Journal of Cellular Physiology

Honokiol Inhibits Signal Transducer and Activator of Transcription-3 Signaling, Proliferation, and Survival of Hepatocellular Carcinoma Cells Via the Protein Tyrosine Phosphatase SHP-1

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The activation of signal transducers and activators of transcription 3 (STAT3) has been closely linked with the proliferation, survival, invasion, and angiogenesis of hepatocellular carcinoma (HCC) and represents an attractive target for therapy. In the present report, we investigated whether honokiol mediates its effect through interference with the STAT3 activation pathway. The effect of honokiol on STAT3 activation, associated protein kinases, and phosphatase, STAT3-regulated gene products and apoptosis was investigated using both functional proteomics tumor pathway technology platform and different HCC cell lines. We found that honokiol inhibited both constitutive and inducible STAT3 activation in a dose- and time-dependent manner in HCC cells. The suppression was mediated through the inhibition of activation of upstream kinases c-Src, Janus-activated kinase 1, and Janus-activated kinase 2. Vanadate treatment reversed honokiol-induced down-regulation of STAT3, suggesting the involvement of a tyrosine phosphatase. Indeed, we found that honokiol induced the expression of tyrosine phosphatase SHP-1 that correlated with the down-regulation of constitutive STAT3 activation. Moreover, deletion of SHP-1 gene by siRNA abolished the ability of honokiol to inhibit STAT3 activation. The inhibition of STAT3 activation by honokiol led to the suppression of various gene products involved in proliferation, survival, and angiogenesis. Finally, honokiol inhibited proliferation and significantly potentiated the apoptotic effects of paclitaxel and doxorubicin in HCC cells. Overall, the results suggest that honokiol is a novel blocker of STAT3 activation and may have a great potential for the treatment of HCC and other cancers.
J. Cell. Physiol. 227: 2184–2195, 2012.

Hepatocellular carcinoma (HCC) is the one of the most commonly occurring malignancies worldwide, with an estimated incidence of half a million new cases per year around the world (Parkin et al., 2005). The disease is more commonly encountered in developing countries with more than half of the patients identified in China (Lin et al., 2011). Available treatment options, including surgical resection and conventional chemotherapy, are often associated with severe morbidity, exhibit severe side effects, and are limited by therapeutic resistance (Avila et al., 2006; Kerr and Kerr, 2009; Alves et al., 2011). Hence, novel therapeutics are urgently needed for the treatment of HCC patients.

The identification of novel anticancer agents derived from existing natural sources provides an enormous opportunity to improve the existing standard of care for HCC and other cancers (Newman, 2008). Honokiol, derived from the stem and bark of the plant *Magnolia officinalis* (Ahn et al., 2006; Fried and Arbiser, 2009), has been reported to modulate various pro-inflammatory cascades in wide variety of tumor cells, for example it was found to inhibit nitric oxide synthesis and tumor necrosis factor (TNF) expression (Son et al., 2000; Lee et al., 2005), inhibit PI3K/mTOR pathway without affecting T-cell function (Crane et al., 2009), induce caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells through down-regulation of the antiapoptotic protein Mcl-1 (Liou et al.,

Contract grant sponsor: National Medical Research Council of Singapore;
Contract grant numbers: R-184-000-201-275, R-713-000-119-275.
Contract grant sponsor: Cancer Science Institute of Singapore, Experimental Therapeutics I Program;
Contract grant number: R-713-001-011-271.

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Received 23 June 2011; Accepted 19 July 2011

Published online in Wiley Online Library (wileyonlinelibrary.com), 25 July 2011.
DOI: 10.1002/jcp.22954

2003), suppress survival signals mediated by Ras dependent phospholipase D activity (Garcia et al., 2008), induce apoptosis in hepatoma cell line through activation of p38 MAP kinase pathway (Deng et al., 2008), and also inhibit cellular FLICE-inhibitory protein and augment death receptor-induced apoptosis in lung cancer cells (Raja et al., 2008). In vivo, honokiol has been found to inhibit skin tumor promotion (Chilampalli et al., 2010; Vaid et al., 2010), suppress the growth of breast cancer through induction of apoptosis and cell cycle (Wolf et al., 2007; Hou et al., 2008), inhibit gastric tumorigenesis by activation of 15-lipoxygenase-1 (Liu et al., 2010); inhibit the growth and metastasis in prostate cancer xenograft models (Shigemura et al., 2007; Hahm et al., 2008), attenuate the growth of human colorectal carcinoma in nude mice (Chen et al., 2004), and enhance the anti-tumor effects of FDA approved EGFR antibody cetuximab in head and neck squamous cell carcinoma xenograft model (Leeman-Neill et al., 2010).

Seven different members of the signal transducer and activator of transcription (STAT) family of transcription factors regulate the expression of various gene products involved in cell survival, proliferation, invasion, angiogenesis, and chemoresistance (Darnell, 1997; Aggarwal et al., 2006). Among the different STATs, STAT3 is perhaps the most intimately linked to tumorigenesis, and is often constitutively activated in many human cancers including HCC (Bromberg and Darnell, 2000; Aggarwal et al., 2009; He and Karin, 2011; Yu et al., 2009). Once activated, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription. STAT3 has been reported to regulate the expression of genes that participate in oncogenesis, such as apoptosis inhibitors (Bcl-xl, Bcl-2, and survivin), cell-cycle regulators (cyclin D1) and inducers of angiogenesis (VEGF) (Grivennikov and Karin, 2010). Thus, small molecule inhibitors of STAT3 activation have the potential for both prevention and treatment of cancer. Moreover, STAT3 has been implicated as a promising target for HCC therapy since inhibition of STAT3 using diverse strategies has been found to induce growth arrest and apoptosis of HCC cells (Li et al., 2006, 2010; Sun et al., 2008; Tan et al., 2010).

Because of the pivotal role of STAT3 in tumor cell survival, proliferation, and angiogenesis, and its expression in various tumor cells, we investigated whether honokiol can mediate its anti-cancer effects in part through the modulation of the STAT3 activation pathway. Alongside testing the effects of honokiol in HCC cell lines, we also tested the hypothesis of inhibition of STAT3 involvement in the honokiol mechanism of action in a virtual predictive tumor cell system. The virtual epithelial tumor cell platform on which predictive studies were conducted to determine the various targets being modulated by honokiol is a comprehensive integrated representation of the pathways representing the key cancer phenotypes of proliferation, apoptosis, angiogenesis, metastasis, and conditions of tumor microenvironment including tumor-associated inflammation (Cirstea et al., 2010). This is a dynamic network of pathways with inter- and intra-cellular crosstalk and associated autocrine and paracrine loops whereby any internal marker can be perturbed through % knockdown and over-expression and impact seen on the whole network. This virtual tumor cell has been used to get an insight into how a particular drug individually or in combination is impacting various cancer phenotypes across different tumor profiles.

(Rajendran et al., 2011; Roy et al., 2011). Thus, using a novel approach of combination of predictive virtual hypothesis testing along with experimental validations we found that honokiol can indeed suppress both constitutive as well as inducible STAT3 expression in HCC cells. This inhibition decreased cell survival and down-regulated expression of proliferative, anti-apoptotic, and angiogenic gene products, leading to suppression of proliferation, induction of apoptosis, and potentiation of the cytotoxic effects of doxorubicin and paclitaxel in HCC cells.

