Celastrol suppresses growth and induces apoptosis of human hepatocellular carcinoma through the modulation of STAT3/JAK2 signaling cascade in vitro and vivo

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Running title: Celastrol inhibits STAT3 signaling in HCC.

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Abbreviations used: STAT3, signal transducer and activator of transcription 3; HCC, hepatocellular carcinoma; FBS, fetal bovine serum; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide; JAK, Janus-like kinase.
Abstract

Cumulative evidences(s) have established that the constitutive activation of signal transducer and activator of transcription 3 (STAT3) plays a pivotal role in the proliferation, survival, metastasis and angiogenesis and thus can contribute directly to the pathogenesis of hepatocellular carcinoma (HCC). Thus, novel agents that can inhibit STAT3 activation have potential for both prevention and treatment of HCC. The effect of celastrol on STAT3 activation, associated protein kinases, STAT3-regulated gene products, cellular proliferation and apoptosis was investigated. The in vivo effect of celastrol on the growth of human HCC xenograft tumors in athymic nu/nu mice was also examined. We observed that celastrol inhibited both constitutive and inducible STAT3 activation and the suppression was mediated through the inhibition of activation of upstream kinases c-Src, as well as Janus-activated kinase-1, and -2. Vanadate treatment reversed the celastrol-induced modulation of STAT3, suggesting the involvement of a tyrosine phosphatase. The inhibition of STAT3 activation by celastrol led to the suppression of various gene products involved in proliferation, survival, and angiogenesis. Celastrol also inhibited the proliferation, and induced apoptosis in HCC cells. Finally, when administered i.p., celastrol inhibited STAT3 activation in tumor tissues and the growth of human HCC xenograft tumors in athymic nu/nu mice without any side effects. Overall, our results suggest for the first time that celastrol exerts its anti-proliferative and pro-apoptotic effects through suppression of STAT3 signaling in HCC both in vitro and in vivo.
Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy ranking fifth in incidence and third in mortality worldwide (1, 2). More than 80% of HCC patients present at advanced disease stages where surgery is not possible, and in cases where surgical resections are performed the two year recurrence rate is high as much as 50%. Although several chemotherapeutic agents have been used in treatment of HCC no single or combination chemotherapy regimens have been shown to be particularly effective (3, 4). Thus, a great challenge lies in identifying novel agents, including complementary and preventive approaches to treat HCC.

The protein, signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor that resides in the cytoplasm (5, 6). Upon activation by cytokines (like IL-6) or growth factors (EGF, PDGF), STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent modulation of gene transcription. STAT3 phosphorylation is mediated through the activation of non–receptor protein tyrosine kinases family of Janus-like kinase (JAKs), which include four members namely, JAK1, JAK2, JAK3, and TYK2 (7). In addition, the role of c-Src kinase has been shown in STAT3 phosphorylation (5). In normal cells, the duration of STAT3 activation is transient, and it plays an important role in the development of various organs and in cell proliferation (8). However, constitutive activation of STAT3 has been frequently encountered in many kinds of tumors (9, 10), including HCC (11-13) and this persistently active STAT3 is thought to contribute to proliferation and oncogenesis by modulating the expression of a variety of genes required for tumor cell survival (e.g., Bcl-xL, Mcl-1, survivin), proliferation (e.g., cyclin D1, c-Myc), and
angiogenesis (e.g., vascular endothelial growth factor [VEGF]) as well as metastasis (9). Thus, STAT3 can be considered as an important therapeutic target for novel drug development.

In the present report, we analyzed the effect of celastrol derived from the Chinese medicinal plant *Tripterygium wilfordii*, which has attracted great attention recently for its potent anti-cancer effects (10, 14, 15). Celastrol has been found to inhibit the proliferation, induce apoptosis and suppress invasion/migration and angiogenesis in a wide variety of tumor models both *in vitro* as well as *in vivo* (16-18). The efficacy of celastrol to modulate the expression of various key mediators of tumorigenesis such as pro-inflammatory cytokines, adhesion molecules, potassium channels, NF-κB, transforming growth factor activated kinase 1 (TAK1), CXCR4, VEGFR, STAT3, proteasome, and heat shock response has been reported previously (14, 17, 19-25). However, the potential anti-cancer effects of celastrol and its mechanism of action(s) have never been investigated before in HCC, which is one of the most lethal cancers.

