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# Honokiol from *Magnolia spp.* induces G1 arrest via disruption of EGFR stability through repressing HDAC6 deacetylated Hsp90 function in lung cancer cells

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ABSTRACT

Honokiol, an active compound derived from *Magnolia spp.* bark, possesses chemopreventive properties in many cancer cell models. However, the chemopreventive mechanism of honokiol in lung cancer cells is still a mystery. We examined histone deacetylase 6 (HDAC6)-mediated epidermal growth factor receptor (EGFR) stability in honokiol-treated lung cancer cells. The results showed that honokiol induced G1 growth arrest was through down-regulation of EGFR expression and thereafter downstream signaling pathways. HDAC6 activity was directly inhibited via honokiol at $IC_{50}$ about 23.55 ± 1.18 μM. Inhibition of HDAC6 activity via honokiol was followed by disrupting HDAC6 and heat shock protein 90 (Hsp90) association and resulting in Hsp90 hyper-acetylation. Meanwhile, hyper-acetylated Hsp90 had been found to disassociate with EGFR and followed by EGFR degradation. Taken together, these results suggested that interruption of EGFR stability by honokiol was through inhibiting HDAC6 activity and consequently suppressing Hsp90 chaperon function in lung cancer cells.



## 1. Introduction

Lung cancer is the leading cause of cancer mortality worldwide (Jemal et al., 2011). Histologically, lung cancers are classified as either non-small cell lung cancers (NSCLCs) or small cell lung cancers (SCLCs). Approximately 80% of lung cancer patients are diagnosed with NSCLCs, especially in adenocarcinoma (Davidson, Gazdar, & Clarke, 2013). Multiple genetic and epigenetic alternations, such as epidermal growth factor receptor (EGFR) mutation, *MYC* gene amplification and promoter hypermethylation of *p16* and *APC*, which activate the growth-related pathways and down-regulate tumor suppressor signaling, are involved in lung tumorigenesis (Cooper, Lam, O'Toole, & Minna, 2013). Overexpression or gene mutation of the EGFR has been indicated in lung adenocarcinoma and is correlated with a poor prognosis (Hirsch et al., 2003; Kosaka et al., 2004). EGFR belongs to the EGFR family of proteins consisting of four related, transmembrane receptor tyrosine kinases: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4

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(Normanno et al., 2006). EGFR family members regulate numerous signaling cascades that modulate cell proliferation, differentiation, apoptosis, cell motility, and survival (Normanno et al., 2006). Activation of EGFR signaling is triggered by ligand-induced receptor homo- or hetero-dimerization followed by autophosphorylation of tyrosine residues in the intrinsic kinase domain and subsequent activation of down-stream Ras-Raf-ERK, PI3K-Akt and STAT signaling (Normanno et al., 2006). Dysregulation of EGFR tyrosine kinase activity occurs by overexpression, amplification or mutation, all resulting in poor prognosis in lung cancers (Arteaga, 2003; Kosaka et al., 2004; Meert et al., 2005). Small molecules that inhibit EGFR activity have been developed for lung cancer treatment. Despite the initial dramatic responses of EGFR-mutant lung tumors to these small-molecule tyrosine kinase inhibitors (TKIs), resistance universally emerges over time (Kobayashi et al., 2005). For example, high response rates are observed with EGFR TKI therapy in lung adenocarcinomas with one of the most common EGFR activating mutations, an in-frame deletion in exon 19 and a point mutation in exon 21 (L858R) (Wu et al., 2008). However, a secondary mutation in EGFR's exon 20 (T790M) has been observed and shows poor prognosis after the same treatment (Kobayashi et al., 2005). Recent studies have noted that molecules that induce the down-regulation of EGFR rather than inhibit kinase activity may be linked to better clinical outcomes (Ahsan et al., 2010; Sawai et al., 2008). Inhibition of histone deacetylases (HDACs) has been demonstrated to down-regulate EGFR expression, resulting in colorectal cancer cell apoptosis (Chou, Wu, Huang, & Chen, 2011). Furthermore, stable knock-down of HDAC6 expression also causes a decrease in EGFR expression (Kamemura et al., 2008). Thus, an investigation into the relationship between HDAC and EGFR is important for lung cancer treatment.

Histone deacetylases are the enzymes that remove an acetyl group from histones at conserved amino-terminal lysine residues to regulate gene expression (Cress & Seto, 2000). Several studies demonstrated that some non-histone proteins are also regulated by HDACs, such as p53, STAT3, and Hsp90 (Bali et al., 2005; Gu & Roeder, 1997; Kovacs et al., 2005; Scroggins et al., 2007; Sun et al., 2009). Generally, HDACs can be subdivided into class I (HDAC1, 2, 3, and 8), class IIa (4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (SIRT1–7) and Class IV HDAC (HDAC11) (Yang & Seto, 2008). Class I HDACs are primarily localized in the nucleus and display specificity for histones. Class II HDACs, which primarily deacetylate non-histone substrates, shuttle between the nucleus and cytoplasm (Marks & Xu, 2009; Yang & Seto, 2008). Previous studies also demonstrate that HDACs are either aberrantly expressed or mislocalized in different tumors. HDAC1 over-expression has been shown to correlate with a poor prognosis in lung cancer patients (Minamiya et al., 2011; Sasaki et al., 2004). Meanwhile, HDAC1, 2, and 3 can repress $p21^{WAF1/CIP1}$ expression and inhibit HDAC activity, leading to tumor cell growth arrest and apoptosis (Wilson et al., 2006; Yamaguchi et al., 2010). Class IIb HDACs have been proven to have oncogenic activities (Kamemura et al., 2008; Lee et al., 2008). For example, HDAC6 enhances cell motility and regulates cell migration through the deacetylation of α-tubulin (Hubbert et al., 2002; Kaluza et al., 2011). Inhibition of HDAC6 and HDAC10 down-regulates vascular endothelia growth factor receptor (VEGFR) expression by disrupting VEGFR's association with Hsp90 (Park et al., 2008). Additionally, HDAC6 regulates the formation of chaperone complexes and the maturation of Hsp90 target proteins by altering Hsp90's acetylation level (Bali et al., 2005; Scroggins et al., 2007). Targeting HDAC6 has more selective effects than inhibition of class I HDACs (Aldana-Masangkay & Sakamoto, 2011; Santo et al., 2012). Accordingly, searching for small molecules that affect HDAC6 may provide a new strategy for cancer therapy.

Hsp90 is an ATP-dependent chaperone that is required for the stability and maturation of numerous conditionally activated target proteins (Scroggins et al., 2007). Over-expressed Hsp90 exists in an active, multi-chaperone complex that is found more frequently in tumor cells than in normal tissues (Ferrarini, Heltai, Zocchi, & Rugarli, 1992; Pick et al., 2007). The tumorigenic role of Hsp90 is to protect an array of mutated and over-expressed oncoproteins, such as the glucocorticoid receptor, VEGFR and EGFR, from misfolding and degradation (Park et al., 2008; Trepel, Mollapour, Giaccone, & Neckers, 2010). Hsp90 has been verified to stabilize wild-type and mutant EGFR (Ahsan et al., 2012; Shimamura, Lowell, Engelman, & Shapiro, 2005). Furthermore, inhibition of Hsp90 by geldanamycin down-regulates an exon 19 deletion and L858R EGFR mutant in NSCLCs (Shimamura et al., 2005) and has been demonstrated to impede proliferation of TKI resistant NSCLCs (Kobayashi et al., 2012). Post-translational modification of Hsp90, specifically phosphorylation and acetylation, plays an important role in regulating its chaperone function and target protein maturation (Mollapour & Neckers, 2012). Treatment with a HDAC inhibitor induces Hsp90 hyperacetylation and blocks Hsp90's association with p23 and the target proteins as well as its ATP binding site (Kovacs et al., 2005; Yang et al., 2008). The resulting loss or weakening of the associations of Hsp90 with its target proteins gives rise to instability and degradation of the complexes (Bali et al., 2005; Kovacs et al., 2005; Mollapour & Neckers, 2012; Park et al., 2008; Yang et al., 2008).

