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Honokiol Enhances Paclitaxel Efficacy in Multi-Drug Resistant Human Cancer Model through the Induction of Apoptosis

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Abstract

Resistance to chemotherapy remains a major obstacle in cancer therapy. This study aimed to evaluate the molecular mechanism and efficacy of honokiol in inducing apoptosis and enhancing paclitaxel chemotherapy in pre-clinical multi-drug resistant (MDR) cancer models, including lineage-derived human MDR (KB-8-5, KB-C1, KB-V1) and their parental drug sensitive KB-3-1 cell lines. In vitro analyses demonstrated that honokiol effectively inhibited proliferation in KB-3-1 cells and the MDR derivatives (IC50 ranging 3.35±0.13 μg/ml to 2.77±0.22 μg/ml), despite their significant differences in response to paclitaxel (IC50 ranging 1.66±0.09 ng/ml to 6560.9±439.52 ng/ml). Honokiol induced mitochondria-dependent and death receptor-mediated apoptosis in MDR KB cells, which was associated with inhibition of EGRF-STAT3 signaling and downregulation of STAT3 target genes. Combined treatment with honokiol and paclitaxel synergistically augmented cytotoxicity in MDR KB cells, compared with treatment with either agent alone in vitro. Importantly, the combined treatment significantly inhibited in vivo growth of KB-8-5 tumors in a subcutaneous model. Tumor tissues from the combination group displayed a significant inhibition of Ki-67 expression and an increase in TUNEL-positive cells compared with the control group. These results suggest that targeting multidrug resistance using honokiol in combination with chemotherapy drugs may provide novel therapeutic opportunities.

Honokiol is a natural component isolated from the bark of magnolia tree [2,3], which has been traditionally used to treat anxiety-related disorders and digestive complaints [2,4]. Interestingly, honokiol also exhibited potent anticancer activities [5,6,7] and further enhanced conventional chemotherapies in a variety of preclinical models of human cancer, including chronic lymphocytic leukemia [3], prostate cancer [8] and multiple myeloma [9]. These relatively wide-ranging anticancer capabilities and favorable safety profile make honokiol an attractive adjunct therapy to enhance conventional chemotherapy in clinical settings.

Overexpression of anti-apoptotic proteins is an underlying mechanism that contributes to the acquisition of therapeutic resistance, recurrence and metastasis. A growing body of evidence suggests that three anti-apoptotic proteins, i.e., survivin, Mcl-1, and Bcl-2, may be directly related to drug resistance in cancer [10,11]. Inhibition of these crucial survival factors has been shown to trigger apoptosis and sensitize cancer cells to drug treatment [5–

Introduction

Chemotherapy remains one of the major treatment options for many types of cancer, however, a significant percentage of patients develop drug resistance during the course of chemotherapy, and inevitably progress without cure [1,2]. Despite the remarkable progress in drug development, paclitaxel with its broad anticancer spectrum has solidified the disruption of microtubule dynamics as one of the most effective anticancer strategies in use today. However, the emergence of drug-resistant cancer cells has greatly limited its clinical efficacy. It is therefore imperative to develop novel strategies to reduce or overcome chemoresistance in cancer. Various approaches to enhance chemoresponse in chemoresistant cancer models have been explored, including those combining natural products or compounds with paclitaxel or other standard chemotherapy reagents.
Figure 1. Honokiol inhibits growth and induces apoptosis in multidrug-sensitive and -resistant cells. (a, b) Growth inhibition effect of paclitaxel and p-gp expression in KB-3-1, KB-8-5, KB-C1 and KB-V1 cells. The IC₅₀ values of paclitaxel in drug resistant KB-8-5, KB-C1 and KB-V1 cells.
The experiments were repeated 3 times independently. Software (Tree Star, Ashland, OR) was used for apoptosis analysis. Cytometer (Becton Dickinson, Franklin Lakes, NJ). FlowJo fluorescence-activated cell sorting (FACS) caliber bench-top flow cytometer was maintained in room temperature. The stained samples were measured using a fluorescence-activated cell sorting (FACS) caliber bench-top flow cytometer (Becton Dickinson, Franklin Lakes, NJ). FlowJo software (Tree Star, Ashland, OR) was used for apoptosis analysis. The experiments were repeated 3 times independently.

