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The anticancer effects of hispolon on lung cancer cells

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ABSTRACT

Hispolon is isolated from *Phellinus igniarius* and exhibits antitumor activity. Here, we explored the effects of hispolon on the lung cancer A549 and H661 cells. Cells were incubated with various concentrations of hispolon (0, 5, 10, 20, 40, 80 or 160 μM) for 12, 24, 48 or 72 h. Cell viability was examined by MTT assay. Cell cycle and apoptosis assay were assessed by flow cytometry. Hispolon decreased cell viability in a dose- and time-dependent manner. The cell cycle distribution showed that hispolon enhanced the accumulations of the cells in G0/G1 phase. Mechanically, hispolon decreased the expression of G1-S transition-related proteins: Cyclin D1, cyclin E, CDK2, CDK4 and CDK6, but increased the expression of CDK inhibitor $p21^{CIP1}$ and $p27^{KIP1}$. Moreover, hispolon induced cell apoptosis through activation of the mitochondrial pathway, evidenced by the loss of mitochondrial membrane potential, the release of cytochrome c into cytosol, and the cleavage of caspase-9, caspase-3 and poly (ADP-ribose) polymerase (PARP) in hispolon-treated cells. Additionally, hispolon enhanced the expression of p53, specific silencing of which almost completely reversed hispolon-mediated antitumor activity. Moreover, hispolon treatment was more effective on H661 cells than on A549 cells in inhibiting cell viability and inducing cell apoptosis. Our results indicate that hispolon inhibits the cell viability, induces G0/G1 cell cycle arrest and apoptosis in lung cancer cells and p53 plays a critical role in hispolon-mediated antitumor activity.

1. Introduction

Lung cancer is a malignant tumor and accounts for approximately 28% of all cancer death [1,2]. Approximately 75% lung cancer is non-small cell lung cancer (NSCLC), which is with high occurrence and a low five-year survival rate of ~15% [2]. Adenocarcinoma and squamous cell carcinoma are the two most common histological subtypes of NSCLC [3,4]. Poor prognosis and chemotherapeutic resistance of NSCLC may be due to the modulation of key cell signaling pathways [3,4]. Many previous studies have focused on the molecular mechanisms underlying lung cancer initiation and progression [1–4]. Apoptosis defect is a hallmark of cancer and is implicated in lung tumorigenesis and drug resistance [1]. Thus, induction of cell apoptosis provides a strategy for cancer treatment.

Hispolon (6-(3,4-dihydroxy-phenyl)-4-hydroxy-hexa-3,5-dien-2-one; $C_{12}H_{12}O_4$), a yellow pigment, is isolated from the *Phellinus igniarius* [5]. Hispolon has hepatoprotective [6], anti-inflammatory [7], antimetastatic [8], and antiproliferative effects [9]. The antitumor activity of this phenol compound (hispolon) has attracted many attentions [8–12]. It has been shown that hispolon exhibits anticancer effects through inhibition of cell growth, induction of cell cycle arrest, and suppression of metastasis in various types of cancer cells [8–12]. However, there were no reports about the effects of hispolon on NSCLC cells. In the present study, we explored the antitumor effects of hispolon on A549 and H661 cells, two human non-small cell lung carcinoma cell lines.

2. Material and methods

2.1. Cell culture

LKB1-defective A549 cells and LKB1-wild type H661 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA).

2.2. Cell viability assays

1×10^4 cells were seeded in 96-well plates and allowed to adhere for 12 h. The cultures were exposed to various concentrations of hispolon (Enzo Life Sciences, Inc., Farmingdale, NY, USA; 0, 5, 10, 20, 40, 80 and 160 μM) or 0.1% dimethyl sulfoxide (DMSO),

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the vehicle control, for 12, 24, 48 and 72 h, followed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 0.5 mg/ml MTT (Sigma–Aldrich, St. Louis, MO, USA) in 100 μl of RPMI1640 medium was added to the cultured cells for 2 h. Cells were washed by phosphated buffered saline (PBS) and lysed by 50 μl of DMSO. The concentrations of MTT were examined colorimetrically. Absorbance was determined at 570 nm.

2.3. Cell cycle analysis

Serum starvation was conducted for 18 h to induce cell quiescence of lung cancer cells. Cells were cultured in fresh complete RMPI 1640 medium at the concentration of 5×10^5 cells/ml and treated with various concentrations of hispolon (0, 5, 10, 20 and 40 μM) or 0.1% DMSO for 24 h. Cells were harvested by trypsinization, washed by PBS, and fixed in 70% ethanol at −20 °C overnight. Fixed cells were washed by PBS, resuspended in 1 ml of PBS containing 1 mg/ml RNase and 50 μg/ml of propidium iodide (Sigma), and incubated for 30 min in the dark at room temperature. The cell cycle distribution of living cells was measured with Cytomic FC 500 (Beckman Coulter, Pasadena, USA).

2.4. Apoptosis assay

Cells were treated with different concentrations of hispolon (0, 5, 10, 20 and 40 μM) or 0.1% DMSO for 24 h. Cells were collected for apoptosis assay with Annexin V-FITC apoptosis detection kit (BD Biosciences Clontech, CA). After centrifugation at 1000 rpm for 5 min, the pellet was resuspended in 100 μl of 1× binding buffer with 2.5 μl Annexin V and 5 μl PI (final concentration, 10 μg/ml). The reaction complex was incubated for 15 min in the dark. Samples were subjected to apoptosis assay by flow cytometry.

