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Honokiol inhibits EGFR signaling and enhances the antitumor effects of EGFR inhibitors

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Abstract

Purpose—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 employed to test the effects of honokiol treatment.

Results—Honokiol was found to inhibit growth in human HNSCC cell lines, with EC₅₀s ranging from 3.3–7.4 μM, and to induce apoptosis, as shown through annexin V staining. These effects were associated with inhibition of EGFR signaling, including downstream inhibition of MAPK, Akt and 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. While HNSCC xenograft models did not demonstrate significant inhibition of *in vivo* tumor growth with honokiol treatment alone, the combination of honokiol plus cetuximab, an FDA-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 where it can augment the effects of EGFR inhibitors and overcome drug resistance.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most commonly occurring malignancies world-wide. 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,

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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 used in traditional Chinese medicine. Studies have demonstrated 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 fill in cancer therapy began with a study demonstrating prevention of skin papillomas in mice (3). Subsequent studies demonstrated 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 nuclear factor kappa B (NFkB) signaling pathway. NFkB is a transcription factor that contributes to several physiological processes (e.g. inflammation) but also regulates the expression of genes that are involved in cancer, including genes that control the cell cycle, apoptosis, tumor angiogenesis, and invasion (15). NFkB 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, NFkB has been reported to interact with another transcription factor, signal transducer and activator of transcription (STAT)-3 (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 NFkB, 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) (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 FDA-approved for use in the treatment of HNSCC in 2006. 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 NFkB (9,11,16,17) as well as gp130, a subunit of the 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

HNSCC cell lines Cal-33, derived from an oral squamous cell carcinoma (SCC) (37) and 1483, from an oropharyngeal SCC (38), were maintained in DMEM medium with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. 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 (MD 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) (39). All the cell lines were genotyped and validated in 2008 using the AmpFISTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster city, CA). 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, Deerfield, IL) for animal treatments. Erlotinib (Chemietek, Indianapolis, IN) was dissolved in 100% dimethylsulfoxide (DMSO), as a vehicle.

Proliferation assay

HNSCC cells ($1-3 \times 10^4$ per well) were cultured overnight and treated with honokiol, erlotinib or the corresponding vehicles, in DMEM/1% serum, the following day. After 72 hours, cells were harvested by trypsinization and live cells counted after staining for trypan blue dye exclusion. Each experiment was performed with triplicate samples and the average percent survival calculated as a comparison to cells treated with the vehicle alone. The EC₅₀ was calculated using Prism software version 4.03 (GraphPad Software Inc).

Apoptosis assay

Cal-33 cells (5×10^4 per well) were seeded and, the following day, treated with either honokiol (10 μ M) 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 counted (at least 3 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 μ M) and/or erlotinib (5 μ M) or the corresponding vehicles, in a matrigel invasion chamber insert (BD Biosciences, San Diego, California). The outer well contained DMEM F12/10% FBS, 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 $\times$ magnification.

Western blotting

For cell lysates used in western blots, cells were cultured in DMEM/10% FBS 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, Hercules, CA) by a semi-dry transfer apparatus (Bio-Rad,