Materials and Methods

Reagents

Honokiol, Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, EGF, BSA, doxorubicin, and paclitaxel were purchased from Sigma–Aldrich (St. Louis, MO). Honokiol was dissolved in dimethylsulfoxide as a 10 mM stock solution and stored at 4°C. Further dilution was done in cell culture medium. RPMI 1640, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen. Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho- STAT3 (Tyr 705) and phospho-Akt, Akt, Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, SHP-1, VEGF, procaspase-3, and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-specific Src (Tyr 416), Src, phospho-specific JAK1 (Tyr 1022/1023), JAK1, phospho-specific JAK2 (Tyr 1007/1008), and JAK2 were purchased from Cell Signaling Technology (Beverly, MA). The siRNA for SHP-1 (sc-29478) and scrambled control (sc-37007) was obtained from Santa Cruz Biotechnology. SHP-1 siRNA is a pool of 3 sequences. Sense Strand (A): CUGGUGGAGCAUUUCAAGATT, (B): CGCAGUACAAGUUCAUCUATT and (C): CAACCCUUCUCCUCUUGUATT. Goat anti-rabbit-horse radish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Sigma–Aldrich. Bacteria-derived recombinant human IL-6 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).

Cell lines

Human HCC cell line HepG2 was obtained from American Type Culture Collection (Manassass, VA). HUH-7, PLC/PRF5, and Hep3B cells were kindly provided by Prof. Kam Man Hui, National cancer Centre, Singapore. All the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1X antibiotic-antimycotic solution with 10% FBS.

Western blotting

For detection of phopho-proteins, honokiol-treated whole-cell extracts were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM $NaVO_4$). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 7.5% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-STAT antibodies (1:1,000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

To detect STAT3-regulated proteins and PARP, cells (2×10^6/ml) were treated with honokiol for the indicated times. The cells were then washed and protein was extracted by incubation for 30 min on ice in 0.05 ml buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% NP-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine, 1 mM DTT, and 1 mM sodium vanadate. The lysate was centrifuged and the supernatant was collected. Whole-cell extract protein (30 μg) was resolved on 12% SDS–PAGE, electrotransferred onto a nitrocellulose membrane, blotted with antibodies against survivin, Bcl-2, Bcl-xl, cyclin D1, Mcl-1, VEGF, procaspase-3, and PARP and then detected by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunocytochemistry for STAT3 localization

HepG2 cells were plated in chamber slides in DMEM containing 10% FBS and allowed to adhere for 24 h. On the next day, the cells were fixed with cold acetone for 10 min, washed with PBS, permeabilized in 0.2% Triton-X for 15 min and blocked with 5% normal goat serum for 1 h. The cells were then incubated with rabbit polyclonal anti-human STAT3 Antibody (dilution, 1/100). After overnight incubation, the cells were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1/100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained cells were mounted with mounting medium (Sigma–Aldrich) and analyzed under an fluorescence microscope (DP 70, Olympus, Tokyo, Japan).

STAT3 luciferase reporter assay

PLC/PRF5 cells were plated in 96-well plates with 1×10^4/well in DMEM containing 10% FBS. The STAT3-responsive elements linked to a luciferase reporter gene were transfected with wild-type or dominant-negative STAT3-Y705F (STAT3F). These plasmids were a kind gift from Dr. Bharat B. Aggarwal at M D Anderson Cancer Center. Transfections were done according to the manufacturer's protocols using Fugene-6 (Roche). At 24 h post-transfection, cells were pretreated with different concentrations of honokiol for 6 h and then induced by EGF for additional 2 h before being washed and lysed in luciferase lysis buffer (Promega, Madison, WI). Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega) and the experiments were done in triplicate and repeated three or more times.

DNA binding assay

DNA binding was performed using a STAT3 DNA binding ELISA kit (Active Motif, Carlsbad, CA). Briefly, nuclear extracts (5 μg) from honokiol treated cells were incubated in a 96-well plate coated with oligonucleotide containing the STAT3 specific DNA probe. Bound STAT3 was then detected by a specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a microplate reader (Tecan Systems, San Jose, CA). Specificity of this assay was tested by the addition of wild-type or mutated STAT3 consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of the nuclear extracts.

Predictive experiments on virtual tumor cell

Predictive experiments were performed using the human physiologically aligned and extensively validated Virtual Tumor Cell technology (Cellworks Group Inc (CWG), CA) (Roy et al., 2011). The Cellworks Tumor cell platform provides a dynamic and transparent view of tumor cell physiology at the functional proteomics abstraction level. The platform's open-access architecture provides a framework for different "what-if" experiments and analysis in an automated high-throughput methodology.

Platform description

The virtual Tumor Cell Platform consists of a dynamic and kinetic representation of the signaling pathways underlying tumor physiology at the bio-molecular level with coverage on all the key tumor phenotypes including proliferation, viability, angiogenesis, metastasis, apoptosis, tumor metabolism, and tumor microenvironment related to associated inflammation. The technology is a comprehensive coverage of protein players; associated gene and mRNA species with regard to tumor related signaling. The platform sub-set coverage includes signaling pathways comprising growth factors like EGFR, PDGFRA, FGFR, c-MET, VEGFR and IGF-1R, cell cycle regulators, mTOR signaling, p53 signaling cascade, cytokine pathways like IL1, IL4, IL6, IL12, TNF; TGF-β, hypoxia mediated regulation, angiogenic promoters, lipid mediators and tumor metabolism and others as described recently by our group (Rajendran et al., 2011). It has a wide coverage of kinases and transcription factors associated with tumor physiology network. The modeling of the time-dependent changes in the fluxes of the constituent pathways has been done utilizing modified ordinary differential equations (ODE) and mass action kinetics. The current version of the technology includes over 5,000 biological species with over thirty thousand cross-talk interactions. The platform has been prospectively and retrospectively validated against an extensive set of pre-defined in vitro and in vivo studies.

The starting control state of the system is based on normal epithelial cell physiology that is non-tumorigenic. The user can control the transition of the normal system to a neoplastic disease state aligning with specific tumor mutation profiles. This is accomplished as an example through over-expression of the tumorigenic genes like EGFR, IGF-1R; knock-downs of the tumor-suppressors like p53, PTEN; and increased states of hypoxia and oxidative stress. Knockdowns or over-expressions of biological species can be done at the expression or activity levels. Drugs are represented in this technology through explicit mechanism of action specification and the drug concentration in the virtual experiments is explicitly assumed to be post ADME (absorption, distribution, metabolism, and excretion).

Predictive study experimental protocol

The virtual Tumor cell is simulated in the proprietary Cellworks computational backplane and triggered to align to the HCC cell line SNU387 having KRAS mutation, CDKN2A deletion and mutation in the P53 gene.

Study details

Honokiol was tested on the above base line and the biomarker trends evaluated as percentage changes from cell baseline values. Honokiol mechanism of action has been shown to inhibit AKT, ERK, and SRC in the system with a concentration of 1 μM and Ki of 0.124 μM for AKT, 5 μM for ERK and 10 μM for SRC, respectively. The on-target inhibition achieved was 75% for AKT and 35% for ERK and SRC, AKT being the primary target. The effect of honokiol was also compared with STAT3 inhibition alone on the referenced base line. STAT3 activation was inhibited by 80% and the effects on the downstream markers analyzed.

