Cancer Therapy: Preclinical

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

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Abstract

Purpose: Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third cause of global cancer mortality. Increasing evidence suggest that 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, the 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 intraperitoneally, 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 antiproliferative and proapoptotic effects through suppression of STAT3 signaling in HCC both in vitro and in vivo. Clin Cancer Res; 17(6); 1425–39. ©2010 AACR.

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 hepatoxic 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 STAT3 was originally identified as a DNA-binding protein that responds to stimulation by epidermal growth factor (EGF) and interleukin-6 (IL-6) and has an important role in their signaling (6, 7). On 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-activated kinase 2 (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...
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 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 whether 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 useful in an integrated analysis of understanding the mechanism of action and efficacy of novel compounds on physiologic endpoints.

Materials and Methods

Reagents

Butein, Hoechst 33342, MIT, Tris, glycine, NaCl, SDS, BSA, EGF, N-acetyl-l-cysteine (NAC), doxorubicin, and paclitaxel were purchased from Sigma-Aldrich. Butein was dissolved in dimethylsulfoxide as a 20 mmol/L stock solution and stored at 4°C. Further dilution was done in cell culture medium. RPMI 1640, FBS, 0.4% trypan blue vital stain, and antibiotic–antimycotic mixture were obtained from Invitrogen. Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (p-STAT3; Tyr 705), p-Akt, Akt, Bcl-2, Bcl-xl, cyclin D1, survivin, Mcl-1, VEGF, caspase-3, and PARP were obtained from Santa Cruz Biotechnology. Antibodies to phospho-specific Src (Tyr 416), Src, phospho-specific Jak2 (Tyr 1007/1008), and Jak2 were purchased from Cell Signaling Technology. Goat anti-rabbit–horseradish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Sigma-Aldrich. Bacteria-derived recombinant human IL-6 was purchased from ProSpec-Tany TechnoGene Ltd.

Cell lines

HCC cell lines HepG2, SNU-387, and PLC/PRF5 were obtained from American Type Culture Collection. HepG2 and SNU-387 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 × antibiotic–antimycotic solution with 10% FBS. PLC/PRF5 cells were cultured in DMEM supplemented with 10% charcoal-treated fetal bovine serum (FBS) and antibiotics.
cultured in DMEM containing 1 × penicillin–streptomycin solution, nonessential amino acids, sodium pyruvate, and l-glutamine with 10% FBS. HCCLM3 was a kind gift of Prof. Zhao-You Tang at the Liver Cancer Institute (Zhongshan Hospital, Fudan University, Shanghai, China). HCCLM3 were cultured in DMEM (high glucose) containing 1 × antibiotic–antimycotic solution with 10% FBS.

Western blotting

For detection of phosphoproteins, butein-treated whole-cell extracts were lysed in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonylfluoride (PMSF), and 4 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 enhanced chemiluminescence (ECL; GE Healthcare).

To detect STAT3-regulated proteins and PARP, HepG2 cells (2 × 10⁶/mL) were treated with butein for the indicated times. The cells were then washed and extracted by incubation for 30 minutes on ice in 0.05 mL buffer containing 20 mmol/L HEPEs, pH 7.4, 2 mmol/L EDTA, 250 mmol/L NaCl, 0.1% NP-40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L PMSF, 0.5 μg/mL benzamidine, 1 mmol/L DTT, and 1 mmol/L 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, Mcl-1, pro-caspase-3, and PARP, and then detected by chemiluminescence (ECL; GE Healthcare).

Immunocytochemistry for STAT3 localization

HepG2 cells were plated in chamber slides in DMEM containing 10% FBS and allowed to adhere for 24 hours. On next day, following treatment with butein for 6 hours, the cells were fixed with cold acetone for 10 minutes, washed with PBS, and blocked with 5% normal goat serum for 1 hour. The cells were then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1/200). After overnight incubation, the cells were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1/100) for 1 hour and counterstained for nuclei with Hoechst (50 ng/mL) for 5 minutes. Stained cells were mounted with mounting medium (Sigma-Aldrich) and analyzed under a fluorescence microscope (Olympus DP 70).

STAT3 luciferase reporter assay

PLC/PRF5 cells were plated in 96-well plates with 1 × 10⁵ per well in DMEM containing 10% FBS. The STAT3-responsive elements linked to a luciferase (Luc) 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. At 24 hours posttransfection, cells were pre-treated with butein 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) and was normalized to β-galactosidase activity. All luciferase experiments were done in triplicate and repeated 3 or more times.

