Suppression of Signal Transducer and Activator of Transcription 3 Activation by Butein Inhibits Growth of Human Hepatocellular Carcinoma in vivo

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Running title: Butein inhibits STAT3 signaling in vitro and in vivo 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; ROS, reactive oxygen species.
Abstract

Purpose: Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third cause of global cancer mortality. Increasing evidence suggest that signal transducer and activator of transcription 3 (STAT3) is a critical mediator of oncogenic signaling in HCC and controls the expression of several genes involved in proliferation, survival, metastasis and angiogenesis. Thus, novel agents that can suppress STAT3 activation have potential for both prevention and treatment of HCC.

Experimental Design: The effect of butein on STAT3 activation, associated protein kinases, STAT3-regulated gene products, cellular proliferation and apoptosis was investigated. The in vivo effect of butein on the growth of human HCC xenograft tumors in male athymic nu/nu mice was also examined.

Results: We tested an agent, butein, for its ability to suppress STAT3 activation in HCC cells and nude mice model along with prospectively testing the hypothesis of STAT3 inhibition in a virtual predictive functional proteomics tumor pathway technology platform. We found that butein inhibited both constitutive and inducible STAT3 activation in HCC cells. The suppression was mediated through the inhibition of activation of upstream kinases c-Src, and Janus-activated kinase 2. Butein inhibited proliferation, and significantly potentiated the apoptotic effects of paclitaxel and doxorubicin in HCC cells. When administered i.p., butein inhibited the growth of human HCC xenograft tumors in male athymic nu/nu mice.

Conclusions: Overall, cumulative results from experimental and predictive studies...
suggest that butein exerts its anti-proliferative and pro-apoptotic effects through suppression of STAT3 signaling in HCC both \textit{in vitro} and \textit{in vivo}.
Translational Relevance

Despite advances in earlier detection and therapy for HCC, it still remains the fifth most common cancer and causes more than 600,000 deaths annually worldwide. Existing drugs lack efficacy and yet are highly toxic. Thus, finding an agent that is both pharmacologically safe and efficacious is needed. Because STAT3 activation has been shown to mediate HCC cell survival, proliferation, metastasis, and angiogenesis, we investigated the effect of butein (3, 4, 2, 4-tetrahydroxychalcone) using HCC cell lines and in vivo mice model. Our experimental and predictive results clearly indicate that anti-proliferative and pro-apoptotic effects of butein in HCC are mediated through suppression of constitutive and inducible STAT3 activation and provide a sound basis for pursuing the use of butein further, either alone or in combination with existing therapy, to reduce toxicity and enhance treatment efficacy for HCC.
Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant tumors, ranking fifth in incidence and third in mortality worldwide (1). Although epidemiologic studies have shown that chronic viral infections and hepatotoxic agents are the major risk factors, the molecular pathogenesis of HCC is quite complex with involvement of several oncogenes and tumor suppressor genes (2, 3). HCC is often diagnosed at an advanced stage when the curative therapies such as surgical resection and liver transplantation are of limited efficacy. Unresectable or metastatic HCC patients have median survival of a few months (4, 5). A great need exists, therefore, for new therapies, including complementary and preventive approaches to treating HCC.

The transcription factor signal transducer and activator of transcription 3 (STAT3) was originally identified as a DNA binding protein that responds to stimulation by epidermal growth factor and interleukin-6 (IL-6) and has an important role in their signaling (6, 7). Upon activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription. The phosphorylation is mediated through the activation of non-receptor protein tyrosine kinases called Janus-like kinase (JAK). JAK1, JAK2, JAK3, and TYK2 have been implicated in the activation of STAT3 (8, 9). In addition, the role of c-Src kinase has been shown in STAT3 phosphorylation (6). In normal cells, the duration of STAT3 activation is temporary, usually lasting from a few minutes to several hours (8). In these cells, STAT3 plays crucial roles in the development of various organs and in
cell proliferation (8). In contrast, constitutive activation of STAT3 has been observed in many kinds of tumors (10-12), including HCC (13-16) and this persistently active STAT3 is thought to contribute to proliferation and oncogenesis by modulating the expression of a variety of genes (17). Moreover, STAT3 has been implicated as a promising target for HCC therapy since inhibition of STAT3 induces growth arrest and apoptosis of human HCC cells (18-25).