2.5. JC-1 assay

Cells were treated with different concentrations of hispolon (0, 5, 10, 20 and 40 μM) or 0.1% DMSO for 24 h. Cells were washed with PBS, resuspended in 1 ml of PBS buffer at the concentration of 1×10^6 cells/ml, and incubated with 5,5′,6,6′-Tetrachloro-1, 1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (final concentration, 2.5 mg/ml) (Life Technologies, Grand Island, NY, USA) for 10 min at 37 °C. The stained cells were washed with PBS and analyzed immediately with Cytomic FC 500 (Beckman Coulter). Cellular with high positive red fluorescence was quantified. Cells with collapsed mitochondrial membrane potential exhibit a decrease in the red/green fluorescence intensity ratio.

2.6. Cell transfection

80 nM of p53 siRNA (GenBank number: NM_000546; sequence: forward, 5′-GACUCCAGUGGUAAUCUACTT-3′; reverse, 5′-GUAGAUUACCACUGGAGUCTT-3′) was used to transfected A549 or H661 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. After 24 h of incubation, a fresh RMPI 1640 complete medium containing 40 μM of hispolon or 0.1% DMSO was added to the transfected cells and incubated with cells for another 24 h. Non-targeted control siRNA was used as a negative control.

2.7. Western blot analysis

A549 or H661 cells were treated with various concentrations of hispolon (0, 5, 10, 20 and 40 μM) or 0.1% DMSO for 24 h. Cells were harvested by trypsinization, washed by cold PBS. Total proteins and cytoplasmic proteins were prepared by ReadyPrep Protein Extraction Kit (Total Protein) and ReadyPrep Protein Extraction Kit (cytoplasmic/nuclear) (Bio-Rad laboratories, Inc., USA). The protein concentrations were detected by Bio-Rad Protein Assay (Bio-Rad). Equal amounts of proteins (20 μg) were electrophoresed for 2 h on 10% sodium dodecyl sulfatepolyacrylamide gels and then transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Membranes were blocked in PBS with 0.1% Tween 20 (PBST) containing 5% non-fat dried milk power for 1 h. Immunoblots were probed with antibodies specific to cyclin E (Cell Signaling Technology, Beverly, MA, USA), cyclin D1 (Cell Signaling Technology), cyclin-dependent kinase 4 (CDK4) (Cell Signaling Technology), CDK2 (Cell Signaling Technology), CDK6 (Cell Signaling Technology), $p21^{CIP1}$ (Cell Signaling Technology), $p27^{KIP1}$ (Cell Signaling Technology), p53 (Cell Signaling Technology), cleaved caspase-9 (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), cleaved poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology). Signals were visualized by an enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ, USA). β-Actin was used as an internal control.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). Statistical analysis of the results was performed by one way analysis of variance (ANOVA). $P < 0.05$ was considered as statistically different.

3. Results

3.1. Hispolon inhibits the growth of A549 and H661 cells

To determine the effect of hispolon on the cell viability of non-small cell lung cancer cells, A549 or H661 cells were treated with various concentrations of hispolon (0, 5, 10, 20, 40, 80 and 160 μM) for different time periods (12, 24, 48 and 72 h). Cells with 0.1% DMSO were served as a control. The MTT assay showed that hispolon inhibited the growth of A549 or H661 cells in a time- and dose-dependent manner (Fig. 1). Hispolon did not modify cell viability compared to DMSO control group after 12 h treatment in A549 cells ($P > 0.05$), but ⩾ 40 μM of hispolon profoundly inhibited the proliferation of H661 cells ($P < 0.05$). At 24 h, 20 μM of hispolon started to decrease the viability of A549 cells ($P < 0.05$, $n = 3$), but 10 μM of hispolon for H661 cells ($P < 0.01$, $n = 3$). At 48 h, all of the concentrations of hispolon (5–160 μM) markedly suppressed the cell viability of H661 cells ($P < 0.01$, $n = 3$), but not 5μM of hispolon for A549 cells ($P > 0.05$). The half maximal inhibitory concentration (IC_{50}) of hispolon on A549 cells were about 35.9 ± 6.9, 28.8 ± 3.1 and 8.1 ± 2.3 μM for 24, 48 and 72 h, respectively (Fig. 1A). However, the IC_{50} of hispolon on H661 cells were about 14.7 ± 6.9, 3.7 ± 0.5, 2.1 ± 0.2 μM (Fig. 1B). Our results indicate that higher concentrations of hispolon and longer treatment durations induce more cell death of A549 and H661 cells. Moreover, H661 cells seem more sensitive to hispolon-induced cytotoxicity than A549 cells.

3.2. Hispolon induces G0/G1 cell cycle arrest in A549 and H661 cells

To explore how hispolon inhibits the proliferation of A549 and H661 cells, flow cytometry was performed to determine cell cycle distribution. Serum starvation was conducted to induce cell quiescence of lung cancer cells. After lung cancer cells were treated with 0, 5, 10, 20 and 40 μM of hispolon for 24 h, the percentage of A549 cells staying at the G0/G1 phase were 43.7 ± 3.8%,