Transfection with SHP-1 siRNA

HepG2 cells were plated in each well of six-well plates and allowed to adhere for 24 h. On the day of transfection, 4 μl of lipofectamine Invitrogen (Carlsbad, CA) was added to 50 nM SHP-1 siRNA in a final volume of 100 μl of culture medium. After 48 h of transfection, cells were treated with honokiol, and whole-cell extracts were prepared to investigate SHP-1, phospho-STAT3, and STAT3 expression by Western blot analysis.

MTT assay

The antiproliferative effect of honokiol against HCC cells was determined by the MTT dye uptake method as described previously (Rajendran et al., 2011). Briefly, the cells (5×10^3/ml) were incubated in triplicate in a 96-well plate in the presence or absence of different concentrations of honokiol in a final volume of 0.2 ml for indicated time intervals at 37°C. Thereafter, 20 μl MTT solution (5 mg/ml in PBS) was added to each well. After a 2-h incubation at 37°C, 0.1 ml lysis buffer (20% SDS, 50% dimethylformamide) was added; and further incubation was done for 2 h at 37°C.

Flow cytometric analysis

To determine the effect of honokiol on the cell cycle, cells were exposed to honokiol for 24 h. Thereafter cells were washed, fixed with 70% ethanol for 30 min at -20°C, and then stained with 10 ug/ml PI and 1 ug/ml RNase A. Cells were then washed again, resuspended, and stained in PBS containing 25 μg/ml propidium iodide (PI) for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako, Denmark).

Live/dead Assay

Apoptosis of cells was also determined by live/dead assay (Molecular Probes, Eugene, OR) that measures intracellular esterase activity and plasma membrane integrity as described previously (Rajendran et al., 2011). Briefly, 1×10^6 cells were incubated with honokiol/doxorubicin/paclitaxel alone or in combination for 24 h at 37°C. Cells were stained with the live/dead reagent (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (DP 70, Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as the mean ± SEM. In all figures, vertical error bars denote the SEM. The significance of differences between groups was evaluated by Student's *t*-test and one way analysis of variance, (ANOVA) test. A *P*-value of <0.05 was considered statistically significant.

Results

We investigated the effect of honokiol on constitutive and inducible STAT3 activation in HCC cells. We also evaluated the effect of honokiol on various mediators of cellular proliferation, cell survival, and apoptosis. The structure of honokiol is shown in Figure 1A. The dose and duration of honokiol used to modulate STAT3 activation did not affect cell viability, indicating that down-regulation of STAT3 was not due to cell killing (data not shown).

Predictive analysis for the effect of honokiol on HCC cells

We first tested the potential effect of honokiol on the STAT3 activation pathway in the virtual tumor cell system aligned to the human HCC cell line SNU387. Both the phosphorylated forms of upstream kinases JAK2 and SRC involved in STAT3 activation showed an equivalent reduction with both honokiol and STAT3 inhibition alone as in Figure 1B. The reduction in survival markers is also more or less similar with both honokiol and STAT3 inhibition alone (Fig. 1C). An increase in both CASP3 and cleaved PARP1 was seen with both honokiol and STAT3 inhibition (Fig. 1D).

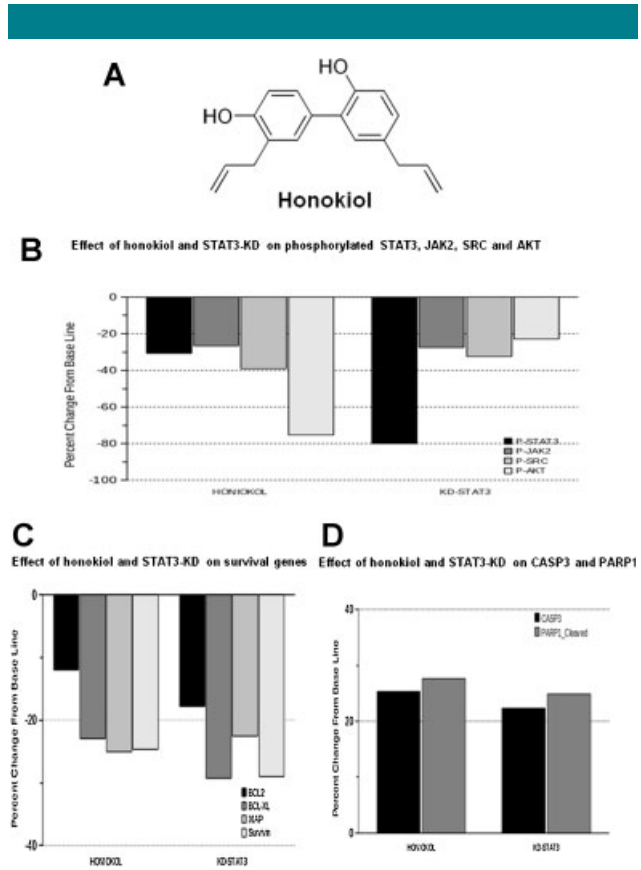

Fig. 1. Predictive proteomics in silico virtual tumor platform generated results. A: The chemical structure of honokiol. B: The graph illustrates the percentage reduction in phosphorylated levels of STAT3, JAK2, and SRC kinase. STAT3 shows a 30% reduction with honokiol treatment. The percentage reduction is around 30% for JAK 2 and 35–40% for SRC with honokiol and STAT3 inhibition alone, respectively. C: The graph illustrates the percentage change in survival markers with honokiol and STAT3 inhibition. Both BCL2 and BCL-xL show a higher reduction in levels ~20% and 34%, respectively with STAT3 inhibition alone as compared to 10% and 22% with honokiol treatment. XIAP shows a slightly higher reduction of ~25% with honokiol as compared to ~21% with STAT3 inhibition. Survivin shows a reduction of approx. 25% with honokiol treatment and 30% with STAT3 inhibition alone. D: The graph illustrates the percentage change in CASP3 and cleaved PARP1. CASP3 is showing an increase of 25% and 21% with honokiol and STAT3, respectively. Cleaved PARP1 is showing an increase of 27% and 24% with honokiol and STAT3, respectively.

Honokiol inhibits constitutive STAT3 phosphorylation in HepG2 cells

The ability of honokiol to modulate constitutive STAT3 activation in HCC cells was investigated. HepG2 cells were incubated with different concentrations of honokiol for 6 h, whole cell extracts were prepared and the phosphorylation of STAT3 was examined by Western blot analysis using antibodies, which recognize STAT3 phosphorylation at tyrosine 705. As shown in Figure 2A, honokiol inhibited the constitutive activation of STAT3 in HepG2 cells in a dose-dependent manner, with maximum inhibition occurring at around 50 μM. Honokiol had no effect on the expression of STAT3 protein (Fig. 2A; lower panel). We also determined the incubation time with honokiol required for the suppression of STAT3 activation in HepG2 cells. As shown in Figure 2B, the inhibition was time-dependent, with complete inhibition occurring at around 6 h, again with no effect on the expression of STAT3 protein (Fig. 2B; lower panel).

Honokiol inhibits binding of STAT3 to the DNA

Because tyrosine phosphorylation causes the dimerization of STATs and their translocation to the nucleus, where they bind to DNA and regulate gene transcription (Yu et al., 1995), we determined whether honokiol suppresses the DNA binding activities of STAT3. Analysis of nuclear extracts prepared from HepG2 cells using ELISA-based TransAM NF-κB assay kit showed that honokiol inhibited STAT3-DNA binding activities in a time-dependent manner (Fig. 2C). These results suggest that honokiol abrogates the DNA binding ability of STAT3.

