

Identification of β -escin as a novel inhibitor of STAT3/JAK2 signaling pathway that suppresses proliferation and induces apoptosis in human hepatocellular carcinoma cells

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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.

ABSTRACT

The activation of signal transducers and activators of transcription 3 (STAT3) has been linked with the proliferation, survival, invasion and angiogenesis of a variety of human cancer cells, including hepatocellular carcinoma (HCC). Agents that can suppress STAT3 activation have potential for prevention and treatment of HCC. In the present report, we tested an agent, β -escin, for its ability to suppress STAT3 activation. We found that β -escin, a pentacyclic triterpenoid, inhibited both constitutive and interleukin-6-inducible STAT3 activation in a dose- and time-dependent manner in HCC cells. The suppression was mediated through the inhibition of activation of upstream kinases c-Src, Janus-activated kinase 1, and Janus-activated kinase 2. Vanadate treatment reversed the β -escin -induced down-regulation of STAT3, suggesting the involvement of a tyrosine phosphatase. Indeed, we found that β -escin induced the expression of tyrosine phosphatase SHP1 that correlated with down-regulation of constitutive STAT3 activation. β -escin also downregulated the expression of STAT3-regulated gene products, such as cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1 and vascular endothelial growth factor. Finally, β -escin inhibited proliferation, and also substantially potentiated the apoptotic effects of paclitaxel and doxorubicin in HCC cells. Overall, these results suggest that β -escin is a novel blocker of STAT3 activation that may have a potential in suppression of proliferation and chemosensitization in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid tumors, ranking fifth in incidence and third in mortality worldwide (Bruix et al., 2004). 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 (Thorgeirsson et al., 2006). Surgery remains to be the first choice for HCC, however, tumor size, hepatic functional reserve and/or portal hypertension may all restrict surgical ablation (Kerr and Kerr, 2009). Currently, first line drugs used for HCC include doxorubicin, fluorouracil, cisplatin, and mitomycin, but most of these are non-selective cytotoxic molecules with significant side effects (Kerr and Kerr, 2009). Hence, novel agents that are cheap, non-toxic and efficacious are urgently needed.

STAT proteins were originally discovered as latent cytoplasmic transcription factors a decade ago (Ihle, 1996). There are seven known mammalian STAT proteins, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, which are involved in cell proliferation, differentiation, and apoptosis (Aggarwal et al., 2009; Yu and Jove, 2004). One STAT family member, STAT3, is often constitutively active in many human cancer cells, including multiple myeloma, leukemia, lymphoma, and solid tumors (Yu et al., 2009). STAT3 can also be activated by certain interleukins (e.g., IL-6) and growth factors (e.g., EGF). 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 (Yue and Turkson, 2009). In addition, the role of c-Src kinase has been shown in STAT3 phosphorylation (Ihle, 1996). Among the STATs, STAT3 is perhaps the most intimately linked to tumorigenesis, is often constitutively activated in many human cancer cells including HCC (Niwa et al., 2005). 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 (Sun et al., 2008).

One potential source of STAT3 inhibitors is agents derived from natural sources as around 70% of all anti-cancer drugs being used in clinical therapy are isolated from natural sources or bear a close structural relationship to compounds of natural origin (Newman, 2008). We describe here the identification of a compound derived from the seeds of horse chestnut (*Aesculus hippocastanum*), called, β -escin or aescin which has a potential in prevention and treatment of cancer. β -escin is a pentacyclic triterpenoid that has been previously reported to exhibit anti-oedematous, anti-inflammatory and anti-carcinogenic properties in various disease models (Sirtori, 2001). For example, it was found that β -escin sodium can inhibit the growth of various tumor cell lines (a human oral mucosal cell line (KB cells), a mice liver cancer cell line (H22), and a mice sarcoma cell line (S180)) and their transplant tumors (Guo et al; 2003). It has also been reported that β -escin can suppress colonic aberrant crypt foci formation in rats and inhibit growth of colon cancer cells (Patlolla et al., 2006). Moreover, this pentacyclic triterpenoid was recently found to exhibit significant anti-tumor effects in human

hepatocellular carcinoma both *in vitro* and *in vivo* (Zhou et al., 2009). These reports suggest that β -escin may be a suitable candidate for cancer treatment.

