed by LSD Test for comparisons of group means. All statistical analyses were performed using SPSS for Windows, version 10 (SPSS, Inc.); a P value <0.05 is considered statistically significant.

RESULTS

Isolation of hispolon from PL and its Structural characterization. PL was isolated via extensive chromatographic purification of the ethyl acetate-soluble fraction of the dried fruiting body. The chemical structure of the purified yellow powder was elucidated by NMR spectroscopy and mass spectrometry studies and was identified as hispolon.

Inhibitory Effects of Hispolon on Tumor Cell Growth. To examine whether hispolon would alter malignant proliferation, inhibitory effects on the growth of J5, HepG2, and Hep3B tumor cells were determined by a MTT colorimetric assay. As

shown in Fig. 1, hispolon inhibited cellular growth of J5, HepG2, and Hep3B cells in a time-dependent and dose-dependent manner, and treatment for 24, 48 and 72 h induced marked inhibition of cellular growth. The IC_{50} values (50% cell growth inhibitory concentration) at 72 h for human hepatoma cancer cells J5, HepG2, and Hep3B cells were 54.53 ± 0.63, 87.59 ± 1.42, and 35.90 ± 1.10 μM, respectively (Table 1). As compared to J5 and HepG2 cells, hispolon seemed to have a stronger death effect toward Hep3B liver cancer cells. The results indicate that hispolon was more cytotoxic to Hep3B cells.

Effects of Hispolon on Nuclear DNA Fragmentation of Hep3B Cells. We assessed the effect of hispolon on the induction of apoptosis in Hep3B cells by DNA fragmentation assay. Hep3B cells treated with 22.5, 45, and 66.5 μM hispolon for 48 h, or treated with 45 μM for 24, 48 and 72 h showed that hispolon treatment resulted in the formation of DNA fragments. Nucleosomal DNA fragmentation was observed in cells treated with 45 μM of hispolon for 0, 24, 48, and 72 h or treated with 22.5, 45, and 66.5 μM hispolon for 48 h (Fig. 2A). The profile for hispolon-induced apoptosis closely correlated with its growth suppressive effect. Thus, growth suppression induced by hispolon in Hep3B cells may be related to the induction of apoptosis.

Effects of Hispolon on Phenotypic Changes in Cell Nucleus. This study further elucidated whether hispolon also induces DNA fragmentation and chromatin condensation in Hep3B cells. Treatment with hispolon resulted in changes in nuclear morphology, as demonstrated by DAPI staining. Condensation and fragmentation were seen in cells 24, 48, and 72 h after 45 μM of hispolon treatment (Fig. 2B). The phenotypic characteristic of hispolon-treated Hep3B cells was also evaluated by microscopic inspection of overall morphology. Apoptotic bodies were observed after Hep3B cells were treated with hispolon for 24 h. Based on the above data, DNA condensation and formation of apoptotic bodies indicated that hispolon-induced Hep3B cell death was a typical apoptotic cell death.

Effects of Hispolon on Cell Cycle Distribution. Induction of apoptosis has been reported to be a potentially promising approach for cancer therapy. Exhibition of the biological phenomena (cell cycle redistribution, DNA fragmentation, and chromatin condensation) represents the proceeding of apoptosis (*22*). The apoptotic effect of hispolon was confirmed by flow cytometric analysis. As shown in Fig. 3A, concomitant with the growth inhibitory effect, hispolon treatment induced a strong S-phase arrest in a time-dependent manner. When Hep3B cells were incubated with 45 μM of hispolon for 0, 6, 12, and 24 h, the relative percentage of cells staying at the

S phase were 17.31%, 43.95%, 70.98% and 70.89%, respectively (Fig. 3B). This increase in the S-phase cell population was accompanied by a concomitant decrease in the G0/G1 and G2/M phase cell populations. Meanwhile, the sub-G1 population was slightly increased in cells exposed to 45 μM hispolon. These results indicated that hispolon caused cell cycle arrest at the S phase, followed by apoptosis.

Effects of Hispolon on Cell Apoptosis. To further confirm and quantify the apoptosis of Hep3B cells triggered by hispolon, cells were stained with both Annexin V-FITC and PI, and subsequently analyzed by flow cytometry (*5*). Fig. 4 shows the annexin V-FITC/PI analysis of Hep3B cells cultured with 45 μM of hispolon for 0, 6, 12 and 24 h. Annexin V positive cells were considered as the relative amount of apoptoic cells. Early apoptotic cells appeared in the annexin V+/PI− fraction, whereas cells damaged by scraping appeared in the annexin V−/PI+ fraction, and late apoptosis or necrotic cells were evident in the annexin V+/PI+ fraction. After treatment with 45 μM of hispolon for 0, 6, 12, and 24 h, the corresponding quantities of necrosis and apoptosis were 1.2%, 5.4%, 14.4%, and 19.6%, respectively (Annexin V+/PI+ fraction).