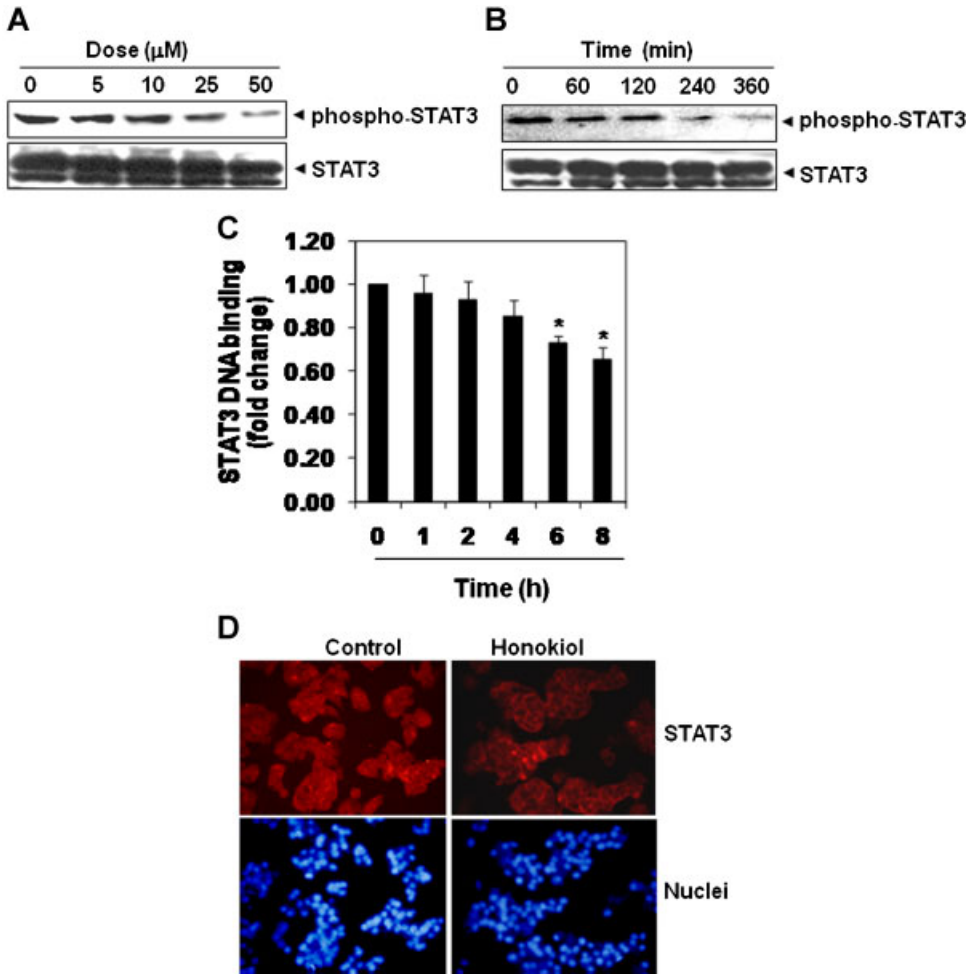

Fig. 2. Honokiol inhibits constitutively active STAT3 in HepG2 cells. A: Honokiol suppresses phospho-STAT3 levels in a dose dependent manner. HepG2 cells (2×10^6/ml) were treated with the indicated concentrations of honokiol for 6 h, after which whole-cell extracts were prepared, and 30 μg of protein was resolved on 7.5% SDS–PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. B: Honokiol suppresses phospho-STAT3 levels in a time-dependent manner. HepG2 cells (2×10^6/ml) were treated with the 50 μM honokiol for the indicated times, after which Western blotting was performed as described for panel B. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. C: Honokiol suppresses STAT3 DNA binding ability in HepG2 cells. HepG2 cells were treated with 50 μM honokiol for indicated time points; nuclear extracts were prepared, and 20 μg of the nuclear extract protein was used for ELISA-based DNA binding assay as described in Materials and Methods Section. The results shown are representative of two independent experiments $^*P < 0.05$. D: Honokiol causes inhibition of translocation of STAT3 to the nucleus. HepG2 cells (1×10^5/ml) were incubated with or without 50 μM honokiol for 6h and then analyzed for the intracelullar distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. The results shown are representative of three independent experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

Honokiol reduces nuclear pool of STAT3 in HCC cells

Because the active dimer of STAT3 is capable of translocating to the nucleus and inducing transcription of specific target genes (Bowman et al., 2000), we determined whether honokiol suppresses nuclear translocation of STAT3. Figure 2D clearly demonstrates that honokiol inhibited the translocation of STAT3 to the nucleus in HepG2 cells.

Honokiol inhibits inducible STAT3 and JAK2 phosphorylation in HCC cells

Because IL-6 induces STAT3 phosphorylation (Bromberg and Wang, 2009), we next determined whether honokiol could inhibit IL-6-induced STAT3 phosphorylation in HCC cells. In HUH-7 cells incubated with honokiol for different times, IL-6-induced STAT3 and JAK2 phosphorylation was suppressed by honokiol in a time-dependent manner. Exposure of cells to honokiol for 3–4 h was sufficient to substantially suppress IL-6-induced STAT3 and JAK2 phosphorylation in HUH-7 cells (Fig. 3A,B). These results suggest that honokiol can down-regulate both constitutive and inducible STAT3 activation and corroborate with the predictive data on STAT3 inhibition as shown in Figure 1B.

Honokiol inhibits IL-6-Inducible Akt phosphorylation in HCC cells

Activated Akt has been shown to play a critical role in the mechanism of action of IL-6. Moreover, activation of Akt has also been linked with STAT3 activation (Chen et al., 1999). We also examined whether honokiol could modulate IL-6-induced Akt activation. Treatment of HUH-7 cells with IL-6 induced phosphorylation of Akt and treatment of cells with honokiol