Honokiol is a bioactive natural compound derived from *Magnolia spp*. Bark. Numerous studies showed that honokiol possesses multiple biological activities such as anti-inflammatory, anti-oxidative, anti-angiogenesis and anti-tumor properties (Chuang et al., 2013; Hu et al., 2008; Leeman-Neill et al., 2010; Zhang et al., 2013). Recent evidence indicates that honokiol down-regulates the expression and phosphorylation of EGFR and causes a reduction of down-stream signaling pathways in tumor cells (Leeman-Neill et al., 2010; Park et al., 2009). Moreover, honokiol also enhances the effect of EGFR TKI-suppressed breast and head and neck squamous cell carcinoma cell growth (Leeman-Neill et al., 2010; Liu et al., 2008). Induction of growth arrest in honokiol-treated NSCLC and suppression of xenograft growth in mice have been demonstrated to be associated with class I HDAC inhibition (Singh, Prasad, & Katiyar, 2013). However, the relationship between HDAC inhibition and EGFR down-regulation by honokiol in NSCLC is still unclear. We hypothesized that repression of NSCLC growth by honokiol may be related to HDAC-mediated down-regulated EGFR expression and signaling. Our results revealed that down-regulation of EGFR expression via honokiol was due to repression of HDAC6 activity, thereby disrupting Hsp90 chaperone function and EGFR stability in NSCLC.

## 2. Materials and methods

### 2.1. Cell culture and cell viability assay

NSCLCs H23, A549 and HCC827 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in 5% fetal bovine serum-containing RPMI-1640 (HyClone Laboratories, Logan, UT, USA) and cultured at 37 °C in a 5% $CO_2$ atmosphere. The three types of cells ($1 \times 10^4$/well) were seeded in 96-well plates for 24 h and then incubated with honokiol (0, 5, 10, 15, 20 and 40 μM) for 24, 48, and 72 h. The honokiol-containing media were renewed every two days. After treatment, cell viability was examined by MTT assay.

### 2.2. Chemicals, reagents and antibodies

Honokiol, tubacin and HDAC6 activity assay kits were purchased from Biomol/Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA). Anti-EGFR, p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-Akt, Akt, HDAC6, acetyllysine, and anti-acetyl-α-tubulin antibodies were obtained from Cell Signaling (Beverly, MA, USA). Anti-Hsp90, anti-ubiquitin, anti-α-tubulin and protein A/G plus agarose were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.3. Cell cycle analysis

H23, A549 and HCC827 cells were seeded and synchronized for 24 h. After synchronization, the serum-free media were replaced by honokiol-containing 5% fetal bovine serum media for 24 h. After incubation, cells were harvested, stained with 50 μg/mL of propidium iodide (Sigma Chemical, St. Louis, MO, USA), and a FACScan laser flow cytometer analysis system (Beckman Coulter, Fullerton, CA, USA) was used to detect DNA contents.

### 2.4. Western blot analysis

Western blot analysis was performed as described previously (Yu et al., 2014). Briefly, cell lysates were prepared via RIPA extraction buffer. Cell lysates were then quantitated, electrophoresed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA). After transfer, the membranes were blocked and incubated with the indicated antibodies. The signals were detected by chemiluminescence (ECL kit, Amersham Pharmacia Biotech, IL, USA). The intensities of protein expression were then quantitated by a UVP BioSpectrum Imaging System ChemiDoc-It2 810 (UVP, LLC, CA, USA). The expression of β-actin was used as the internal control.

### 2.5. HDAC activity detection

HDAC6 activity was measured by the *Fluor-de-Lys*® HDAC6 fluorometric drug discovery kit (Biomol/Enzo Life Sciences International, Inc.) according to the manufacturer's protocol. Briefly, A549 cells were incubated with honokiol for 24 h and then lysed with RIPA extraction buffer. The cell lysates were incubated with the assay buffer containing a HDAC6 assay substrate at 37 °C for 15 min. The reaction was then terminated with HDAC6 developer solution at 37 °C for 45 min and fluorescence was measured at 460 nm using an excitation wavelength of 360 nm. The direct inhibitory effects of honokiol on class I HDACs and HDAC6 activity were examined using HeLa cell nuclear extracts (BML-AK500) and human HDAC6 recombinant protein (BML-AK516), respectively, from an Enzo HDAC assay kit following the manufacturer's protocol. We also analyzed trichostatin A (TSA) and dimethyl sulfoxide (DMSO) as positive and negative controls, respectively.

### 2.6. Immuno-precipitation

Immuno-precipitation experiments were performed as previously described (Yu et al., 2014). Briefly, honokiol-treated A549 cell lysates (1 mg in 200 μL of RIPA extraction buffer) were incubated with an anti-Hsp90 antibody and protein A/G plus agarose at 4 °C for 18 h. The immune-complexes were washed twice with immuno-precipitation buffer and then resuspended in 25 μL of protein loading buffer-containing RIPA extraction buffer. The immune-complexes were then separated by SDS-PAGE electrophoresis, transferred to a membrane, and blotted with anti-ubiquitin, anti-EGFR (Santa Cruz Biotechnology), anti-HDAC6 or anti-acetyllysine primary antibodies. For internal control, blots were then stripped and reprobed with an anti-Hsp90 antibody. The densities representing protein expression are shown as the relative densities compared to controls, which were taken as 1-fold.

### 2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as described previously (Yu et al., 2014). Briefly, honokiol-treated A549 cells were harvested and the total RNA was isolated by an RNA mini kit (Qiagen, Taipei, Taiwan). cDNAs were prepared using a high capacity cDNA reverse transcription kit (Invitrogen, Taipei, Taiwan) following the manufacturer's protocol. Briefly, EGFR and GAPDH were amplified by a thermal cycler in a 25 μL of the PCR reaction mixture that contained dNTPs, reaction buffer, 2 μL of RT-cDNA products, 50 unit/mL pro Taq DNA polymerase (Promega, WI, USA), and either EGFR or GAPDH specific primers. The EGFR, forward primer 5′-GAGAGGAGAACTGCCAGAA-3′ and reverse primer 5′-GTAGCATTTATGGAGAGTG-3′ yielded an amplicon of 454 bp, while the GAPDH forward primer 5′-TGAAGGTCGGAGTCAACGGGTGAGTT-3′ and reverse primer 5′-CATGTAGACCCCTTGAAGAGG-3′ yielded an amplicon of 983 bp. The amplification conditions were as follows: an initial denaturation at 95 °C for 5 min, 30 cycles of amplification for EGFR (95 °C for 50 s, 50 °C for 45 s, and 72 °C for 45 s) or 30 cycles of amplification for GAPDH (94 °C for 50 s, 60 °C for 45 s, and 72 °C for 120 s), and a final extension step at 72 °C for 10 min. The PCR products were separated on a 1.8% agarose gel and visualized by SYBR Safe (Life Technologies, Taipei, Taiwan) staining. Gene expression of EGFR and GAPDH were quantitated using an UVP BioSpectrum Imaging System ChemiDoc-It2 810 (UVP, LLC).

