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RESEARCH PAPER

γ-Tocotrienol is a novel inhibitor of constitutive and inducible STAT3 signalling pathway in human hepatocellular carcinoma: potential role as an antiproliferative, pro-apoptotic and chemosensitizing agent

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BACKGROUND AND PURPOSE
Activation of signal transducer and activator of transcription 3 (STAT3) play a critical role in the survival, proliferation, angiogenesis and chemoresistance of tumour cells. Thus, agents that suppress STAT3 phosphorylation have potential as cancer therapies. In the present study, we investigated whether the apoptotic, antiproliferative and chemosensitizing effects of γ-tocotrienol are associated with its ability to suppress STAT3 activation in hepatocellular carcinoma (HCC).

EXPERIMENTAL APPROACH
The effect of γ-tocotrienol on STAT3 activation, associated protein kinases and phosphatase, STAT3-regulated gene products, cellular proliferation and apoptosis in HCC cells was investigated.

KEY RESULTS
γ-Tocotrienol inhibited both the constitutive and inducible activation of STAT3 with minimum effect on STAT5. γ-Tocotrienol also inhibited the activation of Src, JAK1 and JAK2 implicated in STAT3 activation. Pervanadate reversed the γ-tocotrienol-induced down-regulation of STAT3, suggesting the involvement of a protein tyrosine phosphatase. Indeed, we found that γ-tocotrienol induced the expression of the tyrosine phosphatase SHP-1 and deletion of the SHP-1 gene by small interfering RNA abolished the ability of γ-tocotrienol to inhibit STAT3 activation. γ-Tocotrienol also down-regulated the expression of STAT3-regulated gene products, including cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1 and vascular endothelial growth factor. Finally, γ-tocotrienol inhibited proliferation, induced apoptosis and significantly potentiated the apoptotic effects of chemotherapeutic drugs (paclitaxel and doxorubicin) used for the treatment of HCC.

CONCLUSIONS AND IMPLICATIONS
Overall, these results suggest that γ-tocotrienol is a novel blocker of the STAT3 activation pathway, with a potential role in future therapies for HCC and other cancers.

Abbreviations

FBS, fetal bovine serum; HCC, hepatocellular carcinoma; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide; STAT3, signal transducer and activator of transcription 3

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with an estimated incidence of half a million new cases per year around the world (El-Serag and Rudolph, 2007; Berasain *et al.*, 2009). The HCC is characterized as a highly chemoresistant cancer with several aetiological factors being classified as high-risk factors, including exposure to aflatoxin B1, and infection with hepatitis B virus and hepatitis C virus (Kern *et al.*, 2002; Farazi and DePinho, 2006). First line chemotherapeutic drugs currently being used for HCC therapy include doxorubicin, fluorouracil, cisplatin, and mitomycin, but most of these exhibit significant side effects (Avila *et al.*, 2006; Kerr and Kerr, 2009; Rampone *et al.*, 2009). Therefore, the need to develop more therapeutic strategies for HCC that are non-toxic and efficacious is of paramount importance.

Anticancer drug discovery from agents derived from natural sources provides a great opportunity to improve the existing standard of care for HCC and other cancers (Newman, 2008). One such agent is, γ-tocotrienol, a member of the vitamin E superfamily derived from palm oil and rice bran that has attracted great attention for its antiproliferative and anticarcinogenic potential (Ahn *et al.*, 2007; Sen *et al.*, 2007; Aggarwal *et al.*, 2010). For example, γ-tocotrienol has been reported to suppress the proliferation of a wide variety of tumour cells, including breast (Samant and Sylvester, 2006; Elangovan *et al.*, 2008; Park *et al.*, 2010; Pierpaoli *et al.*, 2010; Samant *et al.*, 2010), colorectal (Eitsuka *et al.*, 2006; Xu *et al.*, 2009), gastric (W. Sun *et al.*, 2008; 2009; Bi *et al.*, 2010; Liu *et al.*, 2010), HCC (Sakai *et al.*, 2004; 2006), melanoma (Chang *et al.*, 2009) and prostate (Srivastava and Gupta, 2006; Yap *et al.*, 2008). *In vivo* studies have demonstrated that γ-tocotrienol can suppress the growth of breast tumour (Sundram *et al.*, 1989; Gould *et al.*, 1991; Iqbal *et al.*, 2004), prostate (Kumar *et al.*, 2006; Yap *et al.*, 2010a), melanoma (He *et al.*, 1997; McAnally *et al.*, 2007) and inhibit liver and lung carcinogenesis (Ngah *et al.*, 1991; Iqbal *et al.*, 2004; Wada *et al.*, 2005, Hiura, 2009) pancreatic cancer (Kunnumakkara *et al.*, 2010) either alone or in combination with other chemotherapeutic drugs and radiation. How γ-tocotrienol mediates its anticancer effects is not fully understood, but the roles of mitogen-activated protein kinases (Sun *et al.*, 2008), PI3K/Akt (Samant and Sylvester, 2006), NF-κB (Ahn *et al.*, 2007), telomerase (Eitsuka *et al.*, 2006), peroxisome proliferators-activated receptor γ (Fang *et al.*, 2010; Li *et al.*, 2010b), epidermal growth factor (Yap *et al.*, 2008) and inhibitors of the differentiation family proteins (Yap *et al.*, 2010b) have been implicated.

