Research Article

Garcinol, a Polyisoprenylated Benzophenone Modulates Multiple Proinflammatory Signaling Cascades Leading to the Suppression of Growth and Survival of Head and Neck Carcinoma

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Abstract

Constitutive activation of proinflammatory transcription factors such as STAT3 and NF- κ B plays a pivotal role in the proliferation and survival of squamous cell carcinoma of the head and neck (HNSCC). Thus, the agents that can modulate deregulated STAT3 and NF- κ B activation have a great potential both for the prevention and treatment of HNSCC. In the present report, we investigated the potential effects of garcinol, an active component of *Garcinia indica* on various inflammatory mediators involved in HNSCC progression using cell lines and xenograft mouse model. We found that garcinol inhibited constitutively activated STAT3 in HNSCC cells in a time- and dose-dependent manner, which correlated with the suppression of the upstream kinases (c-Src, JAK1, and JAK2) in HNSCC cells. Also, we noticed that the generation of reactive oxygen species is involved in STAT3 inhibitory effect of garcinol. Furthermore, garcinol exhibited an inhibitory effect on the constitutive NF- κ B activation, mediated through the suppression of TGF- β -activated kinase 1 (TAK1) and inhibitor of I κ B kinase (IKK) activation in HNSCC cells. Garcinol also downregulated the expression of various gene products involved in proliferation, survival, and angiogenesis that led to the reduction of cell viability and induction of apoptosis in HNSCC cells. When administered intraperitoneally, garcinol inhibited the growth of human HNSCC xenograft tumors in male athymic nu/nu mice. Overall, our results suggest for the first time that garcinol mediates its antitumor effects in HNSCC cells and mouse model through the suppression of multiple proinflammatory cascades. *Cancer Prev Res*; 6(8); 843–54. ©2013 AACR.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC), a complex disease that comprises a broad range of epithelial malignancies, including nasal cavity, oral cavity, pharynx, and larynx accounts for approximately 650,000 new cases and 350,000 cancer-related deaths worldwide annually (6.0% of cases and 5.2% deaths; ref. 1). Current treatments of HNSCC are complicated, depending on the primary tumor sites, stages of the disease, and

considerations for organ preservation, thus multidisciplinary approaches involving surgery, radiotherapy, chemotherapy, and targeted agents are applied (2). Platinum-based regimens are considered most valuable and are used as standard approaches for multi-agent chemotherapy in the HNSCC treatment (3). However, two major drawbacks exist for the platinum agents: serious adverse effect (e.g., organ toxicity, myelotoxicity, and neuropathy) by affecting the healthy cells (4), and the occurrence of intrinsic or acquired resistance (5).

Several dysregulated proinflammatory signaling cascades including NF- κ B, STAT3, and serine/threonine protein kinase B (AKT) have been linked to the carcinogenesis of HNSCC and play a pivotal role in the growth, proliferation, and survival of the head and neck cancer (6–10). For example, Sen and colleagues recently reported that STAT3 activation can even contribute to EGF receptor inhibitor resistance in HNSCC and thereby targeting of STAT3 may represent an effective treatment strategy (10). Moreover, Yan and colleagues reported that a higher level of activated NF- κ B contributed to tumor hematologic and lymphatic metastasis in HNSCC patient specimens (11). Thus, identification of pharmacologic drugs, which can inhibit these

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inflammatory signal pathways, may have the potential for the prevention and treatment of HNSCC.

One such agent derived from natural sources that can have a great potential for head and neck cancer prevention and treatment is garcinol (camboginol) extracted from the dried rind of the fruit *Garcinia indica*. Garcinol has gained much attention in the field of cancer research in recent years, and several reports have shown the antineoplastic and chemopreventive role of garcinol in different cancers, such as leukemia, colorectal, gastrointestinal, and breast cancers (12–16). Although there is a prior evidence showing that garcinol can inhibit 4-nitroquinoline 1-oxide-induced tongue carcinogenesis (17), mechanisms through which garcinol elicits its antitumor activity, specifically in HNSCC, is unknown till date. Given the pivotal role of NF- κ B and STAT3 signaling cascades in the proliferation, survival, and chemoresistance in HNSCC, we speculate that garcinol may exhibit antineoplastic effects in HNSCC by modulating these proinflammatory pathways.

We found that garcinol can indeed suppress both constitutive NF- κ B and STAT3 activation in HNSCC. This inhibition decreased cell survival and downregulated expression of proliferative, antiapoptotic, and angiogenic gene products, leading to the suppression of proliferation and induction of apoptosis. Furthermore, garcinol also inhibited the growth of human HNSCC in a xenograft mouse model and modulated the activation of NF- κ B and STAT3 in tumor tissues.