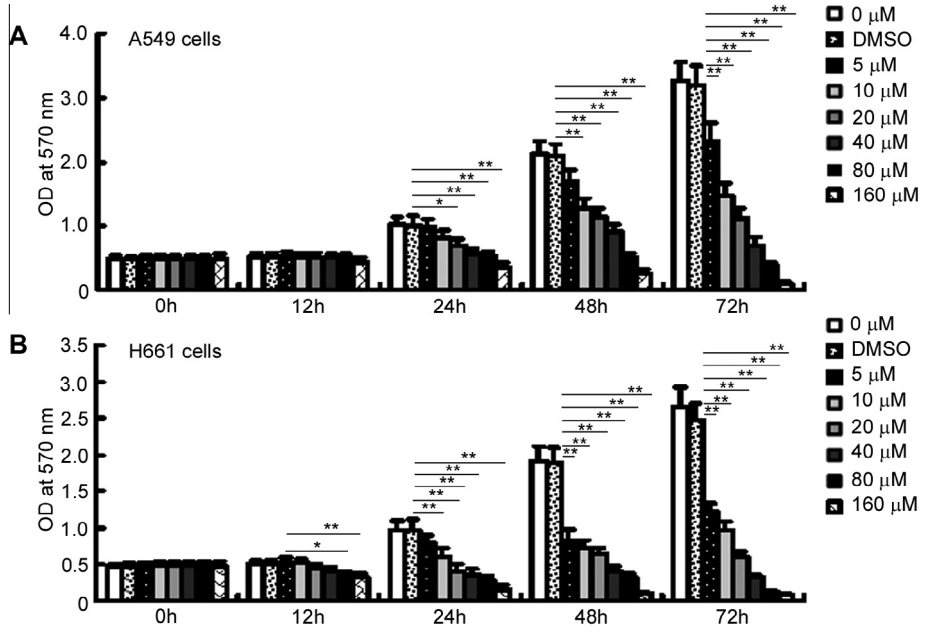

Fig. 1. The effects of hispolon on the cell viability of A549 and H661 cells. A549 (A) or H661 (B) cells were incubated with hispolon (0–160 μM) for 0, 12, 24, 48, and 72 h. Hispolon inhibited the cell viability of A549 and H661 cells in dose- and time-dependent manners in A549 and H661 cells, as determined by MTT assay. Absorbance was determined at 570 nm. Values are expressed as means ± SD of three representative experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding control values.

46.6 ± 3.9%, 55.8 ± 4.1%, 60.9 ± 4.9 and 66.8 ± 5.1%, respectively (Fig. 2A). ⩾10 μM of hispolon significantly increased the percentage of A549 cells in the G0/G1 phase ($P < 0.05$ for 10 μM; $P < 0.01$ for 20 and 40 μM, relative to DMSO control group, $n = 3$) (Fig. 2A). The increase of G0/G1 phase cell population was accompanied by a concomitant decrease in the S and G2/M phase cell populations (Fig. 2A). Similar effects of hispolon on H661 cells were observed in Fig. 2B. Our results indicated that hispolon induces cell cycle arrest at the G0/G1 phase.

To assess the molecular mechanism of hispolon-induced cell-cycle arrest in the G0/G1 phase, we investigated the expression levels of G1-S transition-related proteins in hispolon-treated A549 and H661 cells (Fig. 2C and D). Dose-dependent decreases of cyclin D1 and cyclin E were observed in both A549 and H661 cells with the treatment of hispolon, accompanied by a reduction in the expression of CDK4, CDK2 and CDK6 (Fig. 2C and D). However, the expressions of $p21^{CIP1}$ and $p27^{KIP1}$ proteins, CDK inhibitors [13], were dose-dependently increased in hispolon-treated A549 and H661 cells (Fig. 2C and D).

3.3. Hispolon induces apoptosis in A549 and H661 cells

To analyze the effects of hispolon on the apoptosis of A549 and H661 cells, Annexin V and PI double staining was performed on hispolon-treated A549 and H661 cells. Annexin V^{+}/PI^{-} cells were considered as the apoptotic cells. Hispolon induced a dose-dependent apoptosis of A549 and H661 cells. The apoptosis ratio was ranged from 3.16 ± 1.1% (DMSO group) to 29.06 ± 3.8% (40 μM of hispolon) for A549 cells, and from 3.08 ± 1.3% (DMSO group) to 69.06 ± 5.6% (40 μM of hispolon) for H661 cells (Fig. 3A and B). H661 cells are more sensitive than A549 cells to hispolon-induced apoptosis. To analyze the mechanism of hispolon-mediated apoptosis, the JC-1 assay was used to examine the mitochondrial membrane potential (ΔΨm). Fig. 3C and D showed that ΔΨm was dose-dependently disrupted by hispolon. Consistently, the mitochondrial release of cytochrome c into the cytosol in hispolon-treated cells was increased as compared to DMSO control cells (Fig. 3E and F). Our results indicate that hispolon induces apoptosis at the mitochondrial level. Furthermore, exposure to hispolon enhanced the protein levels of cleaved caspase-3, caspase-9 (key executioners of apoptosis) and cleaved PARP (Fig. 3G and H), indicating that hispolon-mediated lung cancer cell apoptosis is through the intrinsic pathway.

3.4. p53 plays an important role in hispolon-mediated antitumor effects on A549 cells and H661 cells

p53 has been demonstrated to be involved in the regulation of cell cycle and apoptosis by causing mitochondrial dysfunction [14,15]. Western blot analyses revealed that hispolon dose-dependently enhanced the expression of p53, suggesting that p53 is involved in hispolon-induced apoptosis of lung cancer cells (Fig. 4A and B). A p53-targeting siRNA (psiRNA) was used to knockdown the expression of endogenous p53 in lung cancer cells. Compared to the control siRNA (csiRNA), psiRNA resulted in more than 75% reduction in the expression of endogenous p53 (Fig. 4C and D). Moreover, psiRNA did not affect the cell viability of lung cancer cells without hispolon ($P > 0.05$, $n = 3$, Fig. 4C and D). Interestingly, psiRNA almost completely reversed hispolon-mediated suppression of cell viability (Fig. 4E and F), revealing that hispolon-mediated proliferation inhibition effect is dependent on p53. Similarly, psiRNA did not modify the G0/G1 phase population in lung cancer cells without hispolon treatment ($P > 0.05$, $n = 3$, Fig. 4G and H), and significantly attenuated hispolon-induced G0/G1 cell cycle arrest. Additionally, p53 silencing reduced hispolon-induced lung cancer cell apoptosis (Fig. 4I and J).

