Garcinol, a polyisoprenylated benzophenone modulates multiple pro-inflammatory signaling cascades leading to suppression of growth and survival of head and neck carcinoma

Feng Li¹, Muthu K. Shanmugam¹, Luxi Chen¹,², Snehajyoti Chatterjee³, Jeelan Basha³, Alan Prem Kumar¹,²,⁴,⁵, Tapas K. Kundu³, Gautam Sethi ¹,²*

¹Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, ²Cancer Science Institute of Singapore, Centre for Translational Medicine, 14 Medical Drive, #11-01M, Singapore 117599, ³Jawaharlal Nehru Centre for Advanced Scientific Research, Molecular Biology and Genetics Unit, Transcription and Disease Laboratory, Jakkur P.O., Bangalore 560064 India, ⁴School of Biomedical Sciences, Faculty of Health Sciences, Curtin University, Western Australia, 6009, Australia and ⁵Department of Biological Sciences, University of North Texas, Denton, Texas, 76203, United States of America.
*To whom correspondence should be addressed:

Dr. Gautam Sethi, Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Phone: +65-65163267; Fax: +65-68737690; Email: phcgs@nus.edu.sg

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Abbreviations used: STAT3, signal transducer and activator of transcription 3; HNSCC, head and neck squamous cell carcinoma; NF-κB, nuclear factor kappa B; IκBα, inhibitor of NF-κB alpha; IKK, IκB kinase; TAK1, TGF beta activated kinase 1; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; ROS, reactive oxygen species.
Abstract

Constitutive activation of pro-inflammatory transcription factors such as signal transducers and activators of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) plays a pivotal role in the proliferation and survival of head and neck squamous cell carcinoma (HNSCC). Thus, agents which can modulate deregulated STAT3 and NF-κB 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 constitutive NF-κB activation, mediated through the suppression of TGF beta activated kinase 1 (TAK1), and IκB kinase (IKK) activation in HNSCC cells. Garcinol also down-regulated the expression of various gene products involved in cell proliferation, survival, and angiogenesis that led to the suppression of cell proliferation and induction of apoptosis in HNSCC cells. When administered i.p., 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 anti-tumor effects in HNSCC cells and mouse model through the suppression of multiple pro-inflammatory cascades.


**Introduction**

Head and neck squamous cell carcinoma (HNSCC), a complex disease which 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 deaths worldwide annually (6.0% of cases and 5.2% deaths) (1). Current treatments for HNSCC are complicated, depending on the primary tumor sites, stages of the disease and considerations for organ preservation, multidisciplinary approaches involving surgery, radiotherapy, chemotherapy and targeted agents are applied (2). Platinum-based regimens are considered most valuable and 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 pro-inflammatory signaling cascades including NF-κB, STAT3 and serine/threonine protein kinase B (AKT) have been linked to carcinogenesis of HNSCC and play a pivotal role in the growth, proliferation and survival of head and neck cancer (6-10). For example, Sen and coworkers recently reported that STAT3 activation can even contribute to epidermal growth factor receptor inhibitor resistance in HNSCC and thereby targeting of STAT3 may represent an effective treatment strategy (10). Moreover, Yan et al. reported that a higher level of activated NF-κB contributed to tumor hematologic and lymphatic metastasis in HNSCC patient specimens (11).
identification of pharmacological drugs which can inhibit these 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 demonstrated the anti-neoplastic and chemopreventive role of garcinol in different cancers such as leukemia, colorectal, gastrointestinal and breast cancers (12-16). Although there is prior evidence showing that garcinol can inhibit 4-nitroquinololone 1-oxide induced tongue carcinogenesis (17), mechanisms through which garcinol elicits its anti-tumor activity, specifically in HNSCC, is unknown till date. Given the pivotal role of NF-κB and STAT3 signaling cascades in the proliferation, survival and chemoresistance in HNSCC, we speculate that garcinol may exhibit anti-neoplastic effects in HNSCC by modulating these pro-inflammatory pathways.

