Cancer Therapy: Preclinical

Clinical Cancer Research

Honokiol Inhibits Epidermal Growth Factor Receptor Signaling and Enhances the Antitumor Effects of Epidermal Growth Factor Receptor Inhibitors

Rebecca J. Leeman-Neill[1], Quan Cai[2], Sonali C. Joyce[2], Sufi M. Thomas[2], Neil E. Bhola[3], Daniel B. Neill[4], Jack L. Arbiser[5], and Jennifer R. Grandis[1,2,3]

Abstract

Purpose: This study aimed to investigate the utility of honokiol, a naturally occurring compound, in the treatment of head and neck squamous cell carcinoma (HNSCC) as well as its ability to target the epidermal growth factor receptor (EGFR), a critical therapeutic target in HNSCC, and to enhance the effects of other EGFR-targeting therapies.

Experimental Design: Human HNSCC cell lines and the xenograft animal model of HNSCC were used to test the effects of honokiol treatment.

Results: Honokiol was found to inhibit growth in human HNSCC cell lines, with 50% effective concentration (EC_{50}) values ranging from 3.3 to 7.4 μmol/L, and to induce apoptosis, as shown through Annexin V staining. These effects were associated with inhibition of EGFR signaling, including downstream inhibition of mitogen-activated protein kinase, Akt, and signal transducer and activator of transcription 3 (STAT3), and expression of STAT3 target genes, Bcl-X_L and cyclin D1. Furthermore, honokiol enhanced the growth inhibitory and anti-invasion activity of the EGFR-targeting agent erlotinib. Although HNSCC xenograft models did not show significant inhibition of *in vivo* tumor growth with honokiol treatment alone, the combination of honokiol plus cetuximab, a Food and Drug Administration–approved EGFR inhibitor for this malignancy, significantly enhanced growth inhibition. Finally, HNSCC cells rendered resistant to erlotinib retained sensitivity to the growth inhibitory effects of honokiol.

Conclusions: These results suggest that honokiol may be an effective therapeutic agent in HNSCC, in which it can augment the effects of EGFR inhibitors and overcome drug resistance. *Clin Cancer Res; 16(9); 2571–9. ©2010 AACR.*

Head and neck squamous cell carcinoma (HNSCC) is one of the most commonly occurring malignancies worldwide. Advances in therapy for HNSCC have only modestly improved the mortality rate, which has remained at 50% for the past several decades (1). Available therapies, including surgical resection, radiation, and conventional chemotherapy, are often associated with severe morbidity affecting the vital structures of the head and neck, have side effects, and are limited by therapeutic resistance. The need exists, therefore, for the discovery of new therapies, including complementary therapies that can be given in combination with available treatments to allow for lower doses of toxic drugs and to overcome drug resistance.

Honokiol is a natural compound derived from the bark of the magnolia tree and is used in traditional Chinese medicine. Studies have shown various ways in which honokiol may have a therapeutic benefit, including its ability to behave as a muscle relaxant; to have anti-inflammatory, antimicrobial, and antioxidant activity; and indications that it may be useful in protecting against hepatotoxicity, neurotoxicity, thrombosis, and angiopathy (2). Interest in the role that honokiol may play in cancer therapy began with a study showing the prevention of skin papillomas in mice (3). Subsequent studies showed the anticancer activities of honokiol in a variety of cancer cell lines (4–11) and xenograft models (4, 6, 7, 9, 10, 12–14).

In several cancer models, honokiol has been found to alter molecular targets that are known to affect tumor cell growth and survival. One of the most commonly proposed mechanisms of honokiol's antitumor activity is inhibition of the NFκB signaling pathway. NFκB is a transcription factor that contributes to several physiologic processes (e.g., inflammation) but also regulates the expression of genes that are involved in cancer, including

Authors' Affiliations: Departments of [1]Pathology, [2]Otolaryngology, and [3]Pharmacology, University of Pittsburgh and University of Pittsburgh Cancer Institute, and [4]Heinz College, School of Public Policy and Management, Carnegie Mellon University, Pittsburgh, Pennsylvania; and [5]Department of Dermatology, Emory University, Atlanta, Georgia

Note: R.J. Leeman-Neill and Q. Cai contributed equally to this work.

Corresponding Author: Jennifer R. Grandis, 200 Lothrop Street, Eye and Ear Institute, Suite 500, Pittsburgh, PA 15213. Phone: 412-647-5280; Fax: 412-383-5409; E-mail: grandisjr@upmc.edu.

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Translational Relevance

Treatments of head and neck squamous cell carcinoma (HNSCC), a common and frequently fatal malignancy, are currently limited by toxicity, therapeutic resistance, and therapy-related morbidity. Using several HNSCC cell lines and xenograft models we found that honokiol has anticancer activity and inhibits the epidermal growth factor receptor (EGFR) signaling pathway, an important therapeutic target in HNSCC. Furthermore, honokiol was found to enhance the effects of EGFR-inhibiting therapeutics, including cetuximab, which is Food and Drug Administration approved for use in HNSCC. These preclinical results provide the critical biological rationale for future clinical investigations using honokiol in HNSCC patients.

genes that control the cell cycle, apoptosis, tumor angiogenesis, and invasion (15). NFκB and upstream signaling mediators have been found to be inhibited by honokiol treatment of human monocytes (16), embryonic kidney cells (17), endothelial cells (9), lymphoma (11, 17), promyelocytic leukemia (11), multiple myeloma (17), breast cancer (11), cervical cancer (11), and HNSCC cells (17).