In the present report, we analyzed the effect of butein (3, 4, 2, 4-tetrahydroxychalcone) derived from numerous plants including stem bark of *Semecarpus anacardium*, *Rhus verniciflua Stokes* and the heartwood of *Dalbergia odorifera* (26, 27) on HCC cell lines and *in vivo* xenograft mice model. Previous reports have indicated that butein can suppress the proliferation of different human tumor cells, including breast carcinoma, colon carcinoma, osteosarcoma, lymphoma, acute myelogenous leukemia, chronic myeloid leukemia, multiple myeloma, melanoma, and hepatic stellate cells (26-35). Furthermore, it was found to suppress phorbol ester-induced skin tumor formation (36), inhibit carrageenan-induced rat paw edema (37), reduce antibody-associated glomerulonephritis (38), and suppress liver fibrosis induced by carbon tetrachloride (32). Moreover, butein was recently reported to induce G (2)/M phase arrest and apoptosis in HCC cells through reactive oxygen species (ROS) generation and activation of JNK (39) suggesting that butein may have a great potential for HCC treatment.
Because of the critical role of STAT3 in HCC survival, proliferation, invasion, and angiogenesis, we investigated whether butein can mediate its anti-proliferative and pro-apoptotic effects in HCC cells through the suppression of the STAT3 pathway. We found that butein 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, induction of apoptosis, and enhancement of the response to the apoptotic effects of doxorubicin and paclitaxel in HCC cells. Butein also inhibited the growth of human HCC cells in a xenograft mouse model.

Alongside testing the effects of butein in HCC cells and xenograft mouse model, we also tested the hypothesis of STAT3 inhibition in a virtual predictive tumor cell system to explore if butein is mediating its effects primarily through STAT3 inhibition. The virtual epithelial tumor cell platform on which predictive STAT3 inhibition studies were conducted is a comprehensive integrated representation of the pathways representing the key cancer phenotypes of proliferation, apoptosis, angiogenesis, metastasis and conditions of tumor microenvironment including tumor-associated inflammation (40-42). This virtual tumor cell has been used to get an insight into how a particular drug individually or in combination is impacting various cancer phenotypes across different tumor profiles (43). Thus, novel approach of combination of predictive virtual hypothesis testing along with guided experimental validations is extremely
helpful in an integrated analysis of understanding the mechanism of action and efficacy of novel compounds on physiological endpoints.
Materials & Methods

Reagents

Butein, Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, BSA, EGF, N-Acetyl-L-cysteine, doxorubicin, and paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO). Butein was dissolved in dimethylsulfoxide as a 10 mM stock solution and stored at 4°C. Further dilution was done in cell culture medium. RPMI 1640, 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, 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) and JAK2 were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit-horse radish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Sigma-Aldrich (St. Louis, MO). Bacteria-derived recombinant human IL-6 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).

Cell lines

Human hepatocellular carcinoma cell lines HepG2, SNU-387 and PLC/PRF5 were obtained from American Type Culture Collection (Manassass, VA). HepG2 and SNU-387 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 1 X...
antibiotic-antimycotic solution with 10% FBS. PLC/PRF5 cells were cultured in DMEM containing 1x penicillin-streptomycin solution, non-essential amino acids, sodium pyruvate, and L-glutamine with 10% FBS. HCCLM3 was a kind gift of Professor Zhao-You Tang at the Liver Cancer Institute (Zhongshan Hospital, Fudan University, Shanghai). HCCLM3 were cultured in high glucose DMEM containing 1X antibiotic-antimycotic solution with 10% FBS.

**Western blotting**

For detection of phopho-proteins, butein -treated whole-cell extracts were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 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, HepG2 cells (2x10⁶/ml) were treated with butein for the indicated times. The cells were then washed and extracted by incubation for 30 min on ice in 0.05 ml buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% NP-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF,
0.5 μg/ml benzamidine, 1 mM DTT, and 1 mM sodium vanadate. 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, VEGF, procaspase-3, and PARP and then detected by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Immunocytochemistry for STAT3 localization**

HepG2 cells were plated in chamber slides in DMEM containing 10% FBS and allowed to adhere for 24 h. On next day, following treatment with butein for 6 h, the cells were fixed with cold acetone for 10 min, 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 cells were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1/100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained cells were mounted with mounting medium (Sigma-Aldrich) and analyzed under an fluorescence microscope (Olympus DP 70, Japan).