Because of the critical role of STAT3 in HCC survival, proliferation, invasion, and angiogenesis, we investigated whether celastrol can mediate its anti-proliferative and pro-apoptotic effects in HCC cells through the suppression of the STAT3 pathway. We found that celastrol can indeed suppress both constitutive as well as inducible STAT3 expression in HCC cells. This inhibition decreased cell survival and downregulated expression of proliferative, anti-apoptotic and angiogenic gene products, leading to suppression of proliferation and the induction of apoptosis. Celastrol also inhibited the growth of human HCC cells in a xenograft mouse model and modulated the activation of STAT3 in tumor tissues.
Materials & Methods

Reagents

Celastrol with purity greater than 98% was purchased from Alexis Biochemicals (San Diego, CA). A 50 mM stock solution of celastrol was prepared in dimethyl sulfoxide and stored at -20°C to be used within 3 months after preparation. The stored solution was diluted with DMEM medium and further diluted in cell culture medium to make working concentrations. Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, BSA, and EGF were purchased from Sigma-Aldrich (St. Louis, MO). DMEM, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr 705), phospho-Akt (Ser 473), Akt, Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, VEGF, caspase-3, and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-specific Src (Tyr 416), Src, phospho-specific JAK2 (Tyr 1007/1008), JAK2, phospho-specific JAK1 (Tyr 1022/1023), and JAK1 were purchased from Cell Signaling Technology (Beverly, MA). Ki67 was purchased from BD Biosciences, PharMingen. Goat anti-rabbit-horse radish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Sigma-Aldrich (St. Louis, MO). Nuclear extraction and DNA binding kits was obtained from Active Motif (Carlsbad, CA). STAT3 and scrambled control siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bacteria-derived recombinant human IL-6 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).
Cell lines

Human hepatocellular carcinoma cell lines (C3A, HepG2, Hep3B and PLC/PRF5) were obtained from American Type Culture Collection (Manassass, VA). HUH-7 cells were kindly provided by Prof. Kam Man Hui, National cancer Centre, Singapore. All the five HCC cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 1 X antibiotic-antimycotic solution with 10% FBS.

Western blotting

For detection of phopho-proteins, celastrol -treated whole-cell extracts were lysed in lysis buffer (250 mM NaCl, 50 mM HEPES, 5 mM EGTA, 20 mM EDTA (pH 8.0), 0.1% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, and 2 mM NaVO₄). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 7.5% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-STAT antibodies (1:1000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

To detect STAT3-regulated proteins and PARP, C3A cells (2x10⁶/ml) were treated with celastrol for the indicated times. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 ml buffer 250 mM NaCl, 50 mM HEPES, 5 mM EGTA, 20 mM EDTA (pH 8.0), 0.1% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, and 2 mM NaVO₄. The lysate was centrifuged and the supernatant was collected. Whole-cell extract protein (30 μg) was resolved on 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with antibodies against survivin, Bcl-2, Bcl-xl, cyclin D1, Mcl-1, VEGF, procaspase-3, and...
PARP and then detected by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Immunocytochemistry for STAT3 localization**

C3A cells were plated in chamber slides in DMEM containing 10% FBS and allowed to adhere for 24 h. On next day, following treatment with celastrol for 4 h, the cells were fixed with cold acetone for 10 min, permeabilized with 0.2% Triton X-100 for 15 minutes after washing using 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 cells were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1/100) for 1 h and counterstained for nuclei with DAPI (0.5 μg/ml) for 5 min. Stained cells were mounted with mounting medium (Sigma-Aldrich) and analyzed under an fluorescence microscope (Olympus DP 70, Japan).

**DNA binding assay**

DNA binding was performed using a STAT3 DNA binding ELISA kit (Active Motif, Carlsbad, CA, USA). Briefly, nuclear extracts (5 μg) from celastrol treated cells were incubated in a 96-well plate coated with oligonucleotide containing the STAT3 specific DNA probe. Bound STAT3 was then detected using a specific primary antibody. 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 microplate reader (Tecan Systems, San Jose, CA, USA). Specificity of this assay was tested by the addition

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of wild-type or mutated STAT3 consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of the nuclear extracts.

**STAT3 luciferase reporter assay**

PLC/PRF/5 cells were plated in ninety six-well plates with $1 \times 10^4$ per well in DMEM containing 10% FBS. The STAT3-responsive elements linked to a luciferase reporter gene were transfected with wild-type or dominant-negative STAT3-Y705F (STAT3F). These plasmids were a kind gift from Dr. Bharat B. Aggarwal at M D Anderson Cancer Center, Houston, TX. Transfections were done according to the manufacturer's protocol using Lipofectamine™ 2000 purchased from Invitrogen (Carlsbad, CA). At 48 h post-transfection, cells were pretreated with celastrol for 4 h and then induced by EGF for additional 2 h before being washed and lysed in luciferase lysis buffer from Promega (Madison, WI, USA). Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega). All the luciferase experiments were done in triplicate and repeated three or more times.