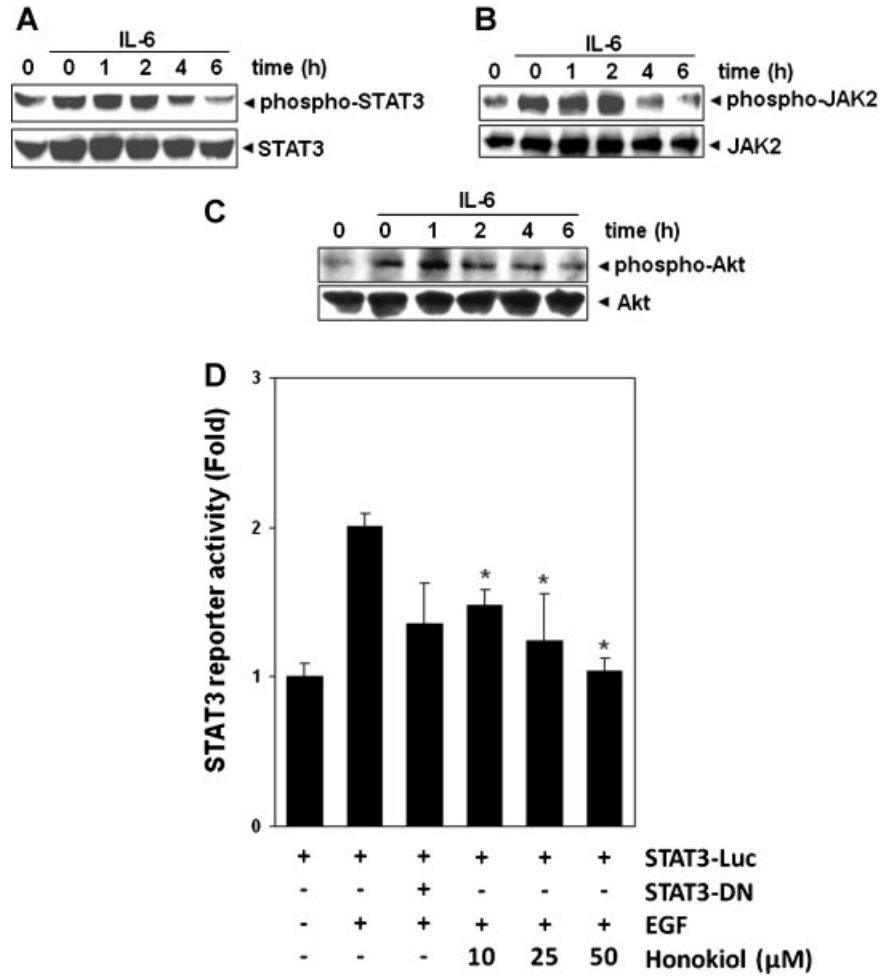

Fig. 3. A: HUH-7 (2×10^6/ml) were treated with 50 μM honokiol for the indicated times and then stimulated with IL-6 (10 ng/ml) for 15 min. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by Western blotting. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. The results shown are representative of three independent experiments. B: HUH-7 (2×10^6/ml) were treated with 50 μM honokiol for the indicated times and then stimulated with IL-6 (10 ng/ml) for 15 min. Whole-cell extracts were then prepared and analyzed for phospho-JAK2 by Western blotting. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. C: HUH-7 (2×10^6/ml) were treated with 50 μM honokiol for the indicated times and then stimulated with IL-6 (10 ng/ml) for 15 min. Whole-cell extracts were then prepared and analyzed for phospho-Akt by Western blotting. The same blots were stripped and reprobed with Akt antibody to verify equal protein loading. D, PLC/PRF-5 cells (5×10^5/ml) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with 10, 25, and 50 μM honokiol for 6 h and then stimulated with EGF (100 ng/ml) for 2 h. Whole-cell extracts were then prepared and analyzed for luciferase activity. The results shown are representative of three independent experiments. (*P-value < 0.05). P-value indicates comparison between EGF and honokiol treated groups was determined by Student's t-test.

suppressed the activation (Fig. 3C). Under these conditions, honokiol had no effect on the expression of Akt protein.

Honokiol suppresses EGF–induced STAT3-dependent reporter gene expression

Our above results showed that honokiol inhibited the phosphorylation and nuclear translocation of STAT3. We next determined whether honokiol affects STAT3-dependent gene transcription. When PLC/PRF5 cells transiently transfected with the pSTAT3-Luc construct were stimulated with EGF, STAT3-mediated luciferase gene expression was increased. Dominant-negative STAT3 blocked this increase, indicating specificity. When the cells were pretreated with honokiol, EGF-induced STAT3 activity was inhibited in a dose-dependent manner (Fig. 3D).

Honokiol suppresses constitutive activation of c-Src

STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (Schreiner et al., 2002). Hence, we determined whether honokiol on constitutive activation of Src kinase in HepG2 cells. We found that honokiol suppressed the constitutive phosphorylation of c-Src kinases (Fig. 4A). The levels of non-phosphorylated Src kinases remained unchanged under the same conditions.

Honokiol suppresses constitutive activation of JAK1 and JAK2

Because STAT3 is also activated by soluble tyrosine kinases of the Janus family (JAKs) (Ihle, 1996), so we next determined whether honokiol affects constitutive activation of JAK1 in HepG2 cells. We found that honokiol suppressed the

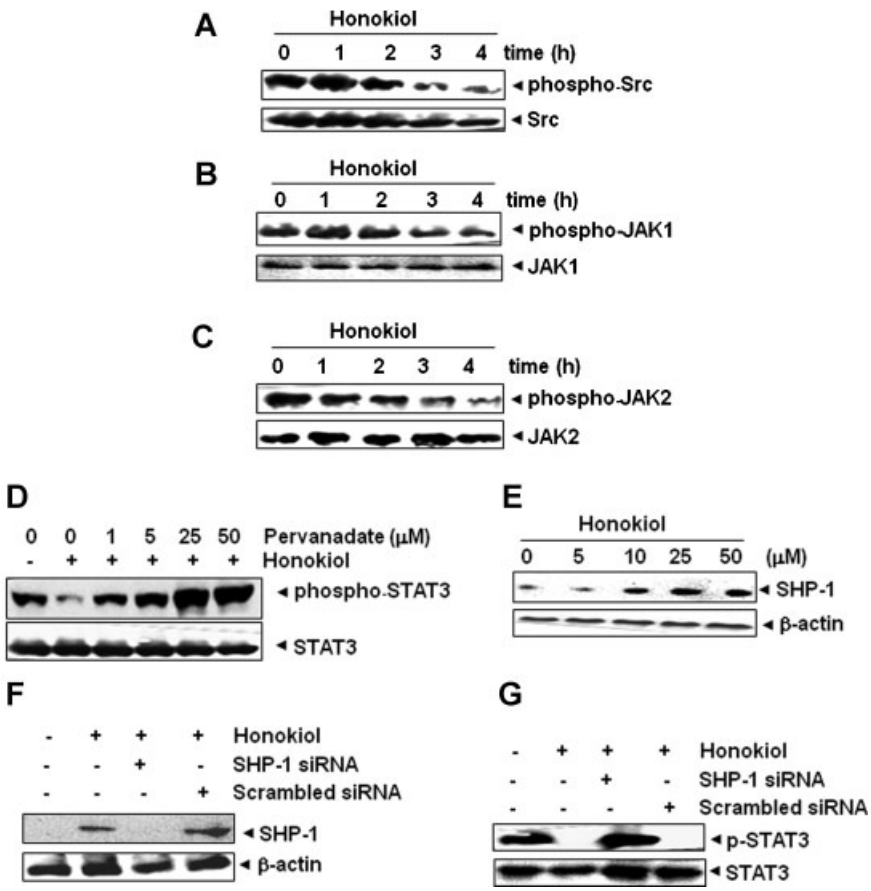

Fig. 4. A: Honokiol suppresses phospho-Src levels in a time-dependent manner. HepG2 cells (2×10^6/ml) were treated with 50 μM honokiol, after which whole-cell extracts were prepared and 30 μg aliquots of those extracts were resolved on 10% SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. B: Honokiol suppresses phospho-JAK1 levels in a time-dependent manner. HepG2 cells (2×10^6/ml) were treated with 50 μM honokiol for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho- JAK1 antibody. The same blots were stripped and reprobed with JAK1 antibody to verify equal protein loading. C: Honokiol suppresses phospho-JAK2 levels in a time-dependent manner. HepG2 cells (2×10^6/ml) were treated with 50 μM honokiol for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK2 antibody. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. D: Pervanadate reverses the phospho-STAT3 inhibitory effect of honokiol. HepG2 cells (2×10^6/ml) were treated with the indicated concentrations of pervanadate and 50 μM honokiol for 6 h, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 7.5% SDS–PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. E: Honokiol induces the expression of SHP-1 protein in HepG2 cells. HepG2 cells were treated with indicated concentrations of honokiol for 6 h, after which Western blotting was performed. F: Effect of SHP-1 knockdown on honokiol induced expression of SHP-1. HepG2 cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 24 h, cells were treated with 50 μM honokiol for 6 h and whole-cell extracts were subjected to Western blot analysis. G: HepG2 cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 24 h, cells were treated with 50 μM honokiol for 6 h and whole-cell extracts were subjected to Western blot analysis for phosphorylated STAT3. The results shown are representative of three independent experiments.

constitutive phosphorylation of JAK1 (Fig. 4B). The levels of non-phosphorylated JAK1 remained unchanged under the same conditions (Fig. 4B, bottom panel). To determine the effect of honokiol on JAK2 activation, HepG2 cells were treated for different time intervals with honokiol and phosphorylation of JAK2 was analyzed by Western blot. As shown in Figure 4C, JAK2 was constitutively active in HepG2 cells and pretreatment with honokiol suppressed this phosphorylation in a time-dependent manner.

