The natural product honokiol preferentially inhibits cellular FLICE-inhibitory protein and augments death receptor–induced apoptosis

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Abstract

Targeting death receptor–mediated apoptosis has emerged as an effective strategy for cancer therapy. However, certain types of cancer cells are intrinsically resistant to death receptor–mediated apoptosis. In an effort to identify agents that can sensitize cancer cells to death receptor–induced apoptosis, we have identified honokiol, a natural product with anticancer activity, as shown in various preclinical studies, as an effective sensitizer of death receptor–mediated apoptosis. Honokiol alone moderately inhibited the growth of human lung cancer cells; however, when combined with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), greater effects on decreasing cell survival and inducing apoptosis than TRAIL alone were observed, indicating that honokiol cooperates with TRAIL to enhance apoptosis. This was also true to Fas-induced apoptosis when combined with Fas ligand or an agonistic anti-Fas antibody. Among several apoptosis-associated proteins tested, cellular FLICE-inhibitory protein (c-FLIP) was the only one that was rapidly down-regulated by honokiol in all of the tested cell lines. The down-regulation of c-FLIP by honokiol could be prevented by the proteasome inhibitor MG132. Moreover, honokiol increased c-FLIP ubiquitination. These results indicate that honokiol down-regulates c-FLIP by facilitating its degradation through a ubiquitin/proteasome-mediated mechanism. Enforced expression of ectopic c-FLIP abolished the ability of honokiol to enhance TRAIL-induced apoptosis. Several honokiol derivatives, which exhibited more potent effects on down-regulation of c-FLIP than honokiol, showed better efficacy than honokiol in inhibiting the growth and enhancing TRAIL-induced apoptosis as well. Collectively, we conclude that c-FLIP down-regulation is a key event for honokiol to modulate the death receptor–induced apoptosis. [Mol Cancer Ther 2008;7(7):2212–23]

Introduction

It is well known that cells can die of apoptosis primarily through the extrinsic death receptor–induced pathway and/or the intrinsic mitochondria-mediated pathway. Cross-talk between these two pathways is mediated by the truncated proapoptotic protein Bid (1). The activation of the extrinsic death receptor–mediated apoptotic pathway relies on binding of a death ligand [e.g., tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)] to its corresponding death receptor(s) or aggregation (e.g., trimerization) of death receptors, which induces for the formation of the death-inducing signaling complex followed by the activating cleavage of caspase-8 in the death-inducing signaling complex. Because Bid serves as a caspase-8 substrate, activation of the extrinsic death receptor apoptotic pathway also turns on the intrinsic apoptotic pathway.

The death ligand TRAIL has recently received much attention because it preferentially induces apoptosis in transformed or malignant cells, but not in most normal cells, showing potential as a tumor-selective apoptosis-inducing cytokine for cancer treatment (2). Currently, recombinant human TRAIL is being tested as an anticancer agent in phase I clinical trials. In addition, agonistic antibodies against death receptor 4 (DR4) and death receptor 5 (DR5), respectively, which directly activate the extrinsic apoptotic pathway, have been developed and tested in phase I or II trials with well tolerance (3). Thus, the death receptor–mediated, particularly the TRAIL death receptor–mediated, apoptosis has been under intense research as a cancer therapeutic target (4). An important issue in this regard is the intrinsic resistance of certain cancer cells to TRAIL/death receptor–induced apoptosis.

Activation of the extrinsic death receptor–mediated apoptotic pathway is primarily inhibited by cellular FLICE-inhibitory

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protein (c-FLIP), which inhibits caspase-8 activation by preventing recruitment of caspase-8 to the death-inducing signaling complex (5, 6). c-FLIP has multiple splice variants; however, only two of them have been well characterized at the protein levels: the 26-kDa short form (c-$FLIP_S$) containing two death effector domains and the 55-kDa long form (c-$FLIP_L$) containing an inactive caspase-like domain in addition to the two death effector domains (7, 8). The levels of c-FLIP, including both $FLIP_L$ and $FLIP_S$, are subject to regulation by ubiquitin/proteasome-mediated degradation (9–11). It has been well documented that elevated c-FLIP expression protects cells from death receptor–mediated apoptosis, whereas down-regulation of c-FLIP by chemicals or small interfering RNA sensitizes cells to death receptor–mediated apoptosis (7). Moreover, overexpression of c-FLIP also protects cells from apoptosis induced by cancer therapeutic agents, such as etoposide and cisplatin (12–16).

Honokiol (Fig. 1A) is an active component purified from magnolia, a plant used in traditional Chinese and Japanese medicine. It has been shown that honokiol induces apoptosis and inhibits the growth of certain types of cancer cells (17–22) and has excellent *in vivo* antitumor activity against skin tumors (23), angiosarcoma (24), breast cancer (21), and bone metastasis of prostate cancer (25). However, the precise mechanism of growth inhibition and apoptosis by honokiol is largely unknown, although it seems to be associated with the inhibition of nuclear factor-κB (NF-κB; ref. 22) and down-regulation of Bcl-X_L and Mcl-1 (18, 19). In an effort to identify agents that sensitize cancer cells to the death receptor–mediated apoptosis, we have identified that honokiol is a potent sensitizer of death receptor–induced apoptosis, primarily through inducing c-FLIP degradation. Thus, our findings highlight a novel mechanism by which honokiol modulates apoptosis in human cancer cells.

Materials and Methods

Reagents

Honokiol and its derivatives used in this study were synthesized in Dr. J. Arbiser's laboratory (Emory University, Atlanta, GA). These compounds were dissolved in DMSO at the concentration of 30 mmol/L, and aliquots were stored at −80°C. Stock solutions were diluted to the appropriate concentrations with growth medium immediately before use. The soluble recombinant human TRAIL and TNFα were purchased from PeproTech, Inc. The proteasome inhibitor MG132 was purchased from Sigma Chemical Co. The agonistic anti-Fas antibody (EOS9.1) was purchased from BioLegend. Soluble recombinant human *Super*Fas ligand and mouse monoclonal anti-FLIP antibody (NF6) was purchased from Alexis Biochemicals. Rabbit polyclonal anti-DR5 antibody was purchased from ProSci, Inc. Mouse monoclonal anti-DR4 antibody (B-N28) was purchased from Diaclone. Mouse monoclonal anti-caspase-3 antibody was purchased from Imgenex. Rabbit polyclonal anti-XIAP, anti-caspase-8, anti-Mcl-1, anti-c-Jun, anti-phospho-c-Jun (Ser^{63}), and anti–poly(ADP-ribose) polymerase (PARP) antibodies and mouse monoclonal anti-survivin antibody were purchased from Cell Signaling Technology, Inc. Rabbit anti–glyceraldehyde-3-phosphate dehydrogenase polyclonal antibody and mouse anti-Bax monoclonal antibody were purchased from Trevigen. Mouse anti-Bcl-2 and rabbit anti-Bcl-X_L antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-β-actin antibody was purchased from Sigma Chemical.