**RNA Isolation and Reverse Transcription**

Total cellular RNA was extracted from untreated and celastrol treated cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Cells were lysed with TRIZOL before the adding in 0.5ml water-saturated chloroform. The cells were then vortexed and incubated for 3 minutes before it was centrifuged at 13,000rpm for 30 minutes. RNA was contained in the top aqueous phase and was transferred to a new microfuge tube. An equal volume of chloroform: isoamyl alcohol solution (24:1) was added and the content was mixed and centrifuged for 10 minutes at 13,000rpm. The top aqueous phase was transferred to a new tube. Three units of DNaseI (Sigma-
Aldrich, LO, USA) were added to the RNA and incubated at 37°C for 20 minutes to digest any DNA contaminations. After incubation, an equal volume of isopropanol was added and the RNA was allowed to precipitate at -80°C for at least 20 minutes. Subsequently, samples were centrifuged for 30 minutes at 4°C to pellet down the RNA. After the supernatant was discarded, RNA was washed with 1ml of cold 75% ethanol and centrifuged for another 30 minutes at 4°C. The resulting RNA pellet was air dried and dissolved in 15μl of RNase-free sterile water. RNA was quantified by measuring absorption of light at 260 and 280nm (A260/280). Ratio close to 2 represents nucleic acid of high quality. For the generation of cDNA via Reverse Transcription (RT), 1μg of RNA sample was reverse transcribed with 1.1U/μl MultiScribe™ reverse transcriptase in the presence of 1× RT buffer, 5mM MgCl₂, 425μM of each dNTPs, 2μM random hexamers, 0.35U/μl RNase inhibitor, and was made up to a final volume of 10μl with RNase-free sterile water. RT reaction was carried out in Mastercycler gradient (Eppendorf, USA) at 25°C for 10 minutes, followed by 37°C for 60 minutes and a terminating step of 95°C for 5 minutes.

**Real Time Polymerase Chain Reaction (PCR)**

For real time PCR, 100ng/μl of total RNA was transcribed as described above. For a 50μl reaction, 10μl of RT product was mixed with 1× TaqMan® Universal PCR Master mix, 2.5μl of 20× TaqMan probes for cyclin D1, Bcl-2, Mcl-1, and survivin respectively, 2.5μl of 20× HuGAPDH TaqMan probe as the endogenous control for each targeting gene, and topped up to 50μl 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 7500 Fast Real-Time PCR System (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA) with a protocol
that consists of 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. Results were analyzed using Sequence Detection Software version 1.3 obtained from Applied Biosystems. Relative gene expression was obtained after normalization with endogenous HuGAPDH and determination of the difference in threshold cycle (Ct) between treated and untreated cells using 2-ΔΔCt method.

**MTT assay**

The antiproliferative effect of celastrol against HCC cells was determined by the MTT dye uptake method as described previously (26). Briefly, the cells (5x10³/ml) were incubated in triplicate in a 96-well plate in the presence or absence of indicated concentration of celastrol in a final volume of 0.2 ml for different time intervals at 37 °C. Thereafter, 20 µl MTT solution (5 mg/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; incubation was done for 1 h at 37 °C; and then the optical density (OD) at 570 nm was measured by Tecan plate reader.

**Flow cytometric analysis**

To determine the effect on the cell cycle, C3A cells were exposed to celastrol for 48 h. Thereafter cells were washed, fixed with 70% ethanol. Cells were then washed, resuspended, and stained in PBS containing 10 µg/ml propidium iodide (PI) and 1 µg/ml RNase A in PBS for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako Cytomation).
**Transfection with Constitutive STAT3 Construct**

Hep3B cells were plated in chamber slides in DMEM containing 10% FBS. After 24 h, the cells were transfected with constitutive STAT3 plasmid by lipofectamine according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were treated with celastrol for 24 h, and viability of the cells was determined by Live/Dead assay as described previously (13). STAT3 constitutive plasmid was kindly provided by Dr. Xinmin Cao at Institute of Molecular and Cell Biology, Singapore.

**STAT3 siRNA transfection**

C3A cells were plated in 6-well plates and allowed to adhere for 24 h. On the day of transfection, lipofectamine obtained from Invitrogen (Carlsbad, CA) was added to 50 nM control or STAT3 siRNA in a final volume of 1 ml of culture medium. After 48 h of incubation following transfection, the cells were treated with celastrol for 24 h and apoptosis was analyzed with CyAn ADP flow cytometer (Dako Cytomation) as described above.

**HCC tumor model**

All procedures involving animals were reviewed and approved by NUS Institutional Animal Care and Use Committee. Six week-old athymic nu/nu female mice were implanted subcutaneously in the right flank with (3 X 10^6 PLC/PRF/5 cells/100 μl saline) as described previously (27). When tumors have reached 0.3 cm in diameter, mice received intra-peritoneal injection of 1 mg/kg and 2 mg/kg celastrol in 200 μl corn oil (n = 5) or corn oil alone (n = 4), 5 doses per week for 3 consecutive weeks. Animals were euthanized at day 30 after first
therapeutic dose injection. Tumor dimensions were measured using a digital caliper, and the tumor volume (V) calculated using the formula: \( V = \frac{\pi}{6} \times \text{length} \times (\text{width})^2 \). Growth curves were plotted using average relative tumor volume within each experimental group at the set time points.