RNA isolation and reverse transcription

Total cellular RNA was extracted from untreated and butein-treated cells by using TRizol reagent (Invitrogen). Cells were lysed with TRizol before adding in 0.5 mL water-saturated chloroform. The cells were then vortexed and incubated for 3 minutes before centrifugation 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 Multi-Scribe reverse transcriptase in the presence of 1 × RT buffer, 5 mmol/L MgCl₂, 425 μmol/L of each dNTPs, 2 μm 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 40 minutes and a terminating step of 95°C for 5 minutes.

Real-time PCR Analysis

For real-time PCR, 100 ng/μL of total RNA was transcribed as described previously. 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 Bcl-2, Bcl-xl, cyclin D1, VEGF, and Mcl-1, 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, in which 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) 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 (ΔCt) 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 (5 × 10⁴/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-hour 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 at 570 nm was measured by Tecan plate reader.

Live/Dead assay
Apoptosis of cells was determined by Live/Dead assay (Molecular Probes), which measures intracellular esterase activity and plasma membrane integrity as described previously (44). Briefly, 1 × 10⁵ cells were incubated with butein/doxorubicin/paclitaxel alone or in combination for 24 hours at 37°C. Cells were stained with the Live/Dead reagent (5 μmol/L ethidium homodimer, 5 μmol/L calcine-AM) and then incubated at 37°C for 30 minutes. Cells were analyzed under a fluorescence microscope (Olympus DP 70).

In silico study
In silico analysis was done using the physiologically aligned and qualified Virtual Tumor Cell technology, Cellworks Group Inc. (43). The Cellworks Tumor cell platform provides a dynamic and transparent view of human cellular physiology at the proteomics abstraction level. The open-access architecture of the platform provides a framework for different "what-if" analysis and studies in an automated high-throughput methodology. The Cellworks platform is implemented using a 3-layered architecture. The top layer 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 biomolecular level. All the key relevant protein players, 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 3,900 intracellular molecules. The platform includes important signaling pathways comprising growth factors such as EGF receptor, platelet-derivated growth factor receptor A, fibroblast growth factor receptor, c-MET, VEGF receptor, and insulin-like growth factor I receptor, cell-cycle regulators, mTOR signaling, p53 signaling cascade, cytokine pathways such as IL-1, IL-4, IL-6, IL-12, and TNF; lipid mediators, and tumor metabolism (Fig. 3A). The platform has been correlated against an extensive set of predefined 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 semiquantitative 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) were implanted subcutaneously in the right flank with (3 × 10⁵) HCCLM3 cells/100 μL saline. When tumors have reached 0.5 cm in diameter, mice received intraperitoneal 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) was calculated using the formula: V = π/6 × length × width². 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, deparaffinized in xylene, dehydrated in graded alcohol, and finally hydrated in water. Antigen retrieval was carried out by boiling the slide in 10 mmol/L sodium citrate (pH 6.0) for 30 minutes. Immunohistochemistry was done following manufacturer’s instructions (Dako LSAB kit). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Nonspecific binding was blocked by incubation in the blocking reagent in the LSAB kit (Dako), according to the manufacturer’s instructions. Sections were incubated overnight with primary antibodies as follows: anti–p-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 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 tetrahydrochloride 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: 20×). Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

Statistical analysis

Statistical analysis was done by Student’s t test and 2-way ANOVA. A value of P < 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. In addition, 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 Figure 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 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 Figure 1B, butein inhibited the constitutive activation of STAT3 in HepG2 cells in a dose-dependent manner, with maximum inhibition occurring at 50 μmol/L. Butein had no effect on the expression of STAT3 protein (Fig. 1D, bottom). Whether butein affects the activation of other STAT proteins in HepG2 cells was also investigated. Under the conditions in which 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 shown in Figure 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. Figure 1F clearly shows 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 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 hour before treatment with butein and STAT3 activation was monitored by Western blot analysis. We found that decrease in phosphorylated STAT3 observed on 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 affects 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 nonphosphorylated Src kinases remained unchanged under the same conditions. The predictive data in Figure 3C also shows a significant inhibition of Src kinase in the tumor cell and clearly correlates with the experimental data trends in Figure 1H.

**Butein suppresses constitutive activation of JAK2**

STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (JAKs; ref. 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 blotting. As shown in Figure 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, which 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 minutes, with maximum phosphorylation observed at 30 to 60 minutes (Fig. 2A). It 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, the phosphorylation of IL-6-induced STAT3 and JAK2 was suppressed by butein in a time-dependent manner. Exposure of cells to butein for 6 hours was sufficient to completely suppress IL-6-induced STAT3 and JAK2 phosphorylation (Fig. 2C and D).