Cell Lines and Cell Culture

Human non–small cell lung cancer (NSCLC) cell lines used in this study were purchased from the American Type Culture Collection. The stable H157-Lac Z-5, H157-$FLIP_L$-21, H157-$FLIP_S$-1, H460-Lac Z-9, and H460-$FLIP_L$-15 transfectants were established as described previously (26, 27). The NF-κB reporter stable cell line A549/NFκB-luc, which maintains a chromosomal integration of a luciferase reporter construct regulated by multiple copies of the NF-κB response element, was purchased from Panomics, Inc. These cell lines were cultured in RPMI 1640 containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

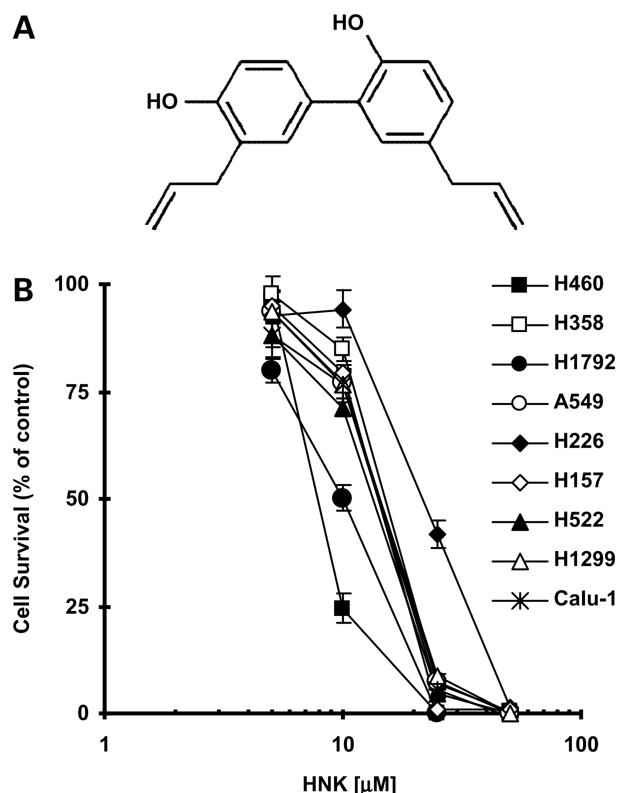

Figure 1. Chemical structure of honokiol (**A**) and its effects on the growth of human NSCLC cells (**B**). The indicated NSCLC cell lines were seeded in 96-well cell culture plates and treated the next day with the given concentrations of honokiol (*HNK*). After 3 d, cell number was estimated using the SRB assay. Cell survival was expressed as the percent of control (DMSO treated) cells. *Points,* mean of four replicate determinations; *bars,* SD.

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Cell Survival Assay

Cells were seeded in 96-well cell culture plates and treated the next day with the agents indicated. The viable cell number was determined using the sulforhodamine B (SRB) assay, as previously described (28).

Detection of Apoptosis

Apoptosis was evaluated by Annexin V staining using Annexin V-PE apoptosis detection kit purchased from BD Biosciences following the manufacturer's instructions. We also detected caspase activation by Western blotting (as described below) or by fluorometric assay as described previously (29) as an additional indicator of apoptosis.

Western Blot Analysis

Whole-cell protein lysates were prepared and analyzed by Western blotting as described previously (30, 31).

Immunoprecipitation for Detection of Ubiquitinated c-FLIP

H157-$FLIP_L$-21 cells, which stably express $FLIP_L$, were transfected with HA-ubiquitin plasmid using the FuGENE 6 transfection reagent (Roche Diagnostics Corp.) following the manufacturer's instruction. After 24 h, the cells were treated with honokiol or MG132 plus honokiol for 4 h and then lysed for immunoprecipitation of Flag-$FLIP_L$ using Flag M2 monoclonal antibody (Sigma Chemical) as previously described (32) followed by the detection of ubiquitinated $FLIP_L$ with Western blotting using anti-HA antibody (Abgent).

Results

Honokiol Inhibits the Growth of Human NSCLC Cells

We first determined the effects of honokiol as a single agent on the growth of a panel of human NSCLC cell lines. In this experiment, nine NSCLC cell lines, including H460, H358, H1792, A549, H226, H157, H522, H1299, and Calu-1, were treated with increasing concentrations of honokiol. After 3 days, we found that honokiol effectively inhibited the growth of the tested cell lines in a dose-dependent manner with IC_{50}s ranging from 10 to 20 μmol/L, except for H226 cells, the IC_{50} of which was ~30 μmol/L (Fig. 1B).

Honokiol Augments Death Receptor – Mediated Apoptosis in Human NSCLC Cells

It was previously shown that the anticancer activity of honokiol can be antagonized by anti-TRAIL antibody, suggesting the involvement of TRAIL in honokiol-mediated anticancer activity (24). We were interested in whether honokiol in combination with exogenous recombinant TRAIL augmented induction of apoptosis. To this end, we treated four NSCLC cell lines (i.e., H226, A549, H157, and H460) with TRAIL alone, honokiol alone, or both drugs combined and then assessed cell survival and apoptosis. As presented in Fig. 2A, the combination of honokiol at concentrations of >10 μmol/L with either dose of TRAIL (5, 10, 20, or 40 ng/mL) was more effective in decreasing tumor cell survival than either single agent alone. For example, in H226 cells, honokiol alone at 30 μmol/L decreased cell survival by ~10%, and TRAIL (40 ng/mL) alone decreased cell survival by ~20%, but the combination of the two agents reduced cell survival by >60%, which is greater than the sum of the effects of each agent alone. We detected the highest caspase-3 activity in all four tested cell lines when exposed to honokiol plus TRAIL compared with cells treated with either honokiol alone or TRAIL alone, which only minimally or moderately increased caspase-3 activity (Fig. 2B). In Western blot analysis, the combination of honokiol and TRAIL induced the highest levels of cleaved caspase-8, caspase-3, and PARP in comparison with honokiol alone or TRAIL alone in all three tested cell lines (A549, H157, and H226; Fig. 2C). In accordance with these findings, we detected 80% apoptotic cells when treated with honokiol plus TRAIL but only 10% and 25% apoptosis in cells exposed to honokiol alone and TRAIL alone, respectively (Fig. 2D). Collectively, these results clearly show that honokiol combined with TRAIL augments induction of apoptosis in human NSCLC cells.

We also examined whether honokiol enhances apoptosis when combined with Fas ligand or an agonistic anti-Fas antibody, both of which trigger Fas-mediated apoptosis. Similarly to the TRAIL findings, we found that Fas ligand or anti-Fas antibody at the tested concentrations (25–200 ng/mL) did not decrease cell survival. However, the presence of honokiol substantially reduced cell survival (see Supplementary Fig. S1),[3] indicating that honokiol also augments Fas-mediated apoptosis.