Materials and Methods

Reagents

MTT, Tris, glycine, NaCl, SDS, bovine serum albumin (BSA), monothioglycerol (MTG), and β -actin antibody were purchased from Sigma-Aldrich. Garcinol with purity more than 98% was prepared from *Garcinia indica* fruit rind as described previously (18). Garcinol was dissolved in dimethyl sulfoxide (DMSO) as a 50 mmol/L stock solution and stored at -20°C for the experiments. Further dilution was done in cell culture medium. Dulbecco's modified Eagle medium (DMEM), FBS, 0.4% Trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen. Antibodies against p65, I κ B α , cyclin D1, Bcl-2, Bcl-xL, Mcl-1, survivin, VEGF, caspase-3, PARP, goat anti-rabbit-horseradish peroxidase (HRP) conjugate, Ki-67, goat anti-mouse HRP, and annexin V-fluorescein isothiocyanate (FITC) assay kit were obtained from Santa Cruz Biotechnology. Antibodies to phospho-specific p65 (Ser536), phospho-specific I κ B α (Ser32), phospho-specific TGF- β -activated kinase 1 (TAK1; Thr187), TAK1, phospho-specific inhibitor of I κ B kinase (IKK) α / β (Ser180/Ser181), IKK α , phospho-specific AKT (Ser473), AKT, phospho-specific mTOR (Ser2448), mTOR, phospho-specific p70S6K (Thr389), p70S6K, phospho-specific STAT3 (Tyr705), STAT3, phospho-specific Src (Tyr416), Src, phospho-specific JAK1 (Tyr1022/1023), JAK1, phospho-specific JAK2 (Tyr1007/1008), JAK2, and CD31 were purchased from Cell Signaling Technology. Bradford reagent was purchased

from Bio-Rad. The Cell Death Detection ELISA^{PLUS} DNA Fragmentation Kit was purchased from Roche Diagnostics. Nuclear extract kit and DNA-binding kit was obtained from Active Motif.

Cell lines

Human HNSCC cell line CAL27 was purchased in the year 2009 from American Type Culture Collection. UMSCC1 cells were kindly provided to us by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) in the year 2009 and have been characterized previously (19). These cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. However, no authentication of these cell lines was done by the authors.

Western blotting

Vehicle or garcinol-treated whole-cell extracts were lysed in lysis buffer [250 mmol/L NaCl, 20 mmol/L HEPES, 2 mmol/L EDTA (pH 8.0), 0.5 mmol/L EGTA, 0.1% Triton X-100, 1.5 $\mu\text{g/mL}$ aprotinin, 1.5 $\mu\text{g/mL}$ leupeptin, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 1.5 mmol/L NaVO₄]. Lysates were then spun at 13,300 rpm for 10 minutes to remove insoluble material and resolved on a 10% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad), blocked with Blocking One (Nacalai Tesque, Inc.), and probed with antibodies of interest overnight at 4°C . The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 hour, and finally examined by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

DNA-binding assay

To determine NF- κ B and STAT3 activation, we conducted DNA-binding assay using TransAM NF- κ B Kit or TransAM STAT3 Kit according to the manufacturer's instructions and as previously described (20, 21). Briefly, 20 μg of nuclear proteins were added into a 96-well plate coated with an unlabeled oligonucleotide containing the consensus binding site for NF- κ B (5'-GGGACTTCC-3') or STAT3 (5'-TTCCCGGAA-3') and incubated for 1 hour. The wells were washed and incubated with antibodies against NF- κ B p65 subunit or STAT3, respectively, for 1 hour. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and it provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a reference wavelength of 655 nm by microplate reader (Tecan Systems).

Immunocytochemistry for STAT3 localization

CAL27 cells were plated in Nunc Lab-Tek Chamber Slide (Thermo Fisher Scientific) in DMEM containing 10% FBS and allowed to adhere overnight. On the next day, the cells were fixed with cold acetone for 15 minutes, washed with PBS, and blocked with 5% normal goat serum for 1 hour. The cells were then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1/100). After overnight incubation, the slides were washed and then

incubated with goat anti-rabbit immunoglobulin G (IgG)-Alexa Fluor 594 (dilution, 1/100) for 1 hour and counterstained for nuclei with 4',6-diamidino-2-phenylindole (DAPI; 0.5 µg/mL) for 15 minutes. Stained slides were mounted with mounting medium (Sigma-Aldrich) and analyzed under an fluorescence microscope (Olympus DP 70).

MTT assay

The antiproliferative effect of garcinol against HNSCC cells was determined by the MTT dye uptake method as described previously (22). Briefly, the cells (1×10^4 /mL) were incubated in triplicate in a 96-well plate in the presence or absence of different concentrations of garcinol in a final volume of 0.2 mL for indicated time intervals at 37°C. Thereafter, 20 µL MTT solution (5 mg/mL in PBS) was added to each well. After a 2-hour incubation at 37°C, 0.1 mL lysis buffer (20% SDS, 50% dimethylformamide) was added after removal of the medium, incubation was continued at 37°C for 1 hour, and then the optical density (OD) at 570 nm was measured by the Tecan plate reader.

Flow-cytometric analysis

To determine the effect of garcinol on the cell cycle, CAL27 cells (5×10^5 /mL) were seeded in a 6-well plate and then exposed to 25 µmol/L garcinol for 24 hours. Thereafter, cells were trypsinized, washed with PBS, and fixed with 70% ethanol for 30 minutes on ice. Cells were then washed again, resuspended, and stained in PBS containing 10 µg/mL propidium iodide (PI) and 1 µg/mL RNase A for 30 minutes at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (DakoCytomation).