We found that garcinol can indeed suppress both constitutive NF-κB and STAT3 activation in HNSCC. This inhibition decreased cell survival and downregulated expression of proliferative, anti-apoptotic 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-κB and STAT3 in tumor tissues.
Materials and Methods

Reagents

MTT, Tris, glycine, NaCl, SDS, BSA, monothioglycerol, β-actin antibody were purchased from Sigma-Aldrich. Garcinol with purity greater than 98% was prepared from *Garcinia indica* fruit rind as described previously (18). Garcinol was dissolved in dimethylsulfoxide (DMSO) as a 50 mM stock solution and stored at -20°C for the experiments. Further dilution was done in cell culture medium. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic–antimycotic mixture were obtained from Invitrogen. Antibodies against p65, IκBα, cyclin D1, Bcl-2, Bcl-xL, Mcl-1, survivin, VEGF, caspase-3, PARP, goat anti-rabbit–horseradish peroxidase (HRP) conjugate and goat anti-mouse HRP, annexin V-FITC assay kit were obtained from Santa Cruz Biotechnology. Antibodies to phospho-specific p65 (Ser536), phospho-specific IκBα (Ser32), phospho-specific TAK1 (Thr187), TAK1, phospho-specific IKKα/β (Ser180/Ser181), IKKα, 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) and JAK2 were purchased from Cell Signaling Technology. Bradford reagent was purchased from Bio-Rad. Cell Death Detection ELISAPLUS DNA fragmentation kit was purchased from Roche Diagnostics. Nuclear extract kit and DNA binding kit was obtained from Active Motif.
Cell lines

Human head and neck squamous cell carcinoma 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 in the year 2009 and have been characterized previously (19). These cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/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 mM NaCl, 20 mM HEPES, 2 mM EDTA (pH 8.0), 0.5 mM EGTA, 0.1% Triton X-100, 1.5 μg/ml aprotinin, 1.5 μg/ml leupeptin, 1 mM PMSF, and 1.5 mM NaVO4). Lysates were then spun at 13,300 rpm for 10 min to remove insoluble material and resolved on a 10% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane (Biorad), 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 h, and finally examined by chemiluminescence (ECL; Amersham Pharmacia Biotech).

DNA binding assay

To determine NF-κB and STAT3 activation, we performed DNA binding assay using TransAM NF-κB 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 96-well plate coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5′-GGGACTTTCC-3′) or STAT3 (5′-TTCCCGGAA-3′) and incubated for 1h. The wells were washed and incubated with antibodies against NF-κB p65 subunit or STAT3 respectively for 1 h. An HRP conjugated secondary antibody was then applied to detect the bound primary antibody and 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 for overnight. On next day, the cells were fixed with cold acetone for 15min, washed with PBS and blocked with 5% normal goat serum for 1 h. 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 IgG-Alexa 594 (dilution, 1/100) for 1 h and counterstained for nuclei with DAPI (0.5 μg/ml) for 15 min. Stained slides were mounted with mounting medium (Sigma–Aldrich) and analyzed under an fluorescence microscope (Olympus DP 70, Japan).

**MTT assay**

The anti-proliferative effect of garcinol against HNSCC cells was determined by the MTT dye uptake method as described previously (22). Briefly, the cells (1x10⁴/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 (5mg/ml in PBS) was added to each well. After a 2 h 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 h; and then the optical density (OD) at 570 nm was measured by Tecan plate reader.

**Flow cytometric analysis**

To determine the effect of garcinol on the cell cycle, CAL27 cells (5x10^5/ml) were seeded in 6-well plate and then exposed to 25 μM garcinol for 24 h. Thereafter cells were trypsinized, washed with PBS, and fixed with 70% ethanol for 30 min 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 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako Cytomation).

**Annexin V assay**

CAL27 cells (5x10^5/ml) were seeded in a 6-well plate and allowed to incubate at 37°C overnight. After treatment with 25 μM garcinol for 24 h, cells were trypsinized, washed with PBS, and resuspended in FITC-conjugated annexin V and PI-containing binding buffer for 15 min in dark. Samples were then analyzed immediately by flow cytometer.