Honokiol Rapidly Reduces c-FLIP Levels in Human NSCLC Cells

It is well known that c-FLIP is a major inhibitor of the extrinsic death receptor–mediated apoptotic pathway (6). Thus, c-FLIP down-regulation is an important mechanism underlying the enhancement of death receptor–induced apoptosis by some anticancer drugs (26, 33, 34). To understand the mechanism by which honokiol sensitizes cells to death receptor–mediated apoptosis, we determined whether honokiol modulated c-FLIP levels. Given that c-FLIPs, particularly $FLIP_S$, are quickly turnover proteins with very short half-lives (10), we had DMSO-treated cells as a control for each indicated time point when we did time course analysis of c-FLIP modulation by honokiol. By Western blot analysis, we detected time-dependent reduction of c-FLIP (both $FLIP_L$ and $FLIP_S$) levels in the three tested cell lines, which occurred at 3 h after treatment and was sustained up to 24 h (Fig. 3A). Moreover, the effect of honokiol on the reduction of c-FLIP was dose dependent and decreased the levels of c-FLIP, particularly $FLIP_S$, at concentrations as low as 5 μmol/L (Fig. 3B). Collectively, these findings indicate that honokiol exerts a potent and rapid effect on the down-regulation of c-FLIP.

We also determined whether honokiol modulates the levels of other proteins known to be involved in the regulation of apoptosis. To this end, we made whole-cell protein lysates from cells exposed to honokiol for a short (3 h) or longer (12 h) time period and then analyzed the levels of proteins as presented in Fig. 3C. As expected,

[3] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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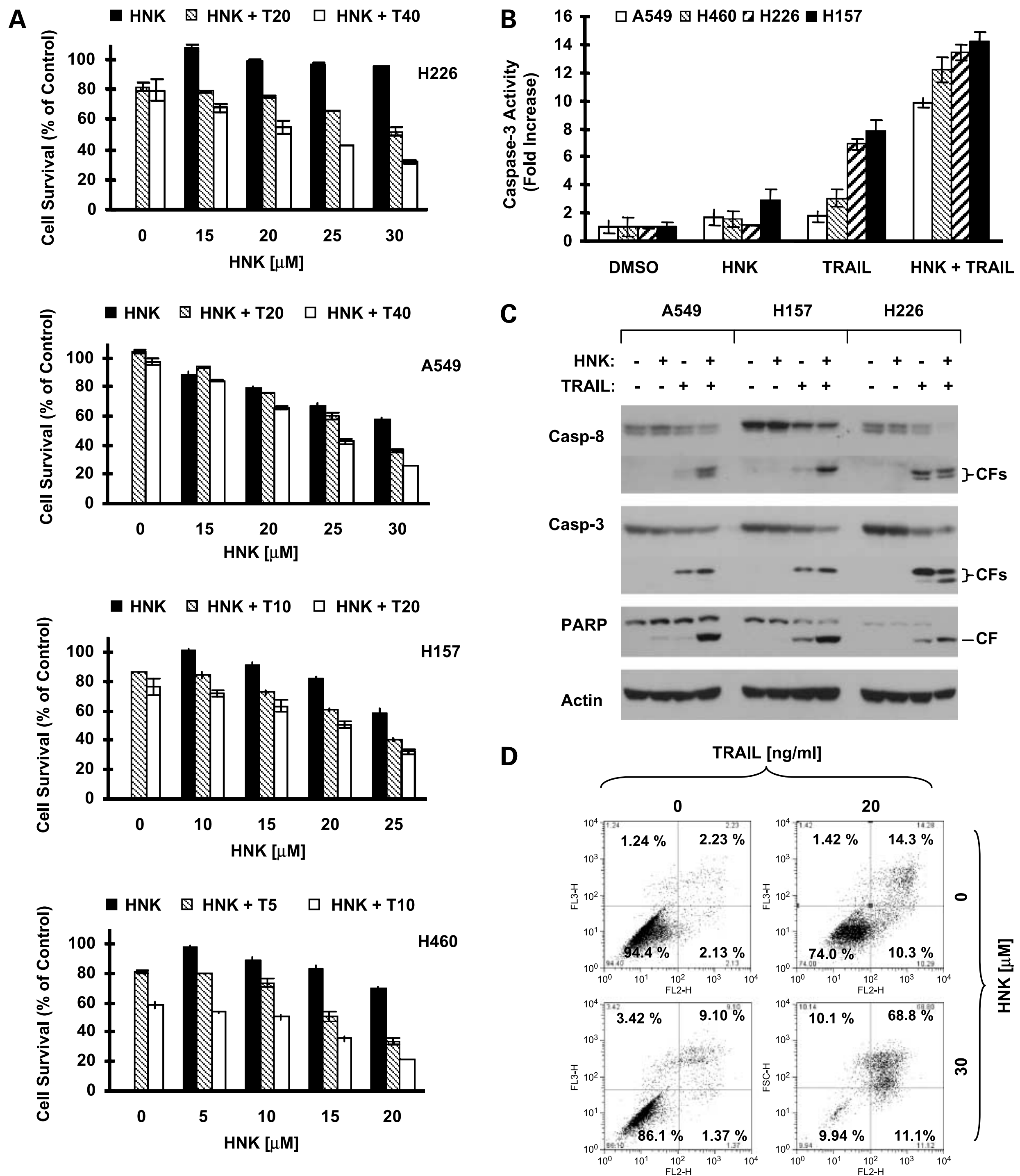

Figure 2. Effects of honokiol combined with TRAIL on cell survival (**A**), caspase activation (**B** and **C**), and apoptosis induction (**D**). **A,** the indicated cell lines were seeded in 96-well cell culture plates and treated the following day with increasing concentrations of honokiol alone, TRAIL alone at the two given concentrations, and their individual combinations. After 24 h, cell number was estimated using SRB assay for calculation of cell survival. *Columns,* mean of four replicate determinations; *bars,* SD. **B** and **C,** the indicated cell lines were treated with DMSO control; honokiol alone at 20 μmol/L (H460), 25 μmol/L (H157), or 30 μmol/L (A549 and H226); TRAIL alone at 5 ng/mL (H460), 10 ng/mL (H157), or 20 ng/mL (A549 and H226); or honokiol plus TRAIL. After 24 h, the cells were subjected to preparation of whole-cell protein lysates for measuring caspase-3 activity using fluorometric assay (**B**) or for detecting caspase (*Casp*) cleavage using Western blotting (**C**). *Columns,* mean of triplicate determinations; *bars,* SD. *CF,* cleaved fragment. **D,** H226 cells were treated with 20 ng/mL TRAIL alone, 30 μmol/L honokiol alone, or their combination for 24 h. The cells were then subjected to measurement of apoptosis using Annexin V staining. The percent positive cells in the top right and bottom right quadrants represent the total apoptotic cell population.