### Materials and Methods

#### Cell Culture
The KB-3-1 and its MDR derivative cell lines were generously provided by Dr. Michael M. Gottesman (NCI, NIH, Bethesda, MD) and have been characterized previously [12]. KB-3-1 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. KB-8-5 and KB-C1 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and 0.01 and 1 μg/ml colchicine respectively. KB-V1 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and 1 μg/ml vinblastine. To eliminate impact of colchicine or vinblastine on experiment result, resistant cells were cultured in drug free medium for one week before any experiment.

#### Cell Growth Assay
Cells were seeded at a density of 5 × 10^3 cells per well into 96-well plates in quadruplet. Twenty-four hours later, drugs were added in various concentration ranges as single agents in two-drug combinations and then incubated for 72 h. The concentration of honokiol was from 0.625 to 20 μg/ml for all four cell lines. For paclitaxel, the concentration ranges were 0.19 to 97.5 ng/ml, 3.02 ng/ml to 3.12 μg/ml, 12.1 ng/ml to 25.0 μg/ml, and 97.5 ng/ml to 100 μg/ml for KB-3-1, KB-8-5, KB-C1, and KB-V1 cells, respectively. Cell growth inhibition was measured by determining cell density with the sulforhodamine B assay. The percentage of inhibition was determined by comparison of cell density in the drug-treated cells with that of the untreated control cells. All experiments were repeated at least three times.

#### Apoptosis Analysis
Apoptosis was analyzed in all four cell lines. Cells were treated with honokiol, paclitaxel, or their combination as indicated in the figure legends, trypsinized, and washed in cold 1× PBS. The cells were resuspended in 1× Annexin binding buffer (BD Pharmingen), and then stained with Annexin V-phycocerythrin ( Annexin V-PE; BD Pharmingen) and 7-AAD (BD Pharmingen) for 15 min at room temperature. The stained samples were measured using a fluorescence-activated cell sorting (FACS) caliber bench-top flow cytometer (Becton Dickinson, Franklin Lakes, NJ). FlowJo software (Tree Star, Ashland, OR) was used for apoptosis analysis. The experiments were repeated 3 times independently.

### Immunoblotting
Thirty microgram protein from whole-cell extracts or cytoplasmic and mitochondrial fractions were quantified, separated on SDS-PAGE gels and transferred to nitrocellulose membranes. After being blocked with 5% nonfat dry milk in TBS-T buffer, the membranes were incubated with specific antibodies overnight at 4°C. Mouse anti-β-actin antibody (Treivgen, Gaithersburg, MD) was used as a sample loading control. Immunostained protein bands were detected with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). The experiments were repeated 3 times.

#### In vivo Anti-tumor Efficacy Assay
The animal experiment was approved by the Institutional Animal Care and Use Committee of Emory University. KB-8-5 cells (5 × 10^6) were injected s.c. into 4–5 week-old female nude mice (Athymic nu/nu, Taconic NY). When the tumors had developed to about 100 mm³, the mice were divided into four groups (n = 5 or 8) in a way to minimize weight and tumor size differences among the groups: control group treated with 20% intralipid (Baxter Healthcare), honokiol group (1.0 mg/mouse or 50 mg/kg), paclitaxel group (20 mg/kg), and honokiol (1.0 mg/mouse or 50 mg/kg) plus paclitaxel (20 mg/kg) combination group. Honokiol was dissolved in 100% ethanol and mixed with 20% intralipid in a 1:14 (v/v) ratio. Honokiol was administered to the mice 3 times per week at 1.0 mg/mouse (or 50 mg/kg) via intraperitoneal injection. Paclitaxel was administered to the mice once per week at 20 mg/kg through tail vein injection. The therapy was continued for 4 weeks. The body weight and tumor size were measured three times per week. The tumor volume was calculated using the formula: \( V = \frac{4}{3} \pi \times \text{larger diameter} \times \text{smaller diameter} \) [13]. The mice were sacrificed 4 weeks after the initiation of treatment. Tumor and organ tissues (liver, heart, lung, spleen, and kidney) were collected for H&E staining and immunostaining analyses.