The transcription factor signal transducer and activator of transcription 3 (STAT3) belongs to a family consisting of seven members (STAT1–4, STAT5α, STAT5β and STAT6) and its constitutive activation has been reported to regulate the expression of gene products involved in cell survival, proliferation, angiogenesis and chemoresistance in various solid tumours, as well as haematological malignancies (Aggarwal *et al.*, 2006; 2009a; Gao and Bromberg, 2006; Costantino and Barlocco, 2008; Yu *et al.*, 2009). STAT3 can be activated by diverse stimuli, including interleukins (e.g. IL-6) and growth factors (e.g. epidermal growth factor). On activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription (Liu *et al.*, 2002; Niwa *et al.*, 2005; Waris *et al.*, 2005; Aggarwal *et al.*, 2009b). The phosphorylation is mediated through the activation of non-receptor protein tyrosine kinases called Janus-activated kinases (JAK). Four members of JAK family (JAK1, JAK2, JAK3 and TYK2) have been implicated to play a role in the activation of STAT3 (Aggarwal *et al.*, 2009a; Devarajan and Huang, 2009). Moreover, the role of c-Src kinase has been reported in STAT3 phosphorylation (Yoshida *et al.*, 2002; Grivennikov and Karin, 2010). Interestingly, STAT3 has also been implicated as a promising target for HCC therapy since inhibition of STAT3 has been reported to induce growth arrest and apoptosis in HCC (Li *et al.*, 2006; 2010a; Choudhari *et al.*, 2007; Kusaba *et al.*, 2007; Tatebe *et al.*, 2008; Lin *et al.*, 2009; Tan *et al.*, 2010).

Because of the crucial role of STAT3 in tumourigenesis, we investigated whether γ-tocotrienol can mediate its effects in part through the suppression of the STAT3 pathway. We found that γ-tocotrienol can indeed suppress both constitutive as well as inducible STAT3 expression in HCC cells, through inhibition of upstream kinases and induction of a phosphatase. This inhibition decreased cell survival and down-regulated the expression of proliferative, anti-apoptotic and angiogenic gene products, leading to suppression of proliferation, induction of apoptosis, and potentiation of the cytotoxic effects of doxorubicin and paclitaxel in HCC cells.

Methods

Reagents

γ-Tocotrienol with purity >97% was obtained from Davos Life Science, Singapore.

Hoechst 33342, epidermal growth factor (EGF), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Tris, glycine, NaCl, sodium dodecyl sulphate (SDS), BSA, doxorubicin and paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO, USA). γ-Tocotrienol was dissolved in dimethyl sulphoxide (DMSO) as a 50 mM stock solution and stored at 4°C. Maximum final concentration of DMSO was less than 0.1% and used for control experiments. Further dilution was done in cell culture medium. RPMI 1640, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal antibodies to STAT3 and STAT5 and mouse monoclonal antibodies against phospho-STAT3 (Tyr 705) and phospho-STAT5, phospho-Akt (Ser 473), Akt, Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, SHP-1,

vascular endothelial growth factor (VEGF), procaspase-3 and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The siRNA for SHP-1 (sc-29478) and scrambled control (sc-37007) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SHP-1 siRNA is a pool of 3 sequences. Sense Strand (A): CUGGUGGAGCAUUUCAA GATT (B): CGCAGUACAAGUUCAUCUATT and (C): CAAC CCUUCUCCUCUUGUATT. 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 (Danvers, MA, USA). Goat anti-rabbit horseradish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Invitrogen (Carlsbad, CA, USA). Bacteria-derived recombinant human IL-6 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).