Hispolon Induces Apoptosis via Intrinsically- and Extrinsically-Mediated

Pathways. The effects of hispolon on the protein expression of Fas, FasL, pro-caspase-8, and Bid in Hep3B cells are shown in Fig. 5A and 5B. Treatment of Hep3B cells with hispolon (45 μM) for 0, 3, 6, 12, and 24 h resulted in significant increases in the levels of Fas and FasL expression. Treatment with 45 μM hispolon for 24 h significantly decreased the expression levels of pro-caspase-8 and Bid by 48% and 56%, respectively, as compared to those of the control.

The effects of hispolon on the protein expression of the Bcl-2 family and cytosolic cytochrome *c* in Hep3B cells are shown in Fig. 6A and 6B. After treatment with 45 μM hispolon for 24 h, the level of pro-apoptotic protein expression of Bax was increased by 187.7%, in comparison to the control. Hispolon treatment at 45 μM for 24 h significantly decreased the level of Bcl-2 (antiapoptotic protein) expression by 38% in comparison with the control. Cytochrome *c* release in the cytosolic fraction following hispolon treatment was then investigated. Treatment with hispolon (45 μM, 24 h) resulted in a significant increase in the level of cytosolic cytochrome *c* expression by 177%, as comparison to the control. A significant time-dependent shift in the ratio of Bax to Bcl-2 was observed after hispolon treatment at 45 μM for 0-24 h (Fig. 6B).

The effects of hispolon on the protein expression of pro-caspase-3, caspase-9, and poly (ADP-ribose) polymerase (PARP) in Hep3B cells are shown in Fig.7A and

7B. The results show that exposure of Hep3B cells to hispolon (45 μM, 24 h) caused the degradation of pro-caspase-3 and caspase-9, which generated a fragment of caspase-9 and caspase-3. Hispolon treatment at 45 μM for 24 h significantly increased the level of expression of cleaved PARP by 138%, as comparison to the control. The results indicate that hispolon treatment causes a significant increase in the activity of caspase-9 and caspase-3 and hispolon may have acted through initiator caspase-8 and then executioner caspase-3 to increase the cleavage form of PARP.

Effects of Hispolon on the Expression of Cell Cycle Regulators Involved in S-Phase Arrest. As shown by immunoblot analysis in Fig. 8A, hispolon (45 μM, 24 h) treatment caused a time-dependent decrease in the expression levels of cell cycle regulators including cyclin A, cyclin E and cyclin-dependent kinases CDK 2, which may contribute to the cell cycle progression from G0/G1 to S-phase. Hispolon treatment at 45 μM for 24 h significantly decreased the level of expression of cyclin A, cyclin E, and CDK 2 by 48.3%, 61.2% and 42.2%, respectively, in comparison with the control. Binding of cyclins to CDKs would form active kinase complexes, which are regulated and inhibited by various CKDIs and growth suppressor genes such as p21waf1/Cip1 and p27Kip1. As shown by immunoblot analysis in Fig. 8A, the expression levels of p21waf1/Cip1 and p27Kip1 were up-regulated in a

300 time-dependent manner by hispolon treatment. Hispolon treatment at 45 μM for 24 h
301 significantly increased the level of expression of p21waf1/Cip1 and p27Kip1 by 156%
302 and 144% in comparison with the control.
303
304 **Effects of Hispolon on MAPK Signaling Pathway.** Studies have shown that the
305 MAPK signaling pathway plays an important role in the action of chemotherapeutic
306 drugs (*23*). Therefore, we determined whether the MAPKs were activated in
307 hispolon-treated Hep3B cells by Western blot analysis using specific antibodies
308 against the phosphorylated (activated) forms of the kinases. It was found that hispolon
309 treatment induced differential phosphorylation of JNK, ERK, and p38 MAPK in cells
310 exposed to 45 μM hispolon (Fig. 9A and 9B). Phosphorylation of ERK was detected
311 as a sustained activation from 0 to 24 h, which decreased thereafter and reached the
312 control level at 24 h. Activation of p38 by hispolon was also observed as early as 3 h
313 after hispolon treatment, which peaked at approximately 24 h. A time course study
314 showed that JNK activation displayed a rapid onset after 3h of treatment, followed by
315 a progressive decline, returning to the basal level after 24 h.
316 To study the role of MAPK activation in hispolon-induced growth inhibition, we
317 examined the effects of specific MAPK inhibitors on overall cell death. The results of
318 the MTT assay showed that pretreatment with SP600125 (a JNK inhibitor) or

SB203580 (a p38 inhibitor) had no effect on hispolon-induced cell death (Fig. 9C), although these inhibitors reduced the phosphorylation of their target kinases.