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honokiol decreased c-FLIP levels in all of the tested cell lines after both 3- and 12-h treatments. Increased levels of DR5 and DR4 were detected in these cell lines exposed to honokiol for 12 h; however, in cells treated with honokiol for 3 h, DR5 and DR4 up-regulation was observed only in one cell line (H460) and in two cell lines (H157 and H460), respectively. Similarly, survivin levels were decreased in most cell lines treated with honokiol for 12 h but only in two cell lines (A549 and H157) when exposed to honokiol for 3 h. The levels of XIAP, Bcl-2, and Bax were not altered in any of these cell lines exposed to honokiol for either 3 or 12 h, whereas Bcl-X_L levels were slightly increased in cells exposed to honokiol for 12 h. Mcl-1 levels were effectively decreased in A549 cells exposed to both 15 and 30 μmol/L for 3 or 12 h; however, in other cell lines, Mcl-1 reduction was detected only when treated with 30 μmol/L honokiol

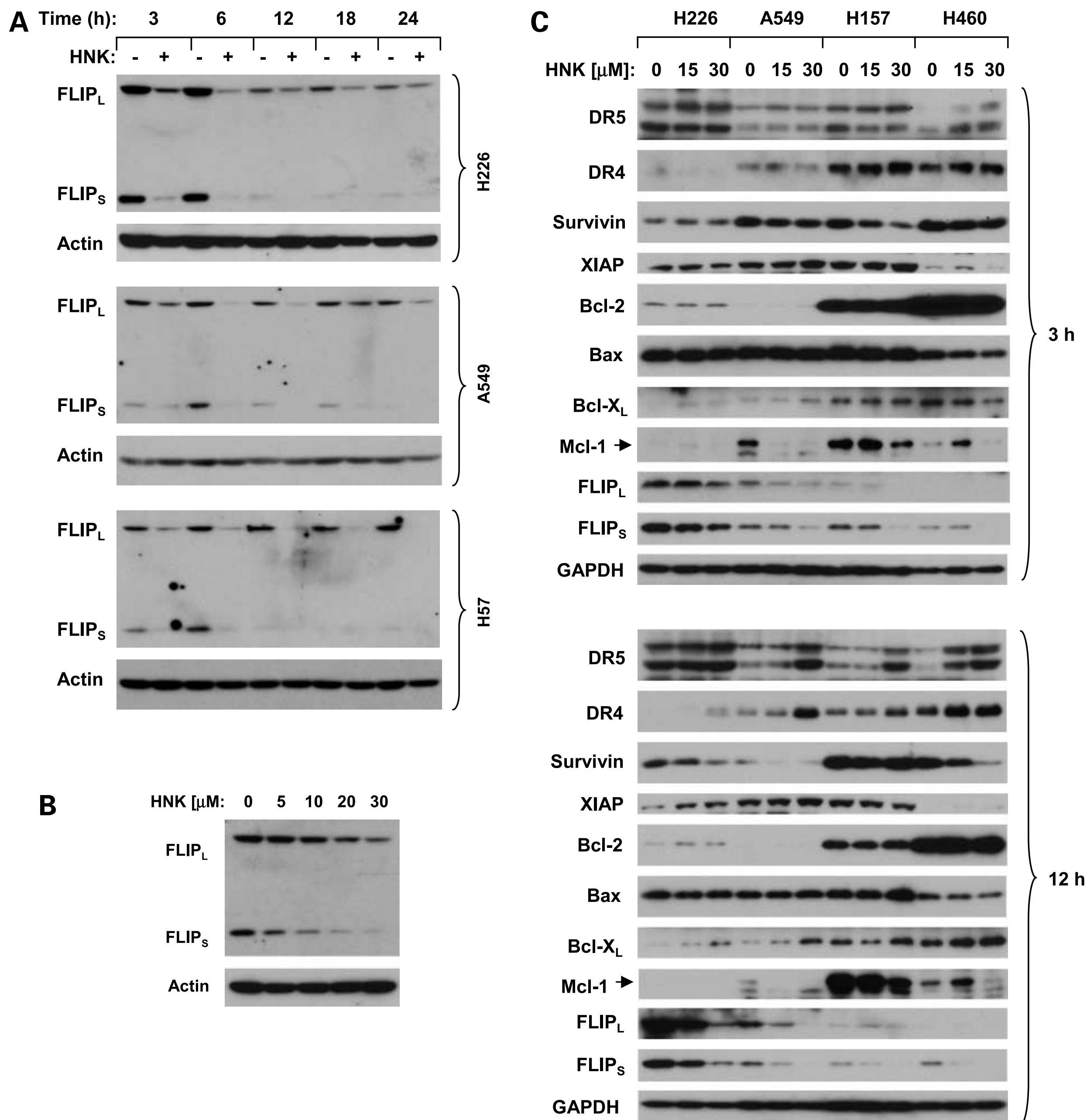

Figure 3. Honokiol modulates c-FLIP levels (**A** and **B**) and the levels of other apoptosis-related proteins (**C**). The given cell lines were treated with 25 μmol/L (H157) or 30 μmol/L (H226 and A549) honokiol for the indicated times (**A**) or with the indicated concentrations of honokiol for 6 h (**B**) or for 3 and 12 h as indicated (**C**). After the treatments, the cell lines were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of the indicated proteins. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

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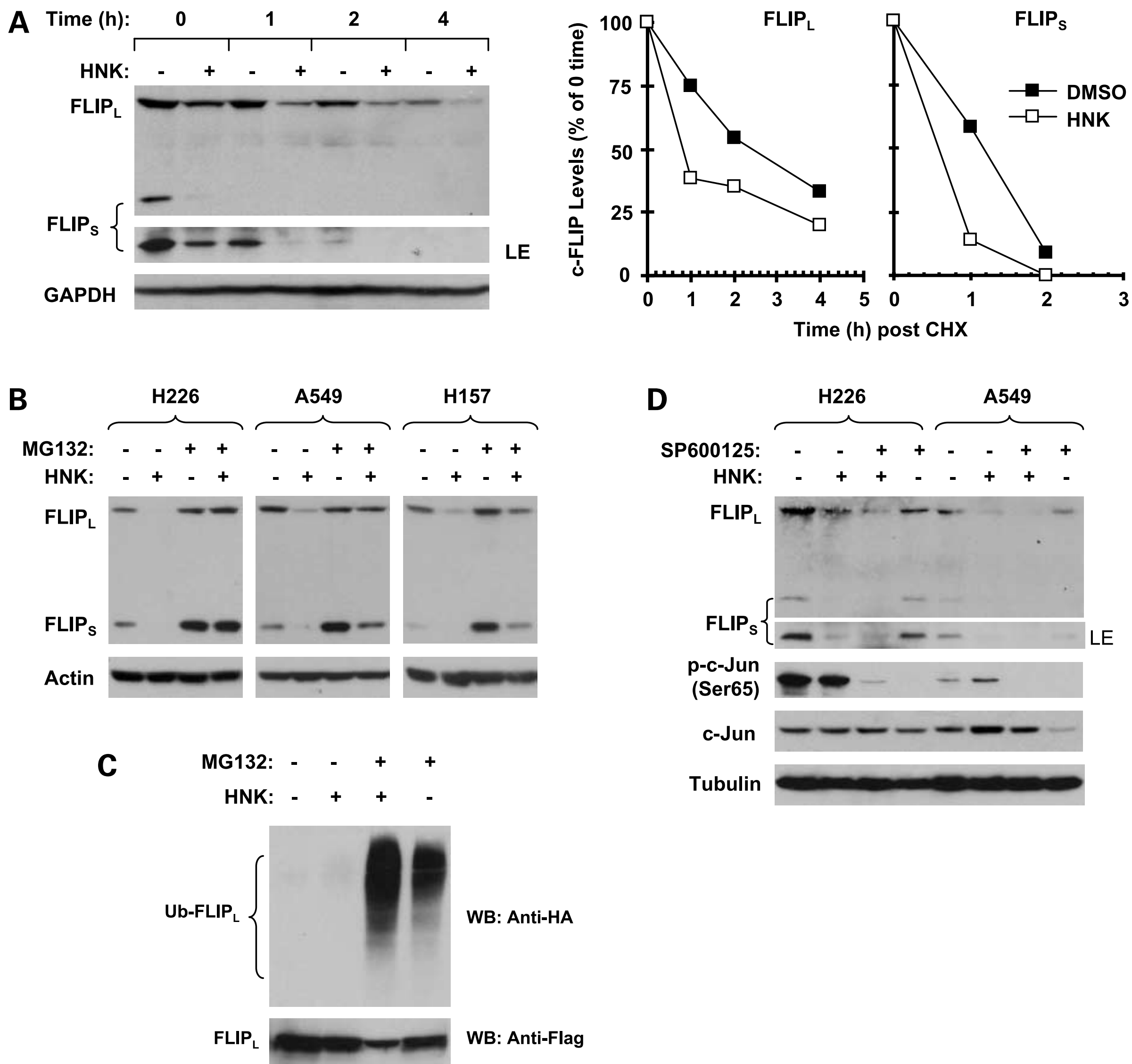