#### Immunohistochemistry and TUNEL Assay
Immunohistochemical analysis for Ki-67 staining on paraffin-embedded mouse xenograft tissue was performed as previously described [13]. Cells staining positive for Ki-67 were counted and the percentage of positive cells was calculated. An average of the 8 readings was used for statistical analysis. TUNEL assay was performed by immunofluorescence using the same specimens as above, following the procedure provided by the manufacturer (Promega, Madison, WI). To analyze the assay results, the total cell number and the positive cell number in the same area were counted for five random areas; the result was presented as an average ratio of positive cell number out of the total cell number.

### Statistical Analysis
The statistical significance of treatment of cells in the in vitro cytotoxicity assay was assessed using the Student’s t-test. For in vivo anti-tumor efficacy assay, a log-linear mixed model with random intercept was used to compare the significance of the mean tumor volumes among each group. The statistical significance of treatment effect on microtubules, apoptosis, and cell proliferation...
Figure 2. Honokiol induces PARP and caspase-3 cleavage and the release of cytochrome c in the cytoplasm in multidrug-sensitive and -resistant cells. (a) KB-3-1 cells and (b) KB-8-5 cells were treated with 5, 10, 15 μg/ml of honokiol for 24, 48 h. Full-length and cleaved PARP, caspase-3, β-actin were detected by western blotting. (c) KB-C1 cells and KB-V1 cells were treated with 5, 10, 15 μg/ml of honokiol for 48 h. Full-length and cleaved PARP, β-actin were detected by western blotting. (d) KB-8-5 cells were treated with 5, 10, 15 μg/ml of honokiol for 24, 48 h. Cyto C, COX4, DR5, β-actin were detected by western blotting.
cleaved caspase-3 and β-actin as a loading control were detected by immunoblotting using whole cell lysates. (c) KB-C1 and KB-V1 cells were treated with 5, 10, 15 μg/ml of honokiol for 48 h. Cleaved PARP was detected by immunoblotting using whole cell lysates. (d) Upper panel, KB-8-5 cells were treated with 5, 10, 15 μg/ml of honokiol for 48 h. Cytoplasmic and mitochondrial fractions were separated and immunoblotted with cytochrome c (Cyto C) antibody. COX4 (a mitochondrial protein) was used to show efficiency of cell fractionation. Lower panel, KB-8-5 cells were treated with 5, 10, 15 μg/ml of honokiol for 24 and 48 h. DR5 was detected by immunoblotting using whole cell lysates. All experiments were repeated at least three times, and representative data are presented.

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Figure 3. Honokiol inhibits the EGFR signaling pathway and downregulates STAT3 target genes in multidrug-sensitive and -resistant cells. KB-3-1 and KB-8-5 cells were treated with 5 μg/ml of honokiol for short time (0.5, 1, or 2 h) or long time (24, 48, or 72 h). Whole cell lysates were immunoblotted for the indicated proteins. The effect of honokiol at early time points: (a) phosphorylation of EGFR and STAT3; (b) phosphorylation and expression of AKT and ERK. The effect of honokiol at late time points: (c) phosphorylation and expression of EGFR and STAT3; (d) phosphorylation and expression of AKT and ERK; (e) expression of survivin, Bcl-2, Mcl-1. (f) Honokiol inhibits the EGFR signaling pathway and downregulates STAT3 target genes in a dose-dependent manner. KB-3-1 and KB-8-5 cells were treated with 5, 10, 15 μg/ml of honokiol for 48 h. Whole cell lysates were immunoblotted for the indicated proteins. (g) Honokiol inhibits the EGFR-STAT3 pathway in KB-C1 and KB-V1 cells. KB-C1 and KB-V1 cells were treated with 5, 10, 15 μg/ml of honokiol for 48 h. All experiments were repeated at least three times, and representative data are presented.