In HNSCC cell lines, NFκB has been reported to interact with another transcription factor, signal transducer and activator of transcription 3 (STAT3; ref. 18), which is a potential molecular target for the treatment of HNSCC. In addition to regulating several genes involved in cancer (19), including some that are also regulated by NFκB, STAT3 signaling has been found to be important for growth and survival of HNSCC cell lines and tumor xenografts (20, 21). In HNSCC, STAT3 mediates signaling through the epidermal growth factor receptor (EGFR; ref. 21), one of the ErbB family of receptors, which is overexpressed in the majority of HNSCC tumors (22–24) where EGFR expression is correlated with poor clinical outcome in HNSCC (25–27). Cetuximab, an antibody that targets the EGFR, was Food and Drug Administration (FDA) approved in 2006 for use in the treatment of HNSCC. Erlotinib, an EGFR-targeting small molecule tyrosine kinase inhibitor (TKI) is currently under clinical evaluation in HNSCC trials (28).

STAT3 has been reported to be a target of several cancer therapies currently under preclinical and clinical investigation (19). Honokiol-induced inhibition of EGFR and STAT3 has recently been reported in a breast cancer cell line (8). Honokiol has also been shown to inhibit several other proteins that are known to interact with STAT3, including NFκB (9, 11, 16, 17) as well as gp130, a subunit of the interleukin 6 (IL-6) receptor (29), and Src (4), which are both known to directly activate STAT3 in HNSCC (30, 31). Honokiol decreases the expression of various STAT3 target genes, including *cyclin D1* (17, 32, 33), *p21Waf1* (34), *c-Myc* (17, 33), *Mcl-1* (5, 7), *Bcl-x_L* (7), *survivin* (7), and *VEGF* (17, 35).

Honokiol has been shown to enhance the effects of a variety of chemotherapeutic agents and small molecule inhibitors including bortezomib (29), fludarabine (5), cladribine (29), chlorambucil (29), doxorubicin (14, 17), adriamycin (36), paclitaxel (14, 17), docetaxel (10), SAHA (14), lapatinib (33), rapamycin (33), or cisplatin (12) in different cancer models. In the current study, we hypothesized that honokiol can be used to target EGFR signaling via STAT3 in the treatment of HNSCC and may also enhance the effects of EGFR-targeting therapies, erlotinib and cetuximab.

Materials and Methods

Reagents and cells. The HNSCC cell lines Cal-33, derived from an oral squamous cell carcinoma (37), and 1483, from an oropharyngeal squamous cell carcinoma (38), were maintained in DMEM medium with 10% heat-inactivated fetal bovine serum at 37°C in a humidified incubator with 5% CO_2. Cal-33 cells were provided by Dr. Gerard Milano in 2006 (Centre Anotoine-Lacassagne, Nice, France) and 1483 cells were obtained in 2007 from Dr. Gary Clayman (M.D. Anderson Cancer Center, Houston TX). 686LN and 686LNR30 cells are isogenic models of acquired EGFR TKI resistance *in vitro* and were obtained from Dr. Georgia Chen (Emory University, Atlanta, GO; ref. 39). All the cell lines were genotyped and validated in 2008 using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems). The cells are genotyped on a regular basis (twice in 2009) to ensure the validity. Honokiol is a natural product extracted from seed cone of *Magnolia grandiflora* as previously described (4). In treatments of cell cultures, honokiol was dissolved in 100% ethanol as a vehicle and in 20% Intralipid (Baxter Healthcare) for animal treatments. Erlotinib (Chemietek) was dissolved in 100% DMSO, as a vehicle.

Proliferation assay. HNSCC cells (1-3 × 10^4/well) were cultured overnight and treated with honokiol, erlotinib or the corresponding vehicles, in DMEM/1% serum, the following day. After 72 hours, the cells were harvested by trypsinization, and live cells were counted after staining for trypan blue dye exclusion. Each experiment was done with triplicate samples, and the average percent survival was calculated as a comparison with cells treated with the vehicle alone. The EC_{50} was calculated using Prism software version 4.03 (GraphPad Software Inc).

Apoptosis assay. Cal-33 cells (5 × 10^4/well) were seeded and, the following day, treated with either honokiol (10 μmol/L) or ethanol, as the vehicle, for 72 hours. Cells were then harvested and stained with Cy3-labeled Annexin V, according to the manufacturer's instructions (Annexin V-Cy3 apoptosis detection kit, BioVision). Stained cells were imaged using a fluorescent microscope (Nikon), and the numbers of Annexin V–positive cells were counted (at least three fields per sample) using ImageJ software (NIH).