Cell lines

Human HCC cell lines HepG2, C3A, SNU-387, and PLC/PRF5 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Hep3B cells were kindly provided by Professor Kam M. Hui, National Cancer Centre, Singapore. HepG2, C3A, Hep3B and SNU-387 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 1 × antibiotic-antimycotic solution with 10% FBS. PLC/PRF5 cells were cultured in DMEM containing 1 × penicillin-streptomycin solution, non-essential amino acids, sodium pyruvate, and L-glutamine with 10% FBS. HepG2 cell lines were used to study the effect of γ-tocotrienol on the constitutive STAT3 signalling cascade because they express constitutively active STAT3; SNU-387 cells were used to study the effect of γ-tocotrienol on inducible STAT3 signalling; PLC/PRF-5 cells were used for transfection experiments because they are easy to transfect; and C3A cells were used for proliferation assays to investigate whether γ-tocotrienol can modulate the proliferation of a wide variety of HCC cells.

Western blotting

For detection of phospho-proteins, γ-tocotrienol-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^{-1} aprotinin, 0.005 mg·mL^{-1} leupeptin, 0.4 mM PMSF and 4 mM $NaVO_4$). Lysates were then spun at 14 000 r.p.m. for 10 min to remove insoluble material and resolved on a 7.5% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with anti-STAT antibodies (1:1000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

To detect STAT3-regulated proteins and PARP, HepG2 cells (2×10^6 mL^{-1}) were treated with γ-tocotrienol for different time intervals. 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^{-1} leupeptin, 2 μg·mL^{-1} aprotinin, 1 mM PMSF, 0.5 μg·mL^{-1} benzamidine, 1 mM dithiothreitol 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, Mcl-1 procaspase-3, and PARP and then detected by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analysis of the scanned blots was performed using Image J software and the results are expressed as fold change relative to the control.

Immunocytochemistry for STAT3 localization

HepG2 cells were plated in chamber slides in DMEM containing 10% FBS and allowed to adhere for 24 h. On the 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 counter-stained for nuclei with Hoechst (50 ng·mL^{-1}) for 5 min. Stained cells were mounted with mounting medium (Sigma-Aldrich) and analysed under an fluorescence microscope (DP 70, Olympus, Tokyo, Japan).

NF-κB DNA binding assay

To determine NF-κB activation, we performed DNA binding assay using TransAM NF-kB p65 transcription factor assay kit (ActiveMotif, Carlsbad, CA, USA) according to the manufacturer's instructions and as described previously (Chua *et al.*, 2010). Briefly, nuclear extracts (5 μg) from γ-tocotrienol-treated cells were incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5′-GGGACTTTCC-3′. Bound NF-κB was then detected by a specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a microplate reader (Tecan Systems, San Jose, CA, USA).

STAT3 luciferase reporter assay

PLC/PRF5 cells were plated in 96-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 instructions using lipofectamine (Invitrogen, Carlsbad, CA, USA). At 24 h post-transfection, cells were pretreated with γ-tocotrienol for 6 h and then induced by EGF before being washed and lysed in luciferase lysis buffer (Promega). Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega). All luciferase experiments were carried out in triplicate and repeated three or more times.

Transfection with SHP-1 siRNA

HepG2 cells were plated in each well of six-well plates and allowed to adhere for 24 h. On the day of transfection, 4 μL of

lipofectamine (Invitrogen, Carlsbad, CA, USA) was added to 50 nM SHP-1 siRNA in a final volume of 100 µL of culture medium. After 48 h of transfection, cells were treated with γ-tocotrienol, and whole-cell extracts were prepared to investigate SHP-1, phospho-STAT3 and STAT3 expression by Western blot analysis.

RNA isolation and reverse transcription

Total cellular RNA was extracted from untreated and γ-tocotrienol-treated cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Cells were lysed with TRIZOL before the addition of 0.5 mL water-saturated chloroform. The cells were then vortexed and incubated for 3 min before being centrifuged at 15 800× *g* for 30 min. 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 min at 15 800× *g*. The top aqueous phase was transferred to a new tube. Three units of DNaseI (Sigma-Aldrich, St. Loius, MO, USA) was mixed with RNA and incubated at 37°C for 20 min to digest any DNA contamination. After incubation, an equal volume of isopropanol was added and the RNA was allowed to precipitate at –80°C for at least 20 min. Subsequently, samples were centrifuged for 30 min 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 min 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 (A260/280). Ratio close to 2 represents nucleic acid of high quality.