Because of the critical role of STAT3 in HCC survival, proliferation, invasion, and angiogenesis, we investigated whether β -escin can mediate its effects in part through the suppression of the STAT3 pathway. We found that β -escin 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.

METHODS

Reagents

β -escin or aescin (Sirtori, 2001) for experiments was kindly supplied by Wuxi Gorunjie Technology Co., Ltd. China. Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, BSA, AG490, epidermal growth factor (EGF), doxorubicin and paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO). Caspase inhibitor zVAD-FMK was obtained from Promega. β -escin 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. Rabbit polyclonal antibodies to STAT3 and STAT5 and mouse monoclonal antibodies against phospho-STAT3 (Tyr 705) and phospho-STAT5, phospho-Akt, Akt, Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, SHP1, VEGF, procaspase-3, cleaved caspase-3, and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-specific Src (Tyr 416), Src, phospho-specific JAK1 (Tyr 1022/1023), JAK1, 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 and PLC/PRF5 were obtained from American Type Culture Collection (Manassass, VA). Human hepatoma HUH-7 cell line was provided by Prof. Kam M. Hui at National Cancer Center, Singapore. Wild type and STAT3 knock out mouse fibroblasts were a kind gift from Dr. Valeria Poli, University of Turin, Italy. HepG2, HUH-7, wild type and STAT3 knock out mouse fibroblasts 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.

Western blotting

For detection of phospho-proteins, β -escin -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 NaVO_4). 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; Amersham Pharmacia Biotech).

To detect STAT3-regulated proteins and PARP, HepG2 cells (2×10^6 /ml) were treated with β -escin 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; Amersham).

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, 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 1×10^4 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 (Roche). At 24 h posttransfection, cells were pretreated with β -escin for 4 h and then stimulated with EGF for additional 2 h before being washed and lysed in luciferase lysis buffer (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 three or more times.

MTT assay

The antiproliferative effect of β -escin against HCC cells was determined by the MTT dye uptake method as described previously (Bhutani et al., 2007). Briefly, the cells (5×10^3 /ml) were incubated in triplicate in a 96-well plate in the presence or absence of indicated concentration of β -escin in a final volume of 0.2 ml for different time intervals at 37 °C. Thereafter, 20 μ l 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 (Bhutani et al., 2007). Briefly, 1×10^6 cells were incubated with β -escin /doxorubicin/ paclitaxel alone or in combination for the indicated time intervals at 37°C. Cells were stained with the Live/Dead reagent (5 μ M ethidium homodimer, 5 μ M calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Olympus DP 70, Japan).

Statistical analysis:

Statistical analysis was performed by one way ANOVA. The probability (p) value less than 0.05 were considered statistically significant.

RESULTS

We investigated the effect of β -escin on constitutive and IL-6-inducible STAT3 activation in HCC cells. We also evaluated the effect of β -escin on various mediators of cellular proliferation, cell survival, and apoptosis. The structure of β -escin is shown in Fig 1A. The dose and duration of β -escin used to modulate STAT3 activation did not affect cell viability, indicating that downregulation of STAT3 was not due to cell killing (data not shown).

β -escin inhibits constitutive STAT3 phosphorylation in HepG2 cells:

The ability of β -escin to modulate constitutive STAT3 activation in HCC cells was investigated. HepG2 cells were incubated with different concentrations of β -escin 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, β -escin inhibited the constitutive activation of STAT3 in HepG2 cells in a dose-dependent manner, with maximum inhibition occurring at around 30 μ M. β -escin had no effect on the expression of STAT3 protein (Fig. 1B; lower panel). As shown in Fig. 1C, the inhibition was time-dependent, with maximum inhibition occurring at around 4-6 h, again with no effect on the expression of STAT3 protein (Fig. 1C; lower panel).