Invasion assay. 686LN cells (1.5 × 10^3) were plated in serum-free DMEM F12 containing epidermal growth factor (EGF) alone (10 ng/mL), EGF with honokiol

(5 μmo/L) and/or erlotinib (5 μmo/L) or the corresponding vehicles, in a matrigel invasion chamber insert (BD Biosciences). The outer well contained DMEM F12/10% fetal bovine serum, as a chemoattractant. After 24 hours' incubation, uninvaded cells were removed and the invaded cells in the matrigel were fixed, stained with Hema 3 (Fisher Scientific), and counted under 200× magnification.

Western blotting. For cell lysates used in Western blots, cells were cultured in DMEM/10% fetal bovine serum for 24 hours and then in serum-free DMEM containing either honokiol or vehicle for another 24 hours. Tumor lysates from animal studies were extracted after homogenization of tumor tissue. The proteins from whole cell lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) by a semidry transfer apparatus (Bio-Rad). The membrane was blocked with 5% skim milk in TBS-Tween (TBS-T) solution (100 mmo/L Tris, 150 mmol/L NaCl, and 0.125% Tween 20). Membranes were incubated overnight with primary antibodies with 5% skim milk in TBS-T. After washing in TBS-T, membranes were incubated with secondary antibodies (antirabbit or antimouse IgG-horseradish peroxidase conjugate from Bio-Rad Laboratories). The blots were washed and developed with a luminol kit (Santa Cruz Biotechnology). Primary antibodies for STAT3, pSTAT3, pEGFR, phospho-p44/p42 mitogen-activated protein kinase (MAPK), AKT, pAKT (Cell Signaling Technology), cyclin D1, Bcl-X_L (Santa Cruz Biotechnology), EGFR (BD Transduction labs) and β-actin (CalBiochem) were used to probe membranes. Relative densitometric values were calculated using DigiDoc software and normalized, for each protein, to the corresponding band representing the house-keeping gene, *β-actin*.

In vivo *tumor xenograft study.* Female athymic nude mice (5-6 weeks old; $n = 30$) were purchased from Harlan and were housed in a pathogen-free animal facility. The animal study protocols were approved by the institutional animal care and use committee. 1483 cells (2×10^6/mouse) were harvested by trypsinization, washed in PBS, resuspended in saline, and s.c. injected into the flank of each mouse. After outgrowth of palpable tumors (7 days), the mice were randomized, by tumor volume, to three treatment groups (8 in the vehicle control group, 14 in the cetuximab group, and 10 in the combination group).

The cetuximab treatment group received 0.8 mg/mouse/day, by i.p. injection, twice per week. The combination treatment group received both cetuximab, twice per week, and honokiol, at 3 mg/mouse/day in 20% Intralipid (Baxter Healthcare), three times per week. Tumors were measured using digital calipers (Control Company) at least three times per week, and tumor volumes were calculated using the following formula: volume = $L \times (W)^2/2$ (L, longest diameter; W, shorter diameter). At the end of the study, the mice were euthanized and tumor tissues were harvested and frozen for analysis by immunoblot.

Statistical analyses. All statistical analyses of *in vitro* results were done using the nonparametric Mann-Whitney or Wilcoxon tests. Analysis of tumor growth rates in the xenograft model was done using a general linear model, assuming that animals are random effects. Tumor volume data were examined for the interaction between treatment group and day of observation to test whether the slopes of the growth curves were significantly different between groups.

Results

Honokiol inhibits growth and induces apoptosis in HNSCC cell lines. The *in vitro* growth inhibitory and proapoptotic activities of honokiol have been shown in several cancer cell lines (4–11). In the current study, two HNSCC cell lines, 1483 and Cal-33, were treated for 72 hours with honokiol at concentrations ranging from 0.01 μmo/L to 100 μmo/L and compared with the vehicle (ethanol) alone. 50% effective concentration (EC_{50}) values were 7.44 μmo/L for 1483 and 3.80 μmol/L for Cal-33 (Fig. 1A). These values are comparable with or lower than EC_{50} values seen in other cancer cell types (5, 7, 10, 33).

To determine the role of programmed cell death in the growth inhibitory effects of honokiol, Cal-33 cells were treated with honokiol followed by Annexin V staining. As shown in Fig. 1B, honokiol increased the number of apoptotic cells by 7.4-fold, suggesting that