The expression SHP-1 was analysed using QIAGEN OneStep RT-PCR kit with GAPDH as an internal control. The RT-PCR reaction mixture contained 10 µL of 5 × QIAGEN OneStep RT-PCR buffer, 1 µg of total RNA, 0.6 µM each of forward and reverse primers, 2 µL of dNTP mix and 2 µL of QIAGEN OneStep RT-PCR enzyme mix in a final volume of 50 µL. The reaction was performed at 50°C for 30 min, 95°C for 5 min, 95°C for 1 min, 61°C for 1 min and 72°C for 1 min for 33 cycles with a final extension at 72°C for 10 min. PCR products were run on 1% agarose gel containing 1 × GelRed nucleic acid gel stain from Biotium (Hayward, CA, USA). Stained bands were visualized under UV light and photographed. The primer sequences for SHP-1 mRNA were as follows: 5′-TGGCGTGGCAGGAGAACAG-3′ (forward) and, 5′-CAGTTGGTCACAGAGTAGGGC-3′ (reverse). The primer sequences for GAPDH were 5′-CCACAGTCCATGCCAT CAD-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

Real-time PCR reaction for cyclin D1, Bcl-xL, Mcl-1 and GAPDH was carried out in Mastercycler gradient (Eppendorf Scientific, Westbury, NY, USA) at 25°C for 10 min, followed by 37°C for 60 min and a terminating step of 95°C for 5 min. Fluorescence was measured with the Sequence Detection Systems 2.0 software. The PCR was performed in multiplex (both target and endogenous control co-amplified in the same reaction with distinct fluorescent dyes). Primers and probe for human GAPDH (part number: 4326317E), cyclin D1 (Assay ID: Hs00765553_m1), BcL-xL (Assay ID: Hs00236329_m1) and Mcl-1 (Assay ID: Hs03043899_m1) were purchased from Applied Biosystems (Assays on Demand). Relative gene expression was obtained after normalization with endogenous GAPDH and determination of the difference in threshold cycle (Ct) between treated and untreated cells ($2^{-\Delta\Delta Ct}$).

MTT assay

The antiproliferative effect of γ-tocotrienol 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^{-1}) were incubated in triplicate in a 96-well plate in the presence or absence of different concentrations of γ-tocotrienol in a final volume of 0.2 mL for indicated time intervals at 37°C. Thereafter, 20 µL MTT solution (5 mg·mL^{-1} 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 at 570 nm was measured by Tecan (Durham, NC, USA) plate reader.

Transfection with constitutive STAT3 construct

Hep3B cells were plated in chamber slides in DMEM containing 10% FBS. After 24 h, the cells were transfected with constitutive STAT3-plasmid by FuGene 6 according to manufacturer's protocol (Roche, Indianapolis, IN, USA). Cells were treated with γ-tocotrienol for 24 h, and viability of the cells was determined by live/dead assay. The STAT3 constitutive plasmid that has been described before (Zhang *et al.*, 2002; Lufei *et al.*, 2007) and was a kind gift from Dr Xinmin Cao at Institute of Moleular and Cell Biology, Singapore.

Live/dead assay

Apoptosis of cells was also determined by the 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 γ-tocotrienol/doxorubicin/paclitaxel alone or in combination for 24 h at 37°C. Cells were stained with the live/dead reagent (5 µM ethidium homodimer, 5 µM calcein-AM) and then incubated at 37°C for 30 min. Cells were analysed under a fluorescence microscope (DP 70, Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as the mean ± SEM. In all figures, vertical error bars denote the SEM. The significance of differences between groups was evaluated by Student's *t*-test and one-way ANOVA, followed by *post hoc* Tukey's test. A *P*-value of less than 0.05 was considered statistically significant.

Results

We investigated the effect of γ-tocotrienol on STAT3 activation and on various mediators of cellular proliferation, cell survival and apoptosis. The structure of γ-tocotrienol is shown in Figure 1A.

γ-Tocotrienol inhibits constitutive STAT3 phosphorylation in HepG2 cells

The ability of γ-tocotrienol to modulate constitutive STAT3 activation in HCC cells was investigated. HepG2 cells were incubated with different concentrations of γ-tocotrienol for 6 h, 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, γ-tocotrienol inhibited the constitutive activation of STAT3 in HepG2 cells in a dose-dependent manner, with maximum inhibition occurring at 50 μM. γ-Tocotrienol had no effect on the expression of STAT3 protein (Figure 1B; lower panel). As shown in Figure 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 (Figure 1C; lower panel).