4. Discussion

Here, we found that hispolon exhibited anticancer effects on *in vitro* cultured lung cancer cells. The underlying mechanisms may be associated with decreased expression of G1-S transition-related proteins, enhanced expression of CDK inhibitors $p21^{CIP1}$ and $P27^{KIP1}$, and the activation of mitochondrial-mediated

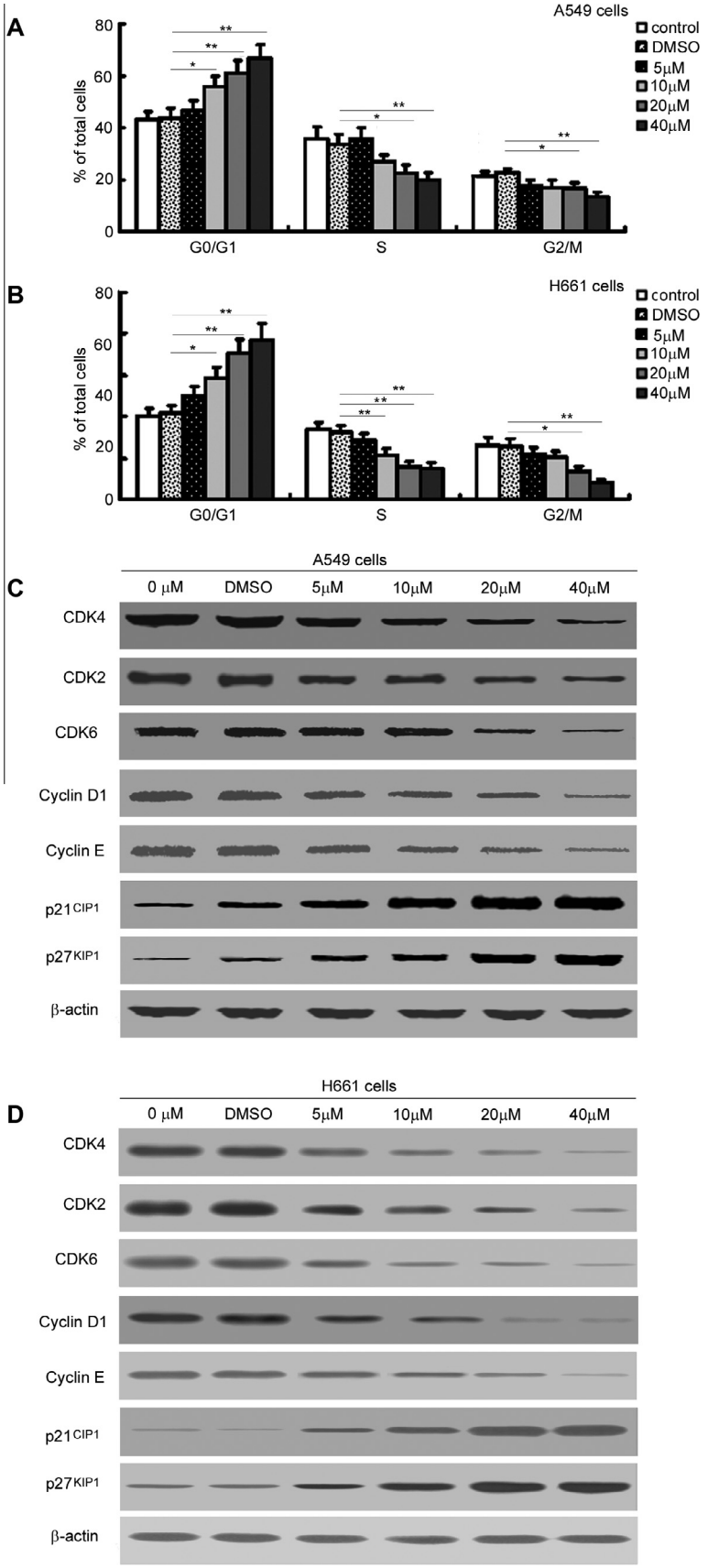

Fig. 2. Hispolon induced cell cycle arrest in the G0/G1 phase in A549 and H661 cells. A549 or H661 cells were treated with different concentrations of hispolon (0–40 μM) for 24 h. Quantitative analysis of the cell cycle distribution of A549 (A) and H661 (B) cells. Expression of cyclin E, cyclin D1, CDK4, CDK2, CDK6, $p21^{CIP1}$, and $p27^{KIP1}$ in A549 (C) and H661 (D) cells. Each bar represents means ± SD of three representative experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding control values.