Effect of β -escin on STAT3 phosphorylation is specific:

Whether β -escin affects the activation of other STAT proteins in HepG2 cells was also investigated. Under the conditions where β -escin completely inhibited STAT3

phosphorylation, it altered neither the levels of constitutively phosphorylated STAT5 nor the expression of STAT5 proteins (Fig. 1D).

β-escin 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 (Brierley and Fish, 2005), we determined whether β-escin suppresses nuclear translocation of STAT3. Fig. 1E clearly demonstrates that β-escin inhibited the translocation of STAT3 to the nucleus in HepG2 cells.

β-escin inhibits inducible STAT3 phosphorylation in HCC cells:

Because IL-6 induces STAT3 phosphorylation (Moran et al., 2008), we determined whether β-escin could inhibit IL-6-induced STAT3 phosphorylation. HUH-7 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 HUH-7 cells incubated with β-escin for different times, IL-6-induced STAT3 phosphorylation was suppressed by β-escin in a time-dependent manner. Exposure of cells to β-escin for 3-4 h was sufficient to completely suppress IL-6-induced STAT3 phosphorylation (Fig. 2C).

β-escin 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 (Chen et al., 1999). We also examined whether β-escin could modulate IL-6-induced Akt activation. Treatment of HUH-7 cells with IL-6 induced phosphorylation of Akt and treatment of cells with β-escin suppressed the activation in a time dependent manner (Fig. 2D). Under these conditions, β-escin had no effect on the expression of Akt protein.

β-escin suppresses EGF-induced STAT3-dependent reporter gene expression:

Our above results showed that β-escin inhibited the phosphorylation and nuclear translocation of STAT3. We next determined whether β-escin 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 β-escin, EGF-induced STAT3 activity was inhibited in a dose-dependent manner (Fig. 2E).

β-escin suppresses constitutive activation of c-Src:

STAT3 has also been reported to be activated by soluble tyrosine kinases of the Src kinase families (Schreiner et al., 2002). Hence, we determined whether β-escin on constitutive activation of Src kinase in HepG2 cells. We found that β-escin suppressed

the constitutive phosphorylation of c-Src kinases (Fig. 3A). The levels of non-phosphorylated Src kinases remained unchanged under the same conditions.

β-escin suppresses constitutive activation of JAK1 and JAK2:

STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (JAKs) (Ihle, 1996), so we determined whether β-escin affects constitutive activation of JAK1 in HepG2 cells. We found that β-escin suppressed the constitutive phosphorylation of JAK1 (Fig. 3B). The levels of non-phosphorylated JAK1 remained unchanged under the same conditions (Fig. 3B, bottom panel). To determine the effect of β-escin on JAK2 activation, HepG2 cells were treated for different time intervals with β-escin and phosphorylation of JAK2 was analyzed by Western blot. As shown in Fig. 3C, JAK2 was constitutively active in HepG2 cells and pretreatment with β-escin suppressed this phosphorylation in a time-dependent manner.

Tyrosine phosphatases are involved in β-escin-induced inhibition of STAT3 activation:

Because protein tyrosine phosphatases have also been implicated in STAT3 activation (Han et al., 2006), we determined whether β-escin -induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a protein tyrosine phosphatase (PTPase). Treatment of HepG2 cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate prevented the β-escin -induced inhibition of STAT3 activation (Fig. 3D). This suggests that tyrosine phosphatases are involved in β-escin -induced inhibition of STAT3 activation.

β-escin induces the expression of SHP1 in HCC cells:

SHP1 is a SH-2 containing tyrosine phosphatase involved in the suppression of a variety of cytokine signals, including STAT3 (Calvisi et al., 2006). We therefore examined whether β-escin can modulate expression of SHP1 in HepG2 cells. Cells were incubated with different concentrations of β-escin for 4 h, whole cell extracts were prepared and examined for SHP1 protein by Western blot analysis. As shown in Fig. 3E, β-escin induced the expression of SHP1 protein in HepG2 cells in a dose-dependent manner, with maximum expression at 15-30 μM. This stimulation of SHP1 expression by β-escin correlated with down-regulation of constitutive STAT3 activation in HepG2 cells (Fig. 1B).