**Apoptosis detection by ELISA**

Apoptosis of cells was also determined using Cell Death Detection ELISA PLUS kit (Roche Inc.) according to manufacturer’s instructions. Briefly, CAL27 cells were treated with 25
μM garcinol for various time points, after which the cells were trypsinized, washed with PBS. 1x10⁴ cells were collected and incubated in 200 μl cell lysis buffer at room temperature for 30 min. The lysates were cleared by centrifugation at 5,000rpm for 10 min and 20 μl of supernatants were pipetted into streptavidin-coated 96-well microtiter plate with 80μl immunoreagent mix, then incubated for 2 h at room temperature with continuous shaking at 200 rpm. The wells were then washed with washing buffer, and color was developed by addition of substrate solution, which was read at 405 nm against the blank, with reference wavelength of 490 nm after 10–15 min. 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 manufacturer’s protocol. Briefly, after treatment with 25 μM garcinol for indicated time intervals, CAL27 cells (5x10⁵/ml) were harvested by trypsinization, washed with PBS, and then incubated with 5 μM MitoSox Red at 37°C for 15 min 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 1x
Taq-Man® Universal PCR Master mix, 2.5μl of 20x TaqMan probes for cyclin D1, Mcl-1, Bcl-xL, and VEGF, respectively, 2.5 μl of 20x 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 done using the 7500 Fast Real-Time PCR System (ABI PRISM 7500; Applied Biosystems, Foster City, CA) with the following protocol: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 s and extension at 60°C for 1 min. The results were analyzed 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 (Ct) between treated and untreated cells using the 2-ΔΔCt 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 of the procedures involving animals were reviewed and approved by NUS Institutional Animal Care and Use Committee. 5-week-old athymic nu/nu male mice (Biological Resource Centre, Biopolis, Singapore) were implanted subcutaneously in the right flank with CAL27 cells (2x10^6 cells/100μl of saline). When tumors have reached 0.25 cm in diameter, the mice were randomized into the following treatment groups (n=5/group): (a) untreated control (DMSO 0.1%, v/v), 100 μl i.p. injection); (b) garcinol (1 mg/kg of body weight, suspended in DMSO 0.1% v/v), i.p. injection) five times/week...
and (c) garcinol (2 mg/kg of body weight, suspended in DMSO 0.1% v/v), i.p. injection) five times/week. Therapy was continued for 4 weeks, and the animals were euthanized at the end of 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 deparafinized in xylene, dehydrated in graded alcohol, and finally hydrated in water. Antigen retrieval was performed by boiling the slide in 10 mM sodium citrate (pH 6.0) for 30 min. Immunohistochemistry was performed following manufacturer instructions (DAKO LSAB kit). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Non-specific 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, anti-Ki67, and anti-CD31 (each at 1:100 dilutions). The slides were subsequently washed several times in Tris-buffered saline with 0.1% Tween 20 and were incubated with biotinylated linker for 30 min, followed by incubation with streptavidin conjugate provided in 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, ×20). Positive cells (brown)
were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

**Statistical analysis**

Statistical analysis was performed by Student’s t-test and a p value of less than 0.05 was considered statistically significant.
Results

We investigated the effects of garcinol on constitutive STAT3 and NF-κB activation in HNSCC cells. We further evaluated the actions of garcinol on various mediators of cellular proliferation, cell survival, and apoptosis. Additionally, 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 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:

Since DNA fragmentation is a key feature associated with apoptosis, we evaluated this process using Cell Death Detection ELISAPLUS kit. Garcinol significantly increased the DNA fragmentation level to 5 folds in CAL27 cells upon treatment with garcinol as compared to 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 to 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 h 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-κB in HNSCC cells:**

NF-κB 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 constitutive NF-κB activation in HNSCC cells was examined. We found that garcinol suppressed phosphorylation and degradation of constitutive IκBα in a time dependent manner (Fig. 2A). We next investigated effect of garcinol on p65 phosphorylation since phosphorylation is required for its transcriptional activity (26). We found that garcinol also suppressed constitutive p65 activation in CAL27 in a time dependent manner (Fig. 2A). To determine whether the inhibition of NF-κB by garcinol resulted from inhibition of upstream kinases TAK1 and IKK, western blot analysis was performed to test TAK1 and IKK phosphorylation. The results showed that garcinol suppressed phospho-TAK1 and phospho-IKKα/β without affecting the levels of TAK1 and IKK protein (Fig. 2B). Consistently, by ELISA-based TransAM NF-κB assay kit, we found that treatment of CAL27 cells with garcinol inhibited DNA binding ability of NF-κB (Fig. 2C). Overall, these results strongly suggest that garcinol can modulate constitutive NF-κB activation through the suppression of upstream TAK1 and IKK.

