Targeting signal transducer and activator of transcription-3 (STAT-3) for the prevention and treatment of hepatocellular carcinoma

Tan M.L. S.\(^1\) and Sethi G.\(^2\)

Department of Pharmacology, Faculty of Science, National University of Singapore
10 Kent Ridge Road, Singapore 117546

ABSTRACT
The aim of the current project is to determine whether \(\beta\)-escin exhibits anti-proliferative effects against hepatocellular carcinoma (HCC) cells through suppression of the signal transducer and activator of transcription 3 (STAT-3) activation pathway. We found that this triterpene inhibited both constitutive and inducible STAT-3 activation in HCC cells. The suppression was mediated through the inhibition of activation of the upstream kinases Janus-like Kinase (JAK) 1, and JAK-2. When compared with AG490, \(\beta\)-escin was a more rapid (4hr vs. 48 hr) and more potent (30\(\mu\)M vs, 100\(\mu\)M) inhibitor of STAT-3 phosphorylation and cell proliferation. Moreover, we found that this triterpenoid also significantly potentiated the apoptotic effects of paclitaxel and doxorubicin in HCC cells. Overall, these results suggest that \(\beta\)-escin is a novel blocker of STAT-3 activation and thus may have potential in suppression of tumor cell proliferation and reversal of chemoresistance in HCC cells.

INTRODUCTION
Hepatocellular carcinoma (HCC) is one of the most lethal malignancies, and is also one of the four most prevalent malignant diseases of adults in Southeast Asian countries like China, Taiwan and Korea (Kern \textit{et. al}, 2002). Several etiologic factors have been classified as high-risk factors in association with HCC, including exposure to aflatoxin B1, and infection with hepatitis B and hepatitis C viruses (Seow \textit{et. al}, 2001). Constitutively active STAT-3 has been demonstrated in approximately 50% of HCC and recent studies have demonstrated that STAT-3 antisense oligonucleotide and STAT-3-specific siRNA can all induce apoptosis and inhibit tumor growth via suppression of STAT-3 activation in HCC cells (Sun \textit{et. al}, 2008). These reports suggest that targeted inhibition of STAT-3 could lead to novel therapy for HCC.

We describe here the effect on the STAT-3 signaling cascade of a novel compound derived from the seeds of horse chestnut (\textit{Aesculus hippocastanum}), known as \(\beta\)-escin which has been previously described to exhibit anti-inflammatory, hypoglycemic, and anti-obesity activities (Sirtori CR, 2001).

MATERIALS AND METHODS
Reagents
\(\beta\)-escin, with the chemical structure shown in Fig. 1A, was obtained from Wuxi Gorunjie Technology Co. Ltd (China), MTT, Tris, AG490, Epidermal Growth Factor (EGF), glycine, NaCl, SDS, \(\beta\)-actin and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Minimum Essential Medium (MEM), Dulbecco’s Modified Eagle Medium (DMEM), fetal
bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Antibodies to p-STAT-3 (Tyr705), STAT-3, p-JAK-1 (Tyr1022/1023) and p-JAK-2, JAK-1, and JAK-2 were purchased from Cell Signaling Technology (Danvers, MA); Goat anti-mouse and anti-rabbit horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Lines**

Human Hepatocellular Carcinoma (HCC) HepG2 (ATCC HB 8065) and PLC/PRF5 (ATCC CRL 8024) were obtained from the American Type Culture Collection (Manassas, VA). HUH-7 cells were a kind gift from Prof. Hui Kam Man at National Cancer Center, Singapore. HepG2 cells were maintained in monolayer culture at 37°C and 5% CO₂ in MEM containing 1x penicillin-streptomycin solution (Invitrogen) with 10% FBS. PLC/PRF5 and HUH-7 cells were cultured in DMEM containing 1x penicillin-streptomycin solution, non-essential amino acids, sodium pyruvate, and L-glutamine with 10% FBS.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay**

The effect of β-escin and AG490 treatment on the viability of HepG2 and PLC/PRF5 cells were measured by the MTT dye uptake method (Sigma-Aldrich) as described previously (Pathak et al., 2007).

**Live/Dead Assay**

Viability of cells was also determined by live/dead assay (Molecular Probes) that measures intracellular esterase activity and plasma membrane integrity as described previously (Pathak et al., 2007).

**Western Blotting**

To detect various proteins, western blot was performed as described previously (Pathak et al., 2007). HepG2 cells (1 x 10⁶/2ml) were treated with either β-escin or AG490. HUH-7 cells (1 x 10⁶/2ml) were pre-treated with β-escin before stimulation with 100ng/ml EGF. Whole-cell protein was resolved on 8 to 10% SDS-PAGE, electrottransferred onto a nitrocellulose membrane, blotted with antibodies, and then detected by enhanced chemiluminescence (GE Health care).