**STAT3 luciferase reporter assay**

PLC/PRF5 cells were plated in ninety six-well plates with 1 x 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 protocols using Fugene-6 obtained from Roche (Indianapolis, IN). At 24 h posttransfection, cells were pretreated with butein 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) and was normalized to β-galactosidase activity. All 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 butein 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 MultiScribeTM reverse transcriptase in the presence of 1xRT 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 1x TaqMan® Universal PCR Master mix, 2.5μl of 20x TaqMan probes for Bcl-2, Bcl-xl, cyclin D1, VEGF and Mcl-1 respectively, 2.5μl of 20x 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 provided by 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 butein against HCC cells was determined by the MTT dye uptake method as described previously (44). Briefly, the cells (5x10^3/ml) were incubated in triplicate in a 96-well plate in the presence or absence of indicated concentration of butein 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 continued overnight at 37 °C; and then the optical density (OD) at 570 nm was measured by Tecan plate reader.

**Live/Dead Assay**
Apoptosis of cells was also determined by Live/Dead assay (Molecular Probes, Eugene, OR, USA) that measures intracellular esterase activity and plasma membrane integrity as described previously (44). Briefly, 1 X10^6 cells were incubated with butein /doxorubicin/ paclitaxel alone or in combination for 24 h at 37°C. Cells were stained with the Live/Dead reagent (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Olympus DP 70, Japan).

In Silico Study

In silico analysis was performed using the physiologically aligned and qualified Virtual Tumor Cell technology, Cellworks Group Inc, CA, USA (43). The Cellworks Tumor cell platform provides a dynamic and transparent view of human cellular physiology at the proteomics abstraction level. The platform’s open-access architecture provides a framework for different ‘what-if’ analysis and studies in an automated high-throughput methodology. The Cellworks platform is implemented using a three-layered architecture. The top later is a TUI/GUI (Text user interface/ graphic user interface) driven user interface. The middle layer is the disease specific system of individual or interacting virtual cell types. The bottom layer is the computational backplane which enables the system to be dynamic and computes all the mathematics in the middle layer.
**Platform Description**

The virtual Tumor Cell Platform consists of a dynamic and kinetic representation of the signaling pathways underlying tumor physiology at the bio-molecular level. All the key relevant protein players and associated gene and mRNA species with regard to tumor related signaling are comprehensively included in the system with their relationship quantitatively represented. Pathways and signaling for different cancer phenotypes comprise 75 major signaling networks with more than 3900 intracellular molecules. The platform includes important signaling pathways comprising growth factors like EGFR, PDGFRA, FGFR, c-MET, VEGFR and IGF-1R, cell cycle regulators, mTOR signaling, p53 signaling cascade, cytokine pathways like IL1, IL4, IL6, IL12, TNF; lipid mediators and tumor metabolism (Fig. 3A). The platform has been correlated against an extensive set of pre-defined *in vitro* and *in vivo* studies.

**Predictive Study Experimental Protocol**

The virtual Tumor cell is simulated in the proprietary Cellworks computational backplane and initialized to a control state wherein all molecules attain the control steady state values, following which the triggers are introduced into the system. The virtual tumor cell technology allows the end user to align the system to a known cancer cell line with perturbations in known markers or mutations that can be used for further analysis (43). In this kinetic based virtual tumor cell platform, there is no statistical variation in the outputs. The system provides predictive information on semi-
quantitative trends. The system predictions have been validated against a large number of experiments and the accuracy of predictions is very high.

**STAT3 Inhibition Study Details:**

STAT3 activity was inhibited by 50% and 100% in disease state and the phenotype associated biomarker trends evaluated as percentage change from disease values.

**Tumor model**

All procedures involving animals were reviewed and approved by SingHealth Institutional Animal Care and Use Committee. Six week-old athymic nu/nu female mice (Animal Resource Centre, Australia) were implanted subcutaneously in the right flank with (3 X 10^6 HCCLM3 cells/100 μl saline). When tumors have reached 0.5 cm in diameter, mice received intra-peritoneal injection of 2 mg/kg butein in 200 μl corn oil (n = 8) or corn oil alone (n = 6), 5 doses per week for 3 consecutive weeks. Animals were euthanized at day 22 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**

Solid tumors from control and butein treated mice were fixed with 10% phosphate buffered formalin, processed and embedded in paraffin. Sections were cut and
deparafinized 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-Bcl-2, 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 two way analysis of variance, (ANOVA). A p value of less than 0.05 was considered statistically significant.
Results

We investigated the effect of butein on constitutive and IL-6-inducible STAT3 activation in HCC cells. We also evaluated the effect of butein on various mediators of cellular proliferation, cell survival, and apoptosis. Additionally, we also tested the hypothesis of butein effects primarily through STAT3 inhibition and compared the predictive trends with the experimental data. The structure of butein is shown in Fig. 1A.