Hercules, CA). The membrane was blocked with 5% skim milk in Tris-buffered saline-Tween (TBS-T) solution (100 mM Tris, 150 mM 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 (anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugate from Bio-Rad Laboratories, Hercules, CA). The blots were washed and developed with a luminol kit (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were for STAT3, pSTAT3, pEGFR, phospho-p44/p42 MAPK, AKT, pAKT (Cell Signaling Technology, Boston, MA), cyclin D1, Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA) EGFR (BD Transduction labs, San Diego, CA) and β -actin (CalBiochem, Gibbstown, NJ) 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 (Indianapolis, Indiana) and were housed in a pathogen free animal facility. The animal study protocols were approved by institutional animal care and use committee. 1483 cells (2×10^6 per mouse) were harvested by trypsinization, washed in PBS, re-suspended in saline, and subcutaneously injected into the flank of each mouse. After outgrowth of palpable tumors (seven days), 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 intraperitoneal 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, Deerfield, IL), three times per week. Tumors were measured using digital calipers (Control Company, Friendswood, TX) at least three times per week and tumor volumes calculated using the following formula: $\text{volume} = L \times (W)^2 / 2$ (L: longest diameter; W: shorter diameter). At the end of the study, mice were euthanized and tumor tissues 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 performed 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 in order 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 pro-apoptotic activities of honokiol have been demonstrated 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 μ M to 100 μ M and compared to the vehicle (ethanol) alone. EC₅₀ values were 7.44 μ M for 1483 and 3.80 μ M for Cal-33 (Figure 1a). These values are comparable or lower than EC₅₀ 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 Figure 1b, honokiol increased the number of apoptotic cells by 7.4-fold suggesting that growth inhibition, at least in part, is due to the induction of apoptosis in HNSCC (Figure 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 μ M) treatment resulted in an 88% decrease in EGFR in Cal-33 cells (Figure 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 (Figure 2b; $p=0.03$), as well as 88% and 87% decreases in expression of Bcl-X_L and cyclin D1, two STAT3 target genes that inhibit apoptosis and promote the cell cycle, respectively (Figure 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 (Figure 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 μ M compared with 10 μ M in our models) (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 EC₅₀ of 134.7 μ M, compared to 15.13 μ M 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 (Figure 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 EC₅₀ in treatment of 686 LN cells (3.3 μ M) with the approximate EC₅₀ for erlotinib (15.1 μ M) resulted in a 77.0% growth inhibition, compared to 52% growth inhibition for erlotinib alone, a 1.5-fold enhancement (Figure 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 μ M), erlotinib (5 μ M), 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 to vehicle control ($p=0.03$). Combined treatment with erlotinib plus honokiol, however, enhanced erlotinib's anti-invasion activity but did not significantly increase the effects of honokiol alone on HNSCC invasion ($p=0.03$; Figure 3C). Neither drug alone induces significant growth inhibition at these concentrations after 24 hours and, in combination, decrease growth by only 23.5%, compared to 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 from angiosarcoma (4), colorectal (6), prostate (7,35), gastric (13), breast (14), lung (12), 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 to the vehicle (14.31 mm³/day versus 19.87 mm³/day; data not shown). These data suggest that honokiol may not be effective as a single agent for *in vivo* treatment of HNSCC.

In order 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 mm³/day, 46.8% slower than the cetuximab group, which grew at a rate of 12.45 mm³/day (p=0.03), and 58.2% slower than the vehicle group, which grew at an average rate of 15.88 mm³/day (p=0.03; Figure 4a). On the last day of the study, the average tumor volume was significantly lower in the combination treatment group as compared to either the vehicle-treated (p=0.02) or cetuximab-treated (p=0.01) groups. 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 to a 1.5 g average weight gain in the vehicle control group. Further investigation demonstrated no weight loss in mice at doses lower than 300 mg/kg/day, approximately twice the dose used in the current study (unpublished observations).

Immunoblots of tumor lysates demonstrated decreased levels of EGFR, tyrosine phosphorylated STAT3 (pSTAT3), and cyclin D1 in mice treated with cetuximab plus honokiol compared to cetuximab alone (p=0.0008, 0.02, and 0.007 for EGFR, pSTAT3 and cyclin D1, respectively; Figures 4b and 4c).

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 also to inhibit NF κ B (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* (Figures 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 demonstrated decreases in heat shock protein (hsp) 90 in breast cancer cells treated with high concentrations of honokiol (100 μ M) (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 an HNSCC treatment may be a useful complementary strategy. In this study, we investigated honokiol's ability to enhance the activity of erlotinib (TarcevaTM), 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 HER2-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 cetuximab's growth inhibitory activity *in vivo*. To our knowledge, this is the first study demonstrating the *in vivo* anticancer activity of honokiol in HNSCC. Our observations of honokiol's ability 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.

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 FDA approved for use in HNSCC. These preclinical results provide the critical biologic rationale for future clinical investigations using honokiol in HNSCC patients.

