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 patients with HCCs present at advanced disease stages where surgery is not possible, and in cases where surgical resections are conducted, the 2-year recurrence rate is as high as 50%. Although several chemotherapeutic agents have been used in treatment of HCCs, 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 HCCs.

Cumulative evidences(s) have established that the constitutive activation of 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 HCCs. 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 intraperitoneally, 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 antiproliferative and proapoptotic effects through suppression of STAT3 signaling in HCC both in vitro and in vivo. Cancer Prev Res; ©2012 AACR.

Research Article

Celastrol Suppresses Growth and Induces Apoptosis of Human Hepatocellular Carcinoma through the Modulation of STAT3/JAK2 Signaling Cascade In Vitro and In Vivo

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Abstract

The protein STAT3 is a latent transcription factor that resides in the cytoplasm (5, 6). Upon activation by cytokines [such as interleukin (IL)-6] or growth factors (EGF, platelet-derived growth factor), 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 (JAK), which include 4 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., 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
Celastrol, which has attracted great attention recently for its potent anticancer 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 and in vivo (16–18). The efficacy of celastrol to modulate the expression of various key mediators of tumorigenesis such as proinflammatory cytokines, adhesion molecules, potassium channels, NF-κB, TGF-activated kinase 1 (TAK1), CXCR4, VEGF receptor (VEGFR), STAT3, proteasome, and heat shock response has been reported previously (14, 17, 19–25). However, the potential anticancer effects of celastrol and its mechanism of action(s) have never been investigated before in HCCs, 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 antiproliferative and proapoptotic effects in HCC cells through the suppression of the STAT3 pathway. We found that celastrol can indeed suppress both constitutive and inducible STAT3 expression in HCC cells. This inhibition decreased cell survival and downregulated expression of proliferative, antiapoptotic, and angiogenic gene products, leading to the 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 and Methods

Reagents

Celastrol with purity greater than 98% was purchased from Alexis Biochemicals. A 50 mmol/L 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 Dulbecco’s Modified Eagle’s Medium (DMEM) and further diluted in cell culture medium to make working concentrations. Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, bovine serum albumin, and EGF were purchased from Sigma-Aldrich. DMEM, FBS, 0.4% trypan blue vital stain, and antibiotic–antimycotic solution with 10% FBS.

For detection of phosho-proteins, celastrol-treated whole-cell extracts were lysed in lysis buffer [250 mmol/L NaCl, 50 mmol/L HEPES, 5 mmol/L EDTA, 20 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 mmol/L NaVO₄]. Lysates were then spun at 14,000 rpm for 10 minutes 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 (1:1,000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 hour, and finally examined by chemiluminescence (ECL; GE Healthcare).

To detect STAT3-regulated proteins and PARP, C3A cells (2 × 10⁵/mL) were treated with celastrol for the indicated times. The cells were then washed and extracted by incubation for 30 minutes on ice in 0.05 mL buffer: 250 mmol/L NaCl, 50 mmol/L HEPES, 5 mmol/L EDTA, 20 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L PMSF, and 2 mmol/L NaVO₄. The lysate was centrifuged and the supernatant was collected by SDS-PAGE. Electrophoresed proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and probed with anti-STAT antibodies (1:1,000). After overnight incubation, the membranes were washed and blocked with 5% normal goat serum for 1 hour. The cells were then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1:100). After overnight incubation, the membranes were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 hour and counterstained for nuclei with DAPI (4′,6-diamidino-2-phenylindole; 0.5 μg/mL) for 5 minutes. Stained cells were mounted with mounting medium (Sigma-Aldrich) and analyzed under a fluorescence microscope (Olympus DP 70).