### 2.8. *Statistical analysis*

The results were expressed as the mean ± SD calculated from at least three independent determinations. One-way ANOVA coupled with Dunnett's *t* tests were used to compare individual experiments with a control value. A probability of $p < 0.05$ was considered to be a significant difference.

## 3. Results

### 3.1. *Honokiol mediated growth inhibition and cell cycle arrest in human NSCLCs*

To investigate the growth inhibitory effects of honokiol in different types of EGFR-positive lung adenocarcinomas, we treated H23, A549 (EGFR-wild type) and HCC827 (EGFR-mutant) with various concentrations (0, 5, 10, 20, and 40 μM) of honokiol for 24, 48 and 72 h, and assessed viability by a MTT assay. As shown in Fig. 1, honokiol treatment exhibited a significant dose- and time-dependent growth inhibitory effect in the three cell types (Fig. 1). To further examine whether the inhibitory effect of honokiol led to growth arrest or cell death, the three cell lines were treated with a serial dosage of honokiol for 24 h and flow cytometric analyses were performed. Our data revealed that honokiol induced a significant dose-dependent G1 growth arrest in H23 and A549 cells (Fig. 2A and B). The percentage of cells in G1 phase increased by approximately 24% (from 45.73 ± 1.66 to 69.17 ± 0.55%) and 14% (from 62.05 ± 1.47 to 76.14 ± 0.85%) in honokiol-treated H23 and A549 cells, respectively. In HCC827 cells, the G1 phase population increased by approximately 8% (from 55.37 ± 2.27 to 63.4 ± 1.44%) after treatment of 20 μM of honokiol for 24 h (Fig. 2C). These results revealed that honokiol enhanced a significant G1 population in the three lung adenocarcinoma cell lines. Treatment of honokiol in EGFR-wild type (H23 and A549) cells was more effective than in EGFR-mutant (HCC827) cells.

### 3.2. *Honokiol down-regulated EGFR expression and downstream signaling in human NSCLCs*

To investigate whether EGFR signaling was involved in honokiol-induced growth arrest, three lung adenocarcinoma cell lines were treated with honokiol and the protein levels of EGFR and EGFR-regulated signaling proteins were assayed by Western blots. The protein level of EGFR was significantly decreased in honokiol-stimulated cells (Fig. 3A). EGFR-regulated ERK activation was also repressed in honokiol-treated cells. However, neither JNK nor p38-MAPK activation was blockaded in the three type cell lines after honokiol addition (Fig. 3B). Interestingly, Akt signaling, another EGFR-regulated survival signaling pathway, exhibited inhibitory effects in honokiol-stimulated A549 and HCC827 but not H23 cells (Fig. 3B).

### 3.3. *Honokiol down-regulated EGFR expression via decreased stability and the ubiquitin–proteasome degradation system*

Repressing EGFR expression has been suggested as another strategy to treat lung cancer (Ahsan et al., 2010; Sawai et al.,

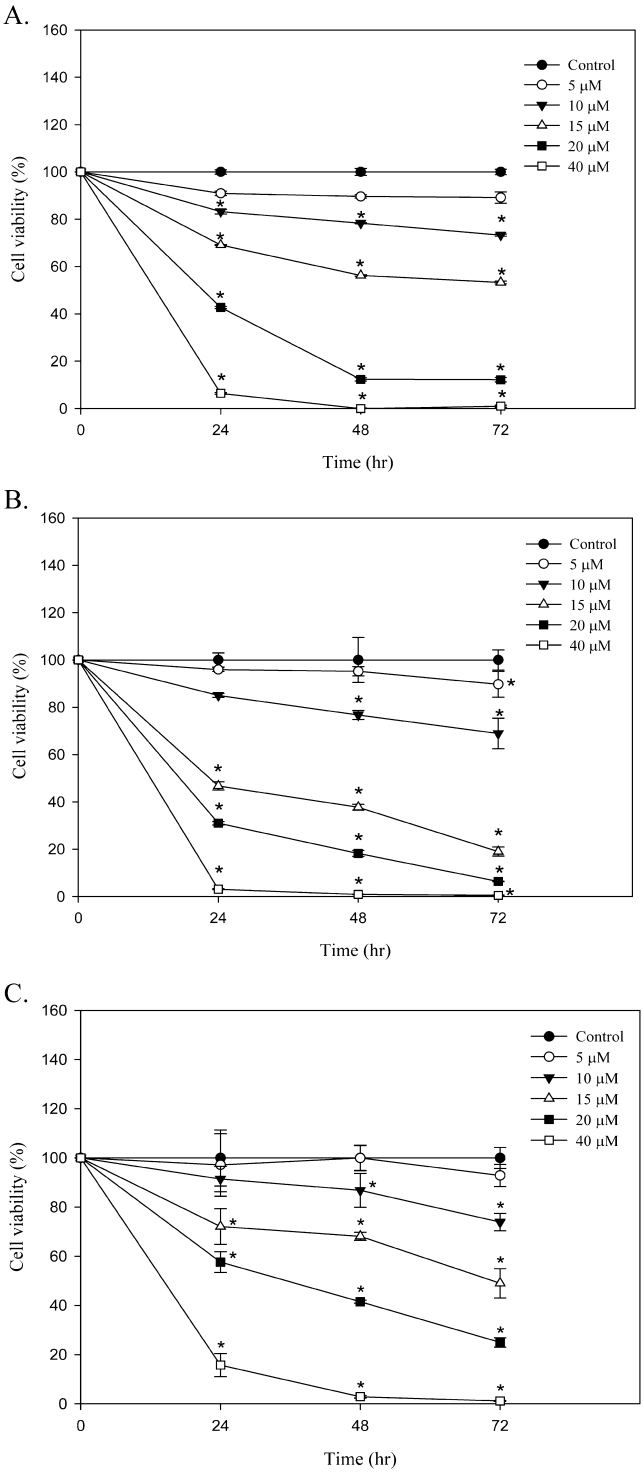


**Fig. 1 – Inhibitory effects of honokiol on viability of human lung carcinoma cancer cell line. (A) H23, (B) A549 and (C) HCC827 cells ($1 \times 10^4$/well) were cultured in 96-well plates and treated with honokiol (5, 10, 15, 20, 40 μM) for 24, 48 and 72 h. Cell viability was detected by a MTT assay. Data were the mean ± S.D. of triplicate samples. *$p < 0.05$ compared with control cells.**