**Immunohistochemical analysis of tumor samples**

Immunohistochemical staining of xenograft tumors was done as described previously (28). In brief, solid tumors harvested from control and celastrol treated mice were fixed with 10% phosphate buffered formalin, processed and embedded in paraffin. Sections were cut and deparaffinized in xylene, and 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, Carpinteria, CA) according to the manufacturer’s instructions. Sections were incubated overnight with primary antibodies as follows: anti-phospho-STAT3, anti-Ki67, and anti-caspase-3 (each at 1:100 dilution). 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 (Dako) according to the manufacturer’s instructions. Immunoreactive species were detected using 3, 3-diaminobenzidine tetrahydrochloride (DAB) as a substrate. Sections were counterstained with Gill’s hematoxylin and mounted under glass cover slips. Images were taken using an Olympus BX51 microscope.
(magnification, 20x). 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 one way analysis of variance, (ANOVA). A p value of less than 0.05 was considered statistically significant.
Results

We investigated the effect of celastrol on STAT3 activation in HCC cells and xenograft mice model. We also evaluated the effect of celastrol on various mediators of cellular proliferation, cell survival, and apoptosis. The structure of celastrol is shown in Fig. 1A.

Celastrol inhibits constitutive STAT3 phosphorylation in C3A cells:

The ability of celastrol to modulate constitutive STAT3 activation in HCC cells was investigated. C3A cells were incubated with different concentrations of celastrol for 6h, whole cell extracts were prepared and the phosphorylation of STAT3 was examined by Western blot analysis using antibodies which recognize STAT3 phosphorylation at tyrosine 705. As shown in Fig. 1B, celastrol inhibited the constitutive activation of STAT3 in C3A cells in a dose-dependent manner, with maximum inhibition occurring at 5 μM. Celastrol had no effect on the expression of STAT3 protein (Fig. 1B; lower panel). We next determined the incubation time with celastrol required for the suppression of STAT3 activation in C3A cells. As shown in Fig. 1C, the inhibition induced by celastrol was also time-dependent, with maximum inhibition occurring at around 4-6 h, again with no effect on the expression of STAT3 protein (Fig. 1C; lower panel).

Celastrol 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 (29), we determined whether celastrol can modulate the DNA binding activity of STAT3. Analysis of nuclear extracts prepared from C3A cells using ELISA based TransAM NF-κB assay kit showed that celastrol inhibited STAT3-DNA binding activities in a time-dependent manner (Fig. 1D). These results suggest that celastrol abrogates the DNA binding ability of STAT3.
Celastrol depletes nuclear pool of STAT3 in HCC cells:

Because nuclear translocation is central to the function of transcription factors and because it is not certain whether phosphorylation is mandatory for nuclear transport of STAT3 and its oncogenic functions (30, 31), we determined whether celastrol can suppress nuclear translocation of STAT3. Fig. 1E clearly demonstrates that celastrol inhibited the translocation of STAT3 to the nucleus in C3A cells.

Celastrol inhibits inducible STAT3 and JAK2 phosphorylation in HCC cells:

Because IL-6 induces STAT3 phosphorylation (32, 33), we determined whether celastrol could inhibit IL-6-induced STAT3 phosphorylation. HUH-7 cells, that have low constitutively active STAT3, were treated with IL-6 for different times and then examined for phosphorylated STAT3. IL-6 induced phosphorylation of STAT3 as early as 5 min, with maximum phosphorylation observed at 30-60 mins (Fig. 2A). IL-6 also induced phosphorylation of STAT3 in a dose-dependent manner with initial activation observed at 5 ng/ml dose (Fig. 2B). In HUH-7 cells incubated with celastrol for different times, IL-6-induced STAT3 and JAK2 phosphorylation was suppressed by celastrol in a time-dependent manner. Exposure of cells to celastrol for 4 h was sufficient to completely suppress IL-6-induced STAT3 and JAK2 phosphorylation (Figs. 2C and 2D).

Celastrol inhibits IL-6-inducible Akt phosphorylation in HCC cells:

Activated Akt has been shown to play a crucial role in the mechanism of action of IL-6. And the activation of Akt has been linked with STAT3 activation (34). We also examined whether celastrol could modulate IL-6-induced Akt activation. Treatment of HUH-7 cells with IL-6
induced phosphorylation of Akt and treatment of cells with celastrol suppressed the activation in a time dependent manner (Fig. 2E). Under these conditions, celastrol had no effect on the expression of Akt protein.