Figure 4. Honokiol modulates c-FLIP levels through ubiquitin/proteasome-mediated protein degradation (**A** – **C**) independent of JNK (**D**). **A,** H226 cells were treated with DMSO or 30 μmol/L honokiol for 5 h. The cells were then washed with PBS thrice and refed with fresh medium containing 10 μg/mL cycloheximide (*CHX*). At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Protein levels were quantitated with NIH ImageJ software (Bethesda, MA) and normalized to glyceraldehyde-3-phosphate dehydrogenase. *Right,* the results were plotted as the relative c-FLIP levels compared with those at the time 0 of cycloheximide treatment. *LE,* longer exposure. **B,** the given cell lines were pretreated with 20 μmol/L MG132 for 30 min before the addition of honokiol (30 μmol/L for H226 and A549; 25 μmol/L for H157). After cotreatment for 4 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. **C,** H157-FLIP$_L$-21 cells, which stably express ectopic Flag-FLIP$_L$, were transfected with HA-ubiquitin plasmid using FuGENE 6 transfection reagent for 24 h. The cells were then pretreated with 20 μmol/L MG132 for 30 min and then cotreated with 25 μmol/L honokiol for 4 h. Whole-cell protein lysates were then prepared for immunoprecipitation using anti-Flag antibody followed by Western blotting (*WB*) using anti-HA antibody for detection of ubiquitinated FLIP$_L$ (*Ub-FLIP$_L$*) and anti-Flag antibody for detection of ectopic FLIP$_L$. **D,** the indicated cell lines were pretreated with 20 μmol/L SP600125 for 30 min and then cotreated with 30 μmol/L honokiol for another 6 h. The cells were then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of the indicated proteins.

(Fig. 3C). Thus, it seems that c-FLIP down-regulation is an early event and likely plays a critical role in mediating death receptor–induced apoptosis.

Honokiol Down-regulates c-FLIP through Promoting Ubiquitin/Proteasome-Mediated Degradation Independent of c-Jun NH_2-Terminal Kinase

Because c-FLIP proteins are regulated by ubiquitin/proteasome-mediated degradation (9, 11), we then determined whether the observed down-regulation of c-FLIP by honokiol would be mediated via this process. Thus, we first examined whether honokiol promotes c-FLIP degradation. To this end, we treated H226 cells with either DMSO solvent control or honokiol for 5 h and then washed away the drug followed by refilling the cells with fresh medium containing the protein synthesis inhibitor cycloheximide. At the indicated times after cycloheximide, the cells were

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harvested for Western blotting for analyzing c-FLIP degradation rate. As presented in Fig. 4A, the half-lives for $FLIP_L$ and $FLIP_S$ in control cells were approximately 145 and 75 min, respectively; however, they were 50 and 40 min, respectively, in honokiol-treated cells. Thus, it is clear that honokiol facilitates c-FLIP degradation. Moreover, we treated cells with honokiol in the presence and absence of the proteasome inhibitor MG132 and then compared c-FLIP modulation under these conditions. We found that the honokiol-induced down-regulation of c-FLIP was inhibited by the presence of MG132 in all of the tested cell lines (Fig. 4B), indicating that honokiol-induced c-FLIP degradation is proteasome dependent. By immunoprecipitation/Western blotting, we detected the highest levels of ubiquitinated $FLIP_L$ in cells treated with honokiol plus MG132 compared with cells exposed to honokiol alone or MG132 alone (Fig. 4C), indicating that honokiol increases c-FLIP ubiquitination. Taken together, we conclude that honokiol initiates ubiquitin/proteasome-mediated c-FLIP degradation, leading to down-regulation of c-FLIP in human NSCLC cells.

Recently, c-Jun NH_2-terminal kinase (JNK) is responsible for TNF-induced, ubiquitin/proteasome-mediated $FLIP_L$ degradation (11). Therefore, we determined whether JNK activation is involved in mediating honokiol-induced c-FLIP degradation. To do so, we examined the effects of honokiol on c-FLIP down-regulation in the presence of the JNK-specific inhibitor SP600125 in H226 and A549 cells. SP600125 at the concentration of 20 μmol/L inhibited both the basal levels of phospho-c-Jun and increased levels of