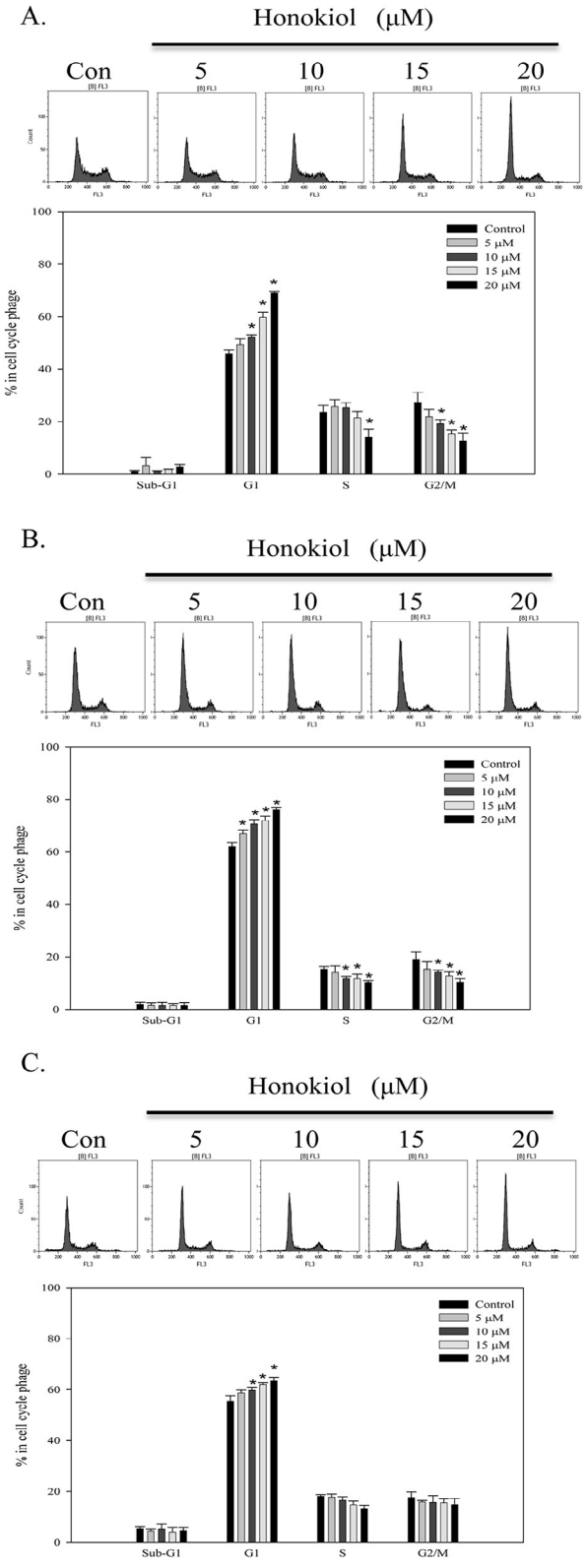


**Fig. 2 – Honokiol induced G1 cell cycle arrest in H23, A549 and HCC827 lung cancer cells. (A) H23, (B) A549 and (C) HCC827 were treated with honokiol (5, 10, 15 and 20 μM) for 24 h. At the end of incubation, cells were collected for cell cycle distribution analyses by flow cytometry. Data were the mean ± S.D. of triplicate samples. Significant difference was observed from the control group (*p < 0.05).**

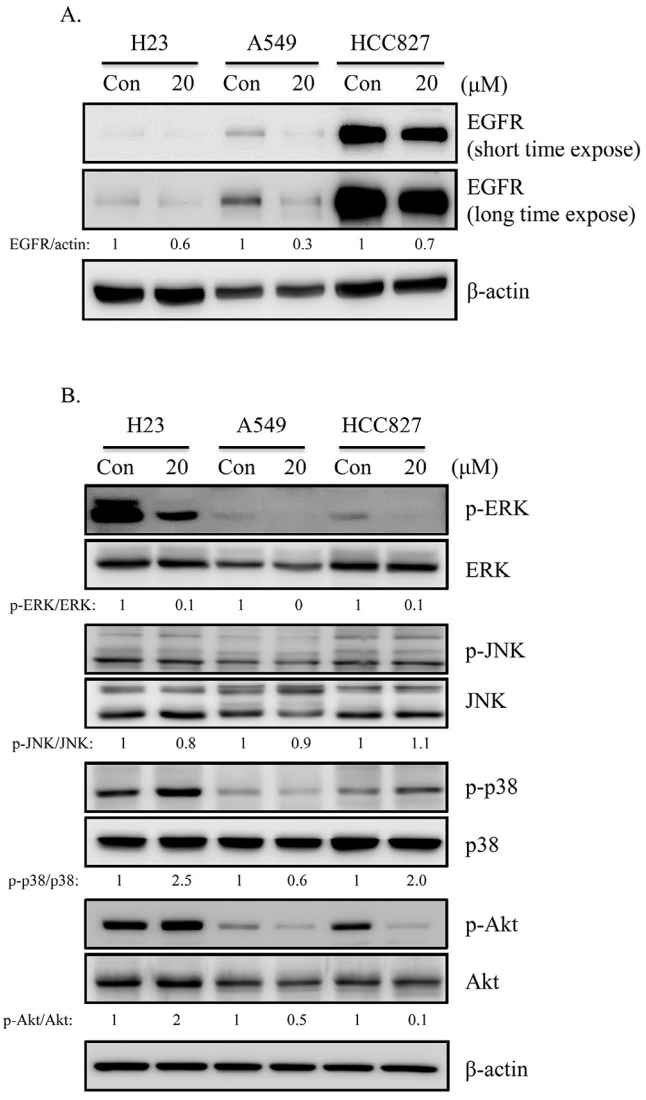


**Fig. 3 – Honokiol down-regulated EGFR expression and downstream signaling in H23, A549 and HCC827 lung cancer cells. H23, A549 and HCC827 cells were treated with 20 μM honokiol for 24 h. After treatment, cells were harvested and western blot analyses were performed with (A) anti-EGFR and (B) anti-p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-Akt, Akt and β-actin antibodies as described in Materials and Methods. Data shown are representative of at least three independent experiments.**

2008). To verify the mechanism of EGFR down-regulation after honokiol treatment, the A549 cell line expressing wild-type EGFR, which is more sensitive to honokiol down-regulated EGFR signaling, was chosen as a model. A549 cells were treated with various concentrations of honokiol for 24 h and the protein level of EGFR was measured. As shown in Fig. 4A, the protein level of EGFR was decreased in a dose-dependent mode after honokiol addition. Down-regulation of EGFR was observed after honokiol treatment for 18 h (Fig. 4B). Furthermore, RT-PCR was performed after A549 cells were incubated with honokiol. The results revealed that there was no significant difference in EGFR gene expression between control and honokiol-stimulated cells