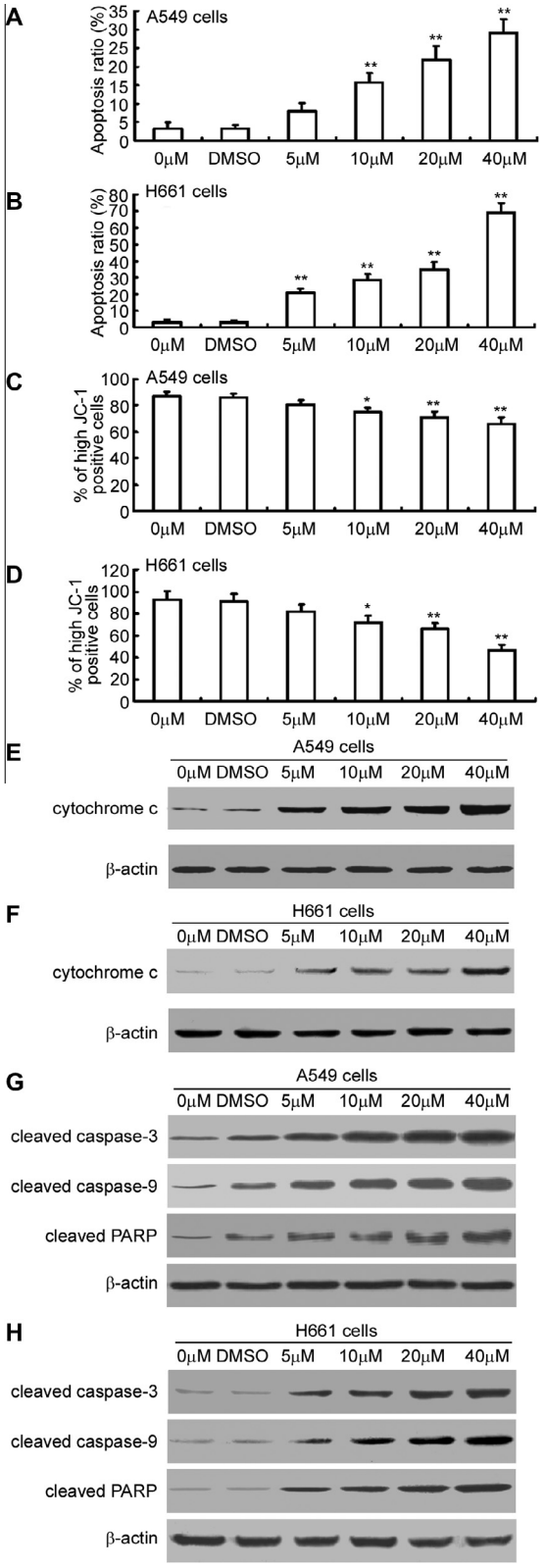

Fig. 3. Effects of hispolon on the apoptosis of A549 and H661 cells. A549 or H661 cells were treated with various concentrations of hispolon (0–40 μM) for 24 h. The apoptosis ratio in A549 cells (A) and H661 cells (B) was analyzed by Annexin-V/PI double staining assay. (C) and (D) Quantitative analysis of the mitochondrial membrane potential with JC-1 assay. (E) and (F) Effects of hispolon on the expression of apoptosis-related proteins in A549 and H661 cells by Western blot analysis. Representative experiments were carried out at least three times. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. related control values.

apoptosis. Additionally, hispolon increased the expression of p53. Specific silencing of p53 abrogated the anticancer effects of hispolon, indicating that p53 plays an important role in hispolon-mediated anti-cancer effects.

Hispolon has been shown antiproliferative effects in several tumor cells [9,16–19]. Previously, hispolon has been found to inhibit the proliferation of estrogen-sensitive ER (+) MCF-7 cells and estrogen-negative ER (−) MDA-MB-231 cells [16], decrease the cell viability of human hepatocellular carcinoma Hep3B cells [9], and reduce the growth of human epidermoid KB cells [19]. Here, we analyzed the effects of hispolon on NSCLC A549 and H661 cell proliferation. We found that hispolon had cell cytotoxicity on A549 and H661 cells. Our results indicate that hispolon might be a novel strategy for lung cancer treatment.

Several studies have demonstrated that hispolon blocks cell cycle progression at the G0/G1 [12,20] or G2/M [21] phase, which depends on the cell context. Similarly, hispolon inhibits cell cycle progression and induces cell cycle arrest in G0/G1 phase in A549 and H661 cells. The cell cycle progression is driven by protein kinase complexes composed of a cyclin and a CDK [22]. During G1-phase progression, the complexes cyclin D-CDK4, cyclin D-CDK6, and cyclin E-CDK2 are activated and move the cell cycle from the G1 phase to the S phase [22]. In the present study, we found that hispolon inhibited the expression of cyclin D1, cyclin E, CDK4, CDK6, and CDK2, indicating that hispolon induces G0/G1 cell cycle arrest through reducing the expression of cyclin-CDK complexes. CDK activity is modulated negatively by CDK inhibitors, including $p21^{CIP1}$ and $p27^{KIP1}$ [15]. To further explore the mechanism of hispolon-induced cell cycle arrest, the expression of $p21^{CIP1}$ and $p27^{KIP1}$ proteins was analyzed by Western blot analysis. Consistently, hispolon increased the expression of $p21^{CIP1}$ and $p27^{KIP1}$. Our results reveal that hispolon-mediated G0/G1 cell cycle arrest might be associated with the downregulation of cyclin D1, cyclin E, CDK4, CDK6, CDK2, and the upregulation of $p21^{CIP1}$ and $p27^{KIP1}$.