β-escin downregulates the expression of cyclin D1, Bcl-2, Bcl-xL, survivin, and VEGF:

STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival, proliferation, angiogenesis and chemoresistance (Aggarwal et al., 2009). 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 β-escin treatment. Their expression decreased in a time-dependent manner, with maximum suppression observed at around 24 h (Fig. 4).

β-escin inhibits the proliferation of HCC cells in a dose and time dependent manner:

Because β -escin down regulated the expression of cyclin D1, the gene critical for cell proliferation, we investigated whether β -escin inhibits the proliferation of HCC cells by using the MTT method. β -escin inhibited the proliferation of HepG2, HUH-7 and PLC/PRF5 cells in a dose and time dependent manner (Fig. 5A).

β -escin activates caspase-3 and induces cleavage of PARP:

Whether suppression of constitutively active STAT3 in HepG2 cells by β -escin leads to apoptosis was also investigated. In HepG2 cells treated with β -escin there was a time-dependent cleavage of pro-caspase-3 and increased expression of cleaved-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). Moreover, the treatment with broad-spectrum caspase inhibitor zVAD-FMK prevented β -escin-induced apoptosis as examined by western blot for PARP cleavage (Fig. 5D). These results clearly suggest that β -escin induces caspase-3-dependent apoptosis in HepG2 cells.

β -escin 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 (Jin et al., 2009). We examined whether β -escin can potentiate the effect of these drugs. HepG2 cells were treated with β -escin together with either doxorubicin or paclitaxel, and then apoptosis was measured by the live/dead assay. As shown in Fig. 6, β -escin substantially

enhanced the apoptotic effects of doxorubicin from 18% to 60% and of paclitaxel from 15 to 45%.

STAT3 deletion reduces β -escin-induced apoptosis:

We next determined the apoptotic effect of β -escin on STAT3 gene deleted mouse embryonic fibroblasts that lack activation of STAT3. Apoptotic effects of β -escin were measured through esterase staining (live and dead) assay. Results shown in Fig. 6B indicate that β -escin -induced apoptosis was effectively abolished in the STAT3 gene deleted (18%) as compared to (40%) in wild type fibroblasts. These results suggest that induction of apoptosis is mediated through the suppression of STAT3 by β -escin.

DISCUSSION

The aim of this study was to determine whether β -escin exerts its anti-cancer effects in HCC cells through the abrogation of the STAT3 signaling pathway. We found that this triterpene suppressed constitutive and IL-6-inducible STAT3 activation in human HCC cells in parallel with the inhibition of c-Src, JAK1 and JAK2 activation. β -escin also downregulated the expression of STAT3-regulated gene products including cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, and VEGF. It 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 report for the first time that β -escin could suppress both constitutive and inducible STAT3 activation in HCC cells and that these effects were specific to STAT3, as β -escin had no effect on STAT5 phosphorylation. In comparison, higher dose (100 μ M to 30 μ M) and longer exposure (24 h vs. 6h) of AG490, a rationally designed JAK2 inhibitor (Meydan et al., 1996) was needed to completely suppress STAT3 phosphorylation in HCC cells (Fuke et al., 2007). The effects of β -escin on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases c-Src, JAK1 and JAK2. Previous studies have indicated that Src and JAK1 kinase activities cooperate to mediate constitutive activation of STAT3 (Garcia et al., 2001). Our findings suggest that β -escin may block cooperation of Src and JAKs involved in tyrosyl phosphorylation of STAT3. How β -escin inhibits IL-6 induced STAT3 activation was also investigated. The roles of JAK2, mitogen-activated protein kinase, and Akt have been implicated in IL-6-induced STAT3 activation (Chen et al., 1999). We found

that IL-6-induced Akt activation was also suppressed by β -escin. We also observed that β -escin suppressed nuclear translocation and EGF induced reporter activity of STAT3. Thus, the recent reported anti-tumor effects of β -escin on human hepatocellular carcinoma (Zhou et al., 2009) could be due to inhibition of STAT3 signaling pathway as described here .