Annexin V assay

CAL27 cells (5×10^5 /mL) were seeded in a 6-well plate and allowed to incubate at 37°C overnight. After treatment with 25 µmol/L garcinol for 24 hours, cells were trypsinized, washed with PBS, and resuspended in FITC-conjugated annexin V and PI-containing binding buffer for 15 minutes in dark. Samples were then analyzed immediately by flow cytometer.

Apoptosis detection by ELISA

Apoptosis of cells was also determined using the Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, CAL27 cells were treated with 25 µmol/L garcinol for various time points, after which the cells were trypsinized and washed with PBS. A total of 1×10^4 cells were collected and incubated in 200 µL cell lysis buffer at room temperature for 30 minutes. The lysates were cleared by centrifugation at 5,000 rpm for 10 minutes, 20 µL of supernatants were pipetted into a streptavidin-coated 96-well microtiter plate with 80 µL immunoreagent mix, and then incubated for 2 hours at room temperature with continuous shaking at 200 rpm. The wells were then washed with washing buffer, and the color was developed by addition of a substrate solution,

which was read at 405 nm against the blank, with reference wavelength of 490 nm after 10 to 15 minutes. The enrichment factor (total amount of apoptosis) was calculated by dividing the absorbance of the sample by the absorbance of the controls without treatment.

ROS detection by MitoSox Red

CAL27 cells were analyzed for mitochondrial superoxide by MitoSox Red (Invitrogen) according to the manufacturer's protocol. Briefly, after treatment with 25 µmol/L garcinol for indicated time intervals, CAL27 cells (5×10^5 /mL) were harvested by trypsinization, washed with PBS, and then incubated with 5 µmol/L MitoSox Red at 37°C for 15 minutes in dark. The cells were then washed and resuspended in PBS, and analyzed immediately by flow cytometry.

RNA extraction and real-time PCR analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was then carried out as described previously (23). Briefly, for a 50-µL reaction, 10 µL of RT product was mixed with $1 \times$ TaqMan Universal PCR Master Mix, 2.5 µL of 20 $\times$ TaqMan probes for cyclin D1, Mcl-1, Bcl-xL, and VEGF, respectively, 2.5 µL of 20 $\times$ 18S RNA TaqMan probe as the endogenous control for each targeting gene, and topped up to 50 µL with sterile water. A negative control for RT, in which sterile water replaced the RNA template, was included. Another control, where RT mix was replaced with sterile water, was included to check for DNA contamination. Real-time PCR was conducted using the 7500 Fast Real-Time PCR System (ABI PRISM 7500; Applied Biosystems) with the following protocol: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and extension at 60°C for 1 minute. The results were analyzed by using Sequence Detection Software version 1.3 provided by Applied Biosystems. Relative gene expression was obtained after normalization with endogenous human 18S and determination of the difference in threshold cycle (C_t) between treated and untreated cells using the $2^{-\Delta\Delta C_t}$ method. Primers and probes for human cyclin D1, Mcl-1, Bcl-xL, and VEGF were purchased as kits from Applied Biosystems (Assays-on-Demand).

Xenograft tumor model

All the procedures involving animals were reviewed and approved by National University of Singapore (NUS; Singapore, Singapore) Institutional Animal Care and Use Committee. Five-week-old athymic nu/nu male mice (Biological Resource Centre, Biopolis, Singapore) were implanted subcutaneously in the right flank with CAL27 cells (2×10^6 cells/100 µL of saline). When tumors have reached 0.25 cm in diameter, the mice were randomized into the following treatment groups ($n = 5$ /group): (i) untreated control [DMSO (0.1%; v/v), 100 µL intraperitoneally (i.p.) injection]; (ii) garcinol [1 mg/kg of body weight, suspended in DMSO (0.1%; v/v), i.p. injection] five times/week; and (iii) garcinol [2 mg/kg of body weight, suspended in DMSO (0.1%; v/v), i.p. injection] five times/week. The therapy was

continued for 4 weeks and the animals were euthanized at the end of the therapy. Tumor tissues were thereafter fixed in formalin and embedded in paraffin for immunohistochemistry and hematoxylin and eosin staining.

Immunohistochemical analysis of tumor tissues

Solid tumors from control and garcinol-treated groups were fixed with 10% phosphate-buffered formalin, processed, and embedded in paraffin. The sections were cut and deparaffinized in xylene, dehydrated in graded alcohol, and finally hydrated in water. Antigen retrieval was conducted by boiling the slide in 10 mmol/L sodium citrate (pH 6.0) for 30 minutes. Immunohistochemistry was conducted following the manufacturer's instructions (Dako LSAB Kit). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Nonspecific binding was blocked by incubation in the blocking reagent in the LSAB Kit (Dako) according to the manufacturer's instructions. Sections were incubated overnight with primary antibodies as follows: anti-p65, anti-phospho-STAT3 (p-STAT3), anti-Ki-67, and anti-CD31 (each at 1:100 dilutions). The slides were subsequently washed several times in TBS with 0.1% Tween-20 and were incubated with biotinylated linker for 30 minutes, followed by incubation with streptavidin conjugate provided in the LSAB Kit according to the manufacturer's instructions. Immunoreactive species were detected using 3,3'-diaminobenzidine tetrahydrochloride as a substrate. The sections were counterstained with Gill's hematoxylin and mounted under glass cover slips. Images were taken using an Olympus BX51 microscope (magnification, $\times 20$). Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

Statistical analysis

Statistical analysis was conducted by Student *t* test and a *P* value less than 0.05 was considered statistically significant.