**Garcinol inhibits AKT/mTOR/P70S6K pathway in HNSCC cells:**

AKT/mTOR/S6K1 is the major anti-apoptotic pathway that can confer the survival advantage and also mediate the resistance of HNSCC cells against various chemotherapeutic agents (27, 28). We therefore next investigated whether garcinol can down-regulate 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, constitutive mTOR and S6K1 activation was also suppressed upon garcinol treatment in HNSCC cells. Overall, the data clearly indicates 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 considered as an important therapeutic option for the patients (29). To determine the ability of garcinol modulating STAT3 activation, CAL27 cells were treated with different concentrations of garcinol for various time points and western blot analysis using antibodies which recognize STAT3 phosphorylation at tyrosine 705 was performed. It was found that garcinol down-regulated the phosphorylation of STAT3 in a dose dependent and time dependent manner (Fig. 3A), and the maximum inhibition was observed with 50 µM at 120 min.

**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 established 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. Fig. 3B clearly demonstrates that garcinol inhibited the translocation of STAT3 to the nucleus in CAL27 cells.
**Garcinol inhibits binding of STAT3 to the DNA:**

Since 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 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 μM 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 monothioglycerol (MTG), a well known ROS scavenger can restore the inhibitory effect of garcinol on STAT3 activation. For this, CAL27 cell were pre-treated with 10 mM antioxidant monothioglycerol (MTG) for 120 min and then subjected to 120 min incubation with 25 μM garcinol. We found that 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 constitutive activation of c-Src, JAK1 and JAK2:

STAT3 activation has been reported to be regulated by soluble tyrosine kinases of Src and 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 μM garcinol for 120 min 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 withdraw of garcinol, phosphorylated STAT3 gradually increased; the reversal was complete by 4 h 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 constitutive expression of these proteins. The results showed that garcinol treatment down-regulated the expression of various gene products,
including the cell cycle regulator Cyclin D1, the anti-apoptotic proteins Bcl-2, Bcl-xL, McI-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, McI-1, Bcl-xL, and VEGF was also examined. Whether the suppression of NF-κB and STAT3-regulated anti-apoptotic 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 NF-κB 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 down-regulated in garcinol-treated mice.
Discussion

The aim of this study was to determine whether garcinol exerts its anti-cancer effects in HNSCC cells through the abrogation of the multiple pro-inflammatory signaling cascades. We found that this polyisoprenylated benzophenone suppressed constitutive STAT3 activation in cells parallel with the suppression of the upstream kinases (c-Src, JAK1 and JAK2) in HNSCC cells. Also, garcinol exerted an inhibitory effect on constitutive NF-κB activation, mediated through the suppression of TAK1 and IKK in HNSCC cells. Garcinol also down-regulated the expression of various STAT3 and NF-κB 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 mouse model. Intra-peritoneal 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 constitutive NF-κB activation in different tumor cell lines (14, 37), but its effect on NF-κB signaling in HNSCC cell lines remains largely unexplored. We first found that NF-κB is constitutively active in HNSCC cells, but the exact reason, why it is persistently active in CAL27 cells is not clear. We also noticed that constitutive activation of IKK can lead to phosphorylation of IκBα in CAL27 cells, which may explain high basal levels of NF-κB in HNSCC cells. Also, our results demonstrate for the first time that garcinol can indeed suppress constitutive NF-
κB 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-κB activation in HNSCC cells through the suppression of IKK phosphorylation. Several kinases, such as MEKK1, MEKK3, PKC, glycogen synthase kinase-3β, TAK1, PDK1 and AKT (38) have been reported to function upstream of IKK. We also report for the first time that garcinol can suppress constitutive TAK1 activation in CAL27 cells. Moreover, a prior study indicates that the constitutive activation of NF-κB 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 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 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 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 activation of STAT3 in breast, prostate and pancreatic cancer cell lines (15),
although the detailed molecular mechanisms underlying its STAT3 inhibitory effects was 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 above, we also noticed that garcinol can inhibit constitutive NF-κB activation through the modulation of upstream kinases (TAK1 and IKK) in HNSCC cells. Interestingly, a prior study had indicated that STAT3 can prolong NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export (46). Thus it is possible that suppression of STAT3 activation may mediate inhibition of NF-κB 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-rictor complex that phosphorylates AKT at Ser 473. AKT is also reported to regulate the master transcription factor NF-κB through
the phosphorylation of p65 to enhance the transcriptional activity of NF-κB (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-κB inhibitory effects of garcinol as observed in HNSCC cells.