Butein inhibits constitutive STAT3 phosphorylation in HepG2 cells:

The ability of butein to modulate constitutive STAT3 activation in HCC cells was investigated. HepG2 cells were incubated with different concentrations of butein 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, butein inhibited the constitutive activation of STAT3 in HepG2 cells in a dose-dependent manner, with maximum inhibition occurring at 50 µM. Butein had no effect on the expression of STAT3 protein (Fig. 1B; lower panel). AG490 is a well-characterized inhibitor of JAK2 phosphorylation (45). We found that exposure to AG490 also suppressed STAT3 phosphorylation in a dose-dependent manner in HepG2 cells (Fig. 1C). As shown in Fig. 1D, the inhibition induced by butein was also time-dependent, with maximum inhibition occurring at around 6-8 h, again with no effect on the expression of STAT3 protein (Fig. 1D; lower panel). Whether butein affects the activation of other STAT proteins in HepG2 cells was also
investigated. Under the conditions where butein completely inhibited STAT3 phosphorylation, it had minimal effect on the levels of constitutively phosphorylated STAT5 and the expression of STAT5 proteins (Fig. 1E). This result supports the predictive data on STAT3 inhibition which had minimal to no impact on the other STAT proteins – STAT5 and STAT6 as demonstrated in Fig. 3B.

**Butein 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 (46, 47), we determined whether butein can suppress nuclear translocation of STAT3. Fig. 1F clearly demonstrates that butein inhibited the translocation of STAT3 to the nucleus in HepG2 cells.

**Butein-induced inhibition of STAT3 activation is ROS-dependent:**

Because ROS generation blocks STAT3 signaling (48, 49) and butein has been found to induce ROS production in HepG2 cells (39), we next determined whether N-Acetyl-L-cysteine (NAC), a well known ROS scavenger can restore the inhibitory effect of butein on STAT3 activation. For this, HepG2 cells were pretreated with NAC for 1 h before treatment with butein and STAT3 activation was monitored by western blot analysis. We found that decrease in phosphorylated STAT3 observed upon butein treatment is ROS dependent, as levels reverted to control levels in the presence of NAC (Fig. 1G).
Butein treatment had no effect on the level of total STAT3 either in the presence or absence of NAC (Fig. 1G).

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

STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (50). Hence, we determined whether butein on constitutive activation of Src kinase in HepG2 cells. We found that butein suppressed the constitutive phosphorylation of c-Src kinases (Fig. 1H). The levels of non-phosphorylated Src kinases remained unchanged under the same conditions. The predictive data in Fig. 3C also shows a significant inhibition of Src kinase in the tumor cell and clearly correlates with the experimental data trends in Fig.1H.

**Butein suppresses constitutive activation of JAK2:**

STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (JAKs) (6), so we determined whether butein affects constitutive activation of JAK2 in HepG2 cells. To determine the effect of butein on JAK2 activation, HepG2 cells were treated for different time intervals with butein and phosphorylation of JAK2 was analyzed by Western blot. As shown in Fig. 1I, JAK2 was constitutively active in HepG2 cells and pretreatment with butein suppressed this phosphorylation in a time-dependent manner.
**Butein inhibits inducible STAT3 and JAK2 phosphorylation in HCC cells:**

Because IL-6 induces STAT3 phosphorylation (51, 52), we determined whether butein could inhibit IL-6-induced STAT3 phosphorylation. SNU-387 cells, that lack 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 SNU-387 cells incubated with butein for different times, IL-6-induced STAT3 and JAK2 phosphorylation was suppressed by butein in a time-dependent manner. Exposure of cells to butein for 6 h was sufficient to completely suppress IL-6-induced STAT3 and JAK2 phosphorylation (Fig. 2C and 2D).