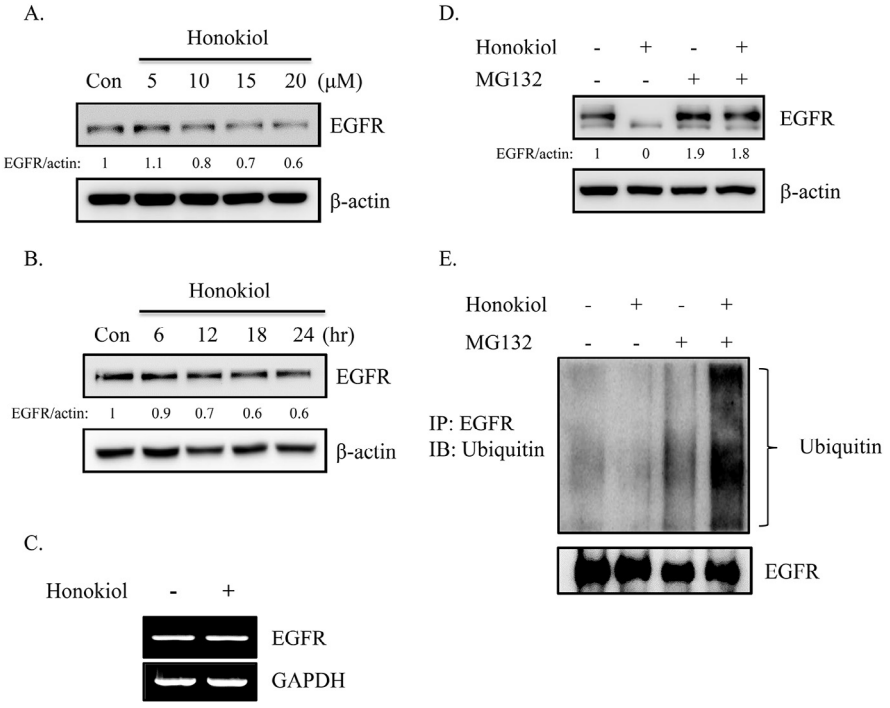


**Fig. 4 – Down-regulated EGFR expression via honokiol was through ubiquitin/proteasome degradation system in A549 lung cancer cells. A549 cells were treated with (A) various dosages of honokiol (5, 10, 15 and 20 μM) for 24 h or (B) 20 μM honokiol for different time intervals (0, 6, 12, 18 and 24 h). After treatment, cells were harvested and western blot analyses were used to detect EGFR expression. (C) A549 cells were treated with 20 μM honokiol for 24 h and then reverse transcription polymerase chain reaction (RT-PCR) was performed to analyze EGFR mRNA expression. (D) A549 cells were incubated with or without 10 μM MG132 for 30 min before 20 μM honokiol stimulation for 24 h. Cells were harvested to detect EGFR expression. (E) Immunoprecipitation analysis was performed with an anti-EGFR antibody. The immunoprecipitates were then detected by western blotting with anti-ubiquitin as described in Materials and Methods. The protein levels of each group after normalization with β-actin are shown below the data. Data shown are representative of at least three independent experiments.**

(Fig. 4C). To evaluate the EGFR protein degradation in honokiol-treated cells, A549 cells were incubated with the proteasome specific inhibitor MG132 before honokiol stimulation. At the end of the treatment, the protein level of EGFR was determined. Interestingly, the decrease in EGFR expression caused by honokiol was rescued when cells were incubated with MG132 before honokiol treatment (Fig. 4D). To confirm the poly-ubiquitination of EGFR in honokiol-treated cells, EGFR was immuno-precipitated and poly-ubiquitin was measured by Western blots. As shown in Fig. 4E, the abundant poly-ubiquitin was detected in cells treated with MG132 alone and co-stimulated with MG132 and honokiol (Fig. 4E). These data exhibited that honokiol-inhibition of EGFR expression may occur through ubiquitin–proteasome degradation rather than transcriptional inhibition.

### 3.4. *Honokiol inhibition of class I HDAC activity occurs via down-regulation HDAC protein expression rather than direct activity inhibition*

Inhibition of HDAC has been demonstrated to possess anti-tumor activity by cell cycle arrest and/or apoptosis induction (Marks & Xu, 2009; Singh et al., 2013; Yamaguchi et al., 2010). A direct class I HDAC activity assay was performed to determine the relationship between honokiol-induced growth arrest and class I HDACs inhibition. Honokiol treatment showed no significant effects on class I HDAC activity. Additionally, the protein levels of class I HDACs were assayed to further examine class I HDAC inhibition in honokiol-treated cells. The data showed that honokiol inhibited HDAC1, 2, and 3 expressions in the three NSCLC cell lines (Fig. 5B). Therefore, these results indicate that honokiol down-regulates class I HDAC expression rather than directly inhibiting class I HDAC activity.

### 3.5. *Honokiol directly inhibits class IIb HDAC6 activity*

Recently, class IIb HDAC6 has emerged as an attractive target for cancer treatment due to its low toxicity (Aldana-Masangkay & Sakamoto, 2011; Bali et al., 2005; Lee et al., 2008; Santo et al., 2012). In this study, the expression of HDAC6 was detected by western blot to address whether class IIb HDAC6 signaling was involved in honokiol-induced growth arrest in lung cancer cells. As shown in Fig. 6A, there was no difference in HDAC6 expression in control and honokiol-incubated cells. However, the amounts of acetyl-α-tubulin, a HDAC6 specific substrate, were elevated in honokiol-treated cells. Moreover, A549 cells were incubated with serial concentrations of honokiol and HDAC activity analyses were executed to further investigate the effects

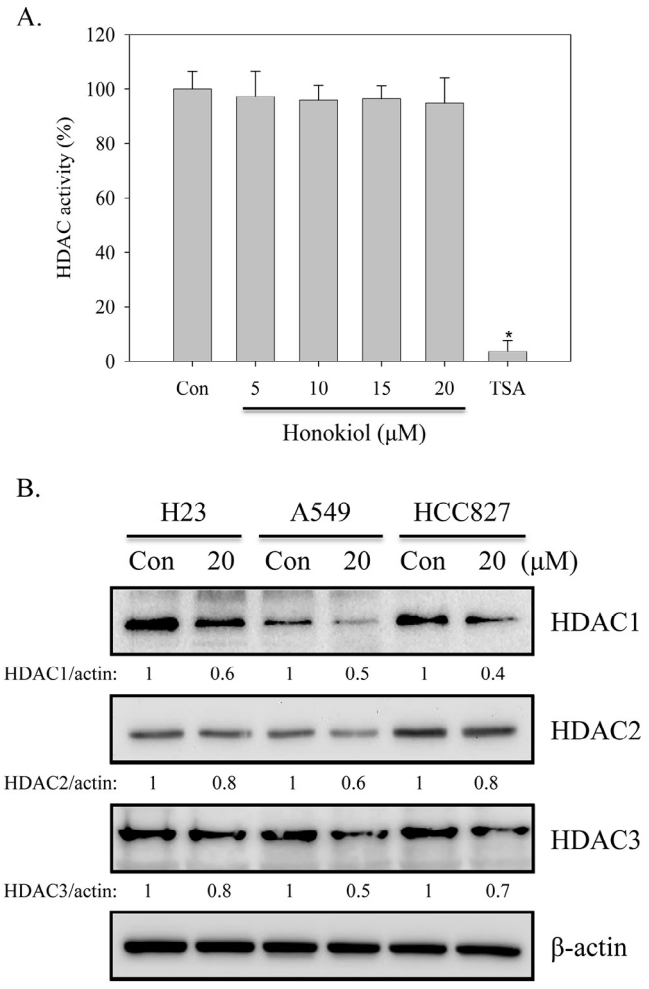


**Fig. 5 – Effect of honokiol on class I HDAC activity and protein expression in lung cancer cells. (A) To examine inhibition by honokiol, a class I HDAC activity assay was performed as described in Materials and Methods. (B) H23, A549 and HCC827 cells were treated with 20 μM honokiol for 24 h. After treatment, cells were harvested and western blot analyses were performed with anti-HDAC1, 2, 3, and β-actin. The protein levels of each group after normalization with β-actin are shown below the data. Data shown are representative of at least three independent experiments.**

of honokiol on HDAC6 activity. As shown in Fig. 6B, HDAC6 activity was inhibited in a dose-dependent manner by honokiol treatment. Interestingly, our data also revealed a dose-dependent inhibition of HDAC6 activities in a cell-free system. The results showed the direct inhibition of HDAC6 activity by honokiol treatment. The $IC_{50}$ of honokiol was approximately 23.55 ± 1.18 μM (Fig. 6C).