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in xenograft tumor tissues was assessed using the Kruskal-Wallis test (one-way ANOVA). $P<0.05$ was considered statistically significant in all analyses.

**Results**

**Honokiol Reduces Viability and Induces Apoptosis in Human MDR Cancer Cells**

The MDR cancer cell lines KB-8-5, KB-C1 and KB-V1 were derived from their drug-sensitive counterpart KB-3-1 cells, and have been widely used as a clinically relevant model in the study of drug resistance [14,15]. These cells exhibited distinct sensitivities to paclitaxel treatment. As shown in Fig. 1a, 1b, the IC$_{50}$ values in MDR cell lines (KB-8-5:26.73±1.01 ng/ml, KB-C1:195.0±11.11 ng/ml, and KB-V1:6360.0±439.52 ng/ml) were increased by 16-, 117- and 4,000-fold, respectively, when compared to the parental KB-3-1 cell line (1.66±0.09 ng/ml). Consistent with their resistant phenotype, MDR cell lines substantially express the classical MDR marker P-glycoprotein (Fig. 1b). Interestingly, a 72 h-treatment with honokiol effectively reduced the viability of MDR cells (KB-8-5:3.22±0.14 μg/ml, KB-C1:3.04±0.30 μg/ml, KB-V1:2.77±0.22 μg/ml), with IC$_{50}$ values similar to those in KB-3-1 cells (3.35±0.13 μg/ml) (Fig. 1c, 1d). Apoptosis analysis further showed that honokiol induced apoptosis in a time- and dose-dependent manner (Fig. 1e). 24 h following honokiol treatment, the percentage of apoptotic KB-3-1 cells was 11.9±3.9% (5 μg/ml of honokiol), 32.9±0.9% (10 μg/ml), and 47.1±2.7% (15 μg/ml); the percentage of apoptotic cells increased to 18.4±2.1%, 51.5±0.6%, and 65.3±1.6%, respectively, following a 48 h-treatment, which was further increased at 72 h. Honokiol also induced a similar degree of apoptosis in other MDR cells, including KB-8-5, KB-C1 and KB-V1 (Fig. 1e). For example, a 48-h treatment with 10 μg/ml of honokiol resulted in apoptosis in 51.5% of KB-3-1 cells, 52.7% of KB-8-5 cells, 52.9% of KB-C1 cells and 67.6% of KB-V1 cells, respectively. These data are consistent with the viability assay (Fig. 1c, 1d), and suggest that honokiol is a potent cytotoxic agent in KB cells regardless of their different paclitaxel sensitivities.

**Honokiol Induces Mitochondria- and Death Receptor (DR)-dependent Apoptosis in Human Cancer Cells**

We investigated the mechanism of honokiol-induced apoptosis in KB cells. As shown in Fig. 2a–c, honokiol treatment induced the cleavage of PARP and caspase-3 in a dose- and time-dependent manner in all the tested KB cells, indicating the
activation of apoptotic signals. Western blot analyses found that honokiol not only induced the release of cytochrome c in the cytoplasm in a dose-dependent manner (Fig. 2d), but also increased the expression of DR5 (Fig. 2d), a surface receptor for...
Figure 6. Inhibition of tumor growth by the combination of honokiol and paclitaxel in a multidrug-resistant xenograft model. (a) The tumor growth of KB-8-5 xenografts was significantly inhibited in the combination-treated group compared with the control (p<0.0001), honokiol
Honokiol Inhibits EGFR-STAT3 Signaling in Human Cancer Cells