**Butein inhibits IL-6-inducible Akt phosphorylation in HCC Cells:**

Activated Akt has been shown to play a critical role in the mechanism of action of IL-6. Moreover, activation of Akt has also been linked with STAT3 activation (53). We also examined whether butein could modulate IL-6-induced Akt activation. Treatment of SNU-387 cells with IL-6 induced phosphorylation of Akt and treatment of cells with butein suppressed the activation in a time dependent manner (Fig. 2E). Under these conditions, butein had no effect on the expression of Akt protein.
**Butein suppresses EGF–induced STAT3-dependent reporter gene expression:**

Our above results showed that butein inhibited the phosphorylation and nuclear translocation of STAT3. We next determined whether butein affects STAT3-dependent gene transcription. When PLC/PRF5 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 blocked this increase, indicating specificity. When the cells were pretreated with butein, EGF–induced STAT3 activity was inhibited in a dose-dependent manner (Fig. 2F).

**Predictive analysis of STAT3 activity knockdown by 50% and 100%:**

To test whether a STAT3 activity inhibition would be the primary target of butein mediated impact in the HCC cells, we tested this hypothesis in the virtual tumor cell system. The STAT3 activity was inhibited by 50% and 100% in a growth-factor over-expressed virtual tumor cell. Fig. 3B shows that knocking down STAT3 activity by 50% and 100% did not have an impact on activities of STAT5 and STAT6 as also seen experimentally with butein studies in Fig. 1. In the 100% STAT3 inhibition experiment (Fig. 3C), Akt shows a reduction of 5% with a very minimal reduction of ERK (<2%) but a much higher reduction of Src to 14% also corroborating with the experimental data shown in Fig 1. In Fig. 3D all of the key proliferation, survival, apoptotic and...
angiogenic markers including, Cyclin D1, Bcl-2, Bcl-xL, Mcl-1, survivin (BIRC5) and VEGF also showed a decrease with STAT3 inhibition as seen experimentally in Fig. 3B. Most of these markers were inhibited in the range of 10 to 25% except for Mcl-1 which showed a much higher inhibition of 74% with a 100% activity knockdown of STAT3. Analyzing the increased predictive trends seen with the apoptotic markers such as P53, Puma (BBC) and Caspases 3, 7 and 9 in Fig. 3E would indicate an increase in the apoptotic phenotype. This again corroborates with the experimental data on the decrease in pro-caspase 3 levels and increase in PARP cleavage. These predictions therefore support the hypothesis that butein effects on the proliferative and apoptotic phenotypes are mainly through inhibiting STAT3 activity.

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

Based on the predictive trends and earlier data, we prospectively tested in the HCC cells if STAT3 activation regulated the expression of various gene products involved in cell survival, proliferation, angiogenesis and chemoresistance (11). 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 butein 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 VEGF were modulated by butein treatment in a time-dependent manner with
maximum reduction observed at around 8 h of treatment (Fig. 4B). These results support the predictive analysis seen with STAT3 inhibition on these markers as seen in Fig 3D.

**Butein inhibits the proliferation of HCC cells in a dose and time dependent manner:**
Because butein down regulated the expression of cyclin D1, the gene critical for cell proliferation, we next investigated whether butein inhibits the proliferation of HCC cells by using the MTT method. Butein inhibited the proliferation of HepG2, SNU-387, and PLC/PRF5 cells in a dose and time dependent manner (Fig. 5A).

**Butein activates pro-caspase-3 and induces cleavage of PARP:**
Whether suppression of constitutively active STAT3 in HepG2 cells by butein leads to apoptosis was also investigated. In HepG2 cells treated with butein there was a time-dependent activation of pro-caspase-3 (Fig. 5B). Activation of downstream caspase-3 led to the cleavage of 116 kDa PARP protein into an 85 kDa fragment (Fig. 5C). These results clearly suggest that butein induces caspase-3-dependent apoptosis in HepG2 cells. An increased apoptotic trend supported by various markers was also seen in the predictive data in Fig. 3E, thereby supporting these effects through butein mediated STAT3 inhibition.
Butein potentiates the apoptotic effect of doxorubicin and paclitaxel in HepG2 cells:
Among chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, and paclitaxel, a mitotic inhibitor, have been used for HCC treatment (54). We examined whether butein can potentiate the effect of these drugs. HepG2 cells were treated with butein together with either doxorubicin or paclitaxel, and then apoptosis was measured by the live/dead assay. As shown in Fig. 5D, butein significantly enhanced the apoptotic effects of doxorubicin from 18% to 55% and of paclitaxel from 15 to 42%.