DNA binding assay

DNA binding was conducted using a STAT3 DNA-binding ELISA kit (Active Motif). 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). Specificity of this assay was tested by the addition 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/PRF5 cells were plated in 96-well plates with 1 × 10⁴ 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 MD Anderson Cancer Center, Houston, TX. Transfections were done according to the manufacturer’s protocol using Lipofectamine 2000 purchased from Invitrogen. At 48 hours posttransfection, cells were pretreated with celastrol for 4 hours and then induced by EGF for additional 2 hours before being washed and lysed in luciferase lysis buffer from Promega. Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega). All the luciferase experiments were carried out in triplicate and repeated 3 or more times.

**RNA isolation and reverse transcription**

Total cellular RNA was extracted from untreated and celastrol-treated cells using TRIzol reagent (Invitrogen). Cells were lysed with TRIzol before the addition in 0.5 mL water-saturated chloroform. The cells were then vortexed and incubated for 3 minutes before it was centrifuged at 13,000 rpm 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 13,000 rpm. The top aqueous phase was transferred to a new tube. Three units of DNase I (Sigma-Aldrich) 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 1 mL of cold 75% ethanol and centrifuged for another 30 minutes at 4°C. The resulting RNA pellet was air dried and dissolved in 15 μL of RNase-free sterile water. RNA was quantified by measuring absorption of light at 260 and 280 nm (A₂₆₀/A₂₈₀). Ratio close to 2 represents nucleic acid of high quality. For the generation of cDNA via reverse transcription (RT), 1 μg of RNA sample was reverse transcribed with 1.1 U/μL MultiScribe reverse transcriptase in the presence of 1 × RT buffer, 5 mmol/L MgCl₂, 425 μmol/L of each dNTPs, 2 μmol/L random hexamers, 0.35 U/μL RNase inhibitor and was made up to a final volume of 10 μL with RNase-free sterile water. RT reaction was carried out in Mastercycler gradient (Eppendorf) 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 PCR**

For real-time PCR, 100 ng/μL of total RNA was transcribed as described above. For a 50 μL reaction, 10 μL of RT product was mixed with 1 × TaqMan Universal PCR Master mix, 2.5 μL of 20× TaqMan probes for cyclin D1, Bcl-2, Mcl-1, and survivin respectively, 2.5 μL of 20× HuGAPDH 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 carried out using 7500 Fast Real-Time PCR System (ABI PRISM 7500; Applied Biosystems) with a protocol that consists of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturation 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 (5 × 10³/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-hour incubation at 37°C, 0.1 mL lysis buffer (20% SDS, 50% dimethylformamide) was added; incubation was done for 1 hour 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 hours. Thereafter, cells were washed and 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 minutes 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 hours, the cells were transfected with constitutive STAT3 plasmid by Lipofectamine according to manufacturer’s protocol (Invitrogen). Cells...
were treated with celastrol for 24 hours, and viability of the cells was determined by Live/Dead Assay as described previously (13). STAT3 constitutive plasmid was kind 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 hours. On the day of transfection, Lipofectamine obtained from Invitrogen was added to 50 mmol/L control or STAT3 siRNA in a final volume of 1 mL of culture medium. After 48 hours of incubation following transfection, the cells were treated with celastrol for 24 hours 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 National University of Singapore Institutional Animal Care and Use Committee. Six-week-old athymic nu/nu female mice were implanted subcutaneously in the right flank with (3 × 10^6) PLC/PRF5 cells/100 μL saline as described previously (27). When tumors have reached 0.3 cm in diameter, mice received intraperitoneal injection of 1 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: \[ V = \frac{\text{width} \times \text{length} \times \text{height}}{2} \]. 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 conducted by boiling the slide in 10 mmol/L sodium citrate (pH 6.0) for 30 minutes. Immunohistochemistry was conducted following manufacturer instructions (DAKO LSAB kit). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Nonspecific binding was blocked by incubation in the blocking reagent in the LSAB Kit (Dako) according to the manufacturer’s instructions. Sections were incubated overnight with primary antibodies as follows: anti-phospho-STAT3, anti-Ki67, and anti-caspase-3 (each at 1:100 dilution). Slides were subsequently washed several times in PBS with 0.1% Tween-20 and were incubated with biotinylated linker for 30 minutes, 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 (DAB) as a substrate. Sections were counterstained with Gill’s hematoxylin and mounted under glass coverslips. 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 conducted by Student t test and one-way 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 6 hours, 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 μmol/L. Celastrol had no effect on the expression of STAT3 protein (Fig. 1B, bottom). 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 to 6 hours, again with no effect on the expression of STAT3 protein (Fig. 1C, bottom).