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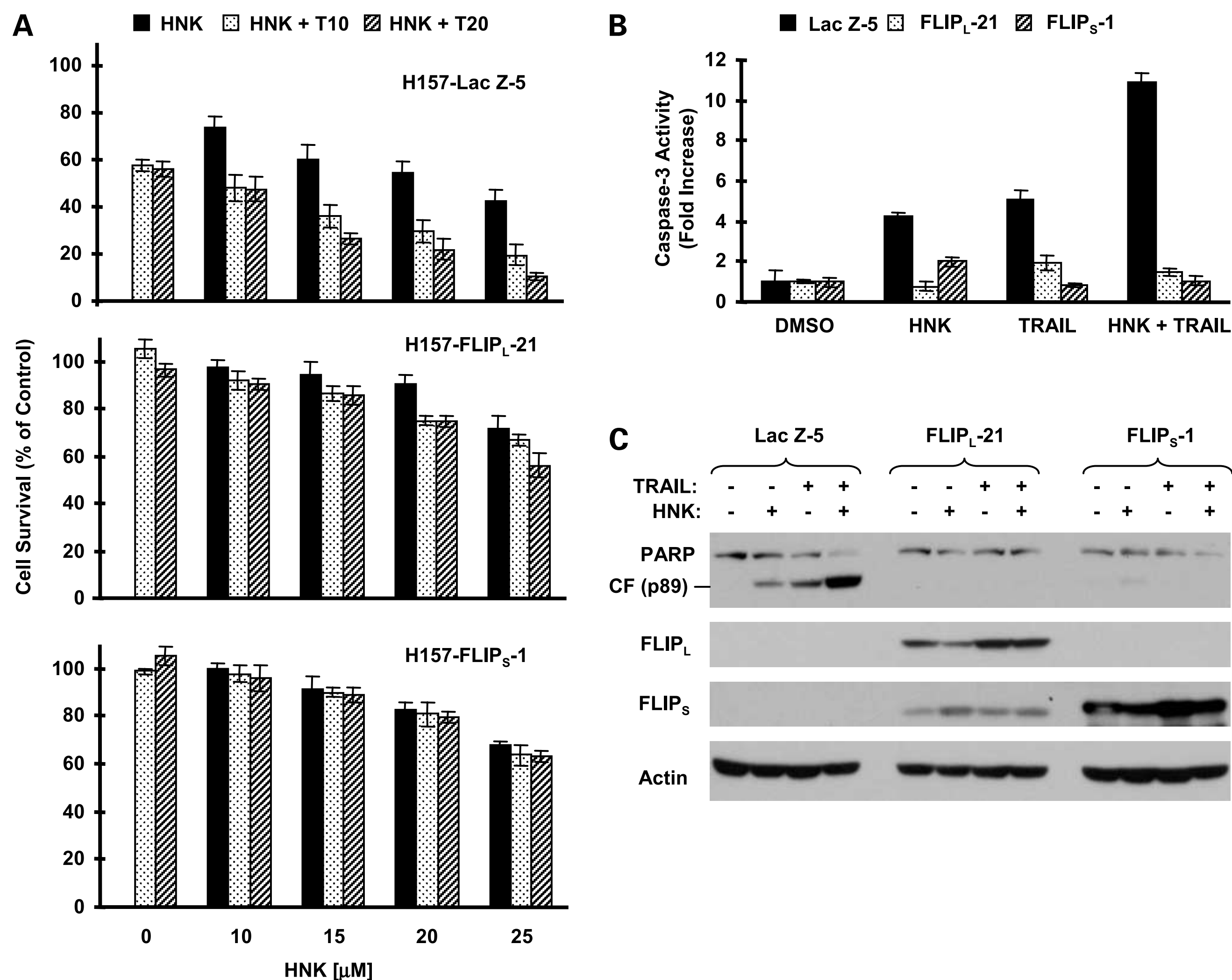

Figure 5. Enforced expression of ectopic c-FLIP confers resistance to induction of apoptosis by the combination of honokiol and TRAIL. **A,** the indicated transfectants were seeded in 96-well plates and treated with the indicated concentrations of honokiol alone, 10 ng/mL TRAIL (*T10*) or 20 ng/mL TRAIL (*T20*) alone, or individual combination of honokiol with TRAIL. After 24 h, the cells were subjected to the SRB assay for measurement of cell survival. *Columns,* mean of four replicate determinations; *bars,* SD. **B** and **C,** the indicated transfectants were treated with DMSO, 25 μmol/L honokiol alone, 10 ng/mL TRAIL alone, or honokiol plus TRAIL for 24 h and then subjected to preparation of whole-cell protein lysates for measuring caspase-3 activity using fluorometric assay (**B**) or for detecting PARP cleavage and c-FLIP using Western blotting (**C**).

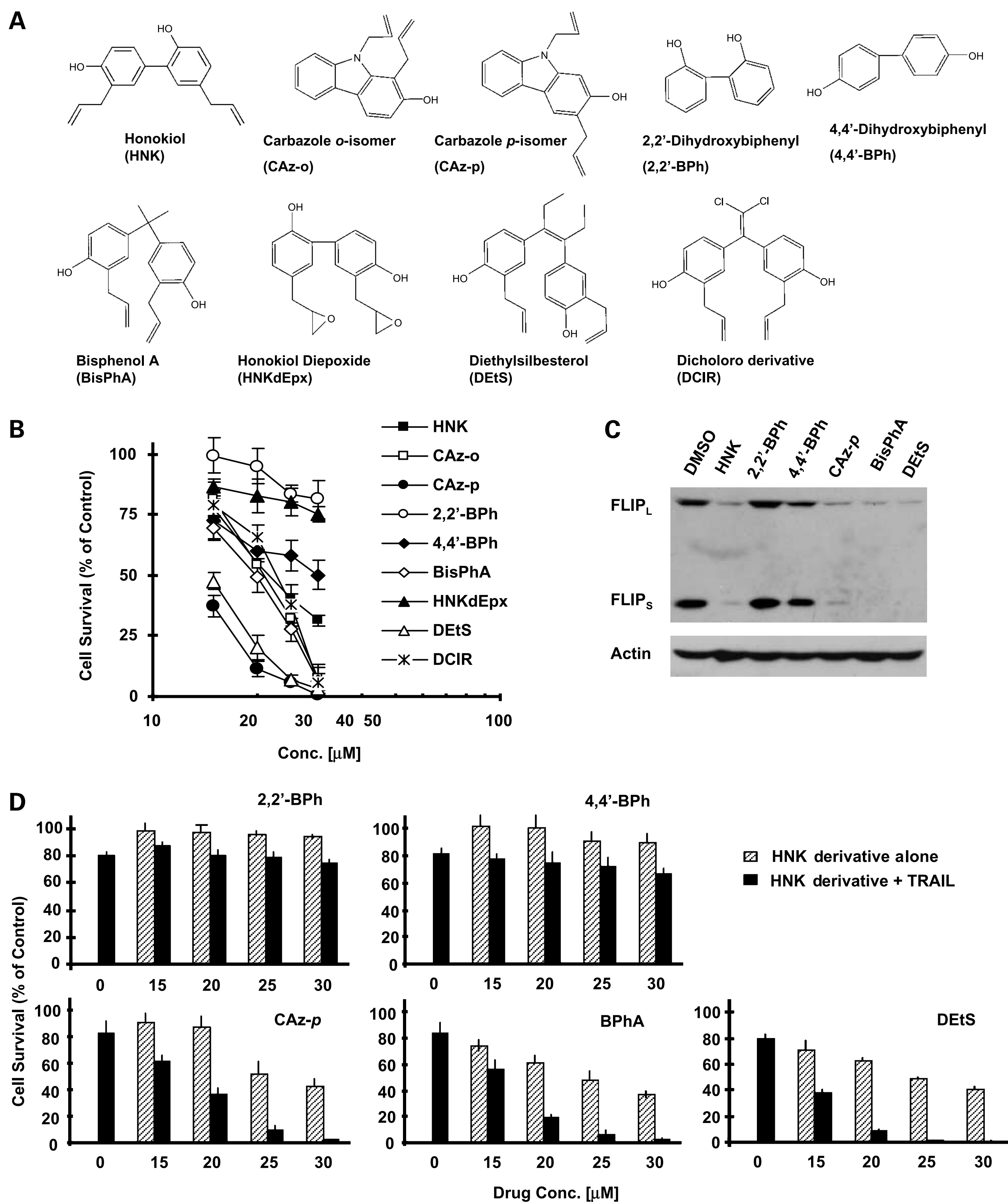

Figure 6. Comparing the effects of honokiol derivatives (**A**) on cell growth (**B**), down-regulation of c-FLIP (**C**), and augmentation of TRAIL-induced apoptosis (**D**). **A,** chemical structures of honokiol derivatives. **B,** H226 cells were seeded in 96-well plates and treated with the indicated concentrations of honokiol. After 3 d, the cells were subjected to the SRB assay for measurement of cell survival. **C,** H226 cells were treated with 30 μmol/L of the indicated honokiol derivatives for 4 h and then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis. **D,** H226 cells were seeded in 96-well plates and treated with the indicated concentrations of honokiol derivative alone, 20 ng/mL TRAIL alone, or their combination. After 24 h, the cells were subjected to the SRB assay for measurement of cell survival. **B,** *points,* mean of four replicate determinations; *bars,* SD. **D,** *columns,* mean of four replicate determinations; *bars,* SD.