The results suggested that JNK and p38 did not play important roles in regulating cell death in Hep3B cells induced by hispolon. However, pretreatment with PD98059 (an ERK inhibitor) significantly decreased the extent of cell death induced by hispolon (Fig. 9C). Only the ERK inhibitor PD98059 significantly blocked hispolon-mediated cell death. These contradictory results imply that the drug actions of hispolon indeed result from the complex interaction of many compounds and many targeted molecules. These results suggested that activation of the ERK pathway was involved in the apoptotic cell death of Hep3B cells induced by hispolon.

Discussion

In the present study, we investigated the apoptosis of human hepatocellular carcinoma cells induced by hispolon. Our data revealed that hispolon, a phenol compound, acts directly on human hepatocellular carcinoma cancer cells to induce cytotoxicity in a manner that causes apoptosis (Table 1). In breast and bladder cancer cells, Lu et al. have reported that hispolon treatment for 72 h inhibits the cell viability at IC_{50} values ranging from 20 μM-40 μM (*24*) as well as inhibits the growth of

human gastric cancer cells cells in a dose- and time-dependent manner, with an IC_{50} of 30 μM at 72 h of incubation (*25*).

A flow cytometic analysis of PI -labeled cells shows that treating Hep3B cells with hispolon (45 μM) induced significant accumulation of cells in the S phase (Fig. 3A). The ratio of G_0/G_1 to S to G_2/M phase in Hep3B cells at 0, 6, 12, and 24 h varied significantly in the presence of 45 μM (Fig. 3B). The G_0/G_1 cell population increased to 70.89% in Hep3B cells treated with 45 μM of hispolon for 24 h. Moreover, a characteristic hypodiploid DNA content peak (sub-G_1) was easily detected after treatment with hispolon 45 μM for 0, 6, 12, and 24 h. A significant increase in sub-G_1 phase is indicative of induction of apoptosis. Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired mutations in genes that directly regulate their cell cycle (*26-27*).

Inhibition of deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth (*28*). Cyclins, CDKs, and CDKIs play essential roles in the regulation of cell cycle progression. CDKIs, such as p21waf1/Cip1 and p27Kip1, are tumor suppressor proteins that downregulate the cell cycle progression by binding with active cyclin-CDK complexes and thereby inhibiting their activities (*29*). Chemopreventive agents usually cause apoptosis or cell cycle arrest at the G0/G1 or G2/M phases. Relatively little is known about mechanisms that control progress

within the S-phase. It has been reported that hispolon elicits cell-cycle arrest at G2-M phases in human breast and bladder cancer cells through the induction of CDKIs and the inhibition of cyclins and CDKs (*24*). Although these results offer much insight for the cell cycle arrest action of hispolon, the detailed molecular mechanisms remain to be clarified. It has been reported that S-phase cell cycle arrest occurs with the loss of Cdk2 activity due to reduced formation of active complex cyclin E/Cdk2 kinase (*30*). We demonstrate here that hispolon-induced cell-cycle arrest was accompanied by down-regulating the protein levels of cyclin A, cyclin E, and CDK2 and up-regulation of p21 and p27 in Hep3B cells. It has been reported that S phase cell-cycle arrest occurs with the loss of Cdk2 activity due to up-regulation of p21 and reduced formation of active complex cyclin E/Cdk2 kinase (*23*). Our findings that hispolon down-regulated cyclin A, cyclin E and CDK2 but up-regulated p21 and p27 suggest that S-phase arrest is responsible for the cell-cycle-arresting effect of hispolon in Hep3B cells.

Hispolon-induced apoptosis in Hep3B cells was also indicated by DNA laddering (Fig. 2A) and DAPI positive staining (Fig. 2B). The induction of apoptosis stimulates endonucleases, which catalyze the breakage of double-stranded DNA to form fragments with oligonucleosome-length, resulting in a typical DNA electrophoresis ladder that signifies apoptotic cell death (*26*). The apoptosis-inducing effect of

hispolon on Hep3B cells appeared to be directly proportional to its concentration. In addition, the apoptosis-inducing efficacy of hispolon was found to be similar to its anti-porliferative activity toward Hep3B cells. Furthermore, using annexin V−FITC to identify apoptotic cells by binding to phosphatidyl serine and a red-fluorescent PI to bind to nucleic acids of necrotic cells, the present study further demonstrated that hispolon induced a significant and dose-dependent increase of annexin V^+/PI^+ apoptotic cells (Fig. 4).