We further noticed that garcinol suppressed the expression of several STAT3 and NF-κB regulated genes; including proliferative (cyclin D1) and anti-apoptotic gene products (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 activation of caspase-3 and the cleavage of PARP. These observations are consistent with a recent study in which garcinol was demonstrated to induce apoptosis in prostate and pancreatic cancer cells through the suppression of NF-κB activation (37). As VEGF expression is also regulated by STAT3, garcinol may mediate anti-angiogenesis 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 nude mouse model without exhibiting any significant toxicity, and downregulated the expression of phospho-STAT3, p65, Ki-67, and CD31 in the treated groups as compared to 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 anti-cancer 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 anti-proliferative and pro-apoptotic effects of garcinol in HNSCC are mediated through suppression of multiple pro-inflammatory signaling cascades and provide a solid rationale for pursuing the application of garcinol further to enhance treatment efficacy for HNSCC.
Acknowledgments

This work was supported by grants from NUS Academic Research Fund [Grants R-184-000-177-112] and National Medical Research Council of Singapore [Grant R-184-000-211-213] to GS. APK was supported by grants from the National Medical Research Council of Singapore [Grant R-713-000-124-213], Ministry of Education, Singapore [MOE2012-T2-2-139] and Cancer Science Institute of Singapore, Experimental Therapeutics I Program [Grant R-713-001-011-271]. TKK is a recipient of Sir JC Bose national fellowship, Department of Science and Technology, Govt. of India.

Conflict of Interest

The authors declare no conflict of interest.
References

34. Kurdi M, Booz GW. Evidence that IL-6-type cytokine signaling in cardiomyocytes is inhibited by oxidative stress: parthenolide targets JAK1 activation by generating ROS. Journal of cellular physiology 2007; 212:424-31.


Figure legends

Figure 1. Garcinol suppresses the proliferation, and induces apoptosis in HNSCC cells. A, The chemical structure of garcinol. B, CAL27 and UMSCC1 cells (1×10⁴/ml) were treated with indicated concentrations of garcinol, and then subjected to MTT assay for 24, 48 and 72 h to analyze proliferation of cells. C, CAL27 were treated with 25 μM garcinol for the indicated times, apoptosis was determined by degree of DNA fragmentation in the cytoplasm of cells. D, CAL27 cells (5×10⁵/ml) were treated with 25 μM garcinol for 24 h, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. E, CAL27 cells (5×10⁵/ml) were treated with 25 μM garcinol for 24 h, after which the cells were collected and stained with PI and FITC-annexin V, and subjected to FACS analysis.

Figure 2. A, Garcinol inhibits constitutive phosphorylation of IκBα and p65. CAL27 cells (1×10⁶/ml) were treated with 50 μM garcinol for the indicated times. Cytoplasmic extracts were prepared and analyzed by western blotting using phospho-specific IκBα (Ser32/36) and total IκBα antibodies. Membrane was stripped and reprobed for GAPDH to confirm equal loading. Nuclear extracts were analyzed for NF-κB activation. B. The effect of garcinol on the constitutive activation of TAK1 and IKKα/β. CAL27 cells (1×10⁶/ml) were treated with 50 μM garcinol for the indicated time, whole-cell extracts 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 (1×10⁶/ml) were treated with different concentrations of garcinol for 6 h. 20 μg of the nuclear protein was used for DNA binding assay as described in “Materials and methods”.
D. Garcinol inhibits AKT/mTOR/P70S6K signaling pathway in CAL27 cells. CAL27 cells (1×10^6/ml) were treated with 50 μM garcinol for the indicated time intervals. Whole cell extracts were analyzed by western with various antibodies as indicated in the “Results”.