Results

We investigated the effects of garcinol on the constitutive STAT3 and NF- κ B activation in HNSCC cells. We further evaluated the actions of garcinol on various mediators of cellular proliferation, cell survival, and apoptosis. In addition, we also tested the potential *in vivo* anticancer effects of garcinol in a xenograft mouse model. The structure of garcinol is shown in Fig. 1A.

Garcinol inhibits the proliferation of HNSCC cells

We first investigated the effect of garcinol on proliferation of HNSCC cell lines by MTT assay. The results showed that the garcinol can inhibit the proliferation of two HNSCC cell lines tested (CAL27 and UMSCC1) in a time- and dose-dependent manner (Fig. 1B).

Garcinol induces apoptosis in HNSCC cells

Because DNA fragmentation is a key feature associated with apoptosis, we evaluated this process using the Cell

Death Detection ELISA^{PLUS} Kit. Garcinol significantly increased the DNA fragmentation level to 5-fold in CAL27 cells upon treatment with garcinol as compared with the solvent control (Fig. 1C). The apoptosis-inducing ability of garcinol was then examined by annexin V staining. Annexin V-positive cell population was found to increase to 26% in garcinol-treated CAL27 cells, compared with 3.6% in DMSO-treated control cells (Fig. 1D). We also analyzed the effect of garcinol on cell-cycle distribution in HNSCC cells and observed that after 24-hour treatment with garcinol, 30% of the cell population had accumulated in sub-G₁-phase (Fig. 1E), which is indicative of apoptosis.

Garcinol inhibits constitutively active NF- κ B in HNSCC cells

NF- κ B has been shown to be constitutively expressed in head and neck cancers and mediates resistance to apoptosis (24, 25); therefore, the effect of garcinol on the constitutive NF- κ B activation in HNSCC cells was examined. We found that garcinol suppressed phosphorylation and degradation of the constitutive I κ B α in a time-dependent manner (Fig. 2A). We next investigated the effect of garcinol on p65 phosphorylation as phosphorylation is required for its transcriptional activity (26). We found that garcinol also suppressed the constitutive p65 activation in CAL27 in a time-dependent manner (Fig. 2A). To determine whether the inhibition of NF- κ B by garcinol resulted from inhibition of upstream kinases TAK1 and IKK, Western blot analysis was conducted to test TAK1 and IKK phosphorylation. The results showed that garcinol suppressed phospho-TAK1 and phospho-IKK α / β without affecting the levels of TAK1 and IKK protein (Fig. 2B). Consistently, by ELISA-based TransAM NF- κ B assay kit, we found that treatment of CAL27 cells with garcinol inhibited DNA-binding ability of NF- κ B (Fig. 2C). Overall, these results strongly suggest that garcinol can modulate the constitutive NF- κ B activation through the suppression of upstream TAK1 and IKK.

Garcinol inhibits AKT/mTOR/P70S6K pathway in HNSCC cells

AKT/mTOR/S6K1 is the major antiapoptotic pathway that can confer the survival advantage and also mediate the resistance of HNSCC cells against various chemotherapeutic agents (27, 28). We therefore investigated whether garcinol can downregulate the constitutive AKT/mTOR/S6K1 activation in HNSCC cells. As shown in Fig. 2D, constitutive activation of the serine/threonine protein kinase AKT was suppressed by garcinol in a time-dependent manner. In addition, the constitutive mTOR and S6K1 activation was also suppressed upon garcinol treatment in HNSCC cells. Overall, the data clearly indicate that the inhibition of AKT/mTOR/S6K1 signaling cascade by garcinol may lead to the apoptosis in HNSCC cells.

Garcinol inhibits constitutively active STAT3 in HNSCC cells

Constitutive activation of STAT3 is frequently encountered in HNSCC cells and STAT3 inhibition can be

Figure 1. Garcinol suppresses the proliferation, and induces apoptosis in HNSCC cells. **A**, the chemical structure of garcinol. **B**, CAL27 and UMSSC1 cells (1×10^4 /mL) were treated with indicated concentrations of garcinol, and then subjected to MTT assay for 24, 48, and 72 hours to analyze proliferation of cells. **C**, CAL27 were treated with 25 μ mol/L garcinol for the indicated times, apoptosis was determined by degree of DNA fragmentation in the cytoplasm of cells. **D**, CAL27 cells (5×10^5 /mL) were treated with 25 μ mol/L garcinol for 24 hours, after which the cells were collected and stained with PI and FITC-annexin V, and subjected to fluorescence-activated cell sorting (FACS) analysis. **E**, CAL27 cells (5×10^5 /mL) were treated with 25 μ mol/L garcinol for 24 hours, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry.

considered as an important therapeutic option for the patients (29). To determine the ability of garcinol to modulate STAT3 activation, CAL27 cells were treated with different concentrations of garcinol for various time points and Western blot analysis using antibodies that recognize STAT3 phosphorylation at tyrosine 705 was conducted. It was found that garcinol downregulated the phosphorylation of STAT3 in a dose- and time-dependent manner (Fig. 3A), and the maximum inhibition was observed with 50 μ mol/L at 120 minutes.