### 3.6. *Inhibition of Hsp90's chaperone function by increasing acetylation in honokiol-stimulated cells via direct inhibition of HDAC6 activity*

It is well understood that EGFR associates with Hsp90 resulting in promotion of EGFR maturation and stability. Down-regulation of Hsp90 expression led to the degradation of EGFR (Ahsan et al., 2012; Kobayashi et al., 2012; Sawai et al., 2008; Shimamura et al., 2005). Western blot analyses were performed to understand the relationship between EGFR degradation and Hsp90 signaling in honokiol-stimulated lung adenocarcinoma cell lines. Variations of Hsp90 protein levels were not observed in the control and honokiol-stimulated cells (Fig. 6A). Acetylation of Hsp90 is another potential approach to regulate chaperone function (Mollapour & Neckers, 2012; Scroggins et al., 2007; Yang et al., 2008). Deacetylation by HDACs, especially HDAC6, has been proven to control Hsp90's chaperone function and target protein maturation (Bali et al., 2005; Kovacs et al., 2005; Scroggins et al., 2007). A549 cells were treated with honokiol for 24 h, Hsp90 was immuno-precipitated and Western blots were used to assess HDAC6 binding and hyperacetylation of Hsp90. As shown in Fig. 7A, the level of bound HDAC6 with Hsp90 was diminished approximately 50% by honokiol treatment. Moreover, the relative densities of Hsp90's acetyllysine in honokiol-incubated cells were enhanced approximately 3-folds. Accordingly, the data implied that honokiol directly inhibited HDAC6 activity and enhanced the hyper-acetylation of Hsp90. Furthermore, immunoprecipitation analyses by Western blotting also indicated that the bound protein levels of EGFR with Hsp90 were significantly lower (approximately 60%) in honokiol-treated cells (Fig. 7B). These results inferred that honokiol-reduced EGFR expression might be caused by disrupting the Hsp90-EGFR interaction. Next, A549 cells were incubated with tubacin, a specific inhibitor of HDAC6, to address the relationship between HDAC6 activity and the degradation of EGFR. As shown in Fig. 7C, EGFR was down-regulated in a dose-dependent mode by tubacin treatment. Honokiol-induced EGFR degradation exhibited a similar cascade as seen in tubacin-treated cells (Fig. 7C). Therefore, the results indicated that honokiol-induced EGFR degradation was caused by directly inhibiting HDAC6 activity.

## 4. Discussion

Reversible acetylation via HDACs regulates broad physiological functions including tumorigenesis. Searching specific HDAC inhibitors from botanicals and investigating their anti-cancer mechanisms might provide a strategy for cancer prevention or therapy. In this study, we demonstrated that honokiol, an active compound from *Magnolia spp*. bark, was an inhibitor of HDAC6. Suppression of HDAC6 activity and hyper-acetylation of Hsp90 by honokiol disrupted EGFR's association with Hsp90, leading to EGFR degradation and G1 growth arrest in NSCLC.

Dysregulation of EGFR gene expression is important in tumorigenesis, especially in NSCLC. Overexpression of EGFR in NSCLC correlates with a poor prognosis (Hirsch et al., 2003; Kosaka et al., 2004). Therefore, small-molecular inhibitors, TKIs, have been developed to target EGFR and down-regulate EGFR signaling, resulting in cell death or growth inhibition (Arteaga, 2003). However, resistance to EGFR TKIs treatments is elevated in patients with secondary mutations (Kobayashi et al., 2005). Furthermore, only approximately 15% of white and African American NSCLC patients have an EGFR mutation (Cote

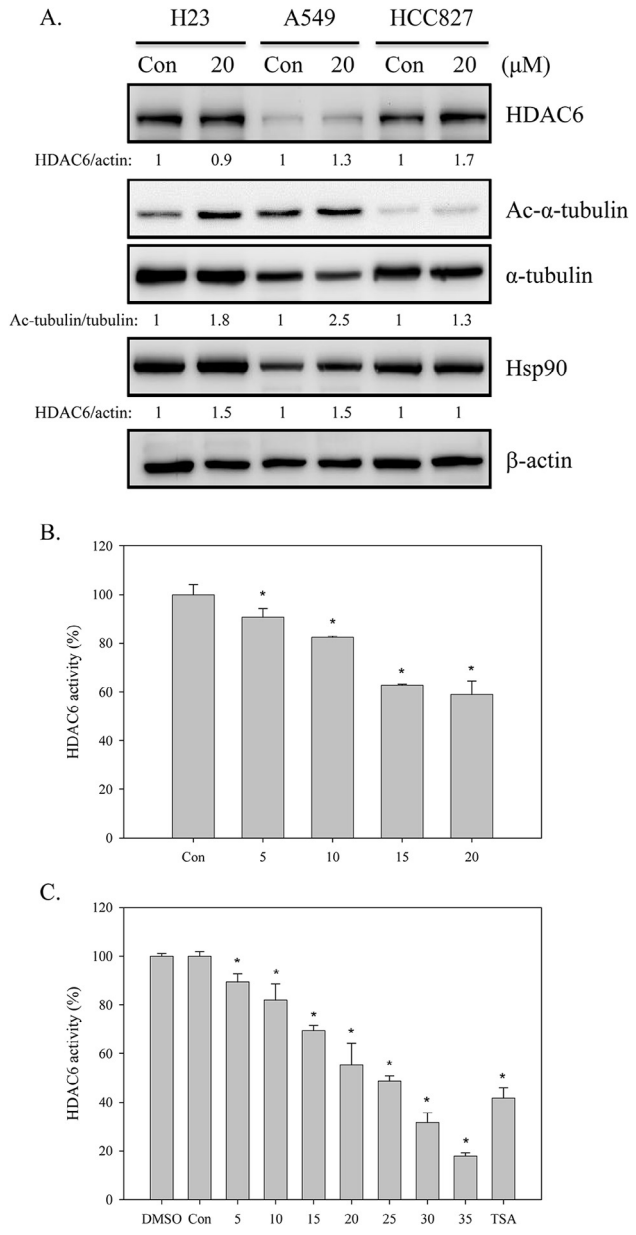


**Fig. 6 – Effect of honokiol on class IIb HDAC6 activity and protein expression in lung cancer cells. (A) H23, A549 and HCC827 cells were treated with 20 μM honokiol for 24 h. After treatment, cells were harvested and western blot analyses were performed with anti-HDAC6, acetyl-α-tubulin, α-tubulin, Hsp90 and β-actin. (B) A549 cells were incubated with various dosages of honokiol (0, 5, 10, 15 and 20 μM) for 24 h. Cells were harvested and HDAC6 activity was determined as described in Material and Methods. (C) Nuclear extracts containing HDAC6 enzymes were incubated with various concentrations of honokiol (0, 5, 10, 15, 20, 25, 30 and 35 μM) and a HDAC6 activity assay was performed as described in Materials and Methods. The vehicle (DMSO) and 2 μM trichostatin A (TSA) were examined as negative and positive control, respectively. The protein levels of each group after normalization with β-actin are shown below the data. Data of HDAC6 activity were the mean ± S.D. of triplicate samples. Significant difference was observed from the control group (*$p < 0.05$).**

et al., 2011; Rosell et al., 2009). Meanwhile, a significant proportion of NSCLC patients express wild-type EGFR (Mok et al., 2009). Thus, studying small molecules that target EGFR maturation and promote EGFR degradation in cancer cells may be a new direction for cancer treatment. In the present study, we showed that honokiol inhibits the growth of different EGFR-expressing lung cancer cell lines. In addition, when treated with honokiol, growth inhibition and G1 phase arrest were more pronounced in cells expressing wild-type EGFR than in cells expressing mutant EGFR (Figs. 1, 2). This may be due to mutation *EGFR* being associated with high levels of *EGFR* gene amplification in HCC827 cells (Amann et al., 2005). Highly *EGFR* gene amplification might overcome or diminish honokiol activity. Furthermore, the protein levels of EGFR and EGFR-regulated signaling proteins were also decreased in honokiol-stimulated cells (Fig. 3B). However, inhibition of EGFR-regulated Akt activation was not observed after treatment with honokiol in H23 cells – lung cancer cells containing a *PTEN*-mutant that leads to constitute Akt activation (Yoon et al., 2010). We speculate that in H23 cells, the *PTEN*-mutation bypasses honokiol-induced Akt inhibition.

