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

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introduction

• 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).

• (1). A review of social and behavioral efforts at oral cancer preventions in India
• Abstract Background.
• Oral cancer is a major public health problem in South-Central Asia, home to one fifth of the world's population. In most regions of India, it is the most common cancer in men and the third most common cancer in women. Prevention is an effective tool to reduce disease burden on society and may offer particular advantages in developing countries.
introduction

• 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).

• (2) Honokiol, a Multifunctional Antiangiogenic and Antitumor Agent
Honokiol, a Multifunctional Antiangiogenic and Antitumor Agent

- Honokiol has been found to have antiangiogenic, antiinflammatory, and antitumor properties in preclinical models, without appreciable toxicity. These findings have increased interest in bringing honokiol to the clinic as a novel chemotherapeutic agent.

- In addition, mechanistic studies have tried to find the mechanism(s) of action of honokiol, for two major reasons. First, knowledge of the mechanisms of action may assist development of novel synthetic analogues. Second, mechanistic actions of honokiol may lead to rational combinations with conventional chemotherapy or radiation for enhanced response to systemic cancers.

- In this review, we describe the findings that honokiol has two major mechanisms of action.

  - First, it blocks signaling in tumors with defective p53 function and activated ras by directly blocking the activation of phospholipase D by activated ras.
  - Second, honokiol induces cyclophilin D, thus potentiating the mitochondrial permeability transition pore, and causing death in cells with wild-type p53.

- Knowledge of the dual activities of honokiol can assist with the development of honokiol derivatives and the design of clinical trials that will maximize the potential benefit of honokiol in the patient setting. *Antioxid. Redox Signal.* 11, 1139–1148.
p53 Signaling

Hypoxia

UV

Chemotherapy

Ionizing Radiation

JNK

HIPk2

CSNK15

p38

p53

Degradation

PTEN

HIF1α

DNA Repair

Acc

p53

MDM2

PIAS1

PML

BRCA1

Cell Survival

CAK

PCAF

Sirt

MDM2

Ub

c-Ab1

HDAC

p300

DNA Damage

ATM

Chk1

Chk2

GSK3β

p53

Ac

Cell Cycle Progression

CyclinD1

CDK2

CyclinE

CDK4

Rb

E2F

p21CIP

c-Fos

GADD45

Gene Expression

Angiogenesis Inhibition

Apoptosis

Caspase

Bax

BGL2

TBP1

BAI1

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• 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).
Three neolignans, known as magnolol [1], honokiol [2] and the new monoterpenylmagnolol [3], were isolated from the bark of Magnolia officinalis as inhibitors of Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). The structure of 3 was determined from 2D nmr spectral data and difference nOe experiments. The MeOH extract of this plant and magnolol exhibited remarkable inhibitory effects on mouse skin tumor promotion in an in vivo two stage carcinogenesis test. This investigation indicates that these neolignans and the extract might be valuable antitumor promoters.
TNF (Tumor Necrosis Factor) Signaling
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 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).
NF-KappaB Family Pathway
• 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).
introduction

• 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-xL (7), survivin (7), and VEGF (17, 35).
EGFR Pathway

• Three major signaling pathways:
  • (1) Ras / Raf / MAPK pathway
  • (2) PI3K / AKT pathway
  • (3) JAK and STAT pathway

• 3 signaling pathway mediates cell differentiation ultimately, survival, migration, invasion, adhesion and cell damage repair and a series of processes, play an important role in the growth of malignant tumors.

• Many tumors have mutant EGFR exists. The role of mutant EGFR may include: a ligand-independent activation of the receptor cells continued; some domain-deleted inhibit the activation of EGFR, which led to the destruction receptor down mechanism, abnormal signal transduction pathways, apoptosis and so on.
Deregulated signaling pathways in HNSCC
introduction

• 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.
Copy number alterations with similar frequency identified between ESCC and HNSCC in JAK–STAT signalling, RTK–Ras signalling and cell cycle pathways.
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),

• 686LN and 686LNR30 cells are isogenic models of acquired EGFR TKI resistance in vitro and were obtained from Dr. Georgia Chen

• Honokiol is a natural product extracted from seed cone of Magnolia grandiflora as previously described (4).