Figure 3. Garcinol suppresses the phosphorylation and nuclear translocation of STAT3. A, Garcinol suppresses phospho-STAT3 levels in a dose dependent and time dependent manner. CAL27 cells (5×10^5/ml) were treated with the indicated concentrations of garcinol for 120 min or treated with 50 μM 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 μM garcinol for 120 min, and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with DAPI (0.5 μg/ml) for 15 min. C, Garcinol suppresses STAT3 DNA binding ability in CAL27 cells. CAL27 cells were treated with 50 μM garcinol for the indicated times, 20 μg of the nuclear protein was used for ELISA-based DNA binding assay as described in “Materials and methods”. D, Garcinol induces ROS production. CAL27 cells were treated with 25 μM 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 pre-treated with antioxidant MTG for 120 min before
exposure to garcinol, after which whole-cell extracts were prepared and analyzed by western blotting using phospho and total STAT3 antibodies.

**Figure 4.** A, Garcinol suppresses phospho-Src levels in a time dependent manner. CAL27 cells (5×10^5/ml) were treated with 50 μM 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 μM garcinol for 120 min, 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.

**Figure 5.** Garcinol suppresses STAT3 and NF-κB 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 μM 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 μM garcinol for indicated
time intervals, after which RNA samples were extracted. 1 μg portions of the respective RNA extracts were subjected to reverse transcription to generate corresponding cDNA. Real time PCR was performed 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 Ct between treated and untreated cells using 2-ΔΔCt method.

**Figure 6.** Garcinol inhibits the growth of human HNSCC *in vivo*. A, athymic mice bearing subcutaneous CAL27 tumors were treated for 5 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 x W^2)/2 where W is the shortest diameter, and L is the longest diameter (n = 6). Data is represented as mean tumor volume ± SE. *p <0.05 for control group versus garcinol treatment groups at the end of 4 weeks treatment. B, Immuno-histochemical 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.
**A.**

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**B.**

- **STAT3**
- **Nucleus**
- **Merged**

- **Control**
  - **STAT3**
  - **Nucleus**
  - **Merged**

- **Garcinol**
  - **STAT3**
  - **Nucleus**
  - **Merged**

**C.**

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**E.**

- **Garcinol**
  - phosho-STAT3
  - STAT3
- **MTG**
  - phosho-STAT3
  - STAT3
**A.**

Garcinol

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Garcinol

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**D.**

- + + + + + 50µM Garcinol

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**Garcinol**

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B. 

C. 

**Cyclin D1 mRNA (Fold change)**

- 0: 1.2
- 2: 0.8
- 4: 0.8
- 6: 0.4
- 8: 0.4

**Mcl-1 mRNA (Fold change)**

- 0: 1.2
- 2: 0.8
- 4: 0.8
- 6: 0.4
- 8: 0.4

**Bcl-xL mRNA (Fold change)**

- 0: 1.2
- 2: 0.8
- 4: 0.8
- 6: 0.4
- 8: 0.4

**VEGF mRNA (Fold change)**

- 0: 1.2
- 2: 0.8
- 4: 0.8
- 6: 0.4
- 8: 0.4

* Indicates significant change.
A. Tumor Volume (mm³) vs. Week

- Vehicle control
- Garcinol (1 mg/kg b.w.)
- Garcinol (2 mg/kg b.w.)

B. Immunohistochemical staining:

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Garcinol, a polyisoprenylated benzophenone modulates multiple pro-inflammatory signaling cascades leading to suppression of growth and survival of head and neck carcinoma

Feng Li, Muthu K. Shanmugam, Luxi Chen, et al.


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