Apoptosis is a programmed cellular process that occurs in physiological and pathological conditions [23]. However, the programmed cell death is disrupted in cancer, which leads to the overgrowth of malignant cells [24]. Induction of tumor cell apoptosis is the final goal of many cancer therapies [25]. Apoptosis is triggered through two signaling pathways: death receptor-mediated extrinsic and mitochondria-mediated intrinsic pathways [25]. The intrinsic apoptotic pathway is characterized by the loss of mitochondrial membrane potential and the release of cytochrome c from mitochondria into the cytoplasm, which lead to the activation of caspase-9. The extrinsic apoptotic pathway is activated by death receptors on the plasma membrane, such as tumor necrosis factor receptor 1 (TNFR1) [25]. Both pathways finally lead to the activation of the executioner caspase-3 and the cleavage of poly (-ADP-ribose) polymerase PARP, a specific substrate for caspase-3 [25]. Here, we found that hispolon enhanced the apoptosis ratio of A549 and H661 cells, indicating a novel strategy for lung cancer treatment. To investigate the pathway(s) involved in hispolon-induced apoptosis of A549 and H661 cells, JC-1 assay was performed to analyze the mitochondria membrane potential. We found that hispolon dose-dependently enhanced the loss of mitochondrial membrane potential, suggesting that hispolon induces A549 and H661 cell apoptosis through intrinsic apoptotic pathway. The collapse of mitochondria membrane potential promotes the release of cytochrome c into the cytoplasm [25]. Western blot analysis showed that the expression of cytochrome c in the cytoplasm was enhanced in hispolon-treated A549 and H661 cells. Furthermore, hispolon increased the expression of cleaved caspase-9, caspase-3 and PARP. These results suggested that hispolon induces A549 and H661 cell apoptosis through intrinsic mitochondrial apoptotic pathway.