**Celastrol suppresses EGF–induced STAT3-dependent reporter gene expression:**

Our above results showed that celastrol inhibited the phosphorylation and nuclear translocation of STAT3. We next determined whether celastrol affects STAT3-dependent gene transcription. When PLC/PRF/5 cells were transiently transfected with the pSTAT3-Luc construct were stimulated with EGF, STAT3-mediated luciferase gene expression was found to be substantially increased. Dominant-negative STAT3 significantly blocked this increase, indicating specificity. When the cells were pretreated with celastrol, EGF–induced STAT3 activity was inhibited in a dose-dependent manner (Fig. 2F).

**Celastrol suppresses constitutive activation of c-Src:**

STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (35). Hence, we determined whether celastrol on constitutive activation of Src kinase in C3A cells. We found that celastrol suppressed the constitutive phosphorylation of c-Src kinases (Fig. 3A). The levels of non-phosphorylated Src kinases remained unchanged under the same conditions.

**Celastrol suppresses constitutive activation of JAK1 and JAK2:**

Because STAT3 is also activated by soluble tyrosine kinases of the Janus family (JAKs) (5), so we next determined whether celastrol affects constitutive activation of JAK1 and JAK2 in C3A
cells. We found that celastrol suppressed the constitutive phosphorylation of JAK1 (Fig 3B). The levels of non-phosphorylated JAK1 remained unchanged under the same conditions (Fig. 3B, bottom panel). To determine the effect of celastrol on JAK2 activation, C3A cells were treated for different time intervals with celastrol and phosphorylation of JAK2 was analyzed by Western blot. As shown in Fig. 3C, JAK2 was constitutively active in C3A cells and pretreatment with celastrol suppressed this phosphorylation in a time-dependent manner.

**Tyrosine phosphatases are involved in celastrol-induced inhibition of STAT3 activation:**

Because protein tyrosine phosphatases have also been implicated in STAT3 activation (36), we determined whether celastrol -induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase (PTPase). Treatment of C3A cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate prevented the celastrol -induced inhibition of STAT3 activation (Fig. 3D). This suggests that tyrosine phosphatases are involved in celastrol-induced inhibition of STAT3 activation.

**Celastrol downregulates the expression of cyclin D1, Bcl-2, Bcl-xL, Mcl-1, survivin, and VEGF:**

STAT3 activation has been reported to regulate the expression of various gene products involved in cell survival, proliferation, angiogenesis and chemoresistance (9). We found that expression of the cell cycle regulator cyclin D1, the antiapoptotic proteins Bcl-2, Bcl-xL, survivin, Mcl-1 and the angiogenic gene product VEGF all of which have been reported to be regulated by STAT3 were modulated by celastrol treatment. Their expression decreased in a time-dependent manner, with maximum suppression observed at around 24 h (Fig. 4A). We also found that mRNA
expression of cyclin D1, Bcl-2, Mcl-1 and survivin was also modulated by celastrol treatment in a time-dependent manner with maximum reduction observed at around 12-24 h post treatment (Fig. 4B).

_Celastrol inhibits the proliferation of HCC cells in a dose and time dependent manner:_
Because celastrol down regulated the expression of cyclin D1, the gene required for cell proliferation, we next investigated whether celastrol inhibits the proliferation of HCC cells by using the MTT method. Celastrol inhibited the proliferation of C3A, HepG2, HUH-7, and PLC/PRF/5 cells in a dose and time dependent manner (Fig. 5A).

_Celastrol causes the accumulation of the cells in the sub-G1 phase of the cell cycle:_
Because D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase and rapid decline in levels of cyclin D1 was observed in celastrol treated cells, we also determined the effect of celastrol on cell cycle phase distribution. We found that celastrol caused increased accumulation of cell population in sub-G1 phase, which is indicative of apoptosis (Fig. 5B).

_Celastrol activates pro-caspase-3 and induces cleavage of PARP:_
Whether suppression of constitutively active STAT3 in C3A cells by celastrol leads to apoptosis was also investigated. In C3A cells treated with celastrol there was a time-dependent activation of pro-caspase-3 (Fig. 5C). Activation of downstream caspase-3 led to the cleavage of 116 kDa PARP protein into an 85 kDa fragment (Fig. 5D). These results clearly suggest that celastrol induces caspase-3-dependent apoptosis in HCC cells.
Overexpression of constitutively active STAT3 prevents celastrol-induced apoptosis

We assessed whether the overexpression of constitutive active STAT3 construct can rescue γ-tocotrienol induced apoptosis. Hep3B cells were transfected with constitutively active STAT3 plasmid, incubated for 48 h, and cells were thereafter treated with celastrol for 24 h and examined for apoptosis by esterase staining assay. The results show that the forced expression of STAT3 reduces celastrol-induced apoptosis significantly from 20% to 12% (Fig. 5E).