Effect of γ-tocotrienol on STAT3 phosphorylation is specific

Whether γ-tocotrienol affects the activation of some other STAT proteins in HepG2 cells was also investigated. Under the conditions where γ-tocotrienol inhibited STAT3 phosphorylation, it had minimum effect on the levels of constitutively phosphorylated STAT5 and the expression of STAT5 proteins (Figure 1D).

γ-Tocotrienol depletes the nuclear pool of STAT3 in HCC cells

As 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 γ-tocotrienol suppresses nuclear translocation of STAT3. Figure 1E clearly demonstrates that γ-tocotrienol inhibited the translocation of STAT3 to the nucleus in HepG2 cells.

γ-Tocotrienol inhibits NF-κB activation in HCC cells

We further investigated whether γ-tocotrienol also regulates signalling pathways other than STAT3 activation. Therefore, we investigated the effect of γ-tocotrienol on NF-κB DNA-binding activity in HepG2 cells by using the ELISA based TransAM NF-κB assay kit. We found that treatment with γ-tocotrienol suppressed binding of NF-κB to the DNA in a time-dependent manner in HepG2 cells (Figure 1F).

γ-Tocotrienol inhibits inducible STAT3 and JAK2 phosphorylation in HCC cells

As IL-6 induces STAT3 phosphorylation (Moran *et al.*, 2008), we next determined whether γ-tocotrienol could inhibit IL-6-induced STAT3 phosphorylation. In SNU-387 cells incubated with γ-tocotrienol for different times, IL-6-induced STAT3 and JAK2 phosphorylation was suppressed by γ-tocotrienol in a time-dependent manner.

Exposure of cells to γ-tocotrienol for 3–4 h was sufficient to completely suppress IL-6-induced STAT3 and JAK2 phosphorylation (Figure 2A,B).

γ-Tocotrienol 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; Kortylewski *et al.*, 2003). We also examined whether γ-tocotrienol could modulate IL-6-induced Akt activation. Exposure of SNU-387 cells with IL-6 induced phosphorylation of Akt and treatment of cells with γ-tocotrienol suppressed the activation in a time-dependent manner (Figure 2C). Under these conditions, γ-tocotrienol had no effect on the expression of Akt protein.

γ-Tocotrienol suppresses EGF-induced STAT3-dependent reporter gene expression

Our above results showed that γ-tocotrienol inhibited the phosphorylation and nuclear translocation of STAT3. We next determined whether γ-tocotrienol affects STAT3-dependent gene transcription. When PLC/PRF5 cells transiently transfected with the pSTAT3-luciferase construct were stimulated with EGF, STAT3-mediated luciferase gene expression was increased. Dominant-negative STAT3 blocked this increase, indicating specificity. When the cells were pretreated with γ-tocotrienol, EGF-induced STAT3 activity was inhibited in a dose-dependent manner (Figure 2D).

γ-Tocotrienol suppresses constitutive activation of 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 γ-tocotrienol can affect constitutive activation of Src kinase in HepG2 cells. We found that γ-tocotrienol suppressed the constitutive phosphorylation of Src kinase in a time-dependent manner (Figure 3A). The levels of non-phosphorylated Src kinase remained unchanged under the same conditions.

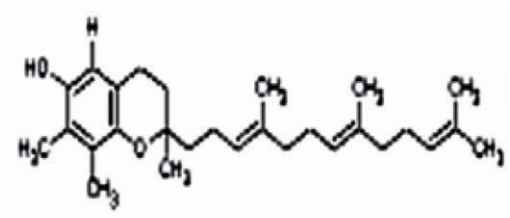
γ-Tocotrienol suppresses constitutive activation of JAK1 and JAK2

The STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (JAKs) (Ihle, 1996), so we determined whether γ-tocotrienol affects constitutive activation of JAK1 in HepG2 cells. We found that γ-tocotrienol suppressed the constitutive phosphorylation of JAK1 (Figure 3B). The levels of non-phosphorylated JAK1 remained unchanged under the same conditions (Figure 3B, bottom panel). To determine the effect of γ-tocotrienol on JAK2 activation, HepG2 cells were treated for different time intervals with γ-tocotrienol and phosphorylation of JAK2 was analysed by Western blot. As shown in Figure 3C, JAK2 was constitutively active in HepG2 cells and pretreatment with γ-tocotrienol suppressed this phosphorylation in a time-dependent manner, with maximum inhibition observed at 3–4 h.