Butein suppresses the growth of human HCC in vivo and STAT3 activation in tumor tissues
We also tested the antitumor potential of butein in vivo via intra-peritoneal administration in a subcutaneous model of human HCC using HCCLM3 cells. Butein at 2mg/kg induced significant inhibition of tumor growth compared with the corn oil-treated controls (Fig. 6A). Two-way repeated measures ANOVA showed a statistically significant difference in tumor growth between the butein-treated and control groups (P treatment = 0.0221, P time < 0.0001, P interaction < 0.0001). At necropsy on day 22 after initial treatment, there was more than 2-fold decreased in tumor growth in the butein-treated group (Mean relative tumor burden= 3.90 ± 0.34) compared with control group (8.46 ± 1.66) (P < 0.01, unpaired t test). We further evaluated the effect of butein on constitutive p-STAT3 levels in HCC tumor tissues by immunohistochemical analysis. And found that butein substantially inhibited the constitutive STAT3 activation in
treated group as compared with control group (Fig. 6B). The effect of butein was also analyzed on the expression of Bcl-2 (marker of survival) and caspase-3 (marker of apoptosis). As shown in Fig. 6B, expression of Bcl-2 was downregulated and that of caspase-3 was substantially increased in butein treated group as compared with control group (Fig. 6B).
4. Discussion and Conclusion

The aim of this study was to determine whether butein exerts its anti-cancer effects in HCC cells through the abrogation of the STAT3 signaling pathway. We found that this chalcone suppressed constitutive and IL-6-inducible STAT3 activation in human HCC cells in parallel with the inhibition of c-Src, JAK1 and JAK2 activation. Butein further downregulated the expression of various STAT3-regulated gene products including, cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and VEGF. This hypothesis was also tested in a virtual predictive tumor cell system and a 50% and 100% STAT3 activity inhibition in the tumor cell generated similar biomarker trends as seen experimentally with butein effects on HCC cells. This chalone also caused the inhibition of proliferation, induced apoptosis as evident by PARP cleavage, and also potentiated the apoptotic effects of doxorubicin and paclitaxel in HCC cells. We subsequently investigated the therapeutic potential of butein therapy in HCC xenograft grown in mouse model. Intra-peritoneal injection of butein into nude mice bearing subcutaneous HCCLM3 xenografts resulted in significant suppression of tumor progression and suppression of expression of p-STAT3 in butein treated tumor tissues.

We observed that butein could suppress both constitutive and inducible STAT3 activation in HCC cells and that these effects were specific to STAT3, as butein had no effect on STAT5 phosphorylation as also confirmed by the predictive studies. The effects of butein on STAT3 phosphorylation 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 (55, 56). Our observations suggest that butein may block cooperation of Src and JAKs involved in tyrosyl phosphorylation of STAT3. We also found that butein-induced inhibition of STAT3 phosphorylation was ROS dependent as treatment with NAC can restore the inhibitory effect of butein on STAT3 activation. Although STAT3 inactivation with increased ROS generation in hematopoietic cells expressing mutant forms of Bcr/Abl has been reported ((57), to the best of our knowledge this is the first report of butein-induced ROS-mediated STAT3 inhibition in HCC cells. We further observed that STAT3, JAK2 and Akt activation induced by IL-6 treatment was also suppressed by butein. We also observed that butein suppressed nuclear translocation and EGF-induced reporter activity of STAT3. This suggests that this chalcone could manifest its effect on both constitutive and inducible STAT3 activation through multiple mechanism(s) or mainly through STAT3 inhibition leading to the other downstream effects as confirmed through the corroboration between the experimental and predictive data. These results are consistent with another report in which butein was found to suppress activation of STAT3 and its regulated gene products in multiple myeloma and head and neck squamous cell carcinoma cell lines(27).

STAT3 phosphorylation plays a critical role in proliferation and survival of tumor cells (9). Several types of cancer, including head and neck cancers (58), multiple myeloma (59), lymphomas, and leukemia (60), 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 cancer cells. Previously, it has been reported that butein can also suppress NF-κB activation in various tumor cells (26). Interestingly, a recent report indicated that STAT3 can prolong NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export (61). Thus it is possible that suppression of STAT3 activation may mediate inhibition of NF-κB activation by butein. Pandey et al. have also reported that butein can induce the expression of a protein tyrosine phosphatase (PTP), SHP-1 with no effect on SH-PTP2 expression in multiple myeloma cells (27). Whether butein-induced inhibition of STAT3 activation involves a protein tyrosine phosphatase (PTP) in HCC cells needs further investigation. Moreover, whether butein also affects other putative inhibitors such as suppressor of cytokine signaling (SOCS1) and protein inhibitors of activated STAT3 (PIAS3) in HCC cells requires further investigation. Also, conditional STAT3 knock-out in different organs and tissues have been developed by various groups (62). For example, the conditional deletion of STAT3 in the liver demonstrated that STAT3 is essential for the induction of all acute phase response genes downstream of IL-6 (63). However, the effects of butein on these conditional STAT3 knock-outs have not been investigated so far.