STAT3 siRNA reduces celastrol-induced apoptosis

We determined whether the suppression of STAT3 expression by siRNA would abrogate the inductive effects of celastrol on apoptosis. Apoptotic effects of celastrol were measured through the increased accumulation of cells in the sub-G1 phase using flow cytometric analysis. Results shown in Fig. 5F indicate that celastrol-induced apoptosis was substantially reduced in the cells transfected with STAT3 siRNA, whereas treatment with scrambled control siRNA had minimal effect (Fig. 5F). These results suggest that induction of apoptosis is mediated through the suppression of STAT3 by celastrol in HCC cells.

Celastrol suppresses the growth of human HCC in vivo and inhibits STAT3 activation in tumor tissues

We also tested the antitumor potential of celastrol in vivo via intra-peritoneal administration in a subcutaneous model of human HCC using PLC/PRF/5 cells. Celastrol at doses of 1mg/kg and 2 mg/kg induced significant inhibition of tumor growth compared with the corn oil-treated controls (Fig. 6A). One way ANOVA showed a statistically significant difference in tumor growth between the celastrol-treated and control groups (*=P<0.001). We further evaluated the effect of
celastrol on constitutive p-STAT3 levels in HCC tumor tissues by immunohistochemical analysis and found that celastrol can substantially inhibit the constitutive STAT3 activation in treated groups as compared with the control group (Fig. 6B). The effect of celastrol was also analyzed on the expression of Ki-67 (marker of proliferation) and caspase-3 (marker of apoptosis). As shown in Fig. 6B, expression of Ki-67 was downregulated and that of caspase-3 was substantially increased in celastrol treated group as compared with control group (Fig. 6B).
Discussion

Targeting STAT3 signaling in cancer cells is an appealing strategy as this pathway is involved in tumor proliferation, anti-apoptosis and angiogenesis. Also, Terence and coworkers recently reported that CD24+ liver stem cells can also drive self renewal and tumor initiation also through STAT3 mediated regulation of *NANOG* gene (37). However, most of the currently available STAT3 inhibitors are notorious of potential cytotoxicity, thus hampering their potential clinical utility for cancer treatment. The aim of this study was to determine whether celastrol exerts its anti-cancer effects in part through the abrogation of the STAT3 signaling pathway in HCC cells.

We found that this triterpene can modulate both constitutive and inducible STAT3 activation in human HCC cells concomitant with the inhibition of c-Src, JAK1 and JAK2 activation. Celastrol further downregulated the expression of various STAT3-regulated gene products including, cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and VEGF, caused the inhibition of proliferation, and induced substantial apoptosis in HCC cells. We subsequently investigated the therapeutic potential of celastrol therapy in HCC xenograft grown in mouse model. Intra-peritoneal injection of celastrol into nude mice bearing subcutaneous PLC/PRF/5 xenografts resulted in significant suppression of tumor progression and suppression of expression of p-STAT3 in celastrol treated tumor tissues.

We observed that celastrol could suppress both constitutive and inducible STAT3 activation in HCC cells and these effects of celastrol 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 (38). Our observations suggest that celastrol may block cooperation of Src and JAKs involved in tyrosyl phosphorylation of STAT3. We also found that STAT3, JAK2 and Akt activation induced by IL-6 treatment was
also suppressed by celastrol. We also observed that celastrol suppressed nuclear translocation and EGF-induced reporter activity of STAT3. This suggests that this triterpene could manifest its effect on both constitutive and inducible STAT3 activation through multiple mechanism(s). These results are consistent with a recent report from our group in which celastrol was found to suppress activation of STAT3 and its regulated gene products in multiple myeloma cells (14).

STAT3 phosphorylation plays a crucial role in proliferation and survival of tumor cells (7). Various types of cancer, including head and neck cancers (39), multiple myeloma (40), lymphomas, and leukemia (41), also have constitutively active STAT3. The suppression of constitutively active STAT3 in HCC cells raises the possibility that this novel STAT3 inhibitor might also inhibit constitutively activated STAT3 in other types of tumor cells. Previously, it has been reported that celastrol can also suppress NF-κB activation in various tumor cells (19). Interestingly, another study suggested that STAT3 can indeed prolong NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export (42). Thus it is possible that suppression of STAT3 activation may mediate inhibition of NF-κB activation by celastrol. We also found that pervanadate treatment reversed the celastrol-induced down-regulation of STAT3, suggesting the involvement of a tyrosine phosphatase. Numerous PTPs have been implicated in STAT3 signaling including SHP1, SH-PTP2, TC-PTP, PTEN, PTP-1D, CD45, PTP-epsilon, low molecular weight (LMW), and PTP (43). Among these, which one of the phosphatase is involved in celastrol-induced inhibition of STAT3 activation in HCC cells needs further investigation. Interestingly, the multikinase protein tyrosine inhibitor sorafenib recently approved by FDA for the treatment of HCC was also found to inhibit STAT3 through activation of a PTP (44).
We also found that celastrol suppressed the expression of several STAT3-regulated genes; including proliferative (cyclin D1) and antiapoptotic gene products (Bcl-2, Bcl-xL, survivin, and Mcl-1) and angiogenic gene product (VEGF). Activation of STAT3 signaling also induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells (45). The downregulation of the expression of Bcl-2, Bcl-xL, survivin and Mcl-1 is likely linked with the celastrol’s ability to induce apoptosis in HCC cells as evident by increased accumulation of cells in the sub-G1 phase, activation of pro-caspase-3 and cleavage of PARP. The downmodulation of VEGF expression as reported here may also explain the anti-angiogenic potential of this triterpene that has been previously described in endothelial and prostate cancer cells (16). Interestingly, we also found that knocking down the expression of STAT3 by siRNA abolished the effect of celastrol on apoptosis, and moreover, overexpression of STAT3 can rescue the apoptotic effects of celastrol, thereby strengthening our hypothesis that anti-proliferative effects of celastrol are mediated through the abrogation of the STAT3 signaling pathway.