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References

1. Sturgis EM. A review of social and behavioral efforts at oral cancer preventions in India. *Head Neck* 2004;26:937–44. [PubMed: 15390198]
2. Fried LE, Arbiser JL. Honokiol, a multifunctional antiangiogenic and antitumor agent. *Antioxidants & redox signaling* 2009;11:1139–48. [PubMed: 19203212]
3. Konoshima T, Kozuka M, Tokuda H, et al. Studies on inhibitors of skin tumor promotion, IX. Neolignans from *Magnolia officinalis*. *J Nat Prod* 1991;54:816–22. [PubMed: 1659613]
4. Bai X, Cerimele F, Ushio-Fukai M, et al. Honokiol, a small molecular weight natural product, inhibits angiogenesis in vitro and tumor growth in vivo. *J Biol Chem* 2003;278:35501–7. [PubMed: 12816951]
5. Battle TE, Arbiser J, Frank DA. The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells. *Blood* 2005;106:690–7. [PubMed: 15802533]
6. Chen F, Wang T, Wu YF, et al. Honokiol: a potent chemotherapy candidate for human colorectal carcinoma. *World J Gastroenterol* 2004;10:3459–63. [PubMed: 15526365]
7. Hahm ER, Arlotti JA, Marynowski SW, Singh SV. Honokiol, a constituent of oriental medicinal herb *magnolia officinalis*, inhibits growth of PC-3 xenografts in vivo in association with apoptosis induction. *Clin Cancer Res* 2008;14:1248–57. [PubMed: 18281560]
8. Park EJ, Min HY, Chung HJ, et al. Down-regulation of c-Src/EGFR-mediated signaling activation is involved in the honokiol-induced cell cycle arrest and apoptosis in MDA-MB-231 human breast cancer cells. *Cancer Lett* 2009;277:133–40. [PubMed: 19135778]
9. Sheu ML, Chiang CK, Tsai KS, et al. Inhibition of NADPH oxidase-related oxidative stress-triggered signaling by honokiol suppresses high glucose-induced human endothelial cell apoptosis. *Free Radic Biol Med* 2008;44:2043–50. [PubMed: 18423412]
10. Shigemura K, Arbiser JL, Sun SY, et al. Honokiol, a natural plant product, inhibits the bone metastatic growth of human prostate cancer cells. *Cancer* 2007;109:1279–89. [PubMed: 17326044]
11. Tse AK, Wan CK, Shen XL, Yang M, Fong WF. Honokiol inhibits TNF-alpha-stimulated NF-kappaB activation and NF-kappaB-regulated gene expression through suppression of IKK activation. *Biochem Pharmacol* 2005;70:1443–57. [PubMed: 16181613]
12. Jiang QQ, Fan LY, Yang GL, et al. Improved therapeutic effectiveness by combining liposomal honokiol with cisplatin in lung cancer model. *BMC Cancer* 2008;8:242. [PubMed: 18706101]
13. Sheu ML, Liu SH, Lan KH. Honokiol induces calpain-mediated glucose-regulated protein-94 cleavage and apoptosis in human gastric cancer cells and reduces tumor growth. *PLoS One* 2007;2:e1096. [PubMed: 17971859]
14. Wolf I, O'Kelly J, Wakimoto N, et al. Honokiol, a natural biphenyl, inhibits in vitro and in vivo growth of breast cancer through induction of apoptosis and cell cycle arrest. *Int J Oncol* 2007;30:1529–37. [PubMed: 17487375]
15. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006;441:431–6. [PubMed: 16724054]
16. Lee J, Jung E, Park J, et al. Anti-inflammatory effects of magnolol and honokiol are mediated through inhibition of the downstream pathway of MEKK-1 in NF-kappaB activation signaling. *Planta Med* 2005;71:338–43. [PubMed: 15856410]