We also found that butein 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). The inhibition of cyclin D1
expression may account for its ability to induce G2/M phase arrest in human hepatoma cancer cells as reported recently (39). Activation of STAT3 signaling also induces *survivin* gene expression and confers resistance to apoptosis in human breast cancer cells (64). The downregulation of the expression of Bcl-2, Bcl-xL, survivin and Mcl-1 is likely linked with the butein’s ability to induce apoptosis in HCC cells as evident by activation of pro-caspase-3 and cleavage of PARP. These observations are consistent with a recent study in which butein was demonstrated to induce apoptosis in hepatoma cells through generation of ROS and activation of JNK (39). The downmodulation of VEGF expression as reported here may also explain the anti-angiogenic potential of this chalcone that can be investigated in detail.

Doxorubicin and paclitaxel are commonly used chemotherapeutic drugs for the treatment of HCC (54). We further demonstrate that butein substantially potentiates the apoptotic effect of doxorubicin and paclitaxel in HCC cells as evident by esterase staining and can be used in combination with existing chemotherapeutic drugs for treatment of HCC. Whether these *in vitro* observations with butein has any relevance to that *in vivo* was also investigated. Our results also show for the first time that butein significantly suppressed HCC growth in nude mice, downregulated the expression of phospho-STAT3 and Bcl-2, and increased the levels of caspase-3 in treated group as compared to control group. To the best of our knowledge, no prior studies with butein in xenograft cancer models have been reported so far, and our overall findings suggest that butein has a tremendous potential for the treatment of HCC.
Butein has been traditionally used for treatment of pain, thrombotic disease, gastritis, stomach cancer, and parasitic infections in Far Eastern countries such as Korea, Japan, and China and so far no potential toxicity of this polyphenol has been previously reported (65). In Korea, butein has also been long used as a food additive, thereby indicating that it is safe and can be consumed by humans (35). Butein as such has never been tested in humans before and hence its clinically relevant doses are not known as yet. However, in a recent study purified extract of *Rhus verniciflua Stokes*, one of the major sources of butein when administered orally daily at a dose of 900 mg to a gastric cancer patient for five months, exhibited significant anti-cancer effect without any toxic side-effects again emphasizing the safety of butein in humans (66). Thus, overall, our experimental and predictive experiment results indicate that anti-proliferative and pro-apoptotic effects of butein in HCC are mediated through suppression of STAT3 activation and provide a sound basis for pursuing the use of butein further, either alone or in combination with existing therapy, to reduce toxicity and enhance treatment efficacy for HCC.
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Figure Legends

Figure 1. Butein inhibits constitutively active STAT3 in HepG2 cells. A, The chemical structure of butein. B, Butein suppresses phospho-STAT3 levels in a dose dependent manner. HepG2 cells (2×10⁶/ml) were treated with the indicated concentrations of butein 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, AG490 suppresses phospho-STAT3 levels in a dose dependent manner. HepG2 cells (2×10⁶/ml) were treated with the indicated concentrations of AG490 for 24h, 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 and STAT3. D, Butein suppresses phospho-STAT3 levels in a time-dependent manner. HepG2 cells (2×10⁶/ml) were treated with the 50 µM butein 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. E, Butein had no effect on phospho-STAT5 and STAT5 protein expression. HepG2 cells (2×10⁶/ml) were treated with 50 µM butein for the indicated times. Whole-cell extracts were prepared, fractionated on SDS-PAGE, and examined by Western blotting using antibodies against phospho-STAT5 and STAT5. F, Butein causes inhibition of translocation of STAT3 to the nucleus. HepG2 cells (1×10⁵/ml) were incubated with or without 50 µM butein for 6h 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. G, Butein-induced inhibition of STAT3 activation is ROS-dependent. HepG2 cells (2×10⁶/ml) were pre-treated with 5mM NAC for 1h followed by treatment with 50 µM butein for 6h. Whole-cell extracts were prepared, fractionated on SDS-PAGE, and examined by Western blotting using antibodies against phospho-STAT3 and STAT3. H, Butein suppresses phospho-Src levels in a time-dependent manner. HepG2 cells (2×10⁶/ml) were treated with 50 µM butein, 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. I, Butein suppresses phospho-JAK2 levels in a time-dependent manner. HepG2 cells (2×10⁶/ml) were treated with 50 µM butein 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.