Caspases are believed to play crucial roles in mediating various apoptotic responses. A model involving two different caspases (caspase-8 and -9) in the mediation of distinct types of apoptotic stimuli has been proposed (*31*). The cascade led by caspase-8 is involved in death receptor-mediated apoptosis such as the one triggered by Fas. Ligation of Fas by Fas ligand results in sequential recruitment of FADD (Fas-associated death domain) and procaspase-8 to the death domain of Fas to form the death-inducing signaling complex, leading to cleavage of procaspase-8, with the consequent generation of active caspase-8. Active caspase-8 in turn activates downstream effecter caspases through the cleavage of Bid, committing the cell to apoptosis (*27*). The present results suggest that hispolon may act through the initiator caspase-8 and then the executioner caspase-3 to increase the cleavage form of PARP for DNA fragmentation (Fig. 5A and 7A).

Many reports have pointed out that the ability of anticancer agents in inducing apoptosis of tumor cells (such as Taxol) correlates with the ability of decreasing the expression of Bcl-2 (*32*). In the present study, we showed that the expression of Bcl-2 decreased as the concentration of hispolon and the percentage of apoptotic Hep3B cells increased. This inverse proportional relationship suggested that Bcl-2 may play a preventive role in hispolon-mediated apoptosis of Hep3B cells. Mitochondrial-dependent apoptosis is often through the activation of a pro-apoptotic factor in the Bcl-2 family. Thus, one possible role of Bcl-2 in the prevention of apoptosis is to block the release of cytochrome *c* from mitochondria (Fig. 6A). On the contrary, increases in the expression of Bax and cytochrome *c* release were observed during hispolon treatment in the present study. Bax is a pro-apoptotic protein that has also been shown to induce cytochrome *c* release and caspase activation recently (*33*). The above findings suggest that hispolon induces apoptosis in Hep3B cells through a mitochondria-mediated pathway.

Several protein kinase pathways have been known to regulate cell proliferation and survival. MAPKs, a family of serine-threonine protein kinases, have been implicated in apoptosis and cell cycle regulation signaling in diverse cell models (*23*). In general, JNK and p38 are activated by diverse stimuli such as oxidative stress, UV irradiation, and osmotic shock and required for the induction of apoptosis. ERK plays

414 vital roles in cell growth and division and is generally considered to be a survival
415 mediator (*34*). In human HCC cell lines, multiple anticancer effects such as inhibition
416 of cellular proliferation as well as induction of cell cycle arrest and apoptosis have
417 been achieved by blocking ERK signaling (*35*). ERK inactivation observed in this
418 study may contribute to the S phase cell cycle arresting and apoptotic activities of
419 hispolon, which need to be investigated further. In addition, different MAPK
420 signaling pathways can be coordinately manipulated to enhance the efficacy of
421 anticancer drug. Cotreatment of anticancer drugs with ERK inhibitors has been found
422 to enhance anticancer effects. In our experiments, as shown in Fig. 9A, hispolon
423 markedly elevated the phosphorylated forms of JNK and p38 and reduced the
424 phosphorylated form of ERK1/2 in a dose dependent manner. Therefore,
425 hispolon-induced apoptosis in Hep3B involves mitochondria caspase pathways,
426 activation of JNK and P38 and inhibition of the ERK MAPK signaling. The use of
427 specific inhibitors revealed that JNK and p38 did not play important roles in
428 regulating cell death induced by hispolon in Hep3B cells. In this study, the MTT
429 method was used to examine the effects of specific MAPK inhibitors, as the same
430 method has often been used to examine the effects of specific MAPK inhibitors (*23*,
431 *36*, *37*). In our previous reports, we also used the same approach to show that hispolon
432 modulates ERK phosphorylation (*20*, *23*).

Our observations that hispolon induced S-phase arrest and p21 overexpression are in agreement with those of a previous report which shows that the transduction of the p21 gene results in S-phase arrest (*40*). P21, an inhibitor of CDKs, directly inhibits CDK2, CDK3, CDK4, and CDK6 activity. Overexpression of p21 usually leads to G_1 or G_2 arrest by inhibiting CDK activity. P21 can also directly inhibit DNA synthesis by binding to proliferating cell nuclear antigen (PCNA) (*41*). The expression of p21 can be regulated at the transcriptional, post-transcriptional, or post-translational levels by p53-dependent and -independent mechanisms (*5*). Indeed, we found that suppression of ERK activation attenuated hispolon-mediated induction of p21 expression and S-phase arrest. Although ERK and p21 are likely to play a role in hispolon-mediated S-phase arrest, it is possible that some other molecules that were not examined here may also be involved in hispolon-mediated S-phase arrest.

In conclusion, this study has provided mechanistic insights into how hispolon regulates the components of cell cycle progression and apoptotic machinery to delay S to G2/M transition and induces apoptosis in Hep3B cells. Our data imply the potential of hispolon as a chemotherapeutic agent because many antbicancer drugs are known to achieve their anticancer function by inducing apoptosis and/or cell cycle arrest in susceptible cells.

ABBREVIATIONS USED

FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; ERK, extracellular signaling-regulating kinase; JNK/SAPK, c-Jun N-terminal kinase/ stress-activated protein kinase;

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