To further examine the EGFR down-regulation mechanism caused by honokiol treatment, A549 cells were chosen as the model. The data revealed a dose- and time-dependent decrease of EGFR expression in honokiol-treated A549 cells (Fig. 4A and B). Moreover, honokiol reduction of EGFR expression had been demonstrated to not occur at the transcriptional level (Fig. 4C). The proteasome specific inhibitor MG132 was utilized to understand honokiol-induced EGFR degradation. As shown in Fig. 4D, the reduction of EGFR expression caused by honokiol was rescued by pretreatment with MG132. Immunoprecipitation analyses revealed that the poly-ubiquitin of EGFR accumulated after MG132 and honokiol co-treatment (Fig. 4E). In conclusion, EGFR was suppressed through proteasome/ubiquitin signaling rather than transcriptional regulation.

Recent studies demonstrated that HDACs regulate EGFR expression in colon cancer (Chou et al., 2011). Honokiol has been shown to suppress NSCLC cell growth and induce cell death through class I HDAC inhibition (Singh et al., 2013). Accordingly, we hypothesized that honokiol would decrease the expression of EGFR protein by inhibiting the HDAC-regulated pathway. To test this hypothesis, honokiol's effect on HDAC activity was examined. As shown in Fig. 5A, honokiol did not inhibit the activity of class I HDAC in a cell free system. To understand the regulatory effects of honokiol on HDAC activity, the protein levels of class I HDACs were examined. The results revealed that HDAC 1, 2 and 3 were reduced after honokiol treatment in three types of lung cancer cell lines (Fig. 5B). These results indicated that class I HDAC activity inhibited by honokiol was caused by down-regulation of class I HDAC protein expression rather than direct class I HDAC activity inhibition. It has been demonstrated that treatment with a high dosage (60 μM) of honokiol in long term (72 h) exposure suppressed the protein levels of class I HDAC through proteasomal degradation (Singh et al., 2013). Our study further demonstrated that class I HDAC down-regulation with a low dosage (20 μM) in short term (24 h) exposure of honokiol was also observed (Fig. 5).

Inhibition of class I HDAC has been demonstrated to regulate EGFR expression. Additionally, class IIb HDAC6 has also

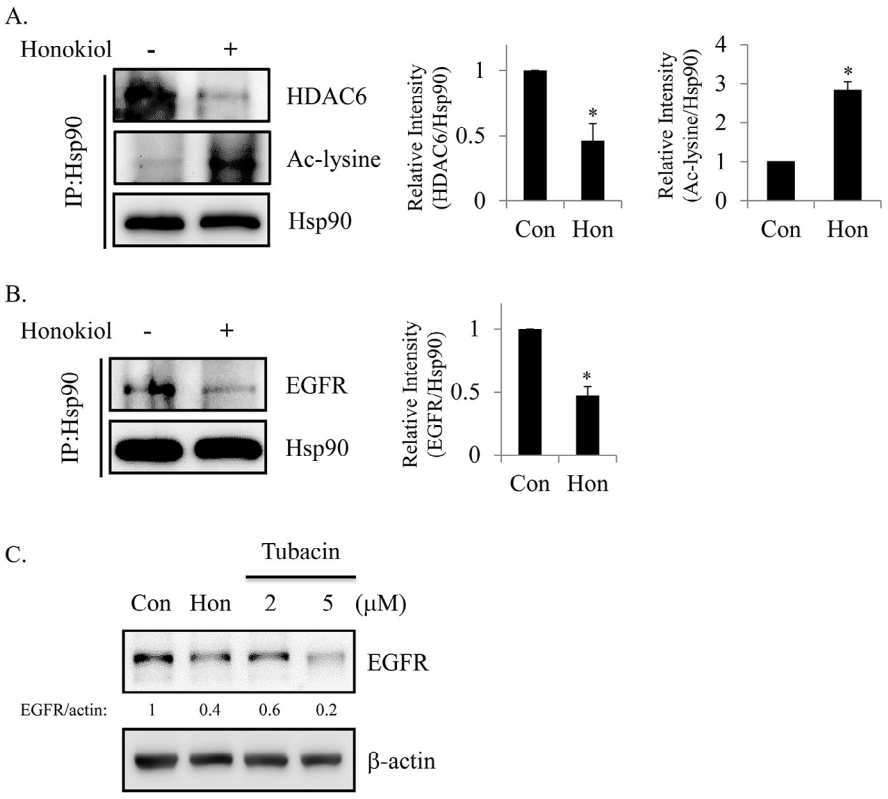


**Fig. 7 – Honokiol-down-regulated EGFR expression was through suppression HDAC6 activity and disruption of Hsp90 function in A549 lung cancer cells. A549 cells were incubated with 20 μM honokiol for 24 h. Cells were then harvested and immunoprecipitation analysis was performed with an anti-Hsp90 antibody. The immunoprecipitates were then detected by western blotting with (A) anti-HDAC6, acetyllysine and (B) anti-EGFR antibodies as described in Materials and Methods. (C) A549 cells were treated with 20 μM honokiol and tubacin (2 and 5 μM) for 24 h. After treatment, cells were collected and western blot analyses were performed with anti-EGFR and β-actin antibodies as described in Materials and Methods. The protein levels of each group after normalization with Hsp90 or β-actin are shown below the data. Data of immunoprecipitative western blot were the mean ± S.D. of triplicate samples. Significant difference was observed from the control group (*$p < 0.05$).**

been demonstrated to control EGFR trafficking and degradation in renal epithelia cells (Liu et al., 2012). Down-regulation of EGFR expression has been observed in HDAC6-knockdown A549 lung cancer cells (Kamemura et al., 2008). Moreover, the expression of HDAC6 was investigated to understand the relationship between honokiol-reduced EGFR expression and HDAC6 signaling. In this study, the protein level of HDAC6 showed insignificant differences between control and honokiol-treated cells (Fig. 6A). However, hyper-acetylation of α-tubulin, a HDAC6 specific substrate, was enhanced in honokiol-treated cells (Fig. 6A). To demonstrate that the hyper-acetylation of α-tubulin was mediated by inhibition of HDAC6, A549 cells were treated with honokiol and cell lysates were harvested for HDAC6 activity analyses. The results showed that HDAC6 activity was inhibited by honokiol in a dose-dependent manner (Fig. 6B). Interestingly, HDAC6 activity was directly inhibited by honokiol with $IC_{50}$ at 23.55 ± 1.18 μM (Fig. 6C). This is the first observation that honokiol is an inhibitor of HDAC6. There are numerous HDAC inhibitors available, although most of them are pan-HDAC inhibitors or class I inhibitors (Witt, Deubzer, Milde, & Oehme, 2009). Among the pan-HDAC inhibitors, some have been approved for clinical use, such as vorinostat (SAHA) and romidepsin (depsipeptide, FK228) (Robey et al., 2011). HDAC6 has emerged as a promising target due to its inability to deacetylate histone substrates and its relevant role in tumorigenesis (Aldana-Masangkay & Sakamoto, 2011). Additionally, targeting HDAC6 is expected to produce less toxic side effects (Santo et al., 2012). Therefore, searching for HDAC6 inhibitors might provide new strategies for cancer therapy.