The EGFR-STAT3 signaling pathway plays an important role in the regulation of growth and survival in cancer cells. We investigated the effects of honokiol on several key components of the EGFR-STAT3 signaling pathway. KB-3-1 and KB-8-5 cells were treated with honokiol (5 μg/ml) for 0.5, 1, and 2 h. Western blot analysis using total cell lysates revealed a markedly reduced phosphorylation of EGFR at Tyr1173, an indicator of activated EGFR (Fig. 3a). We further examined the phosphorylation status of STAT3 (Tyr705), Akt (Ser473), and ERK (Thr202/Tyr204), three known EGFR downstream signaling components. Interestingly, honokiol treatment rapidly inhibited phosphorylation of STAT3 and Akt, but had no effect on ERK phosphorylation (Fig. 3ab). To further examine whether the expression level of EGFR and its downstream target proteins is also inhibited by honokiol treatment (5 μg/ml), we treated cells for 24, 48, and 72 h. As shown in Fig. 3cd, honokiol treatment inhibited protein expression of EGFR, STAT3, ERK, and AKT in a time-dependent manner in KB-3-1 and KB-8-5 cells. Similarly, phosphorylation of those proteins was decreased upon honokiol treatment (Fig. 3cd). Consistently, the expression levels of three known STAT3 target genes, i.e., survivin, Bcl-2 and Mcl-1, were dramatically decreased following honokiol treatment (Fig. 3e).

We next examined the dose-dependent response of EGFR-STAT3 signaling to honokiol treatment in KB-3-1 and KB-8-5 cells. As shown in Fig. 3f, the expression and phosphorylation of EGFR (Try1173) were decreased in the presence of 5 or 10 μg/ml of honokiol (48 h), and were further decreased at a dose of 15 μg/ml of honokiol (Fig. 3f). Consistently, the expression and/or phosphorylation of EGFR downstream signaling components, including ERK, Akt, STAT3, survivin, Bcl-2 and Mcl-1, were also dramatically inhibited in a dose-dependent manner (Fig. 3f). A similar effect of honokiol on EGFR-STAT3 signaling was also observed in both KB-C1 and KB-V1 cells (Fig. 3g). Of particular interest, an RT-PCR analysis found that honokiol inhibition of survivin expression may involve the suppression of survivin transcription at the mRNA level (Figure S1).

Honokiol Enhances the in vitro Cytotoxicity of Paclitaxel in MDR Cancer Cells

We investigated whether the inhibition of survivin, Bcl-2 and Mcl-1 by honokiol could sensitize MDR cancer cells to conventional chemotherapy such as paclitaxel. As shown in Fig. 4, a 24 h treatment with either honokiol (5 μg/ml) or paclitaxel (450 ng/ml) resulted in ≥20% of apoptosis in KB-C1 cells, whereas combined treatment using both honokiol and paclitaxel resulted in approximately 30% apoptosis. Moreover, at the 48 h and 72 h time points, the combined treatment more effectively induced apoptosis (76% and 86%, respectively) in KB-C1 cells than either single agent (≤25%) or the vehicle control. Consistently, the combined treatment was more effective in inducing apoptosis than either single agent in MDR KB-8-5 and KB-V1 cells (Fig. 4). Using KB-8-5 cells as a example, a combination index (CI) assay confirmed the synergistic effect of combined treatment with honokiol and paclitaxel in reducing the viability of MDR cancer cells (Table S1).

Western blot analysis further showed that the combined treatment with honokiol and paclitaxel was more effective than either agent alone in inducing the cleavage of caspase-3, PARP, and the release of cytochrome c from mitochondria in KB-8-5 cells (Fig. 5a, 5b). A 48-h treatment with honokiol alone or the combination of honokiol and paclitaxel decreased the expression and phosphorylation of EGFR, as well as other key EGFR-STAT3 signaling components, including STAT3, p-STAT3(Tyr705), ERK, p-ERK(Thr202/Tyr204), Akt and p-Akt(Ser473). The protein expression of survivin, Bcl-2 and Mcl-1 was also markedly reduced upon the treatment with honokiol or the combination of honokiol and paclitaxel. Interestingly, however, the treatment with paclitaxel alone did not have significant effect on these signaling components or target genes of the EGFR-STAT3 pathway (Fig. 5c).