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phospho-c-Jun (e.g., A549), confirming that SP600125 worked as expected in our cell systems. However, SP600125 did not block honokiol-induced c-FLIP (both $FLIP_L$ and $FLIP_S$) down-regulation (Fig. 4D). Collectively, we suggest that JNK does not play a role in the honokiol-induced down-regulation of c-FLIP.

Enforced Expression of Ectopic c-FLIP Protects Cells from Honokiol/TRAIL-Induced Apoptosis

To determine the involvement of c-FLIP down-regulation in honokiol-mediated sensitization of death receptor–induced apoptosis, we examined the effect of enforced expression of ectopic c-FLIP on the apoptosis-inducing effects by honokiol combined with TRAIL. Thus, we compared the effects of honokiol plus TRAIL on the survival of three transfectants, H157-Lac Z-5, H157-$FLIP_L$-21, and H157-$FLIP_S$-1, which express the control protein Lac Z, $FLIP_L$, and $FLIP_S$, respectively. As presented in Fig. 5A, the combination of honokiol and TRAIL was more effective than each single agent in decreasing cell survival in H157-Lac Z-5 cells, but this enhanced effect was lost in H157-$FLIP_L$-21 and H157-$FLIP_S$-1 cells, indicating that enforced expression of ectopic $FLIP_L$ or $FLIP_S$ confers cell resistance to the combination of honokiol and TRAIL. By measuring caspase-3 activity and PARP cleavage, we detected no increases in caspase-3 activity and PARP cleavage in both H157-$FLIP_L$-21 and H157-$FLIP_S$-1 cells exposed to honokiol alone, TRAIL alone, or in combination compared with H157-Lac Z-5 cells, in which increased caspase-3 activity and PARP cleavage were observed on these treatments, particularly honokiol combined with TRAIL (Fig. 5B and C). These results further indicate that enforced expression of the ectopic c-FLIP abolishes the ability of honokiol to augment TRAIL-induced apoptosis. In addition, we generated identical results in H460 transfectants that express Lac Z and $FLIP_L$, respectively (Supplementary Fig. S2).[3] Together, we conclude that c-FLIP down-regulation is a key event that mediates augmentation of the death receptor–mediated apoptosis by honokiol.

We noted that honokiol alone was less effective in decreasing the survival of H157-$FLIP_L$-21 and H157-$FLIP_S$-1 cells in comparison with that of H157-Lac Z-5 cells (Fig. 5A). In agreement, we found that honokiol increased caspase-3 activity (Fig. 5B) and PARP cleavage (Fig. 5C) in H157-Lac Z-5 cells but not in H157-$FLIP_L$-21 and H157-$FLIP_S$-1 cells. These results show that overexpression of ectopic c-FLIP also protects cells from honokiol-induced apoptosis, suggesting that c-FLIP down-regulation also plays a role in mediating honokiol-induced apoptosis.

The Potencies of Honokiol Derivatives in Decreasing Cell Survival and Enhancing Death Receptor–Mediated Apoptosis Are Associated with Their Abilities to Down-regulate c-FLIP

To improve the anticancer efficacy of honokiol, we synthesized some derivatives of honokiol as presented in Fig. 6A. These derivatives had different activities in decreasing the survival of H226 cells. Among these compounds, 2,2′-dihydroxybiphenyl, 4,4′-dihydroxybiphenyl, and honokiol diepoxide had the weakest activity, whereas diethylsilbesterol and carbazole *p*-isomer had the most potent activity in decreasing cell survival. Bisphenol A, carbazole *o*-isomer, and dichloro derivative had activity in between but slightly more potent than honokiol in decreasing cell survival (Fig. 6B). By comparing their effects on modulation of c-FLIP levels, we found that both 2,2′-dihydroxybiphenyl and 4,4′-dihydroxybiphenyl had minimal effects on reducing c-FLIP levels, whereas carbazole *p*-isomers, particularly bisphenol A and diethylsilbesterol, were even more potent than honokiol in decreasing c-FLIP levels (Fig. 6C). Accordingly, we found that 2,2′-dihydroxybiphenyl or 4,4′-dihydroxybiphenyl, when combined with TRAIL, did not exhibit augmented effects on decreasing cell survival; however, bisphenol A, diethylsilbesterol, or carbazole *p*-isomer in combination with TRAIL showed more potent activity than honokiol in decreasing cell survival (Fig. 6D). Collectively, these results show that there is a tight association between down-regulation of c-FLIP and sensitization of death receptor–mediated apoptosis by honokiol and its derivatives.

Discussion

In this study, we have shown that the natural product honokiol and its derivatives inhibit the growth of human NSCLC cells and, more importantly, augment TRAIL-induced apoptosis in human NSCLC cells. To our knowledge, this is the first study showing that honokiol and its derivatives can function as sensitizers of death receptor–mediated apoptosis. Given that the tumor-selective TRAIL is a potential cancer therapeutic protein and is being tested in phase I clinical trials, our findings suggest a potential strategy for the use of honokiol in combination with TRAIL in the treatment of lung cancer and possibly other types of cancer. Thus, the current finding is of clinical significance and also warrants further evaluation on the efficacy of honokiol combined with TRAIL in animal models.

By comparing the modulatory effects of honokiol on the expression levels of several proteins involved in the regulation of apoptosis, including c-FLIP, DR4, DR5, survivin, XIAP, Mcl-1, Bcl-2, Bcl-X_L, and Bax, c-FLIP, a major inhibitor of the death receptor–induced apoptosis, was rapidly down-regulated in a dose-dependent manner in all of the tested NSCLC cell lines at 3 h after honokiol treatment (Fig. 3C). Thus, it seems that honokiol preferentially decreases the levels of c-FLIP (both $FLIP_L$ and $FLIP_S$). Moreover, we have shown that honokiol down-regulates c-FLIP levels by facilitating ubiquitin/proteasome-mediated degradation of c-FLIP. This is evidenced by enhancement of c-FLIP turnover rates in honokiol-treated cells, by prevention of the honokiol-induced c-FLIP reduction using the proteasome inhibitor MG132, and by the increased levels of ubiquitinated c-FLIPs, which are detected in cells cotreated with MG132 and honokiol using immunoprecipitation-Western blotting (Fig. 4). Although JNK

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activation is suggested in regulating ubiquitin/proteasome-dependent degradation of $FLIP_L$ (11), we failed to show a role of JNK in mediating honokiol-induced c-FLIP degradation based on the following facts. First, honokiol decreases both forms of c-FLIP (i.e., $FLIP_L$ and $FLIP_S$), whereas JNK regulates the degradation of only the long form of c-FLIP (i.e., $FLIP_L$; ref. 11). Second, honokiol only slightly increases JNK activation in one cell line (i.e., A549) but not in another cell line (i.e., H226), in which c-FLIP was still down-regulated. Third, the JNK inhibitor SP600125 does not prevent honokiol-induced down-regulation of c-FLIP.