Garcinol depletes nuclear pool of STAT3 in HNSCC cells

As nuclear translocation is central to the function of transcription factors and because it is not very well estab-

lished whether phosphorylation is mandatory for nuclear transport of STAT3 and its oncogenic functions (30, 31), we determined the effect of garcinol on nuclear translocation of STAT3. Figure 3B clearly shows that garcinol inhibited the translocation of STAT3 to the nucleus in CAL27 cells.

Garcinol inhibits binding of STAT3 to the DNA

Because tyrosine phosphorylation causes the dimerization of STATs and their translocation to the nucleus, where they bind to DNA and regulate gene transcription (31, 32), we determined whether garcinol suppresses the DNA-binding activities of STAT3. Analysis of nuclear extracts prepared from CAL27 cells using ELISA-based TransAM STAT3 assay kit showed that garcinol inhibited STAT3-DNA-binding

Figure 2. A, garcinol inhibits the constitutive phosphorylation of IκBα and p65. CAL27 cells (1×10^6 /mL) were treated with 50 μmol/L garcinol for the indicated times. Cytoplasmic extracts (CE) were prepared and analyzed by Western blotting using phospho-specific IκBα (Ser32/36) and total IκBα antibodies. Membrane was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm equal loading. Nuclear extracts (NE) were analyzed for NF-κB activation. B, the effect of garcinol on the constitutive activation of TAK1 and IKKα/β. CAL27 cells (1×10^6 /mL) were treated with 50 μmol/L garcinol for the indicated time, whole-cell extracts (WCE) were prepared and analyzed by phospho- and total-TAK1 and -IKKα/β antibodies. C, the effect of garcinol on NF-κB DNA-binding ability in HNSCC cells. CAL27 cells (1×10^6 /mL) were treated with different concentrations of garcinol for 6 hours. Twenty microgram of the nuclear protein was used for DNA-binding assay as described in the "Materials and Methods." D, garcinol inhibits AKT/mTOR/p70S6K signaling pathway in CAL27 cells. CAL27 cells (1×10^6 /mL) were treated with 50 μmol/L garcinol for the indicated time intervals. Whole-cell extracts were analyzed by Western blotting with various antibodies as indicated in the "Results."

activities in a time-dependent manner (Fig. 3C). These results suggest that garcinol abrogates the DNA-binding ability of STAT3.

Garcinol-induced inhibition of STAT3 activation is ROS-dependent

To test whether garcinol can generate reactive oxygen species (ROS), CAL27 cells were treated with 25 μmol/L garcinol for different time durations. The results showed that garcinol could induce mitochondrial superoxide (mtO_2^-) production in a time-dependent manner (Fig. 3D). Because ROS generation blocks STAT3 signaling (23, 33, 34) and garcinol was found to induce ROS production in CAL27 cells, we next determined whether MTG, a well-known ROS scavenger, could restore the inhibitory effect of garcinol on STAT3 activation. For this, CAL27 cells were pretreated with 10 mmol/L antioxidant MTG for 120 minutes and were then subjected to 120-minute incubation with 25 μmol/L garcinol. We found that the decrease in phosphorylated STAT3 observed upon garcinol treatment is ROS-dependent, as levels reverted to control levels in the presence of MTG (Fig. 3E). Garcinol treatment had no effect on the level of total STAT3 either in the presence or absence of MTG.

Garcinol suppresses the constitutive activation of c-Src, JAK1, and JAK2

STAT3 activation has been reported to be regulated by soluble tyrosine kinases of Src and Janus-activated kinase (JAK) family (31, 35). Hence, we determined whether garcinol affected the constitutive activation of these kinases. We found that garcinol suppressed the constitutive phosphorylation of Src kinase in a time-dependent manner (Fig. 4A). Similarly, constitutively activated JAK1 (Fig. 4B) and JAK2 (Fig. 4C) were inhibited substantially by garcinol without affecting the total Src, JAK1, and JAK2 protein levels.

Garcinol-induced inhibition of STAT3 phosphorylation is reversible

We also examined whether garcinol-induced inhibition of STAT3 phosphorylation was reversible or not. Cells were first treated with 50 μmol/L garcinol for 120 minutes and then washed twice with PBS to remove the drug. The cells were then cultured in fresh medium for various durations, and the level of phosphorylated STAT3 was measured. Our results showed that after withdrawal of garcinol, phosphorylated STAT3 gradually increased; the reversal was complete by 4 hours and did not involve changes in STAT3 protein levels (Fig. 4D).