**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. Figure 1E clearly shows that celastrol inhibited the translocation of STAT3 to the nucleus in C3A cells.
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 reporter gene expression.
gene transcription. When PLC/PRF5 cells were transiently transfected with the pSTAT3-Luc construct 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 affects 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 nonphosphorylated 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; ref. 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 nonphosphorylated JAK1 remained unchanged under the same conditions (Fig. 3B, bottom). 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 blotting. 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, was modulated by celastrol treatment. Their expression decreased in a time-dependent manner, with maximum suppression observed at around 24 hours (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 to 24 hours posttreatment (Fig. 4B).

Celastrol inhibits the proliferation of HCC cells in a dose- and time-dependent manner

Because celastrol downregulated 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 MIT method. Celastrol inhibited the proliferation of C3A, HepG2, HUH-7, and
PLC/PRF5 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 constitutively active STAT3 construct can rescue celastrol-induced apoptosis. Hep3B cells were transfected with constitutively active STAT3 plasmid, incubated for 48 hours, and cells were thereafter treated with celastrol for 24 hours 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 intraperitoneal administration in a subcutaneous model of human HCCs using PLC/PRF5 cells. Celastrol at...
doses of 1 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 (\( P < 0.05 \)). 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 Ki67 (marker of proliferation) and caspase-3 (marker of apoptosis). As shown in Fig. 6B, expression of Ki67 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, antiapoptosis, and angiogenesis. Also, Lee and colleagues 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 exhibit significant cytotoxicity, thus hampering their potential clinical use for cancer treatment. The aim of this study was to determine whether celastrol exerts its anticancer 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. Intraperitoneal injection of celastrol into nude mice bearing subcutaneous PLC/PRF5 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 JAK2 kinase activities cooperate to mediate constitutive activation of STAT3. 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 downregulation of STAT3, suggesting the involvement of a tyrosine phosphatase. Numerous PTPs have been implicated in STAT3 signaling including SHP1, SH-PTP2, TC-PTP, PTPN1, PTP-1B, CD45, PTP-epsilon, low molecular weight (LMW), and PTP (43). Among these, which 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 U.S. Food and Drug Administration (FDA) for the treatment of HCCs 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

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Figure 5. (Continued) E, overexpression of constitutive STAT3 rescues Hep3B cells from celastrol-induced cytotoxicity. First, Hep3B cells were transfected with constitutive STAT3 plasmid. After 48 hours of transfection, the cells were treated with 1 μmol/L celastrol for 24 hours, 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 nmol/L). After 48 hours, cells were treated with 5 μmol/L celastrol for 24 hours and analyzed for the percentage of apoptosis by flow cytometric analysis.
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 ability of celastrol 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 antiangiogenic 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 antiproliferative 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 Ki67, and increased the levels of caspase-3 in treated group as compared with control group. Interestingly, Venkatesha and colleagues, using the rat adjuvant–induced arthritis model of human rheumatoid arthritis, recently showed that celastrol also exerts its antiarthritic 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 HCCs 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.

Celasrol has been well tolerated in preclinical studies using various inflammatory diseases and cancer models, with no reported toxicity so far (16–19, 47, 48). Celasrol 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 antiproliferative and proapoptotic effects of celasrol in HCCs are mediated through the suppression of STAT3 activation and provide a sound basis for exploring the potential of celasrol to overcome toxicity and enhance treatment efficacy for patients with HCCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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References


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