It has been documented that modulation of c-FLIP levels alters cell sensitivity to death receptor–mediated apoptosis (7, 9, 35–38). In this study, enforced expression of ectopic $FLIP_L$ or $FLIP_S$ abrogated induction of apoptosis by honokiol combined with TRAIL (Fig. 5), suggesting a critical role of c-FLIP down-regulation in mediating the augmentation of TRAIL-induced apoptosis by honokiol. Through studies on the honokiol derivatives, we found that the potencies of these derivatives on enhancing TRAIL-induced apoptosis were tightly associated with their abilities to decrease c-FLIP levels (Fig. 6), further supporting the notion that c-FLIP down-regulation is a key mechanism by which honokiol and its derivatives sensitize TRAIL-induced apoptosis. Given that c-FLIP is a major inhibitor of death receptor–mediated apoptosis, we believe that honokiol can function as a general sensitizer of the pathway. Indeed, honokiol combined with Fas ligand or the agonistic Fas antibody also exhibits augmented effects on decreasing cell survival (Supplementary Fig. S1).[3] Thus, we speculate that honokiol as well as its derivatives can also sensitize cancer cells to agonistic anti-DR4 or anti-DR5 antibody-triggered apoptosis, implying the potential use of honokiol in combination with an agonistic anti-TRAIL death receptor antibody for cancer therapy.

It is known that TRAIL/TRAIL death receptor–mediated and/or Fas ligand/Fas–mediated apoptosis are vital components of immunosurveillance of cancer cell by both T cells and natural killer cells (39, 40). Accordingly, up-regulation of c-FLIP in tumor cells will protect tumor cells from being eradicated by this immunosurveillance mechanism and hence favors tumor immune escape (40). Honokiol down-regulates c-FLIP in cancer cells, resulting in sensitization of cancer cells to both TRAIL-mediated and Fas ligand–mediated apoptosis. These results imply that honokiol may be able to sensitize cancer cell to T cell–mediated and/or natural killer cell–mediated immunosurveillance or immunotherapy through such a mechanism, which needs further investigation in the future.

We noted that honokiol alone had much weaker activity on decreasing cell survival and on increasing caspase-3 activity and PARP cleavage in cells expressing an ectopic $FLIP_L$ or $FLIP_S$ compared with control cells expressing Lac Z (Fig. 5). These data also suggest that c-FLIP down-regulation contributes to honokiol-induced apoptosis. Several recent studies have documented that c-FLIP down-regulation participates in the induction of apoptosis by certain types of anticancer agents, including chemotherapeutic agents. Thus, the relationship between c-FLIP down-regulation and honokiol-induced apoptosis warrants further investigation.

It has been suggested that c-FLIP plays a critical role in anoikis resistance and distant tumor formation (41). Thus, c-FLIP may be a potential therapeutic target against advanced cancer or metastatic cancer. Accordingly, agents that specifically inhibit c-FLIP may have therapeutic potential for these types of cancers, particularly when combined with TRAIL or agonistic anti-DR4 or anti-DR5 antibody that triggers the death receptor–mediated apoptosis. Honokiol is a natural product purified from magnolia, a plant used in traditional Chinese and Japanese medicine and more recently used as a component of dietary supplements and cosmetic products (42). It is a systemically available and well-tolerated compound in mice with potent antitumor activity (21, 24, 25, 43). Importantly, the maximal plasma concentrations of honokiol in mice can safely reach higher than 1 mg/mL (43). Moreover, honokiol has a simple chemical structure and should be easily modified. Therefore, honokiol is an ideal lead compound for synthesizing c-FLIP inhibitors with more potent activity than honokiol in sensitizing the death receptor–mediated apoptosis and better cancer therapeutic efficacy.

We noted that honokiol did modulate the expression of a few other apoptotic proteins, such as DR4, DR5, Mcl-1, and survivin, at a high concentration (e.g., 30 μmol/L) and/or relatively late times (e.g., 12 h). In some cell lines (e.g., H460 or H157), the modulation of these proteins by honokiol also occurred at 3 h after treatment. In A549 cells, Mcl-1 levels were effectively reduced even by 15 μmol/L honokiol for a 3-h exposure (Fig. 3C). Given that these proteins are also involved in modulating death receptor–induced apoptosis (44), it is possible that the modulation of these proteins can also contribute to honokiol-mediated sensitization of the death receptor–induced apoptosis to a certain extent, at least in some cell lines. It is likely that the initial honokiol-mediated removal of c-FLIP by induction of its degradation followed by up-regulation of DR4 and/or DR5 and decreases in the levels of the downstream inhibitors survivin and Mcl-1 make cancer cells sensitive to death receptor–induced apoptosis. Nonetheless, the roles of these proteins in modulating death receptor–induced apoptosis need further investigation.

It has been shown that honokiol enhances TNFα-induced apoptosis by inhibiting TNF-induced NF-κB activation and the expression of certain antiapoptotic genes regulated by NF-κB, including *Mcl-1*, *survivin*, and *c-FLIP* (22). Our study aimed at modulation of basal levels of protein involved in regulation of apoptosis. Indeed, honokiol effectively inhibited NF-κB activation induced by TNFα in our assay (see Supplementary Fig. S3).[3] Although TRAIL activates NF-κB in some cell systems or under certain conditions (45, 46), we failed to show that TRAIL

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increased NF-κB activity under the condition in our assay. Moreover, we found that honokiol only minimally inhibited the basal levels of NF-κB activity (Supplementary Fig. S3).[3] Given that honokiol promotes c-FLIP degradation as shown in our study, we collectively suggest that it is unlikely that honokiol sensitizes NSCLC cells to TRAIL-induced apoptosis through inhibition of NF-κB.

In summary, the present study for the first time shows that the natural product honokiol sensitizes death receptor–mediated apoptosis in human NSCLC cells by facilitating the ubiquitin/proteasome-mediated degradation of c-FLIP, thus warranting further *in vivo* evaluation of honokiol combined with TRAIL or an agonistic TRAIL death receptor antibody as a potential cancer therapeutic regimen.

Disclosure of Potential Conflicts of Interest

J.L. Arbiser: inventor of these compounds for which Emory University has filed a patent. The other authors disclosed no potential conflicts of interest.

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