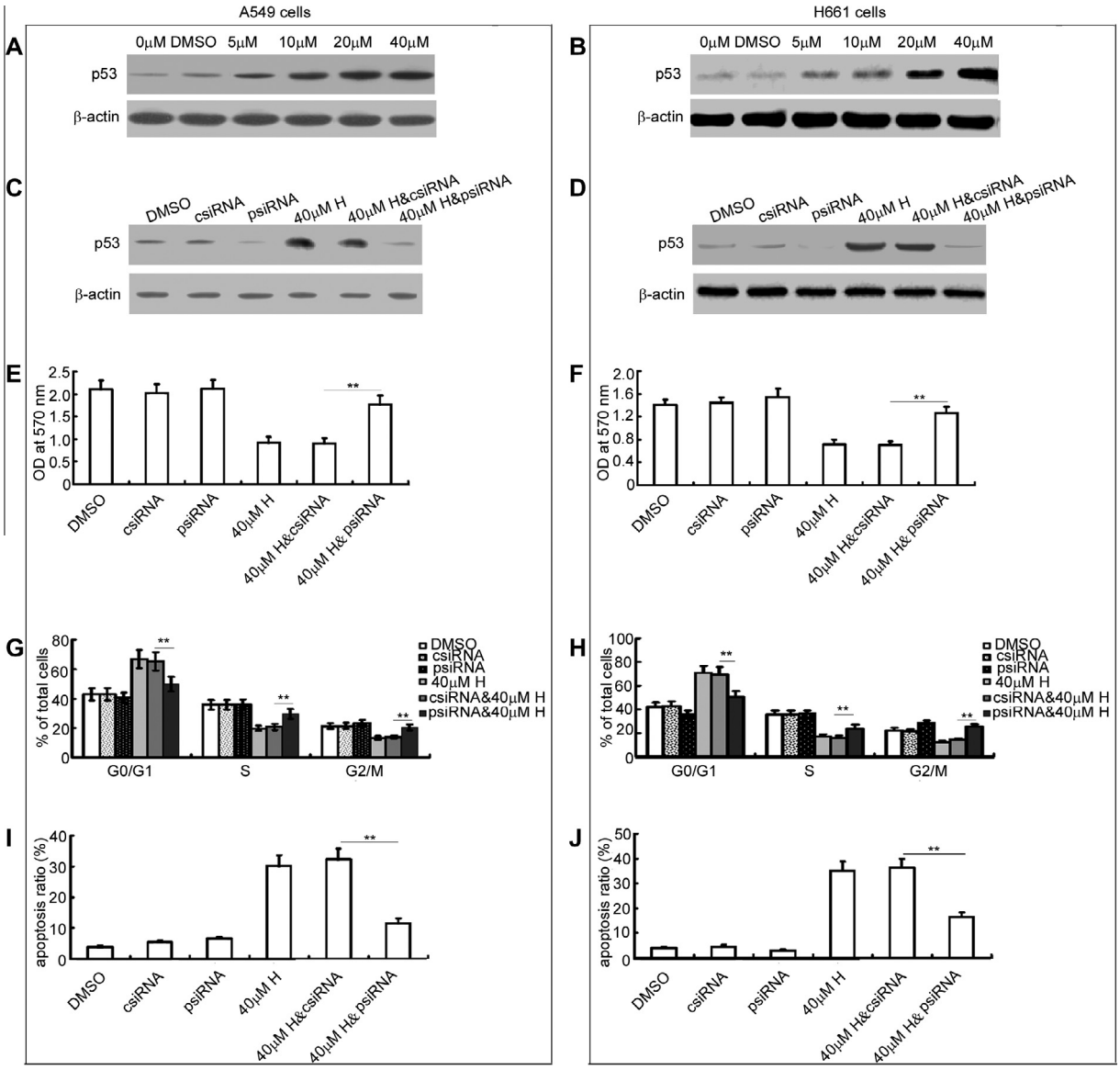

Fig. 4. The involvement of p53 in hispolon-mediated effects on A549 and H661 cells. A549 or H661 cells were treated with hispolon (0–40 μM) for 24 h. Western blot analysis was conducted to examine the expression of p53 in hispolon-treated A549 (A) and H661 (B) cells. (B-J) A549 or H661 cells were transfected with p53-specific siRNA and then incubated with various concentrations of hispolon for 24 h. (C) and (D) the expression of p53 in A549 and H661 cells transfected with p53 siRNA. (E) and (F) MTT assay was performed to analyze the effects of p53 silencing on hispolon-induced cytotoxicity on A549 and H661 cells. (G) and (H) The cell cycle distribution of A549 and H661 cells transfected with p53 siRNA with or without hispolon treatment. (I) and (J) The apoptosis ratio of A549 and H661 cells transfected with p53 siRNA with or without hispolon treatment. Representative experiments were carried out at least three times. Data are presented as mean ± SD. $^{**}P < 0.01$ vs. related control values.

In response to a variety of stresses, p53, a transcription factor, either induce apoptosis (cell death) or cell cycle arrest (cell preservation) to inhibit tumor development [26]. We found that hispolon enhanced the expression of p21, which is tightly controlled by p53 [27]. The expression of p53 was analyzed in hispolon-stimulated A549 and H661 cells. Consistent with the upregulation of p21 by hispolon, the expression of p53 was also elevated by hispolon, indicating the involvement of p53 in hispolon-mediated effects on A549 and H661 cells. Specific silencing of p53 by p53 siRNA significantly attenuated hispolon-induced cell cycle arrest in A549 and H661 cells, indicating that hispolon-mediated G0/G1 cell cycle arrest is p53-dependent. Furthermore, p53 has been suggested to trigger mitochondrial apoptosis [28]. Interestingly, hispolon-mediated loss of mitochondrial membrane potential was inhibited by silencing of p53, suggesting that hispolon-induced apoptosis dependents on p53. The important role of p53 in hispolon-mediated cell cycle arrest and apoptosis might be used to explain why p53 knockdown decreases the cytotoxicity of hispolon on A549 and H661 cells. Thus, p53 plays a critical role in hispolon-mediated effects on A549 and H661 cells.

In the present study, we found that H661 cells are more sensitive to hispolon-induced cytotoxicity and apoptosis. H661 cells are LKB1-wild type cells, while A549 cells are LKB1-defective cells. LKB1 possesses a variety of potentially tumor-suppressive activities, such as suppression of mammalian target rapamycin, regulation of cell polarity, inhibition of the cell cycle, and activation of p53 [29]. It seems that LKB1 might be involved in hisplon-induced cytotoxicity in lung cancer cells. Further investigation should be performed to confirm this hypothesis.

In sum, hispolon shows anticancer effects in A549 and H661 cells through inhibition of cell proliferation and induction of cell cycle arrest and apoptosis. It seems that hispolon-mediated anticancer effects are dependent on p53.