Tyrosine phosphatases are involved in honokiol-induced inhibition of STAT3 activation

Because protein tyrosine phosphatases have also been implicated in STAT3 activation (Han et al., 2006), we determined whether honokiol-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase (PTPs). Treatment of HepG2 cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate prevented the honokiol-induced inhibition of STAT3 activation (Fig. 4D). This suggests that tyrosine phosphatases are involved in honokiol-induced inhibition of STAT3 activation.

Honokiol induces the expression of SHP-1 in HCC cells

SHP-1 is a SH-2 containing tyrosine phosphatase involved in the suppression of a variety of cytokine signals, including STAT3 (Calvisi et al., 2006). We therefore examined whether honokiol can induce the expression of SHP-1 in HepG2 cells. Cells were

incubated with different concentrations of honokiol for 6 h, whole cell extracts were prepared and examined for SHP-1 protein by Western blot analysis. As shown in Figure 4E, honokiol induced the expression of SHP-1 protein in HepG2 cells in a dose-dependent manner, with maximum expression at 25–50 μM. This stimulation of SHP-1 expression by honokiol correlated with down-regulation of constitutive STAT3 activation in HepG2 cells (Fig. 2A). Therefore, these results suggest that stimulation of SHP-1 expression by honokiol may mediate the down-regulation of constitutive STAT3 activation in HepG2 cells. SHP-1 siRNA down-regulated the expression of SHP-1 induced by honokiol.

Whether the suppression of SHP-1 expression by siRNA abrogates the honokiol-induced SHP-1 expression, was also investigated. As observed by Western blot analysis, honokiol-induced SHP-1 expression was effectively abolished in the cells transfected with SHP-1 siRNA but not in those treated with the scrambled siRNA (Fig. 4F).

SHP-1 siRNA reversed the inhibition of STAT3 activation by honokiol

We next determined whether the suppression of SHP-1 expression by siRNA abrogates the inhibitory effect of honokiol on STAT3 activation. We found that honokiol failed to suppress STAT3 activation in the cells transfected with SHP-1 siRNA (Fig. 4G). However, in cells transfected with scrambled siRNA, honokiol caused down-regulation of STAT3 activation. Thus, these results with siRNA demonstrate the critical role of SHP-1 in suppression of STAT3 phosphorylation by honokiol.

Honokiol down-regulates the expression of cyclin D1, Bcl-2, Bcl-xL, Mcl-1, surcivin, and VEGF

STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival, proliferation, angiogenesis, and chemoresistance (Aggarwal et al., 2009). We found that expression of the cell cycle regulator cyclin D1, the antiapoptotic proteins Bcl-2, Bcl-xL, survivin, Mcl-1 and the angiogenic gene product VEGF all of which have been reported to be regulated by STAT3 were modulated by honokiol treatment. Their expression decreased in a time-dependent manner, with maximum suppression observed at around 24 h (Fig. 5). These results also support the predictive analysis seen with STAT3 inhibition on these markers as seen in Figure 1C.

Honokiol inhibits the proliferation of HCC cells in a dose and time dependent manner

Because honokiol down-regulated the expression of cyclin D1, a gene critical for cell proliferation, we investigated whether honokiol inhibits the proliferation of HCC cells by using the MTT assay. Honokiol inhibited proliferation of HepG2, HUH-7, PLC/PRF5, and HepG2 cells in a dose and time dependent manner (Fig. 6A).

Honokiol causes the accumulation of the cells in the sub-G1 phase of the cell cycle

Because D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase (Matsushime et al., 1991) and rapid decline in levels of cyclin D1 was observed in honokiol treated cells, we determined the effect of honokiol on cell cycle phase distribution. We found that honokiol can cause increased accumulation of cell population in sub-G1 phase, which is indicative of apoptosis (Fig. 6B).

Honokiol activates caspase-3 and causes PARP cleavage

Whether suppression of constitutively active STAT3 in HepG2 cells by honokiol leads to apoptosis was also investigated. In HepG2 cells treated with honokiol there was a time-dependent activation of pro-caspase-3 (Fig. 6C). Activation of downstream caspases led to the cleavage of a 118 kDa PARP protein into an 85 kDa fragment (Fig. 6D). These results clearly suggest that honokiol induces caspase-3-dependent apoptosis in HepG2 cells.

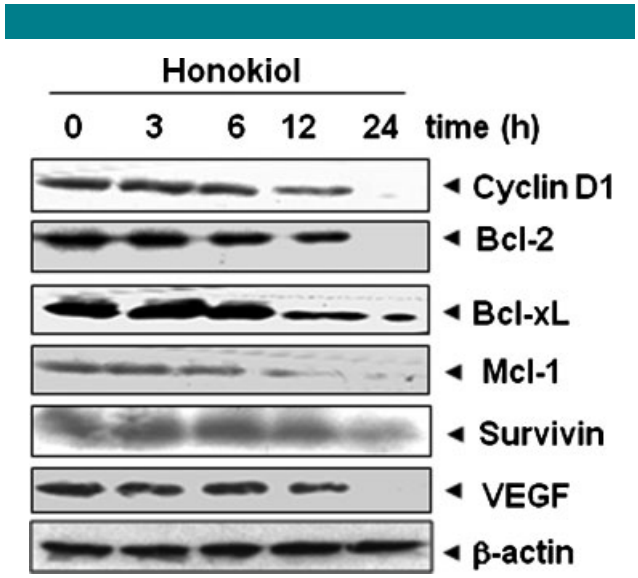

Fig. 5. Honokiol suppresses STAT3 regulated gene products involved in proliferation, survival and angiogenesis. HepG2 cells (2×10^6/ml) were treated with 50 μM honokiol for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS–PAGE, membrane sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-X_L, survivin, Mcl-1, and VEGF antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. The results shown are representative of three independent experiments.

Honokiol potentiates the apoptotic effect of doxorubicin and paclitaxel in HepG2 cells: Among chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, and paclitaxel, a mitotic inhibitor, have been used for HCC treatment (Jin et al., 2009). We examined whether honokiol can potentiate the effect of these drugs. HepG2 cells were treated with honokiol together with either doxorubicin or paclitaxel, and then apoptosis was measured by the live/dead assay. As shown in Figure 7, honokiol significantly enhanced the apoptotic effects of doxorubicin from 12 to 36% and of paclitaxel from 10 to 32%.