**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 aforementioned 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 p-STAT3-Luc plasmid and then stimulated with EGF, STAT3-mediated
Figure 3. Predictive *in silico* Virtual Tumor Cell 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—p-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—cyclin D1, 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 cyclin D1, Bcl-2, Bcl-xL, survivin, and VEGF, respectively. E, the figure illustrates the percentage increase in TP53, PUMA, caspase-3, -7, and -9 with 50% and 100% STAT3 activity inhibition, and the increasing trend of these markers supports the increase in apoptotic end point seen experimentally.
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–overexpressed virtual tumor cell system. Figure 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 Figure 1. In the 100% STAT3 inhibition experiment (Fig. 3C), Akt shows a reduction of 5% with a very minimal reduction of extracellular signal-regulated kinase (ERK; <2%) but a much higher reduction of Src to 14%, also corroborating with the experimental data shown in Figure 1. In Figure 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 Figure 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 caspase-3, -7, and -9 in Figure 3E indicates a pre-apoptotic effect. This again corroborates with the experimental data on the decrease in procaspase-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**

On the basis of the predictive trends and earlier data, we prospectively tested in the HCC cells whether STAT3 activity was modulated by butein. Two-way repeated-measures ANOVA showed a statistically significant difference in tumor growth between the butein-treated and control groups (Fig. 6A). The tumor burden was also analyzed on the expression of Bcl-2 (marker of constitutive STAT3 activation in tumor tissues) and STAT3 expression in both groups (8.46 ± 3.90 vs 1.66; P = 0.34) compared with the control group (Pinteraction < 0.0001, Ptime < 0.0001). At necropsy on day 22 after initial treatment, there was more than 2-fold decrease in tumor growth in the butein-treated group (mean relative tumor burden = 3.90 ± 0.34) compared with the 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 the treated group as compared with the control group (Fig. 6B). The level of butein was also analyzed on the expression of Bcl-2 (marker of constitutive STAT3 activity). 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 decrease in expression of procaspase-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 Figure 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 Figure 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 tissue**

We also tested the antitumor potential of butein in vivo via intraperitoneal administration in a subcutaneous model of human HCC, using HCCLM3 cells. Butein at 2 mg/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 (Pinteraction = 0.0221, Ptime < 0.0001). Because butein downregulated 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).
survival) and caspase-3 (marker of apoptosis). As shown in Figure 6B, expression of Bcl-2 was downregulated and that of caspase-3 was substantially increased in the butein-treated group as compared with the control group (Fig. 6B).

Discussion and Conclusion

The aim of this study was to determine whether butein exerts its anticancer 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 chalcone 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. Intraperitoneal injection of butein into nude mice bearing subcutaneous HCC-LM3 xenografts resulted in significant suppression of tumor progression and inhibition 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 because butein had minimal 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 (53, 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

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 × 10^5/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. *, P < 0.05. B, HepG2 cells were treated with 50 μmol/L 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 μmol/L 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 × 10^5/mL) were treated with 5 μmol/L butein and 10 nmol/L doxorubicin or 5 nmol/L paclitaxel alone or in combination for 24 hours 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. *, P < 0.05.
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 acetyl transferase 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 and colleagues 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 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.

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

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 2 mg/kg butein (n = 8) or corn oil alone (n = 6), *, P < 0.05 (2-way ANOVA). Relative tumor volume observed for the respective treatment groups at necropsy (22 days after initial drug treatment). Students’ 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 20× magnification × . Arrowheads indicate positive staining for a given biomarker.
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 ability of butein to induce apoptosis in HCC cells, as evident by activation of caspase-3 and cleavage of PARP. These observations are consistent with a recent study in which butein was shown 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 antiangiogenic potential of this chalcone that needs further investigation.

Doxorubicin and paclitaxel are commonly used chemotherapeutic drugs for the treatment of HCC (54). We further show 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 the treatment of HCC. Whether these in vitro observations with butein have 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 p-STAT3 and Bcl-2, and increased the levels of caspase-3 in the treated group as compared with the 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 the 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 employed as a food additive, thereby indicating that it is safe and can be consumed by humans (35). 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 5 months, exhibited significant anticancer effect without any toxic side effects, again emphasizing the safety of butein in humans (66). Overall, our results suggest that butein suppresses HCC growth in vitro and in vivo through modulation of STAT3 activation pathway. Further studies are required before its clinical potential is fully realized in HCC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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

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