17. Ahn KS, Sethi G, Shishodia S, Sung B, Arbiser JL, Aggarwal BB. Honokiol potentiates apoptosis, suppresses osteoclastogenesis, and inhibits invasion through modulation of nuclear factor-kappaB activation pathway. *Mol Cancer Res* 2006;4:621–33. [PubMed: 16966432]
18. Squarize CH, Castilho RM, Sriuranpong V, Pinto DS Jr, Gutkind JS. Molecular cross-talk between the NFkappaB and STAT3 signaling pathways in head and neck squamous cell carcinoma. *Neoplasia* 2006;8:733–46. [PubMed: 16984731]
19. Leeman RJ, Lui VW, Grandis JR. STAT3 as a therapeutic target in head and neck cancer. *Expert Opin Biol Ther* 2006;6:231–41. [PubMed: 16503733]
20. Grandis JR, Drenning SD, Chakraborty A, et al. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth in vitro. *J Clin Invest* 1998;102:1385–92. [PubMed: 9769331]
21. Rubin Grandis J, Zeng Q, Drenning SD. Epidermal growth factor receptor--mediated stat3 signaling blocks apoptosis in head and neck cancer. *Laryngoscope* 2000;110:868–74. [PubMed: 10807365]
22. Dassonville O, Formento JL, Francoual M, et al. Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. *J Clin Oncol* 1993;11:1873–8. [PubMed: 8410112]
23. Rubin Grandis J, Melhem MF, Barnes EL, Tweardy DJ. Quantitative immunohistochemical analysis of transforming growth factor- alpha and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck. *Cancer* 1996;78:1284–92. [PubMed: 8826952]
24. Santini J, Formento JL, Francoual M, et al. Characterization, quantification, and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas. *Head Neck* 1991;13:132–9. [PubMed: 2022478]
25. Ang KK, Berkey BA, Tu X, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer research* 2002;62:7350–6. [PubMed: 12499279]
26. Gupta AK, McKenna WG, Weber CN, et al. Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. *Clin Cancer Res* 2002;8:885–92. [PubMed: 11895923]
27. Rubin Grandis J, Melhem MF, Gooding WE, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst* 1998;90:824–32. [PubMed: 9625170]
28. Loeffler-Ragg J, Schwentner I, Sprinzl GM, Zwierzina H. EGFR inhibition as a therapy for head and neck squamous cell carcinoma. *Expert Opin Investig Drugs* 2008;17:1517–31.
29. Ishitsuka K, Hideshima T, Hamasaki M, et al. Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis. *Blood* 2005;106:1794–800. [PubMed: 15870175]
30. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. *Cancer research* 2003;63:2948–56. [PubMed: 12782602]
31. Xi S, Zhang Q, Dyer KF, et al. Src kinases mediate STAT growth pathways in squamous cell carcinoma of the head and neck. *J Biol Chem* 2003;278:31574–83. [PubMed: 12771142]
32. Hahm ER, Singh SV. Honokiol causes G0-G1 phase cell cycle arrest in human prostate cancer cells in association with suppression of retinoblastoma protein level/phosphorylation and inhibition of E2F1 transcriptional activity. *Molecular cancer therapeutics* 2007;6:2686–95. [PubMed: 17938262]
33. Liu H, Zang C, Emde A, et al. Anti-tumor effect of honokiol alone and in combination with other anti-cancer agents in breast cancer. *Eur J Pharmacol* 2008;591:43–51. [PubMed: 18588872]
34. Fong WF, Tse AK, Poon KH, Wang C. Magnolol and honokiol enhance HL-60 human leukemia cell differentiation induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Int J Biochem Cell Biol* 2005;37:427–41. [PubMed: 15474987]
35. Li Z, Liu Y, Zhao X, et al. Honokiol, a natural therapeutic candidate, induces apoptosis and inhibits angiogenesis of ovarian tumor cells. *Eur J Obstet Gynecol Reprod Biol* 2008;140:95–102. [PubMed: 18440692]
36. Hou W, Chen L, Yang G, et al. Synergistic antitumor effects of liposomal honokiol combined with adriamycin in breast cancer models. *Phytother Res* 2008;22:1125–32. [PubMed: 18570244]