STAT3 phosphorylation plays a critical role in proliferation and survival of tumor cells (Yue and Turkson, 2009). Several types of cancer, including head and neck cancers (Song and Grandis, 2000), multiple myeloma (Bhardwaj et al., 2007), lymphomas, and leukemia (Zhang et al., 2002), 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 β -escin can also suppress NF- κ B activation in the brain of rats and different human tumor cells (Xiao and Wei; 2005; Harikumar et.al; 2010). Whether suppression of STAT3 activation by β -escin is linked to inhibition of NF- κ B activation is not clear. However, a recent report indicated that STAT3 prolongs NF- κ B nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF- κ B nuclear export (Lee et al., 2009). Thus it is possible that suppression of STAT3 activation may mediate inhibition of NF- κ B activation by β -escin.

We also found evidence that the β -escin induced inhibition of STAT3 activation involves a protein tyrosine phosphatase (PTP). Numerous PTPs have been implicated in STAT3 signaling including SHP1, SH-PTP2, TC-PTP, PTEN, PTP-1D, CD45, PTP-epi, low molecular weight (LMW), and PTP (Kunnumakkara et al., 2009). SHP1 is

implicated in negative regulation of JAK/STAT signaling pathways (Calvisi et al., 2006) and it has been found that loss of SHP1 may contribute to the activation of JAK or STAT proteins in cancer (Wu et al., 2003). Indeed we observed that β -escin stimulates the expression of SHP1 protein in HCC cells, which correlated, with downregulation of constitutive STAT3 phosphorylation. Whether it also affects other putative inhibitors such as suppressor of cytokine signaling (SOCS1) and protein inhibitors of activated STAT3 (PIAS3) requires further investigation.

We also found that β -escin 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). β -escin has been previously reported to induce growth arrest at the G1-S phase in human colon cancer HT29 cells (Patlolla et al., 2006). Since cyclin D1 has been closely linked with G1-S phase arrest, down regulation of cyclin D1 as shown here may mediate this effect. Mcl-1 is highly expressed in tumor cells (Epling-Burnette et al., 2001), and Niu *et al.* (Niu et al., 2002) reported that inhibition of STAT3 by a Src inhibitor results in down-regulation of expression of the *Mcl-1* gene in melanoma cells. In addition, activation of STAT3 signaling induces *survivin* gene expression and confers resistance to apoptosis in human breast cancer cells (Gritsko et al., 2006). Bcl-2 and Bcl-xL can also block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance (Seitz et al., 2009). Thus, the downregulation of the expression of Bcl-2, Bcl-xL, survivin and Mcl-1 is likely linked with the β -escin's ability to induce apoptosis in HCC cells as evident by activation of caspase-3 and cleavage of PARP. Furthermore,

we observed that apoptotic effects of β -escin were abolished in STAT3 deleted cells, thus strengthening our hypothesis that antiproliferative and pro-apoptotic effects of β -escin are mediated through the abrogation of the STAT3 signaling pathway. The downmodulation of VEGF expression as reported here can explain the anti-angiogenic effects of this triterpene as described previously in rat aortic disk assay (Zhao et al., 2007)

Doxorubicin and paclitaxel are commonly used chemotherapeutic drugs for the treatment of HCC (Jin et al., 2009). We further demonstrate that β -escin 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. Several studies in animals suggest that β -escin is very well tolerated and has potential against inflammatory diseases and cancers (Sirtori, 2001). β -escin has also shown satisfactory evidence for a clinically significant activity in chronic venous insufficiency (CVI), haemorrhoids and post-operative oedema and is currently in clinical trials in HIV patients (Grases et al., 2004; Sirtori, 2001). We contend that the apparent pharmacologic safety of β -escin and its ability to down-regulate the expression of several genes involved in cell proliferation, survival and invasion provides a sufficient rationale for testing β -escin in patients for treatment of HCC and other cancers harboring active STAT3.