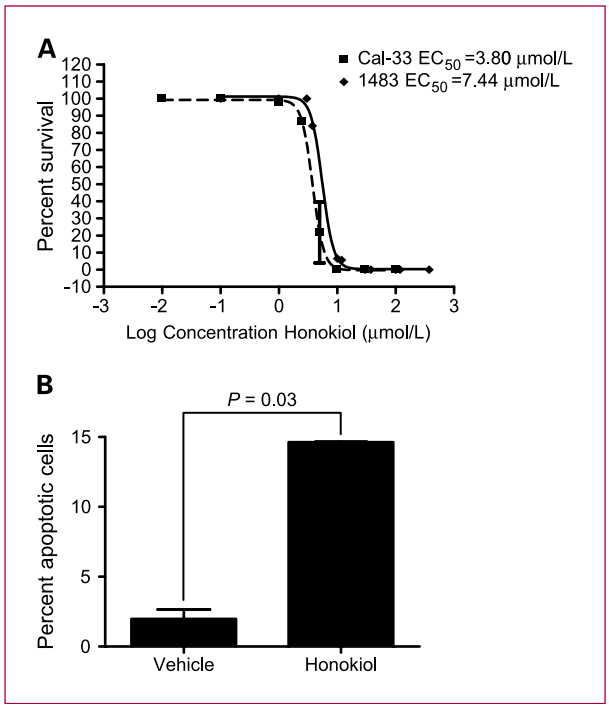

Fig. 1. Honokiol inhibits growth and induces apoptosis in HNSCC cell lines. A, HNSCC cells (1483 and Cal-33) were treated with varying concentrations of honokiol for 72 hours, stained with trypan blue dye, and counted. The experiment was done twice with triplicate samples and similar results. B, Cal-33 cells were treated with either honokiol (10 μmol/L) or vehicle for 72 hours. Cells were then harvested and stained with Cy3-labeled Annexin V. Images of stained cells were obtained with a fluorescent microscope and the percentage of Annexin V–positive cells was determined. The experiment was done four times with triplicate samples and similar results ($P = 0.03$).

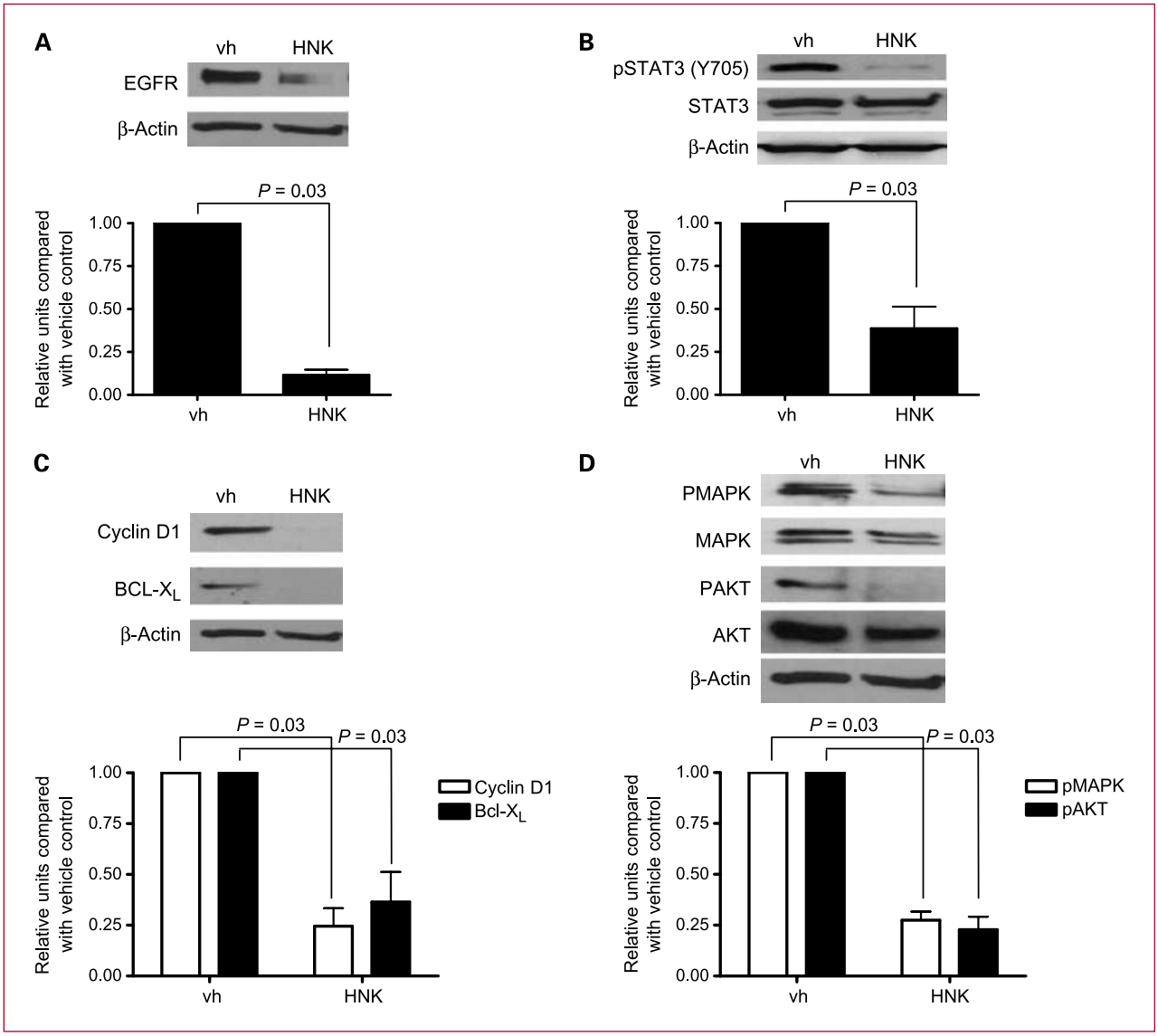

Fig. 2. Honokiol inhibits the EGFR signaling pathway in HNSCC cells. A, B, C, and D, Cal-33 cells were cultured in DMEM/10% fetal bovine serum for 24 hours, then in serum-free DMEM, containing either honokiol (10 μmol/L) or vehicle, for another 24 hours. Whole cell lysates were probed for EGFR, PSTAT3, STAT3, Bcl-X_L, cyclin D1, phospho p42/p44 MAPK, phospho Akt, and Akt, with β-actin as a loading control. HNK, honokiol; vh, vehicle. Each experiment was done four times with similar results (P = 0.03 for all proteins).

growth inhibition, at least in part, was due to the induction of apoptosis in HNSCC (Fig. 1B; P = 0.03).