**RESULTS AND DISCUSSION**

The present study was undertaken to determine the effect of β-escin on STAT-3 signaling pathway. The structure of β-escin is shown in Fig. 1A.

**β-escin inhibits constitutive and inducible STAT-3 Phosphorylation in HCC cells**

Whether β-escin can modulate the constitutive STAT-3 activation in human hepatocellular carcinoma cells was investigated. HepG2 cells were incubated with different concentrations of β-escin for 4 h and whole-cell extracts were prepared and examined for phosphorylated STAT-3 by Western blot analysis. As shown in Fig. 1B-1, β-escin inhibited the constitutive activation of STAT-3 in HepG2 cells in a dose-dependent manner, with maximum inhibition occurring at 30µM. β-escin had no effect on the expression of total STAT-3 protein (Fig. 1B-1, lower panel). We also determined the incubation time β-escin required for suppression of STAT-3 activation in HepG2 cells. As shown in Fig. 1B-2, the inhibition was time dependent, with maximum inhibition occurring at 4-6 h, again with no effect on the expression of total STAT-3 protein (Fig. 1B-2, lower panel). From Fig. 1D, β-escin was also found to suppress EGF-induced STAT-3 phosphorylation in HUH-7 cells, that expressed relatively lower basal levels of STAT-3 phosphorylation.
**β-escin suppresses constitutive activation of JAK-1 and JAK-2**

STAT-3 has been reported to be activated by soluble tyrosine kinases of the JAK family (Ihle, 1996); thus, we determined whether β-escin affects constitutive activation of JAK-1 and JAK-2 in HepG2 cells. We found that β-escin suppressed the constitutive phosphorylation of JAK-1 and JAK-2 (Fig. 1C). The levels of β-actin and total JAK-2 remained unchanged under the same conditions (Fig. 1C, lower panel).

![Figure 1. A) Structure of β-escin. B1 to B2) β-escin inhibits constitutive activation of STAT-3 in HepG2 cells in a dose dependent and time dependent manner. C1 to C2) β-escin suppresses constitutive activation of JAK-1 and JAK-2. D) β-escin inhibits EGF-induced activation of STAT-3 in HUH-7 cells](image)

**β-escin is more effective than AG490 in inhibiting the activation of STAT-3 in HCC cells**

AG490 is the best-known rationally designed inhibitor of JAK-2 kinase linked to STAT-3 activation (Meydan et al., 1996). We examined how the activity of β-escin compares with AG490 for the suppression of cell survival. Cell viability was measured by the MTT and live/dead assay. As shown in Fig. 2B, β-escin was more potent than AG490 against HCC cells. However, it was found to be more effective in suppressing proliferation of HepG2 cells than PLC/PRF5 (Fig. 2B-3). At 30 μM, the apoptotic effects of AG490 and β-escin are 10% and 70% respectively (Fig. 3A). These results coincide with their relative effects on STAT-3 phosphorylation as observed by western blotting (Fig. 2A).

![Figure 2. A1 to A2) AG490 and β-escin inhibit STAT-3 activation in HepG2 cells. B1 to B3) HepG2 and PLC/PRF5 cells were subjected to MTT assay in 12 and 24 hours](image)

**Figure 3. A1 to A2) The apoptotic effects of AG490 and β-escin in HepG2 cells.**

**β-escin potentiates the apoptotic effect of doxorubicin and paclitaxel in HCC cells**

Among chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, and paclitaxel, a mitotic inhibitor, have been widely used for HCC treatment (Burden and Osheroff, 1998). 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. 4, β-escin significantly enhanced the apoptotic effects of doxorubicin from 10 to 40% and of paclitaxel from 12 to 55%.

![Figure 3. A1 to A2) The apoptotic effects of AG490 and β-escin in HepG2 cells.](image)
CONCLUSION
The goal of this study was to determine whether β-escin exerts its anti-cancer effects through the abrogation of STAT-3 signalling pathway in HCC. We found for the first time that β-escin could suppress both constitutive and EGF-induced STAT-3 activation in HCC cells. We also found that β-escin did suppress constitutive activation of JAK-1 and JAK-2. We also observed that β-escin was more potent than AG490 in inhibiting STAT-3 phosphorylation and proliferation of HCC cells. We further found that β-escin potentiated the apoptotic effect of doxorubicin and paclitaxel in HCC cells. Several studies in animals suggest that β-escin is very well tolerated and has potential against inflammatory diseases (Sirtori, 2001). In conclusion, our results clearly demonstrated that the anti-proliferative, and anti-inflammatory activities of β-escin are mediated through the inhibition of STAT-3 signalling cascade.

ACKNOWLEDGEMENTS
I would like to thank Mr. Li Feng and Dr. Sethi for their training, guidance and support.

REFERENCES

Figure 4. A1 to A2) β-escin potentiates the apoptotic effect of doxorubicin and paclitaxel in HepG2 cells