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FOOT NOTES

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Legends for Figures

Figure 1. β -escin inhibits constitutively active STAT3 in HepG2 cells. A, The structure of β -escin. B, β -escin suppresses phospho-STAT3 levels in a dose dependent manner. HepG2 cells (2×10^6 /ml) were treated with the indicated concentrations of β -escin for 4h, after which whole-cell extracts were prepared, and 30 μ g of protein was resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. C, β -escin suppresses phospho-STAT3 levels in a time-dependent manner. HepG2 cells (2×10^6 /ml) were treated with the 30 μ M β -escin for the indicated times, after which western blotting was performed as described for panel B. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. D, β -escin had no effect on phospho-STAT5 and STAT5 protein expression. HepG2 cells (2×10^6 /ml) were treated with 100 μ M β -escin 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. E, β -escin causes inhibition of translocation of STAT3 to the nucleus. HepG2 cells (1×10^5 /ml) were incubated with or without 30 μ M β -escin 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.

Figure 2. β -escin downregulates IL-6-induced phospho-STAT3. A, HUH-7 cells (2×10^6 /mL) were treated with IL-6 (10 ng/ml) for indicated times, whole cell extracts were prepared, and phospho-STAT3 was detected by Western blot as described in

Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. B, HUH-7 cells (2×10^6 /mL) were treated with indicated concentrations of IL-6 for 15 minutes, whole cell extracts were prepared, and phospho-STAT3 was detected by Western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. C, HUH-7 (2×10^6 /ml) were treated with 30 μ M β -escin 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, HUH-7 (2×10^6 /ml) were treated with 30 μ M β -escin 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. E, PLC/PRF5 cells (5×10^5 /mL) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with indicated doses of β -escin for 4 h and then stimulated with EGF (100 ng/mL) for 2 h. Whole-cell extracts were then prepared and analyzed for luciferase activity. The results shown are representative of three independent experiments. * indicates p value <0.05.

Figure 3. A, β -escin suppresses phospho-Src levels in a time-dependent manner. HepG2 cells (2×10^6 /ml) were treated with 30 μ M β -escin, after which whole-cell extracts were prepared and 30 μ g aliquots of those extracts were resolved on 10% SDS-PAGE,

electrotransferred onto nitrocellulose membranes, and probed for phospho-Src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. B, β -escin suppresses phospho-JAK1 levels in a time-dependent manner. HepG2 cells (2×10^6 /ml) were treated with 30 μ M β -escin for indicated time intervals, after which whole-cell extracts were prepared and 30 μ g portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK1 antibody. The same blots were stripped and reprobed with JAK1 antibody to verify equal protein loading. C, β -escin suppresses phospho-JAK2 levels in a time-dependent manner. HepG2 cells (2×10^6 /ml) were treated with 30 μ M β -escin for indicated time intervals, after which whole-cell extracts were prepared and 30 μ g portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK2 antibody. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. D, Pervanadate reverses the phospho-STAT3 inhibitory effect of β -escin. HepG2 cells (2×10^6 /ml) were treated with the indicated concentrations of pervanadate and 100 μ M β -escin for 6 h, after which whole-cell extracts were prepared and 30 μ g portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. E, β -escin induces the expression of SHP1 protein in HepG2 cells. HepG2 cells (2×10^6 /ml) were treated with indicated concentrations of β -escin for 4h, after which whole-cell extracts were prepared and 30 μ g portions of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for

SHP1 antibody. The same blots were stripped and reprobed with β -actin antibody to verify equal protein loading.

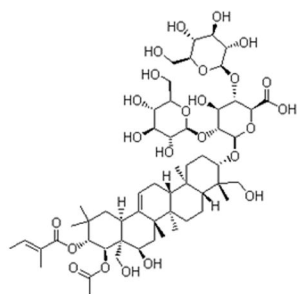
Figure 4. β -escin suppresses STAT3 regulated gene products involved in proliferation, survival and angiogenesis. HepG2 cells (2×10^6 /ml) were treated with 30 μ M β -escin for indicated time intervals, after which whole-cell extracts were prepared and 30 μ g portions of those extracts were resolved on 10% SDS-PAGE, membrane sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-X_L, survivin, Mcl-1, and VEGF antibodies. The same blots were stripped and reprobed with β -actin antibody to verify equal protein loading.