37. Gioanni J, Fischel JL, Lambert JC, et al. Two new human tumor cell lines derived from squamous cell carcinomas of the tongue: establishment, characterization and response to cytotoxic treatment. *Eur J Cancer Clin Oncol* 1988;24:1445–55. [PubMed: 3181269]
38. Sacks PG, Parnes SM, Gallick GE, et al. Establishment and characterization of two new squamous cell carcinoma cell lines derived from tumors of the head and neck. *Cancer research* 1988;48:2858–66. [PubMed: 2452013]
39. Muller S, Su L, Tighiouart M, et al. Distinctive E-cadherin and epidermal growth factor receptor expression in metastatic and nonmetastatic head and neck squamous cell carcinoma: predictive and prognostic correlation. *Cancer* 2008;113:97–107. [PubMed: 18473353]
40. Nagase H, Ikeda K, Sakai Y. Inhibitory effect of magnolol and honokiol from *Magnolia obovata* on human fibrosarcoma HT-1080. Invasiveness in vitro. *Planta Med* 2001;67:705–8. [PubMed: 11731909]
41. Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R, Sehti SM. Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. *Cancer research* 2003;63:1270–9. [PubMed: 12649187]
42. Leeman-Neill RJ, Wheeler SE, Singh SV, et al. Guggulsterone enhances head and neck cancer therapies via inhibition of signal transducer and activator of transcription-3. *Carcinogenesis* 2009;30:1848–56. [PubMed: 19762335]
43. Masuda M, Suzui M, Weinstein IB. Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin Cancer Res* 2001;7:4220–9. [PubMed: 11751523]
44. Nam S, Buettner R, Turkson J, et al. Iridin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc Natl Acad Sci U S A* 2005;102:5998–6003. [PubMed: 15837920]
45. Feng FY, Varambally S, Tomlins SA, et al. Role of epidermal growth factor receptor degradation in gemcitabine-mediated cytotoxicity. *Oncogene* 2007;26:3431–9. [PubMed: 17146438]
46. Mizuno H, Cho YY, Zhu F, et al. Theaflavin-3, 3'-digallate induces epidermal growth factor receptor downregulation. *Mol Carcinog* 2006;45:204–12. [PubMed: 16353237]
47. Supino-Rosin L, Yoshimura A, Yarden Y, Elazar Z, Neumann D. Intracellular retention and degradation of the epidermal growth factor receptor, two distinct processes mediated by benzoquinone ansamycins. *J Biol Chem* 2000;275:21850–5. [PubMed: 10806200]
48. He YY, Huang JL, Gentry JB, Chignell CF. Epidermal growth factor receptor down-regulation induced by UVA in human keratinocytes does not require the receptor kinase activity. *J Biol Chem* 2003;278:42457–65. [PubMed: 12930839]
49. Rubin Grandis J, Zeng Q, Tweardy DJ. Retinoic acid normalizes the increased gene transcription rate of TGF- α and EGFR in head and neck cancer cell lines. *Nat Med* 1996;2:237–40. [PubMed: 8574972]
50. Tong J, Taylor P, Peterman SM, Prakash A, Moran MF. Epidermal growth factor receptor phosphorylation sites Ser991 and Tyr998 are implicated in the regulation of receptor endocytosis and phosphorylations at Ser1039 and Thr1041. *Mol Cell Proteomics* 2009;8:2131–44. [PubMed: 19531499]

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 performed twice with triplicate samples and similar results. (B) Cal-33 cells were treated with either honokiol (10 μM) 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 counted. The experiment was performed 4 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% FBS for 24 hours, in serum free DMEM, containing either honokiol (10 μ M) 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, Akt and with β -actin as a loading control. Each experiment was performed four times with similar results ($p=0.03$ for all proteins).

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 performed twice for each cell line/clone with triplicate samples and similar results. (B) 686 LN cells were treated with either honokiol at its EC₅₀ (3.3 μ M) or erlotinib (15.1 μ M), both drugs, or their corresponding vehicles. After 72 hours, cells were stained with trypan blue dye and counted. The experiment was performed 4 times with triplicate samples and similar results (p=0.03). (C) 686LN cells were plated in serum-free DMEM F12, on top of matrigel inserts in wells containing DMEM F12/10% FBS. Both inserts and outer wells contained EGF (10 ng/ml) and either honokiol (5 μ M) with or

without erlotinib (5 μ m) or the vehicle. Matrigel inserts were fixed and stained after 24 hours. Numbers of cells invading the matrigel were counted. The experiment was performed 6 times, using duplicate samples and counting at least 8 fields per well ($p=0.03$ for both EGF versus honokiol and honokiol versus honokiol+erlotinib), with similar results. Percentages of invaded cells, on the y axis, were calculated by comparison to cells treated with EGF alone, as the control.

Fig. 4.

The *in vivo* effects of honokiol in HNSCC xenograft therapy models. (A) Nude mice were inoculated with 2×10^6 1483 cells, subcutaneously, 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 IP injection on corresponding days. Tumor measurements were performed 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, are shown ($p=0.0008$, 0.02 , and 0.007 for EGFR, PSTAT3 and cyclin D1, respectively).