References

[1] S.W. Han, J. Roman, Targeting apoptotic signaling pathways in human lung cancer, Curr. Cancer Drug Targets 10 (2010) 566–574.
[2] D.E. Midthun, J.R. Jett, Chemotherapy for advanced lung cancer. When to expect a response, Postgrad. Med. 101 (1997) 187–188 (191–2, 194 passim).
[3] R. Salgia, T. Hensing, N. Campbell, A.K. Salama, M. Maitland, P. Hoffman, V. Villaflor, E.E. Vokes, Personalized treatment of lung cancer, Semin. Oncol. 38 (2011) 274–283.
[4] L. West, S.J. Vidwans, N.P. Campbell, J. Shrager, G.R. Simon, R. Bueno, P.A. Dennis, G.A. Otterson, R. Salgia, A novel classification of lung cancer into molecular subtypes, PLoS One 7 (2012) e31906.
[5] P. Toopmuang, C. Khamchum, V. Punsuvon, Detection and confirmation of hispolon in the mushroom *Phellinus linteus*, ScienceAsia 40 (2014) 141–144.
[6] G.J. Huang, J.S. Deng, C.S. Chiu, J.C. Liao, W.T. Hsieh, M.J. Sheu, C.H. Wu, Hispolon protects against acute liver damage in the rat by inhibiting lipid peroxidation, proinflammatory cytokine, oxidative stress and down-regulating the expressions of iNOS, COX-2, and MMP-9, Evid. Based Complement. Alternat. Med. 2012 (2012) 480714.
[7] H.Y. Chang, M.J. Sheu, C.H. Yang, T.C. Lu, Y.S. Chang, W.H. Peng, S.S. Huang, G.J. Huang, Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice, Evid. Based Complement. Alternat. Med. 2011 (2011) 478246.
[8] G.J. Huang, C.M. Yang, Y.S. Chang, S. Amagaya, H.C. Wang, W.C. Hou, S.S. Huang, M.L. Hu, Hispolon suppresses SK-Hep1 human hepatoma cell metastasis by inhibiting matrix metalloproteinase-2/9 and urokinase-plasminogen activator through the PI3K/Akt and ERK signaling pathways, J. Agric. Food Chem. 58 (2010) 9468–9475.
[9] G.J. Huang, J.S. Deng, S.S. Huang, M.L. Hu, Hispolon induces apoptosis and cell cycle arrest of human hepatocellular carcinoma Hep3B cells by modulating ERK phosphorylation, J. Agric. Food Chem. 59 (2011) 7104–7113.
[10] W. Chen, Z. Zhao, L. Li, B. Wu, S.F. Chen, H. Zhou, Y. Wang, Y.Q. Li, Hispolon induces apoptosis in human gastric cancer cells through a ROS-mediated mitochondrial pathway, Free Radic. Biol. Med. 45 (2008) 60–72.
[11] P.C. Hsiao, Y.H. Hsieh, J.M. Chow, S.F. Yang, M. Hsiao, K.T. Hua, C.H. Lin, H.Y. Chen, M.H. Chien, Hispolon induces apoptosis through JNK1/2-mediated activation of a Caspase-8, -9, and -3-dependent pathway in Acute Myeloid Leukemia (AML) cells and inhibits AML Xenograft tumor growth in vivo, J. Agric. Food Chem. 61 (2013) 10063–10073.
[12] Y.C. Chen, H.Y. Chang, J.S. Deng, J.J. Chen, S.S. Huang, I.H. Lin, W.L. Kuo, W. Chao, G.J. Huang, Hispolon from *Phellinus linteus* induces G0/G1 cell cycle arrest and apoptosis in NB4 human leukaemia cells, Am. J. Chin. Med. 41 (2013) 1439–1457.
[13] R.S. Wong, Apoptosis in cancer: from pathogenesis to treatment, J. Exp. Clin. Cancer Res. 30 (2011) 87.
[14] K. Hastak, M.K. Agarwal, H. Mukhtar, M.L. Agarwal, Ablation of either p21 or Bax prevents p53-dependent apoptosis induced by green tea polyphenol epigallocatechin-3-gallate, FASEB J. 19 (2005) 789–791.
[15] A.M. Abukhdeir, B.H. Park, P21 and p27: roles in carcinogenesis and drug resistance, Expert. Rev. Mol. Med. 10 (2008) e19.
[16] J. Wang, F. Hu, Y. Luo, H. Luo, N. Huang, F. Cheng, Z. Deng, W. Deng, K. Zou, Estrogenic and anti-estrogenic activities of hispolon from *Phellinus lonicerinus* (Bond.) Bond. et sing, Fitoterapia 95 (2014) 93–101.
[17] P.C. Hsiao, Y.H. Hsieh, J.M. Chow, S.F. Yang, M. Hsiao, K.T. Hua, C.H. Lin, H.Y. Chen, M.H. Chien, Hispolon induces apoptosis through JNK1/2-mediated activation of a caspase-8, -9, and -3-dependent pathway in acute myeloid leukemia (AML) cells and inhibits AML xenograft tumor growth in vivo, J. Agric. Food Chem. 61 (2013) 10063–10073.
[18] J. Ravindran, G.V. Subbaraju, M.V. Ramani, B. Sung, B.B. Aggarwal, Bisdemethylcurcumin and structurally related hispolon analogues of curcumin exhibit enhanced prooxidant, anti-proliferative and anti-inflammatory activities in vitro, Biochem. Pharmacol. 79 (2010) 1658–1666.
[19] W. Chen, F.Y. He, Y.Q. Li, The apoptosis effect of hispolon from *Phellinus linteus* (Berkeley & Curtis) Teng on human epidermoid KB cells, J. Ethnopharmacol. 105 (2006) 280–285.
[20] G.J. Huang, J.S. Deng, S.S. Huang, M.L. Hu, Hispolon induces apoptosis and cell cycle arrest of human hepatocellular carcinoma Hep3B cells by modulating ERK phosphorylation, J. Agric. Food Chem. 59 (2011) 7104–7113.
[21] T.L. Lu, G.J. Huang, T.J. Lu, J.B. Wu, C.H. Wu, T.C. Yang, A. Iizuka, Y.F. Chen, Hispolon from *Phellinus linteus* has antiproliferative effects via MDM2-recruited ERK1/2 activity in breast and bladder cancer cells, Food Chem. Toxicol. 47 (2009) 2013–2021.
[22] M.T. Park, S.J. Lee, Cell cycle and cancer, J. Biochem. Mol. Biol. 36 (2003) 60–65.
[23] S. Rodriguez-Nieto, B. Zhivotovsky, Role of alterations in the apoptotic machinery in sensitivity of cancer cells to treatment, Curr. Pharm. Des. 12 (2006) 4411–4425.
[24] K. Takeda, J. Stagg, H. Yagita, K. Okumura, M.J. Smyth, Targeting death-inducing receptors in cancer therapy, Oncogene 26 (2007) 3745–3757.
[25] P. Hasty, B.A. Christy, P53 as an intervention target for cancer and aging, Pathobiol. Aging Age Relat. Dis. 3 (2013).
[26] L. Li, H.J. Dai, M. Ye, S.L. Wang, X.J. Xiao, J. Zheng, H.Y. Chen, Y.H. Luo, J. Liu, Lycorine induces cell-cycle arrest in the G0/G1 phase in K562 cells via HDAC inhibition, Cancer Cell Int. 12 (2012) 49.
[27] J.E. Chipuk, D.R. Green, Cytoplasmic p53: bax and forward, Cell Cycle 3 (2004) 429–431.
[28] Y. Lin, J. Xu, H. Liao, L. Li, L. Pan, Piperine induces apoptosis of lung cancer A549 cells via p53-dependent mitochondrial signaling pathway, Tumour Biol. 35 (2014) 3305–3310.
[29] U. Shah, N.E. Sharpless, D.N. Hayes, LKB1 and lung cancer: more than the usual suspects, Cancer Res. 68 (2008) 3562–3565.