Figure 5. β -escin suppresses the proliferation, and activates caspase-3. A, HepG2, HUH-7 and PLC/PRF5 cells (5×10^3 /mL) were plated in triplicate, treated with indicated concentrations of β -escin, and then subjected to MTT assay after 24, 48 and 72 hours to analyze proliferation of cells. Standard deviations between the triplicates are indicated. B, HepG2 cells were treated with 30 μ M β -escin for the indicated times, whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against pro-caspase-3 and cleaved caspase-3 antibodies. The same blot were stripped and reprobed with β -actin antibody to show equal protein loading. C, HepG2 cells were treated with 30 μ M β -escin 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, Caspase inhibitor suppresses the β -escin -induced apoptosis of

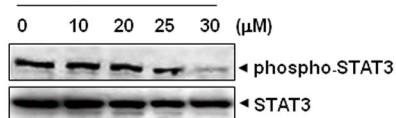
HepG2 cells. HepG2 cells (2×10^6 /ml) were preincubated with 30 μ M β -escin and 10 μ M zVAD-FMK alone or in combination for 24 h at 37°C. Thereafter, whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot against PARP antibody. The same blot was stripped and reprobed with β -actin antibody to show equal protein loading.

Figure 6. β -escin potentiates the apoptotic effect of doxorubicin and paclitaxel. A, HepG2 cells (1×10^6 /ml) were treated with 10 μ M β -escin 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. The results shown are % apoptosis and are representative of three independent experiments. * indicates p value <0.05. B, Deletion of STAT3 inhibited the apoptotic effect of β -escin. Wild type and STAT3 deleted fibroblasts were treated with 30 μ M β -escin for 12 h and analyzed for the percentage of apoptosis by Live/Dead assay. 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.

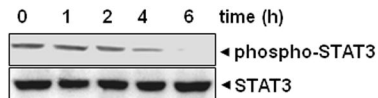
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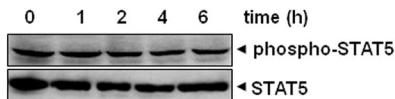
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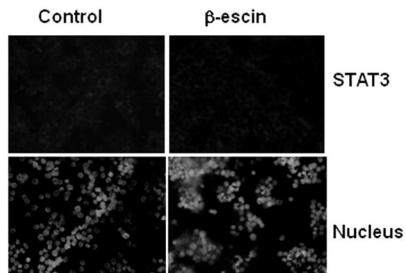
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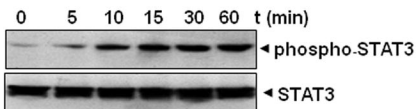
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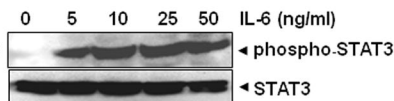
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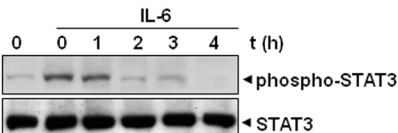
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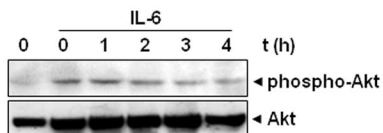
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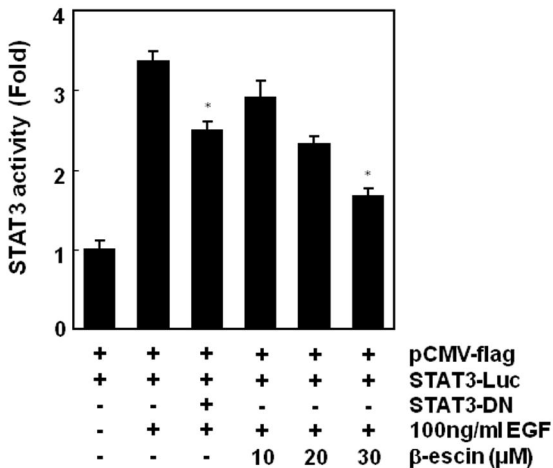
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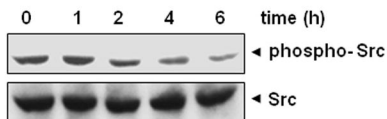
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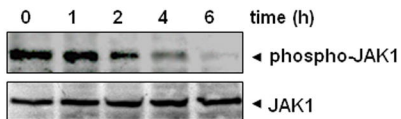
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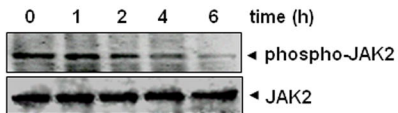
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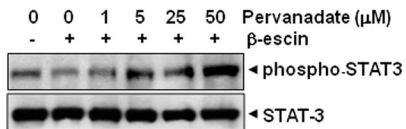
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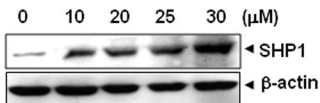
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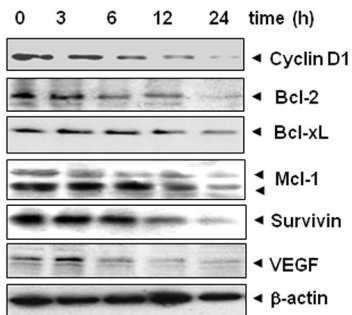