Whether these in vitro observations with celastrol has any relevance to that in vivo was also investigated. Our results also show for the first time that celastrol significantly suppressed HCC growth in nude mice, downregulated the expression of phospho-STAT3 and Ki-67, and increased the levels of caspase-3 in treated group as compared to control group. Interestingly, Venkatesha et al, using the rat adjuvant-induced arthritis model of human rheumatoid arthritis, recently demonstrated that celastrol also exerts its anti-arthritis activity through the suppression of STAT3 transcription factor (46). However, whether the long-term administration of celastrol may be effective for preventing the development of HCC by targeting STAT3 signaling axis in liver cancer tumorigenesis and also if this triterpene can be used in conjunction with existing
chemotherapeutic agents and targeted therapies approved by FDA for HCC treatment warrants further in depth investigation.

Celastrol has been well tolerated in pre-clinical studies using various inflammatory diseases and cancer models, with no reported toxicity so far (16-19, 47, 48). Celastrol as such has never been tested in humans before and hence its clinically relevant doses are not clear as yet. Thus, overall, our *in vitro* and *in vivo* experimental findings clearly indicate that the anti-proliferative and pro-apoptotic effects of celastrol in HCC are mediated through the suppression of STAT3 activation and provide a sound basis for exploring the potential of celastrol to overcome toxicity and enhance treatment efficacy for HCC patients.
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CONFLICT OF INTEREST

No potential conflict of interest is declared.
References


Figure Legends
Figure 1. Celastrol inhibits constitutively active STAT3 in C3A cells.  A, The chemical structure of celastrol.  B, Celastrol suppresses phospho-STAT3 levels in a dose dependent manner. C3A cells (2×10^6/ml) were treated with the indicated concentrations of celastrol for 4h, after which whole-cell extracts were prepared, and 30 μg of protein was resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3.  C, Celastrol suppresses phospho-STAT3 levels in a time-dependent manner.  C3A cells (2×10^6/ml) were treated with the 5 μM celastrol for the indicated times, after which western blotting was performed as described for panel B. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.  D, Celastrol suppresses STAT3 DNA binding ability in C3A cells.  C3A cells were treated with 5 μM celastrol for the indicated time points; nuclear extracts were prepared, and 20 μg of the nuclear extract protein was used for ELISA based DNA binding assay as described in Material and Methods. The results shown are representative of two independent experiments *, p < 0.05. E, Celastrol causes inhibition of translocation of STAT3 to the nucleus. C3A cells (1×10^5/ml) were incubated with or without 5 μM celastrol for 4 h and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with Hoechst (50 ng/ml) for 5 min.

Figure 2. Celastrol downregulates IL-6–induced phospho-STAT3 in HCC cells.  A, HUH-7 cells (2×10^6/mL) were treated with IL-6 (10 ng/ml) for indicated times, whole cell extracts were prepared, and phospho-STAT3 was detected by Western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading.  B, HUH-7 cells (2×10^6/mL) were treated with indicated concentrations of IL-6 for 15 minutes, whole cell extracts were prepared, and phospho-STAT3 was detected by Western
blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. C, HUH-7 (2×10⁶/ml) were treated with 5 μM celastrol for the indicated times and then stimulated with IL-6 (10 ng/ml) for 15 minutes. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by Western blotting. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. D, HUH-7 (2×10⁶/ml) were treated with 5 μM celastrol for the indicated times and then stimulated with IL-6 (10 ng/ml) for 15 minutes. Whole-cell extracts were then prepared and analyzed for phospho-JAK2 by Western blotting. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. E, HUH-7 (2×10⁶/ml) were treated with 5 μM celastrol for the indicated times and then stimulated with IL-6 (10 ng/ml) for 15 minutes. Whole-cell extracts were then prepared and analyzed for phospho-Akt by Western blotting. The same blots were stripped and reprobed with Akt antibody to verify equal protein loading. The results shown are representative of three independent experiments. F, PLC/PRF/5 cells (5 x 10⁵/mL) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with indicated doses of celastrol for 4 h and then stimulated with EGF (100 ng/mL) for 2 h. Whole-cell extracts were then prepared and analyzed for luciferase activity. The results shown are representative of three independent experiments. * indicates p value <0.05.