Discussion

The goal of this study was to determine whether honokiol exerts its anti-cancer effects through the abrogation of the STAT3 signaling pathway in HCC cells. We found that honokiol suppressed constitutive and IL-6-inducible STAT3 activation in human HCC cells in parallel with the inhibition of c-Src, JAK1 and JAK2 activation. Honokiol also down-regulated the expression of STAT3-regulated gene products including cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and VEGF. It also caused the inhibition of proliferation; increased accumulation of cells in sub-G1 phase, and it significantly potentiated the apoptotic effects of doxorubicin and paclitaxel in HCC cells. This hypothesis was also tested in a virtual predictive tumor cell system and the predictive results indicate that honokiol mediates its suppressive effects on STAT3 activation cascade in HCC cells this mechanism of action generated similar biomarker trends as seen experimentally with honokiol effects on HCC cells.

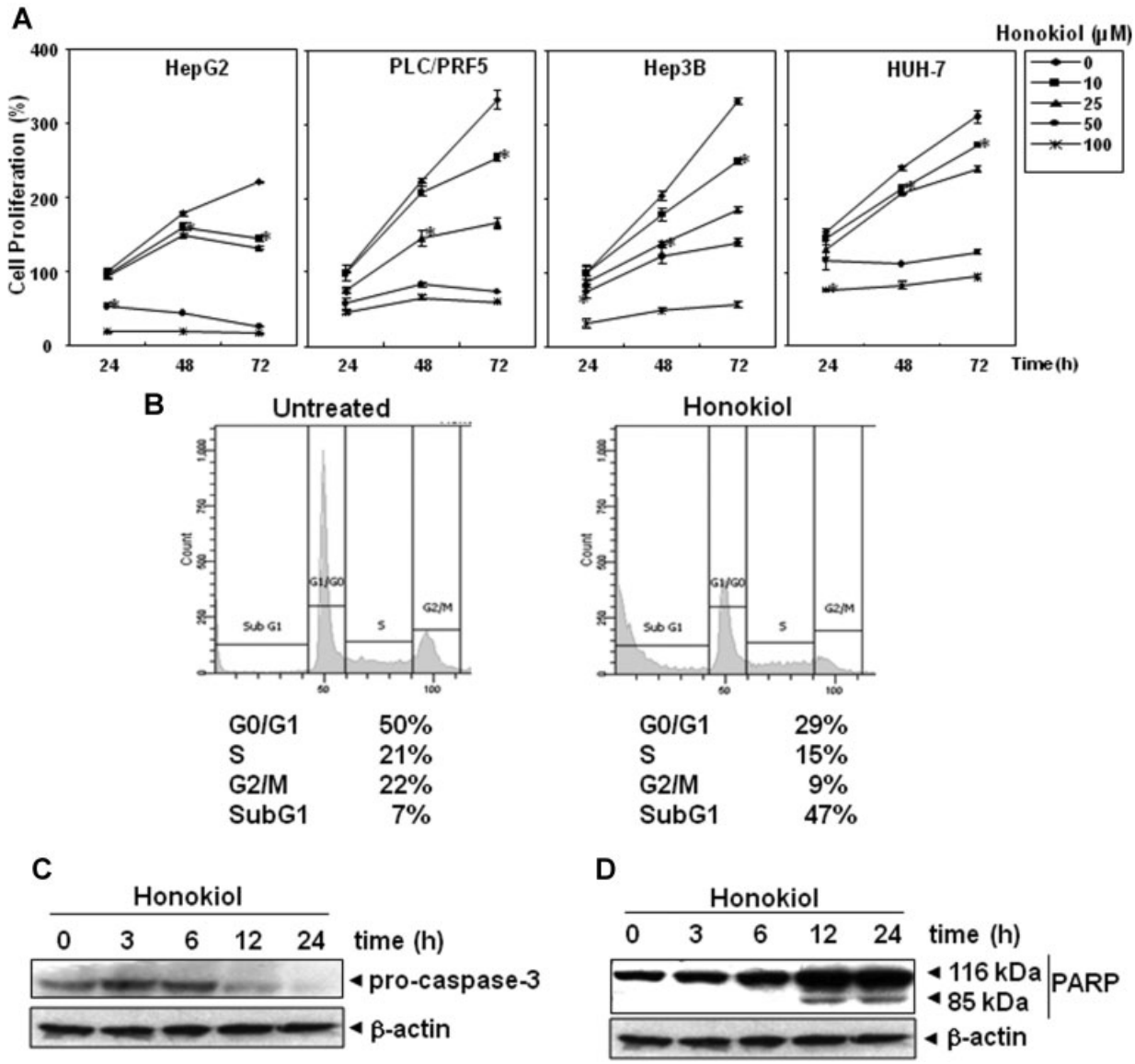

Fig. 6. A: Honokiol suppresses proliferation, causes accumulation of cells in sub-G1 phase, and activates caspase-3. A, HepG2, PLC/PRF5, Hep3B, and HUH-7 cells (5×10^3/ml) were plated in triplicate, treated with indicated concentrations of honokiol, and then subjected to MTT assay after 24, 48, and 72 h to analyze proliferation of cells. Standard deviations between the triplicates are indicated. B: HepG2 cells (2×10^6/ml) were treated with 50 μM honokiol for the indicated times, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. C: HepG2 cells were treated with 50 μM honokiol for the indicated times, whole-cell extracts were prepared, separated on SDS–PAGE, and subjected to Western blotting against pro-caspase-3 antibody. The same blot were stripped and reprobed with β-actin antibody to show equal protein loading. D: HepG2 cells were treated with 50 μM honokiol for the indicated times, and whole-cell extracts were prepared, separated on SDS–PAGE, and subjected to Western blot against PARP antibody. The same blot was stripped and reprobed with β-actin antibody to show equal protein loading. The results shown are representative of three independent experiments.

This is the first report to suggest that biphenolic compounds, such as honokiol can inhibit STAT3 activation in HCC cells. Whether examined by STAT3 phosphorylation at tyrosine 705, by nuclear translocation, and by DNA binding, we found that honokiol suppressed STAT3 activation. We found that honokiol also suppressed STAT3 activation induced by IL-6, one of the many tumor cell growth factors that activate STAT3 (Moran et al., 2008; Bromberg and Wang, 2009). The doses required to inhibit STAT3 activation were very comparable to rationally designed chemical inhibitors that inhibit STAT3 dimerization (Fuke et al., 2007). The effects of honokiol on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases c-Src, JAK1 and JAK2. Previous studies have indicated that Src and JAK1 kinase activities cooperate to mediate constitutive activation of STAT3 (Campbell et al., 1997; Garcia et al., 2001). Our findings suggest that honokiol may block cooperation of Src and JAKs involved in tyrosyl phosphorylation of STAT3. How honokiol inhibits IL-6 induced STAT3 activation was also investigated. The roles of JAK2, mitogen-activated protein kinase, and Akt have been implicated in IL-6-induced STAT3 activation (Ihle, 1996; Chen et al., 1999). We found that IL-6-induced Akt activation was also suppressed by honokiol. We also observed that honokiol suppressed nuclear translocation and IL-6