Garcinol suppresses the expression of NF-κB and STAT3-regulated genes involved in the cell proliferation, survival, and angiogenesis

NF-κB and STAT3 are reported to regulate the expression of various genes involved in proliferation, survival, angiogenesis, and chemoresistance (31, 36); we next investigated whether garcinol could modulate the

Figure 3. Garcinol suppresses the phosphorylation and nuclear translocation of STAT3. **A**, garcinol suppresses p-STAT3 levels in a dose- and time-dependent manner. CAL27 cells (5×10^5 /mL) were treated with the indicated concentrations of garcinol for 120 minutes or treated with 50 μ mol/L garcinol for the indicated times, after which whole-cell extracts were prepared and analyzed by phospho-specific and total-STAT3 antibodies. **B**, garcinol causes inhibition of translocation of STAT3 to the nucleus. CAL27 cells (5×10^5 /mL) were incubated with or without 50 μ mol/L garcinol for 120 minutes, and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with DAPI (0.5 μ g/mL) for 15 minutes. **C**, garcinol suppresses STAT3 DNA-binding ability in CAL27 cells. CAL27 cells were treated with 50 μ mol/L garcinol for the indicated times, 20 μ g of the nuclear protein was used for ELISA-based DNA-binding assay as described in the "Materials and Methods." **D**, garcinol induces ROS production. CAL27 cells were treated with 25 μ mol/L garcinol for indicated time intervals, and then stained with MitoSox Red for flow-cytometric analysis. The data were presented by histograms in terms of the mean intensity of MitoSox fluorescence normalized to untreated control. **E**, MTG restores garcinol-inhibited STAT3 phosphorylation. CAL27 cells were pretreated with antioxidant MTG for 120 minutes before exposure to garcinol, after which whole-cell extracts were prepared and analyzed by Western blotting using phospho- and total-STAT3 antibodies.

constitutive expression of these proteins. The results showed that garcinol treatment downregulated the expression of various gene products, including the cell-cycle regulator cyclin D1, the antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1, and survivin, and the angiogenic gene product VEGF in a time-dependent manner (Fig. 5A). To determine whether garcinol also affects the transcription of these genes, the mRNA expression of cyclin D1, Mcl-1, Bcl-xL, and VEGF was also examined. Whether the suppression of NF- κ B- and STAT3-regulated antiapoptotic gene products in CAL27 cells by garcinol leads to apoptosis was also examined. Cells were treated with garcinol

for different times and then examined for caspase activation. We found a time-dependent activation of caspase-3 by garcinol (Fig. 5B). Activation of this downstream effector caspase led to the cleavage of a 116-kDa PARP protein into an 87-kDa fragment (Fig. 5B). These results clearly suggest that garcinol induces caspase-3-dependent apoptosis in CAL27 cells.

We also found that mRNA of various proliferative, survival, and angiogenic genes was constitutively expressed in CAL27 cells, and garcinol treatment suppressed the expression in a time-dependent manner (Fig. 5C). These results suggest that garcinol modulates the expression of various

Figure 4. A, garcinol suppresses phospho-Src levels in a time-dependent manner. CAL27 cells (5×10^5 /mL) were treated with 50 μ M/L garcinol, after which whole-cell extracts were prepared and analyzed by Western blotting using phospho- and total-Src antibodies. B, garcinol suppresses phospho-JAK1 levels in a time-dependent manner. The same lysates were analyzed by Western blotting using phospho- and total-JAK1 antibodies. C, garcinol suppresses phospho-JAK2 levels in a time-dependent manner. Whole-cell extracts were analyzed by Western blotting using phospho- and total-JAK2 antibodies. D, garcinol-induced inhibition of STAT3 phosphorylation is reversible. CAL27 cells were treated with 50 μ M/L garcinol for 120 minutes, and washed with PBS to remove the drug before adding fresh medium. Whole-cell extracts were prepared and analyzed by Western blotting using phospho- and total-STAT3 antibodies.

NF- κ B- and STAT3-regulated genes also at the transcriptional level.

Garcinol inhibits the growth of HNSCC *in vivo*

Next, we analyzed whether garcinol can inhibit the growth of HNSCC CAL27 xenograft in nude mice. Garcinol at doses of 1 and 2 mg/kg induced significant inhibition of tumor growth compared with the DMSO-treated controls (Fig. 6A). We further evaluated the effect of garcinol on constitutive p65 and p-STAT3 levels in HNSCC tumor tissues by immunohistochemistry and found that garcinol can significantly inhibit the constitutive p65 and STAT3 activation in treated groups as compared with the control group (Fig. 6B). The effect of garcinol was also analyzed on the expression of Ki-67 (marker of cell proliferation) and CD31 (marker of microvessel density). As shown in Fig. 6B, expression of both Ki-67 and CD31 was downregulated in garcinol-treated mice.