**Figure 2.** Butein downregulates IL-6–induced phospho-STAT3 in HCC cells. A, SNU-387 cells (2×10⁶/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, SNU-387 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, SNU-387 (2×10^6/ml) were treated with 50 μM butein 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. The results shown are representative of three independent experiments. D, SNU-387 (2×10^6/ml) were treated with 50 μM butein 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. The results shown are representative of three independent experiments. E, SNU-387 (2×10^6/ml) were treated with 50 μM butein 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/PRF5 cells (5 x 10^5/mL) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with indicated doses of butein for 6 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:** Predictive *In Silico* Virtual tumor platform generated results. A, The figure illustrates a high-level view of the maze of interactions and cross-talks present in the Virtual Tumor Cell platform. B, The figure illustrates the percentage reduction in key STATs following STAT3 inhibition. Both STAT5 and STAT6 show an insignificant reduction with 50% and 100% inhibition of STAT3 activity. C, The figure illustrates the percentage reduction in key kinases – phospho Akt, Erk and Src following 50% and 100% STAT3 activity inhibition. Active SRC shows the maximum reduction as seen experimentally. There is some reduction seen in Akt but minimal to insignificant change seen in ERK. D, The figure illustrates the percentage reduction in tumor markers-CyclinD1, Bcl-2, Bcl-xL, Mcl-1, survivin and VEGF following STAT3 inhibition. Mcl-1 shows a 70% reduction with complete inhibition of STAT3, with the inhibition being 15%, 18%, 17%, 18% and 25% for CyclinD1, Bcl-2, Bcl-xL, survivin and VEGF respectively. E, The figure illustrates the percentage increase in TP53, PUMA, caspase3, 7 and 9 with 50% and 100% STAT3 activity inhibition and the increasing trend of these markers supports the increase in apoptotic endpoint seen experimentally.

**Figure 4.** Butein suppresses STAT3 regulated gene products involved in proliferation, survival and angiogenesis. A, HepG2 cells (2×10⁶/ml) were treated with 50 μM butein 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-X\textsubscript{L}, survivin, Mcl-1, and VEGF antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. B, HepG2 cells (3×10\textsuperscript{5}/ml) were treated with 50μM butein 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 VEGF 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 provided by 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.

**Figure 5.** Butein suppresses the proliferation, activates caspase-3 and potentiates apoptosis induced by doxorubicin and paclitaxel. A, HepG2, SNU-387, and PLC/PRF5 cells(5×10\textsuperscript{3}/mL) were plated in triplicate, treated with indicated concentrations of butein, 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, HepG2 cells were treated with 50 μM butein for the indicated times, whole-cell extracts were prepared, separated on SDS-PAGE, and
subjected to Western blotting against pro-caspase-3 antibody. The same blot were stripped and reprobed with β-actin antibody to show equal protein loading. C, HepG2 cells were treated with 50 μM butein 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. D, Butein potentiates the apoptotic effect of doxorubicin and paclitaxel. HepG2 cells (1×10^6/ml) were treated with 5 μM butein and 10 nM doxorubicin or 5 nM paclitaxel alone or in combination for 24 h at 37°C. Cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. * indicates p value <0.05.

**Figure 6.** Butein inhibits the growth of human HCC in vivo. A, Athymic mice bearing subcutaneous HCCLM3 tumors were treated for 5 times a week for 3 consecutive weeks with 2mg/kg butein (n=8) or corn oil alone (n=6). *=P<0.05 (Two-way ANOVA). Relative tumor volume observed for the respective treatment groups at necroscopy (22 days after initial drug treatment). Student-t- test was used to analyze the data. B, Immunohistochemical analysis of p-STAT3, Bcl-2, and caspase-3 showed the inhibition in expression of p-STAT3, and Bcl-2 and increased levels of caspase-3 expression in butein 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.
Suppression of Signal Transducer and Activator of Transcription 3 Activation by Butein Inhibits Growth of Human Hepatocellular Carcinoma in vivo

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