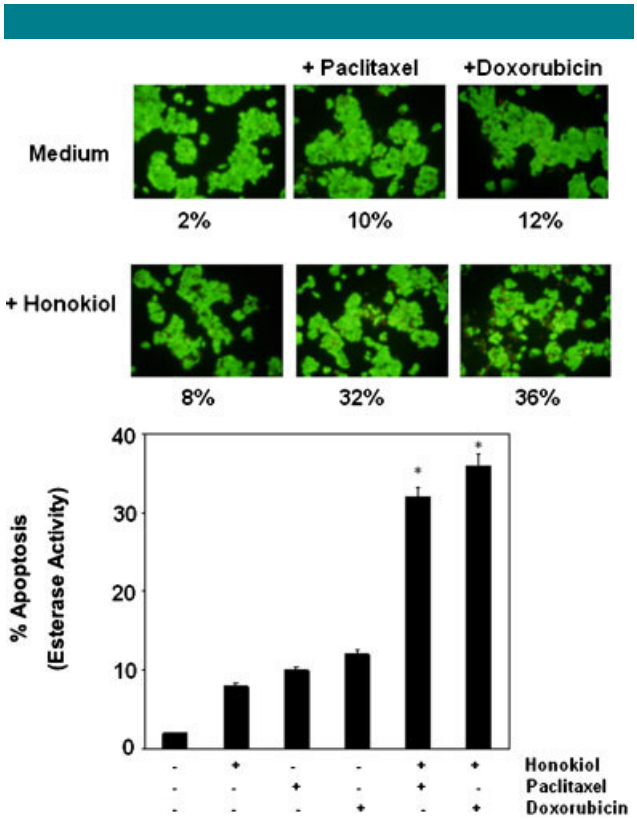

Fig. 7. Honokiol potentiates the apoptotic effect of doxorubicin and paclitaxel. HepG2 cells (1×10^6/ml) were treated with 25 μM honokiol and 10 nM doxorubicin or 5 nM paclitaxel alone or in combination for 24 h at 37°C. Cells were stained with a live/dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in the Materials and Methods Section. The results shown are % apoptosis and are representative of three independent experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

induced reporter activity of STAT3. Our results are in part agreement with another recent study, in which honokiol was reported to enhance the antitumor effects of epidermal growth factor receptor inhibitors in head and neck squamous cell carcinoma through the modulation of STAT3 activation cascade, although the complete mechanism of STAT3 inhibition was not elucidated in this study (Leeman-Neill et al., 2010). Overall, it was found that honokiol could suppress both constitutive and inducible STAT3 activation leading to the other downstream effects as confirmed through the corroboration between the experimental and predictive data.

STAT3 phosphorylation plays a critical role in proliferation and survival of tumor cells (Yue and Turkson, 2009). Several types of cancer, including head and neck cancers (Macha et al., 2011), multiple myeloma (Kannaiyan et al., 2011), lymphomas, and leukemia (Zhang et al., 2002), also have constitutively active STAT3. The suppression of constitutively active STAT3 in HCC cells raises the possibility that this novel STAT3 inhibitor might also inhibit constitutively activated STAT3 in other types of cancer cells. Previously, it has been reported that honokiol can also suppress NF-κB activation (Ahn et al., 2006). Whether suppression of STAT3 activation by honokiol is linked to inhibition of NF-κB activation is not clear. The p65 subunit of NF-κB has been shown to interact with STAT3 (Yu et al., 2002). STAT3 and NF-κB, however, are activated in response to different cytokines: IL-6 is a major activator of STAT3 and TNF is a potent activator of NF-κB. Interestingly, erythropoietin has been shown to activate NF-κB through the activation of JAK2 kinase (Digicaylioglu and Lipton, 2001). Thus, it is possible that the suppression of JAK2 kinase activation is the critical target for the inhibition of both NF-κB and STAT3 activation by honokiol. Also, another study has indicated that STAT3 prolongs NF-κB nuclear retention through acetyltransferase p300-mediated p65 acetylation, thereby interfering with NF-κB nuclear export (Lee et al., 2009). Thus, it is possible that suppression of STAT3 activation may mediate inhibition of NF-κB activation by honokiol.

We also found evidence that the honokiol-induced inhibition of STAT3 activation involves a protein tyrosine phosphatase (PTP). Numerous PTPs have been implicated in STAT3 signaling including SHP-1, SH-PTP2, TC-PTP, PTEN, PTP-1D, CD45, and PTP-episilon (Tan et al., 2010). SHP-1 is implicated in negative regulation of JAK/STAT signaling pathways (Calvisi et al., 2006) and it has been found that loss of SHP-1 may contribute to the activation of JAK or STAT proteins in cancer (Wu et al., 2003). Indeed we found for the first time that honokiol induces the expression of SHP-1 protein in HCC cells, which correlated, with down-regulation of constitutive STAT3 phosphorylation. Transfection with SHP-1 siRNA reversed the STAT3 inhibitory effect of honokiol, thereby further implicating a critical role of this phosphatase in honokiol-induced down-regulation of STAT3 activation. Interestingly, the multikinase protein tyrosine inhibitor sorafenib recently approved by FDA for the treatment of HCC was also found to inhibit STAT3 through activation of a PTP (Yang et al., 2010).

We further found that honokiol suppressed the expression of several STAT3-regulated genes; including proliferative (cyclin D1) and antiapoptotic gene products (Bcl-2, Bcl-xL, survivin, and Mcl-1) and angiogenic gene product (VEGF). The down-regulation of cyclin D1 expression by honokiol correlated with suppression in proliferation as observed in various HCC cell lines. Mcl-1 is highly expressed in tumor cells (Epling-Burnette et al., 2001), and Niu et al. (2002) had reported that inhibition of STAT3 by a Src inhibitor results in down-regulation of expression of the *Mcl-1* gene in melanoma cells. In addition, activation of STAT3 signaling induces *survivin* gene expression and confers resistance to apoptosis in human breast cancer cells (Gritsko et al., 2006). Bcl-2 and Bcl-xL can also block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance (Seitz et al., 2009). Thus, the down-regulation of the expression of Bcl-2, Bcl-xL, survivin, and Mcl-1 is likely linked with the honokiol's ability to induce apoptosis in HCC cells. The downmodulation of VEGF expression is in lines with a recent report in which siRNA targeting VEGF inhibits HCC growth and tumor angiogenesis in vivo (Raskopf et al., 2008).

Doxorubicin and paclitaxel are commonly used chemotherapeutic drugs for the treatment of HCC (Jin et al., 2009). We found that honokiol can significantly enhance the apoptotic effects of doxorubicin and paclitaxel in HCC cells, thereby suggesting that it can also be used in conjunction with chemotherapeutic drugs for the treatment of HCC. Honokiol has been shown to target multiple pathways of tumorigenesis, including proliferation, apoptosis, angiogenesis, invasion, and tumor-induced immuno-suppression in wide variety of tumor cells and in vivo cancer models (Fried and Arbiser, 2009). However, no reports exist in the literature elaborating the effect of honokiol on STAT3/JAK2 signaling cascade in HCC cells. Our results indicate for the first time that honokiol inhibits both inducible and constitutive STAT3 activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, and angiogenesis. Further in vivo studies may provide important leads for exploiting the pharmacological potential of honokiol for treatment of cancers harboring active STAT3 and other inflammatory diseases.

Acknowledgments

This work was supported by grants from National Medical Research Council of Singapore [Grant R-184-000-201-275] to GS. APK was supported by grants from the National Medical Research Council of Singapore [Grant R-713-000-124-213] and Cancer Science Institute of Singapore, Experimental Therapeutics I Program [Grant R-713-001-011-271].

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