HDAC inhibitors are structurally identified by three primary domains, including a zinc binding group, a linker region and a cap domain (Butler & Kozikowski, 2008; Suzuki, 2009). The functional zinc binding group chelates the zinc cofactor at the activate site of the enzyme. Based on the different chemical structure of the zinc binding group, HDAC inhibitors can be divided into hydroxamic acid-based derivates, benzamides, short-chain fatty acid, electrophilic ketones, and cyclic peptides (Butler & Kozikowski, 2008; Suzuki, 2009). Modification of cap domain reveals the most promising strategy to generate specific isoform inhibitors (Somoza et al., 2004). However, chemical functional groups could not be classified into these five categories also to inhibit HDAC activity (Huang et al., 2011, 2012). Although honokiol is not classified into any of these

categories, it showed a direct inhibition of HDAC6 activity in the present study.

Hsp90 is well known to be more highly expressed in tumors than in normal tissues (Ferrarini et al., 1992; Pick et al., 2007). Hsp90 is an ATP-dependent chaperone that regulates the maturation and stability of numerous target proteins, including growth factor receptors, kinases and transcription factors, which participate in cell proliferation, metastasis and survival (Ahsan et al., 2012; Kobayashi et al., 2012; Kovacs et al., 2005; Sawai et al., 2008; Scroggins et al., 2007). The hydrophobic *N*-terminal domain of Hsp90 binds to ATP, altering its conformation and promoting its interaction with co-chaperones p23 and p50/cdc37. Hsp90 and its co-chaperones associate with and stabilize the target proteins. Hydrolysis of ATP to ADP by Hsp90's intrinsic ATPase activity directs misfolded target proteins to be polyubiquitin by E3 ubiquitin ligase and subsequently degraded by the proteasome (Trepel et al., 2010). It has been demonstrated that Hsp90 inhibitors suppress EGFR expression in head and neck squamous cell carcinoma cells and overcome TKI-resistance NSLCL growth (Ahsan et al., 2012; Kobayashi et al., 2012; Shimamura et al., 2005, 2008). Therefore, decreases in Hsp90 expression and/or disruption in its chaperone activity may provide a therapeutic target for NSCLC treatment. However, the protein level of Hsp90 was not influenced in honokiol-treated cells (Fig. 6A). Recently, post-translational modification of Hsp90, specifically acetylation, has been noted to be important for epigenetic regulation and for the control of tumorigenesis (Scroggins et al., 2007; Trepel et al., 2010; Yang et al., 2008). Hsp90 is characterized as a specific substrate of HDAC6 (Bali et al., 2005; Kovacs et al., 2005). Hyper-acetylation of Hsp90 via HDAC6 inhibition regulates Hsp90's function and destabilizes several Hsp90 target proteins (Kovacs et al., 2005; Park et al., 2008; Scroggins et al., 2007; Yang et al., 2008). Targeting HDAC6 may contribute to cancer therapy by leading to the accumulation of hyper-acetylated Hsp90, resulting in the degradation of target proteins. Accordingly, we hypothesized that honokiol down-regulated EGFR expression by the disruption of Hsp90's chaperone function via inhibition of HDAC6. To verify this hypothesis, the HDAC6–Hsp90 interaction and hyper-acetylation of Hsp90 were investigated. As shown in Fig. 7A, the bound protein level of HDAC6 to Hsp90 was decreased approximately 50% in honokiol-treated cells, whereas a 3-fold increase in acetyllysine levels was observed in honokiol-treated cells (Fig. 7A). Interestingly, Hsp90-bound EGFR was decreased in honokiol-treated cells by immuno-precipitation analyses (Fig. 7B). Our results revealed that the repression of EGFR via honokiol was through disrupting the Hsp90–EGFR association rather than inhibiting Hsp90 protein expression. Furthermore, down-regulation of EGFR was also observed after treatment with tubacin, a specific HDAC6 inhibitor (Fig. 7C). These results indicated that inhibition of HDAC6 via honokiol disrupted the HDAC6–Hsp90 interaction, resulting in hyper-acetylation of Hsp90 and the dissociation of Hsp90 and EGFR.

Although treatment with an HDAC6 inhibitor mediates the accumulation of Hsp90 hyper-acetylation, other HDAC inhibitors are also thought to regulate Hsp90's acetylation level. Treatment with HDAC inhibitor FK228, irrelevant to HDAC6, also induced Hsp90 hyper-acetylation (Furumai et al., 2002). Moreover, HDAC1 has been found to deacetylate Hsp90 in the nucleus of breast cancer cells (Zhou, Agoston, Atadja, Nelson, & Davidson, 2008). These publications indicate that HDAC6 may not only be an Hsp90 deacetylase. Our data showed that honokiol reduced HDAC1, 2, and 3 protein expression and directly inhibited HDAC6 activity (Figs. 5 and 6). Immunoprecipitation analysis also showed that honokiol decreased the interaction between HDAC6 and Hsp90 following Hsp90 hyper-acetylation (Fig. 7B). However, the possibility of hyper-acetylation of Hsp90 partially contributed by class I HDAC inhibition via honokiol cannot be excluded. It should be clarified that the members of HDAC affect Hsp90 and its functions. A recent study indicated that at least 11 acetylated-lysine residues of Hsp90 have been discovered (Yang et al., 2008). Scroggins and colleagues demonstrated that K294 in the middle domain of Hsp90 is a critical acetylation site. Acetylation of K294 decreases the affinity of the target protein and certain co-chaperones, while deacetylation increases these interactions (Scroggins et al., 2007). Acetylation of K69 in Hsp90 plays an important role in its extracellular localization and association with MMP2 (Yang et al., 2008). These studies showed that the acetylation of specific lysine residues in Hsp90 might mediate different biological effects. Therefore, it is essential to verify the roles of specific Hsp90 lysine residues in regulating EGFR maturation and the relationship between HDAC6 and Hsp90 hyper-acetylation via honokiol in lung cancer cells.

Natural products have been recognized to have potential chemopreventive properties. Over 60% of clinical anticancer drugs are derived from natural sources (Newman, Cragg, & Snader, 2003). Honokiol, a biological component from the bark of *Magnolia* genus plants that have long been used in traditional Chinese medicine, appears to have multiple biological properties including anti-oxidative, anti-inflammatory, anti-angiogenesis, and antitumor activities (Chuang et al., 2013; Hu et al., 2008; Leeman-Neill et al., 2010; Liu et al., 2008; Park et al., 2009; Singh et al., 2013; Zhang et al., 2013). Honokiol has been identified to cross the blood–brain barrier and does not reveal any toxic effects in animal models (Wang et al., 2011). Moreover, genotoxic experiments also indicate that no mutagenic activity is observed in honokiol-treated cells (Zhang et al., 2008). These studies suggest that honokiol could be a good antitumor or chemopreventive agent because of its safety properties. The antitumor activities of honokiol have been demonstrated in a series of cancer cell lines such as brain, breast and lung cancer cell lines (Park et al., 2009; Singh et al., 2013; Wang et al., 2011). EGFR signaling has been examined as an important target of honokiol for lung cancer treatment (Leeman-Neill et al., 2010). The epigenetic regulation of EGFR has been investigated in many cancer cell lines (Chou et al., 2011; Liu et al., 2012; Marks & Xu, 2009; Singh et al., 2013). However, the molecular mechanism of HDAC-mediated EGFR signaling regulated by honokiol in lung cancer was still unclear. Our results demonstrated that honokiol induced G1 arrest through the down-regulation of EGFR expression via ubiquitin/proteasome degradation in lung cancer cells. Meanwhile, honokiol directly inhibited HDAC6, which led to Hsp90 hyper-acetylation. The hyper-acetylation of Hsp90 disrupted EGFR maturation, leading to EGFR degradation. Our findings suggested that honokiol might consider being a potential chemoprevention agent of lung cancer through epigenetic regulation.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

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