Discussion

The aim of this study was to determine whether garcinol exerts its anticancer effects in HNSCC cells through the

abrogation of the multiple proinflammatory signaling cascades. We found that this polyisoprenylated benzophenone suppressed the constitutive STAT3 activation in parallel with the suppression of the upstream kinases (c-Src, JAK1, and JAK2) in HNSCC cells. Also, garcinol exerted an inhibitory effect on the constitutive NF- κ B activation, mediated through the suppression of TAK1 and IKK in HNSCC cells. Garcinol also downregulated the expression of various STAT3- and NF- κ B-regulated gene products including cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and VEGF, which are involved in the cell proliferation, survival, and angiogenesis. Garcinol also inhibited cell proliferation and induced substantial apoptosis in HNSCC cells. We subsequently investigated the therapeutic potential of garcinol therapy in HNSCC xenograft grown in a mouse model. Intraperitoneal injection of garcinol into nude mice bearing subcutaneous CAL27 xenografts resulted in significant suppression of tumor progression and suppression of expression of p-STAT3 and p65 in garcinol-treated tumor tissues.

Although garcinol has been shown to inhibit the constitutive NF- κ B activation in different tumor cell lines (14, 37), but its effect on NF- κ B signaling in HNSCC cell lines remains largely unexplored. We first found that NF- κ B is constitutively active in HNSCC cells, but the exact reason, why it is persistently active in CAL27 cells is still not clear. We also noticed that the constitutive activation of IKK can lead to phosphorylation of I κ B α in CAL27 cells, which may explain high basal levels of NF- κ B in HNSCC cells. Also, our results show for the first time that garcinol can indeed suppress the constitutive NF- κ B activation in CAL27 cells as also confirmed by DNA-binding and Western blot analysis for phospho-p65 and p65. The inhibition of IKK activation by garcinol suggests that it abolishes NF- κ B activation in HNSCC cells through the suppression of IKK phosphorylation. Several kinases, such as MEKK1, MEKK3, PKC, glycogen synthase kinase-3 β , TAK1, PDK1, and AKT (38), have been reported to function upstream of IKK. We also report for the first time that garcinol can suppress the constitutive TAK1 activation in CAL27 cells. Moreover, a prior study indicates that the constitutive activation of NF- κ B in HNSCC cells is mediated through the TRADD-TRAF2-RIP-TAK1-IKK pathway (39), and our results clearly indicate that garcinol can indeed abrogate TAK1-mediated IKK activation.

We also observed that garcinol could suppress the constitutive STAT3 activation in HNSCC cells that correlated with the suppression of upstream protein tyrosine kinases c-Src and JAK2. Previous studies have indicated that Src and JAK2 kinase activities cooperate to mediate the constitutive activation of STAT3 (31, 40). Our observations suggest that garcinol may block cooperation of Src and JAKs involved in tyrosyl phosphorylation of STAT3. We found that garcinol-induced inhibition of STAT3 phosphorylation was ROS-dependent as treatment with MTG, an antioxidant containing thiol group, can restore the inhibitory effect of garcinol on STAT3 activation. Although STAT3 inactivation with increased ROS generation in hematopoietic cells expressing mutant forms of Bcr/Abl (41) and hepatocellular carcinoma

Figure 5. Garcinol suppresses STAT3- and NF- κ B-regulated gene products involved in proliferation, survival, and angiogenesis and activates caspase-3. The same blots were stripped and reprobed with β -actin antibody to verify equal protein loading (A and B). A, CAL27 cells (5×10^5 /mL) were treated with 25 μ mol/L garcinol for indicated time; whole-cell extracts were prepared, separated by SDS-PAGE, and subjected to Western blotting against cyclin D1, Bcl-2, Bcl-xL, Mcl-1, survivin, and VEGF antibodies. B, the same whole-cell extracts were analyzed by Western blotting using caspase-3 and PARP antibodies. C, CAL27 cells (1×10^6 /mL) were treated with 25 μ mol/L garcinol for indicated time intervals, after which RNA samples were extracted. One microgram portions of the respective RNA extracts were subjected to reverse transcription to generate corresponding cDNA. Real-time PCR was conducted to measure the relative quantities of mRNA. Each RT product was targeted against cyclin D1, Mcl-1, Bcl-xL, and VEGF TaqMan probes, with 18S as endogenous control for measurement of equal loading of RNA samples. The results were analyzed using Sequence Detection Software version 1.3 provided by Applied Biosystems. Relative gene expression was obtained after normalization with endogenous Hu18S and determination of the difference in C_t between treated and untreated cells using $2^{-\Delta\Delta C_t}$ method.

cells (23) has been reported, to the best of our knowledge, this is the first report of garcinol-induced ROS-mediated STAT3 inhibition in HNSCC cells. Our results are consistent with a recent report in which garcinol was found to suppress the activation of STAT3 in breast, prostate, and pancreatic cancer cell lines (15), although the detailed molecular mechanisms underlying its STAT3 inhibitory effects were not elucidated in this study.

Also, STAT3 phosphorylation plays a pivotal role in proliferation and survival of tumor cells (42). Several types of cancer, including hepatocellular carcinoma (31), multiple myeloma (43), prostate (44), and lymphomas/leukemia (45), also display constitutively active STAT3. The suppression of constitutively active STAT3 in HNSCC cells raises the possibility that it might also abrogate constitutively activated STAT3 in other types of cancer cells. As discussed earlier, we also noticed that garcinol can inhibit the constitutive NF- κ B activation through the modulation of upstream kinases (TAK1 and IKK) in HNSCC cells.