Honokiol inhibits the EGFR signaling pathway. The EGFR signaling pathway, including activation of STAT3 and increased expression of STAT3 target genes, plays an important role in HNSCC. One approved HNSCC therapy (cetuximab) and various investigational therapies target EGFR and/or STAT3. By densitometric analysis of immunoblot bands, 24-hour honokiol (10 μmol/L) treatment resulted in an 88% decrease in EGFR in Cal-33 cells (Fig. 2A; P = 0.03). In studies of Cal-33 cells treated with both EGF and honokiol, we found that honokiol treatment resulted in a decrease in phosphorylated EGFR to a lesser degree than the corresponding decrease in total EGFR expression levels (data not shown), which suggests that honokiol likely primarily affects levels of total EGFR.

Honokiol-induced EGFR inhibition was associated with a 61% downstream inhibition of STAT3 in Cal-33 cells (Fig. 2B; P = 0.03), as well as 63% and 75% decreases in expression of *Bcl-X_L* and *cyclin D1*, two STAT3 target genes that inhibit apoptosis and promote the cell cycle, respectively (Fig. 2C; P = 0.03). Honokiol treatment also resulted in decreased levels of phosphorylated p42/p44 MAPK and phosphorylated Akt, by 73% and 77%, respectively, suggesting a global effect on EGFR signaling (Fig. 2D; P = 0.03). As EGFR signaling via STAT3 is known to play a key role in

HNSCC growth both *in vitro* and *in vivo*, these data indicate that by inhibiting both EGFR levels and STAT3 activation, honokiol may have potential utility in the treatment of HNSCC. This effect on EGFR signaling and STAT3 has recently been shown in a breast cancer cell line, albeit at much higher concentrations of honokiol (60 μmol/L compared with 10 μmol/L in our models; ref. 8).

Honokiol enhances the activity of erlotinib in HNSCC cells. Honokiol was tested for its ability to inhibit growth in HNSCC models of EGFR inhibitor resistance. The clone 686 LNR30 was chosen for its relative resistance to erlotinib, having an EC_{50} of 134.7 μmol/L, compared with 15.13 μmol/L in the parental cell line 686 LN (39). Honokiol, on the other hand, was equally effective in inhibiting growth of the parental and erlotinib-resistant cell lines (Fig. 3A). These results suggest that honokiol is able to overcome resistance to erlotinib.

Cetuximab, which is currently FDA approved for treatment of HNSCC, is known to inhibit HNSCC growth *in vivo*, but has minimal effects *in vitro*. Therefore, in our studies of honokiol's effect in combination with EGFR inhibition, we used erlotinib in cell culture experiments and cetuximab in animal studies. Honokiol enhanced the growth inhibition seen with erlotinib treatment *in vitro*. Combining honokiol at its EC_{50} in treatment of 686 LN cells (3.3 μmol/L) with the approximate EC_{50} for erlotinib (15.1 μmol/L) resulted in a 77.0% growth inhibition, compared with 52% growth inhibition for erlotinib alone, a 1.5-fold enhancement (Fig. 3B; $P = 0.03$), further supporting the use of honokiol in combination with EGFR blockade in HNSCC.

Honokiol has been shown to decrease the invasiveness of fibrosarcoma (40) and breast cancer cells (33). We assayed the invasiveness of 686 LN cells using matrigel invasion assays. Cells were plated in serum-free medium containing EGF (10 ng/mL) as a stimulant and either honokiol (5 μmol/L), erlotinib (5 μmol/L), a combination of both drugs, or their corresponding vehicles as controls. Medium containing 10% serum was present in the lower chamber as a chemoattractant. After 24 hours, honokiol was found to inhibit invasion, on its own, by 72.7%, compared with vehicle control ($P = 0.03$). Combined treatment with erlotinib plus honokiol, however, enhanced the anti-invasion activity of erlotinib but did not significantly increase the effects of honokiol alone on HNSCC invasion ($P = 0.03$; Fig. 3C). Neither drug alone induces significant growth inhibition at these concentrations after 24 hours, and, in combination, they decrease growth by only 23.5%, compared with an 83.3% decrease in number of invaded cells (data not shown). It is likely, therefore, that these data represent true decreases in invasiveness rather than just growth inhibition. Considering the large impact of invasion and metastasis on the clinical outcome of HNSCC, these data suggest that honokiol may have a role in HNSCC treatment, especially in combination with EGFR-targeting therapies.