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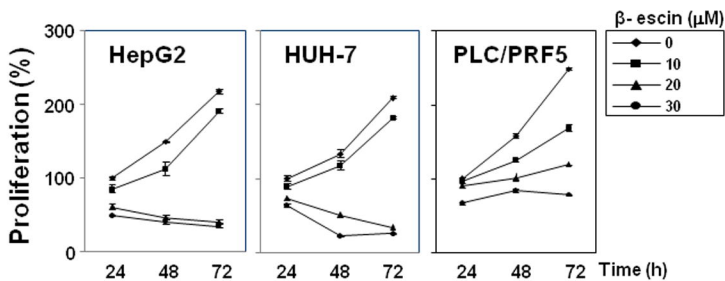


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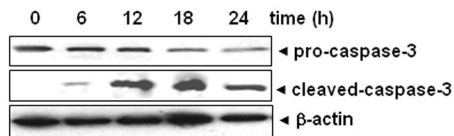




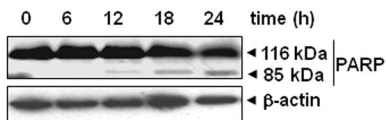
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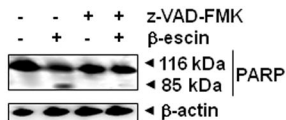
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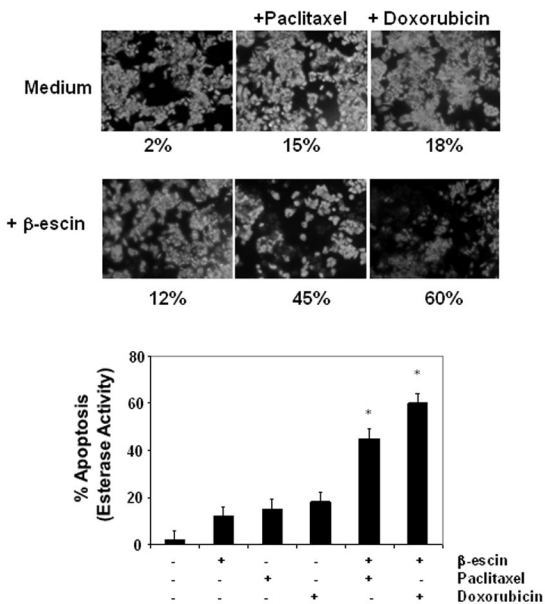
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