Figure 3. A, Celastrol suppresses phospho-Src levels in a time-dependent manner. C3A cells (2×10⁶/ml) were treated with 5 μM celastrol, after which whole-cell extracts were prepared and 30μg aliquots of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-Src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. B, Celastrol suppresses phospho-
JAK1 levels in a time-dependent manner. C3A cells (2×10⁶/ml) were treated with 5 μM celastrol for indicated time intervals, after which whole-cell extracts were prepared and 30μg portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK1 antibody. The same blots were stripped and reprobed with JAK1 antibody to verify equal protein loading. C, Celastrol suppresses phospho-JAK2 levels in a time-dependent manner. C3A cells (2×10⁶/ml) were treated with 5 μM celastrol for indicated time intervals, after which whole-cell extracts were prepared and 30μg portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK2 antibody. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. D, Pervanadate reverses the phospho-STAT3 inhibitory effect of celastrol. C3A cells (2×10⁶/ml) were treated with the indicated concentrations of pervanadate and 5 μM celastrol for 4 h, after which whole-cell extracts were prepared and 30μg portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. The results shown are representative of three independent experiments.

**Figure 4.** Celastrol suppresses STAT3 regulated gene products involved in proliferation, survival and angiogenesis. A, C3A cells (2×10⁶/ml) were treated with 1 μM celastrol for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE, membrane sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and VEGF antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. B, C3A cells (3×10⁵/ml) were treated with 1 μM celastrol for the indicated time intervals, after
which cells were harvested after treatment and RNA samples were extracted. 1µg portions of the respective RNA extracts then proceed for 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, Bcl-2, Mcl-1, and survivin TaqMan probes, with HuGAPDH as endogenous control for measurement of equal loading of RNA samples. Results were analyzed using Sequence Detection Software version 1.3 obtained from Applied Biosystems. Relative gene expression was obtained after normalization with endogenous HuGAPDH and determination of the difference in threshold cycle (Ct) between treated and untreated cells using 2-ΔΔCt method. The results shown are representative of three independent experiments.

**Figure 5.** Celastrol suppresses the proliferation, causes accumulation of cells in sub-G1 phase and activates caspase-3. A, C3A, HepG2, HUH-7, and PLC/PRF/5 cells (5×10^3/mL) were plated in triplicate, treated with indicated concentrations of celastrol, and then subjected to MTT assay after 24, 48 and 72 hours to analyze proliferation of cells. Standard deviations between the triplicates are indicated. * indicates p value <0.05. B, C3A cells (2×10^6/mL) were treated with 1 µM celastrol for 48 h, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. C, C3A cells were treated with 1 µM celastrol for the indicated times, whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against procaspase-3 antibody. The same blot were stripped and reprobed with β-actin antibody to show equal protein loading. D, C3A cells were treated with 1 µM celastrol for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against PARP antibody. The same blot was stripped and reprobed with β-actin antibody to show equal protein loading. E, Overexpression of constitutive STAT3 rescues
Hep3B cells from celastrol-induced cytotoxicity. First, Hep3B cells were transfected with constitutive STAT3 plasmid. After 48 h of transfection, the cells were treated with 1μM celastrol for 24 h, and then the cytotoxicity was determined by Live/Dead assay, and 20 random fields were counted. F, Knockdown of STAT3 siRNA inhibited the apoptotic effect of celastrol. C3A cells were transfected with either STAT3-specific or scrambled siRNA (50 nM). After 48 h, cells were treated with 5μM celastrol for 24 h and analyzed for the percentage of apoptosis by flow cytometric analysis.

**Figure 6.** Celastrol inhibits the growth of human HCC *in vivo*. A, Athymic mice bearing subcutaneous PLC/PRF/5 tumors were treated for 5 times a week for 4 consecutive weeks with 1mg/kg and 2mg/kg celastrol (each group, n=5) or corn oil alone (n=4). *=P<0.001 (One-way ANOVA). B, Immunohistochemical analysis of p-STAT3, Ki-67, and caspase-3 showed the inhibition in expression of p-STAT3, Bcl-2 and increased levels of caspase-3 expression in celastrol treated samples as compared with control group. Percentage indicates positive staining for the given biomarker. The photographs were taken at the magnification of x 20.
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Celastrol suppresses growth and induces apoptosis of human hepatocellular carcinoma through the modulation of STAT3/JAK2 signaling cascade in vitro and vivo

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