***Honokiol enhances the growth inhibitory activity of cetuximab and inhibits EGFR signaling* in vivo.** Honokiol has been found to prevent the formation of skin papillomas *in vivo* (3) and to inhibit growth of xenografts derived

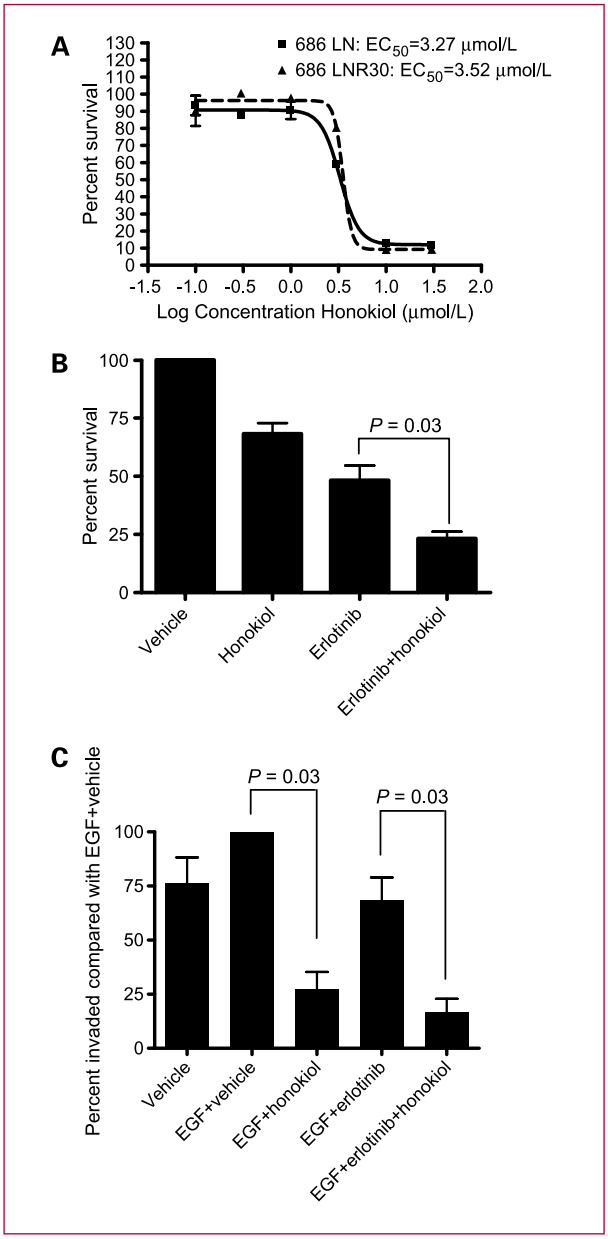

Fig. 3. Honokiol enhances the effects of erlotinib. A, 686 LN cells and the erlotinib-resistant clone 686 LNR30 were treated with varying concentrations of honokiol for 72 hours, stained with trypan blue dye, and counted. The experiment was done twice for each cell line/clone with triplicate samples and similar results. B, 686 LN cells were treated with either honokiol at its EC_{50} (3.3 μmol/L) or erlotinib (15.1 μmol/L), both drugs, or their corresponding vehicles. After 72 hours, cells were stained with trypan blue dye and counted. The experiment was done four times with triplicate samples and similar results ($P = 0.03$). C, 686 LN cells were plated in serum-free DMEM F12, on top of matrigel inserts in wells containing DMEM F12/10% fetal bovine serum. Both inserts and outer wells contained EGF (10 ng/mL) and either honokiol (5 μmol/L) with or without erlotinib (5 μm) or the vehicle. Matrigel inserts were fixed and stained after 24 hours. Numbers of cells invading the matrigel were counted. The experiment was done six times, using duplicate samples and counting at least 8 fields per well ($P = 0.03$ for both EGF versus honokiol and honokiol versus honokiol plus erlotinib), with similar results. Percentages of invaded cells, on the *y* axis, were calculated by comparison with cells treated with EGF +vehicle, as the control.