Paclitaxel exerts its antitumor activity by binding to tubulin inside the lumen of the microtubule, resulting in microtubule polymerization, stabilization and disruption of microtubule dynamics, causing mitotic arrest and apoptosis in proliferating cells. To investigate whether honokiol can enhance paclitaxel induced microtubule polymerization and stabilization, we examined the effect of each treatment on microtubules in KB-8-5 and KB-C1 cells. Drug-induced stabilization of the tumor cells' interphase microtubules, as evidenced by an increase in microtubule polymer mass resulting in bundling, was observed upon treatment with both paclitaxel and the combination; however, honokiol alone was not effective in the formation of microtubule bundles (Fig. 5d). Collectively, these data indicate that honokiol enhances the in vitro cytotoxicity of paclitaxel in MDR cancers, may be through the inhibition of EGFR-STAT3 survival signaling.

Honokiol Enhances the in vivo Efficacy of Paclitaxel in MDR Cancer Xenograft Tumors

We evaluated the antitumor efficacy of honokiol, paclitaxel, and their combination in treating MDR cancer in the KB-8-5 xenograft model. The treatment was continued for 4 weeks. As shown in Fig. 6a, at a dose of 20 mg/kg once per week, a 4-week injection of paclitaxel alone did not significantly inhibit the growth of KB-8-5 subcutaneous tumors, when compared to the vehicle control (p = 0.917). Treatment with honokiol alone (50 mg/kg, 3 times per week, via i.p.) slightly suppressed tumor growth, although the difference was not significant (p = 0.194) (Fig. 6a). In contrast, the combination treatment dramatically inhibited the growth of KB-8-5 tumors, when compared with all other groups (vs. control: p<0.0001; vs. honokiol: p = 0.036; vs. paclitaxel: p = 0.004). The average tumor volume in each treatment group at the endpoint was 2385.4±510.0 mm³ (control), 1810.2±483.2 mm³ (honokiol), 2591.3±726.2 mm³ (paclitaxel), and 573.9±146.1 mm³ (combination). Representative mice from
Honokiol could sensitize MDR cancer cells to paclitaxel through inhibition and regulation of key signaling pathways. The combination treatment significantly reduced the tissue level of MDR-related genes, such as P-gp, and inhibited EGF signaling, thereby downregulating several anti-apoptotic proteins. Honokiol, therefore, is a promising agent in the chemosensitization of MDR cancer cells to paclitaxel, particularly in combination with conventional therapeutic drugs.

**Supporting Information**

Figure S1 Honokiol treatment significantly decreases the levels of survivin mRNA. KB-8-5 cells were treated with 5 μM/mL of honokiol for 48 h downregulated survivin expression and enhanced the intracellular accumulation of paclitaxel (data not shown); thus, downregulation of survivin by honokiol may also contribute to the synergistic effect of honokiol and paclitaxel in the resistant KB cell lines.

In conclusion, we have shown that honokiol can inhibit cell proliferation and induce apoptosis in multi-drug resistant cell lines. Our study also demonstrated that honokiol can synergistically augment the cytotoxicity of paclitaxel by inhibiting the EGFR-STAT3 signaling pathway and downregulating multiple anti-apoptotic proteins. Honokiol, therefore, is a promising agent in the development of novel treatments for drug-resistant cancer, particularly in combination with conventional therapeutic drugs.

Bcl-2 and Mcl-1 are Bcl-2 family member proteins.

**Table S1** Combination index (CI) assay confirmed the synergistic effect of combined treatment with honokiol
and paclitaxel in reducing the viability of MDR cancer cells.

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Methods S1

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Acknowledgments

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Author Contributions

Conceived and designed the experiments: XW DMS. Performed the experiments: XW HW MJL WH LK SN ARA MB. Analyzed the data: XW HCS. Contributed reagents/materials/analysis tools: ZC JLA. Wrote the paper: XW JJB DMS.

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