• Erlotinib (Chemietek) was dissolved in 100% DMSO, as a vehicle.
Erlotinib

- Erlotinib hydrochloride (trade name Tarceva) is a drug used to treat non-small cell lung cancer (NSCLC), pancreatic cancer and several other types of cancer. It is a reversible tyrosine kinase inhibitor, which acts on the epidermal growth factor receptor (EGFR). It is marketed in the United States by Genentech and OSI Pharmaceuticals and elsewhere by Roche.
EGFR-specific ligands (e.g., epiregulin and transforming growth factor α)

HER1 (EGFR)

HER1 (EGFR), HER2, HER3, or HER4

Cell membrane

Tyrosine kinase domains

Cytoplasm

Cell proliferation, cell survival, metastasis, and angiogenesis

Nucleus

HER2

HER3

HER4

SOS

PI3K

AKT

RAS

RAF

MTOR

MAPK

MEK
Honokiol

- **Honokiol** is a lignan isolated from the bark, seed cones, and leaves of trees belonging to the genus *Magnolia*. It has been identified as one the chemical compounds in some traditional eastern herbal medicines along with magnolol, 4-O-methylhonokiol, and obovatol.

- Honokiol has been extracted from a number of species of Magnolia native to many regions of the globe. Magnolia grandiflora, which is native to the American South, as well as Mexican species like Magnolia dealbata have been found to be sources of honokiol.[1] Traditionally in Asian medicine, the Magnolia biondii, Magnolia obovata, and Magnolia officinalis are commonly used.[2] The compound itself has a spicy odor.

- Honokiol belongs to a class of neolignan biphenols. As a polyphenol it is relatively small and can interact with cell membrane proteins through intermolecular interactions like hydrogen bonding, hydrophobic interactions, or aromatic pi orbital co-valency.[1] It is hydrophobic and readily dissolved in lipids. It is structurally similar to propofol.[1]
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Pharmacology

- Antitumorigenic activities
- Neurotrophic activity
- Anti-inflammatory activity
- Antioxidant activity
- Cytotoxicity inhibition
- GABA$_A$ modulation
- Ca$^{2+}$ inhibition
- Antiviral activity
- Metabolic activity
- Pharmacokinetics
- Delivery methods
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.
Cal-33 cells (5 × 10⁴/well) were seeded

Cells were then treated with either honokiol (10 μmol/L) or ethanol, as the vehicle, for 72 hours following day

Cells were then harvested and stained with Cy3-labeled Annexin V

and the numbers of Annexin V–positive cells were counted
686LN cells \((1.5 \times 10^3)\) were plated in serum-free DMEM F12 containing epidermal growth factor (EGF) alone \((10 \text{ ng/mL})\), EGF with honokiol \((5 \text{ μmo/L})\) and/or erlotinib \((5 \text{ μmo/L})\) or the corresponding vehicles, in a matrigel invasion chamber insert. The outer well contained DMEM F12/10\% fetal bovine serum, as a chemoattractant. 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. After 24 hours' incubation.
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 mmol/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.

Primary antibodies for STAT3, pSTAT3, pEGFR, phospho-p44/p42 mitogen-activated protein kinase (MAPK), AKT, pAKT, cyclin D1, Bcl-XL, EGFR and β-actin 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; \( n = 30 \))

1483 cells (\( 2 \times 10^6 \)/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 (received 0.8 mg/mouse/day, by i.p. injection, twice per week)
- 10 in the combination 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: \( \text{volume} = L \times (W)^2/2 \) (\( L \), 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 \textit{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
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 ($P = 0.03$).
Honokiol inhibits the EGFR signaling pathway
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-XL, 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).
Honokiol enhances the activity of erlotinib in HNSCC cells
Honokiol enhances the effects of erlotinib.

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.
Honokiol enhances the growth inhibitory activity of cetuximab and inhibits EGFR signaling in vivo
Cetuximab

- **Cetuximab** is an *epidermal growth factor receptor* (EGFR) inhibitor used for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer [1] and head and neck cancer. Cetuximab is a chimeric (mouse/human) monoclonal antibody given by intravenous infusion that is distributed under the trade name *Erbitux* in the U.S. and Canada by the drug company Bristol-Myers Squibb and outside the U.S. and Canada by the drug company Merck KGaA. In Japan, Merck KGaA, Bristol-Myers Squibb and Eli Lilly have a co-distribution.
The in vivo effects of honokiol in an HNSCC xenograft therapy model.

Fig. 4. The in vivo effects of honokiol in an HNSCC xenograft therapy model. A, nude mice were inoculated with $2 \times 10^6$ 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).
Discussion
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.
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.
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.
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