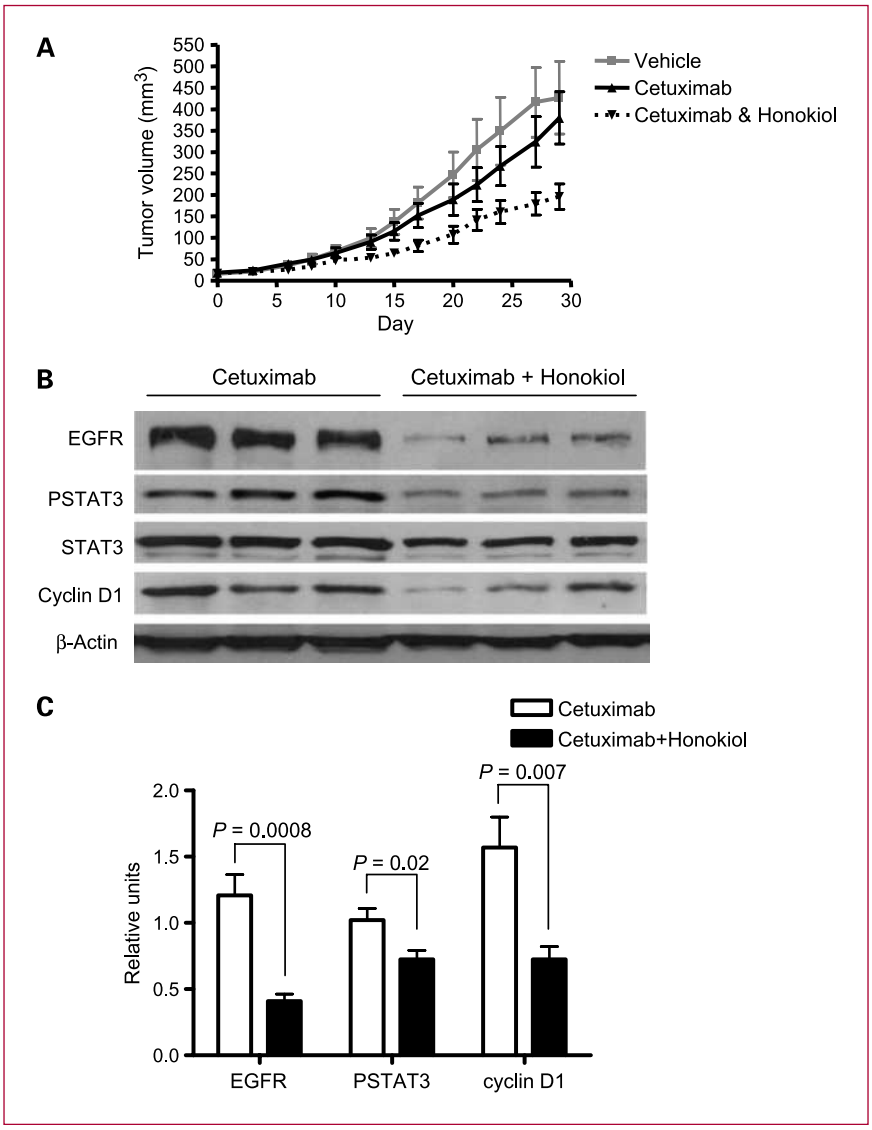

Fig. 4. The *in vivo* effects of honokiol in an HNSCC xenograft therapy model. A, nude mice were inoculated with 2×10^6 1483 cells, s.c., into their flanks. After tumor outgrowth mice were randomized to treatment groups, based on tumor volume. Mice were treated with either cetuximab (0.8 mg/mouse, 2 days/week), or cetuximab plus honokiol (3 mg/mouse, 3 days/week) on alternating days. The vehicle control group received Intralipid and saline by i.p. injection on corresponding days. Tumor measurements were done at least three times per week. B, lysates were extracted from tumors of mice in the cetuximab alone and cetuximab plus honokiol group. Selected lysates, probed by immunoblot for PSTAT3, STAT3, EGFR, and cyclin D1, are shown. C, densitometric values representing averages from lysates of tumors from all mice in each of these two groups, and which have been normalized to β-actin (P = 0.0008, 0.02, and 0.007 for EGFR, PSTAT3, and cyclin D1, respectively).

from angiosarcoma (4), colorectal (6), prostate (7, 35), gastric (13), breast (14), lung (12), and ovarian (35) cancer xenografts, *in vivo*. In a pilot study of honokiol's *in vivo* anticancer activity the average growth rates of HNSCC xenografts were not significantly reduced in animals treated with honokiol compared with the vehicle (14.31 mm^3/day versus 19.87 mm^3/day; data not shown). These data suggest that honokiol may not be effective as a single agent for *in vivo* treatment of HNSCC.

To determine whether or not honokiol enhances the anticancer activity of EGFR-inhibiting therapy *in vivo*, as was seen *in vitro*, female nude mice were inoculated with 1483 HNSCC cells and, after tumor outgrowth and randomization to treatment groups, treated with vehicle, cetuximab alone (0.8 mg/mouse 2 days per week), or honokiol (3 mg/mouse 3 days per week) plus cetuximab on alternating days. Honokiol was found to significantly enhance the growth inhibitory activity of cetuximab. Xenografts in the combination group grew at an average rate of 6.63 mm^3/day, 46.8% slower than the cetuximab group, which grew at a rate of 12.45 mm^3/day (P = 0.03), and 58.2% slower than the vehicle group, which grew at an average rate of 15.88 mm^3/day (P = 0.03; Fig. 4A). On the last day of the study, the average tumor volume was significantly lower in the combination treatment group as compared with either the vehicle-treated (P = 0.02) or cetuximab-treated (P = 0.01) group. Although mice were not weighed in this study, in another experiment, mice treated with the same dose of honokiol (3 mg) for 25 days were found to gain 3.0 g, on average, compared with a 1.5 g average weight gain in the vehicle control group. Further investigation showed no weight loss in mice at doses <300 mg/kg/day, approximately twice the dose used in the current study (unpublished observations).