Interestingly, a prior study had indicated that STAT3 could prolong NF- κ B nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF- κ B nuclear export (46). Thus, it is possible that suppression of STAT3 activation may mediate inhibition of NF- κ B activation by garcinol. Also, whether garcinol also affects other putative inhibitors such as suppressor of cytokine signaling (SOCS) and protein inhibitors of activated STAT3 (PIAS3) in HNSCC cells requires further investigation.

Although previous studies have shown that garcinol can modulate AKT survival signaling cascade in colorectal cancer cells (12), our study is also the first report to examine the effect of garcinol on AKT/mTOR/S6K1 signaling axis in HNSCC cells. We found that garcinol treatment suppressed the expression of phospho-AKT, -mTOR, and -S6K1 in a time-dependent manner in CAL27 cells, which further indicates the possibility that garcinol could interfere with the mTOR-ricor complex that phosphorylates AKT at Ser 473. AKT is also reported to regulate the master

Figure 6. Garcinol inhibits the growth of human HNSCC *in vivo*. A, athymic mice bearing subcutaneous CAL27 tumors were treated for five times a week for 4 consecutive weeks with 1 and 2 mg/kg garcinol or 0.1% DMSO alone (each group; $n = 5$). The diameters were measured twice a week for 6 weeks using Vernier caliper, and the tumor volume was calculated using the formula $(L \times W^2)/2$, where W is the shortest diameter and L is the longest diameter ($n = 6$). Data are represented as mean tumor volume $\pm$ SE. *, $P < 0.05$ for control group versus garcinol treatment groups at the end of 4 weeks treatment. B, immunohistochemical analysis of nuclear p65, p-STAT3, Ki-67, and CD31 showed the inhibition in expression of these proteins in garcinol-treated samples. Percentage indicates positive staining for the given biomarker. b.w., body weight.

transcription factor NF- κ B through the phosphorylation of p65 to enhance the transcriptional activity of NF- κ B (47). Furthermore, AKT is also involved in the regulation of interleukin-6-induced STAT3 signaling pathway (31). Thus, it is possible that the inhibition of AKT/mTOR/S6K pathways may contribute to STAT3/NF- κ B inhibitory effects of garcinol as observed in HNSCC cells.

We further noticed that garcinol suppressed the expression of several STAT3- and NF- κ B-regulated genes, includ-

ing proliferative (cyclin D1), antiapoptotic (Bcl-2, Bcl-xL, survivin, and Mcl-1), and angiogenic gene product (VEGF). The inhibition of cyclin D1 expression may account for its ability to inhibit proliferation in various tumor cell lines (48, 49). Activation of STAT3 signaling also induces *survivin* gene expression and confers resistance to apoptosis in human breast cancer cells (50). The downregulation of the expression of Bcl-2, Bcl-xL, survivin, and Mcl-1 is likely linked with the garcinol's potential to induce apoptosis in HNSCC cells as evidenced by the activation of caspase-3 and the cleavage of PARP. These observations are consistent with a recent study in which garcinol was shown to induce apoptosis in prostate and pancreatic cancer cells through the suppression of NF- κ B activation (37). As VEGF expression is also regulated by STAT3, garcinol may mediate antiangiogenesis in HNSCC cells through the downregulation of VEGF.

Whether the *in vitro* observations with garcinol have any relevance to the *in vivo* context was also investigated. Our results indeed indicate for the first time that garcinol significantly suppressed HNSCC growth in a nude mouse model without exhibiting any significant toxicity, and downregulated the expression of p-STAT3, p65, Ki-67, and CD31 in the treated groups as compared with the control group. To the best of our knowledge, no prior studies with garcinol in xenograft HNSCC or 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis models have been reported previously, and thus our novel findings suggest that garcinol may have a tremendous potential for the treatment of HNSCC. Interestingly, garcinol has been tested before in other tumor cell lines and xenograft cancer models, and has exhibited significant anticancer effects without any reported toxic effects so far (48); however, its activity against HNSCC remains unexplored till date. Moreover, garcinol has not been so far tested in humans as an anticancer agent, and hence its clinically relevant doses are not clear as of yet. Thus, overall, our results clearly validate that the antiproliferative and proapoptotic effects of garcinol in HNSCC are mediated through suppression of multiple proinflammatory signaling cascades and provide a solid rationale for pursuing the application of garcinol further to enhance treatment efficacy for HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Li, M.K. Shanmugam, T.K. Kundu, G. Sethi
Development of methodology: F. Li, M.K. Shanmugam, L. Chen, A.P. Kumar

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Li, M.K. Shanmugam, L. Chen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Li, M.K. Shanmugam, L. Chen, G. Sethi
Writing, review, and/or revision of the manuscript: F. Li, M.K. Shanmugam, L. Chen, S. Chatterjee, A.P. Kumar, T.K. Kundu, G. Sethi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Li, M.K. Shanmugam, S. Chatterjee, J. Basha, A.P. Kumar

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