Immunoblots of tumor lysates showed decreased levels of EGFR, tyrosine phosphorylated STAT3 (pSTAT3), and cyclin D1 in mice treated with cetuximab plus honokiol compared with cetuximab alone (P = 0.0008, 0.02, and 0.007 for EGFR, pSTAT3, and cyclin D1, respectively; Fig. 4B and C).

Discussion

The therapeutic potential, including antitumor activity, of honokiol, a natural product derived from the magnolia plant and used in traditional Chinese medicine, has been reported in various preclinical models. In the current study, we investigated honokiol's potential utility in the treatment of HNSCC. Honokiol was found to inhibit growth and induce apoptosis in HNSCC cell lines and to enhance the growth-inhibitory and anti-invasion activities of the EGFR-targeting TKI erlotinib. Furthermore, EGFR signaling, STAT3 activity, and expression of STAT3 target genes were inhibited upon honokiol treatment. Finally, honokiol was found to enhance the efficacy of the EGFR-targeting antibody cetuximab, and inhibit EGFR signaling *in vivo*.

Many studies have focused on targeting EGFR and STAT3 with a variety of engineered molecules, including antibodies, small molecule inhibitors, oligonucleotides, peptidomimetics, and others (19, 28). Several natural compounds have also been found to inhibit STAT3, mainly via effects on upstream signaling molecules, in different models (19, 41–44). Our rationale for investigating the ability of honokiol to target EGFR and STAT3 signaling included evidence of STAT3 inhibition in honokiol treatment of a multiple myeloma cell line (29) and, more recently, inhibition of EGFR and STAT3 signaling in breast cancer cells (8). Furthermore, honokiol has been found to inhibit other signaling molecules upstream of STAT3, including Src (4) and gp130 (29), and to inhibit NFκB (9, 11, 16, 17), which is known to experience crosstalk with STAT3 in HNSCC (18). Finally, honokiol induces downregulation of various STAT3 target genes (5, 7, 17, 32, 33, 35).

Honokiol was found to decrease expression levels of total EGFR both *in vitro* and *in vivo* (Figs. 2A and 4B). Cellular exposure to various anticancer agents has been shown to affect total EGFR levels through a variety of different mechanisms, including lysosomal and proteosomal degradation (45–47), caspase-mediated degradation (48), and decreased transcription (49), and to affect surface levels through receptor internalization (47). Furthermore, a complex balance between EGFR recycling and degradation can be altered, in multiple ways, by different drug treatments and EGFR mutations (50). Honokiol's structure contains two phenolic groups that can scavenge free radicals (2), suggesting the potential for nonspecific effects on multiple signaling pathways.

A recent study showed decreases in heat shock protein (hsp) 90 in breast cancer cells treated with high concentrations of honokiol (100 μmol/L; ref. 8). It is possible, therefore, that honokiol regulates EGFR degradation and recycling through similar mechanisms to either of the hsp90 inhibitors herbimycin A and geldanamycin (47).

Currently available treatments for HNSCC, including chemotherapy and radiation, can contribute to the morbidity and mortality associated with this disease. In addition, patients can develop resistance to standard chemotherapeutics. Critical regions of the head and neck such as the spinal cord can only tolerate a finite dose of radiation, thereby limiting the repeated clinical use of these therapies. Therefore, administration of a compound that enhances the activity of a HNSCC treatment may be a useful complementary strategy. In this study, we investigated honokiol's ability to enhance the activity of erlotinib (Tarceva), a small molecule inhibitor that has shown promise in clinical trials in HNSCC (28). Honokiol results in decreased levels of pMAPK and pAKT as well, suggesting global inhibition of the EGFR signaling pathway, rather than a specific effect on STAT3 signaling. In addition, as honokiol has been found to inhibit Src, gp130, and EGFR, it is likely that in HNSCC cell lines, STAT3 signaling is inhibited through more than one upstream mechanism. Limited clinical responses to EGFR-targeting therapies, like cetuximab and erlotinib, may be due to activation of STAT3 through alternative signaling pathways, including Src and the IL-6 receptor. An agent that targets one of these alternative pathways, like honokiol, which inhibits Src and the IL-6 receptor, and at the same time enhances EGFR inhibition, may potentially be useful in overcoming the limited clinical responses to EGFR targeting agents seen to date in HNSCC patients. Liu et al. have shown that honokiol synergizes with lapatinib, another EGFR-targeting therapeutic, in the treatment of human epidermal growth factor receptor 2–overexpressing breast cancer cells (33). In the current study, honokiol was found to enhance the growth inhibitory and anti-invasion activities of erlotinib *in vitro* as well as the growth inhibitory activity of cetuximab *in vivo*. To our knowledge, this is the first study showing the *in vivo* anticancer activity of honokiol in HNSCC. Our observations of the ability of honokiol to target EGFR/STAT3 signaling, to enhance the therapeutic effects of EGFR-targeting molecules, both *in vitro* and *in vivo*, and to inhibit growth of a cell line known to be resistant to other EGFR inhibitors suggest a potential role for honokiol in the treatment of HNSCC, particularly in combination with EGFR-inhibiting therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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