Tyrosine phosphatases are involved in γ-tocotrienol-induced inhibition of STAT3 activation

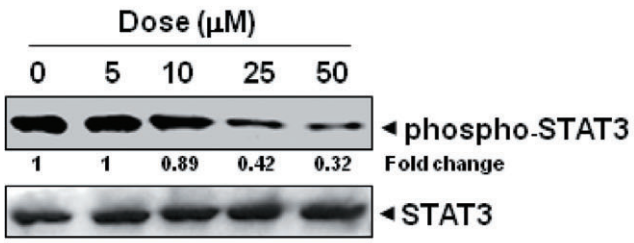
Protein tyrosine phosphatases have also been implicated in STAT3 activation (Han *et al.*, 2006), hence, we determined

A



γ-Tocotrienol

B



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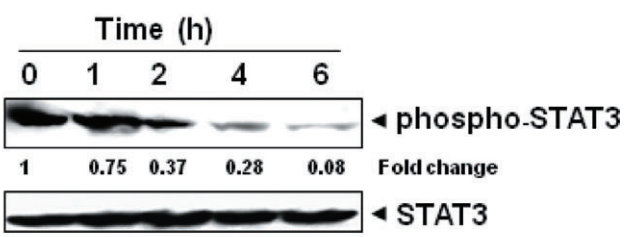

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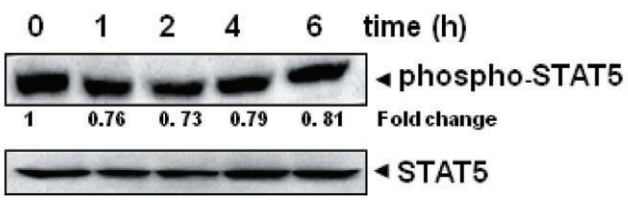

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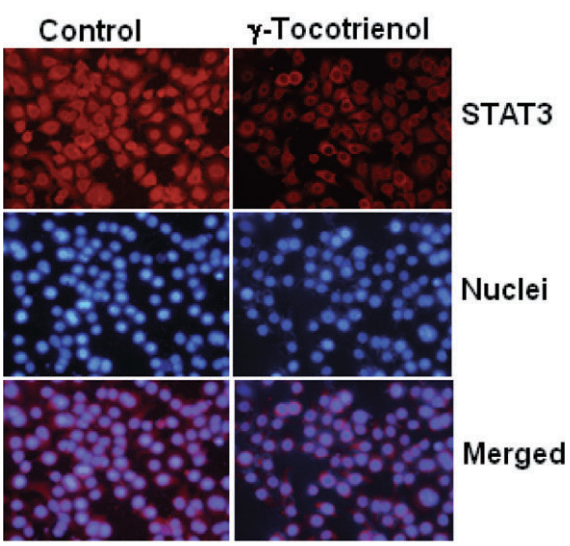


F

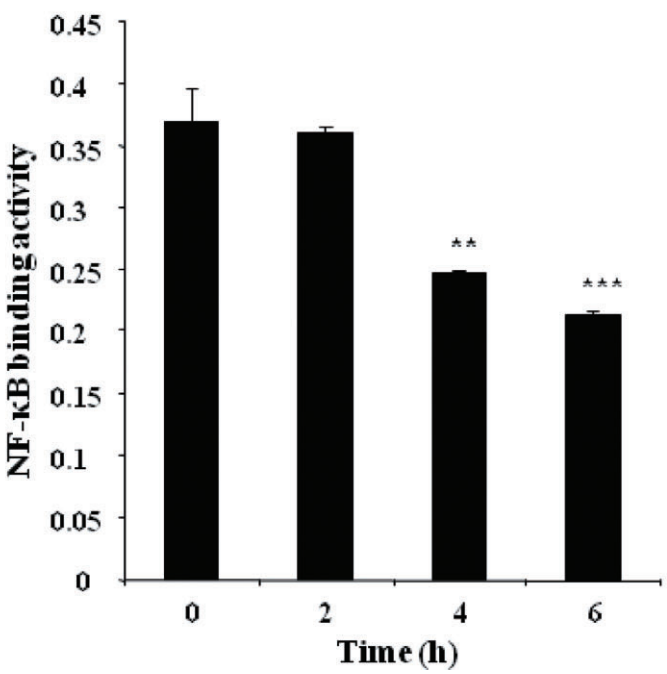
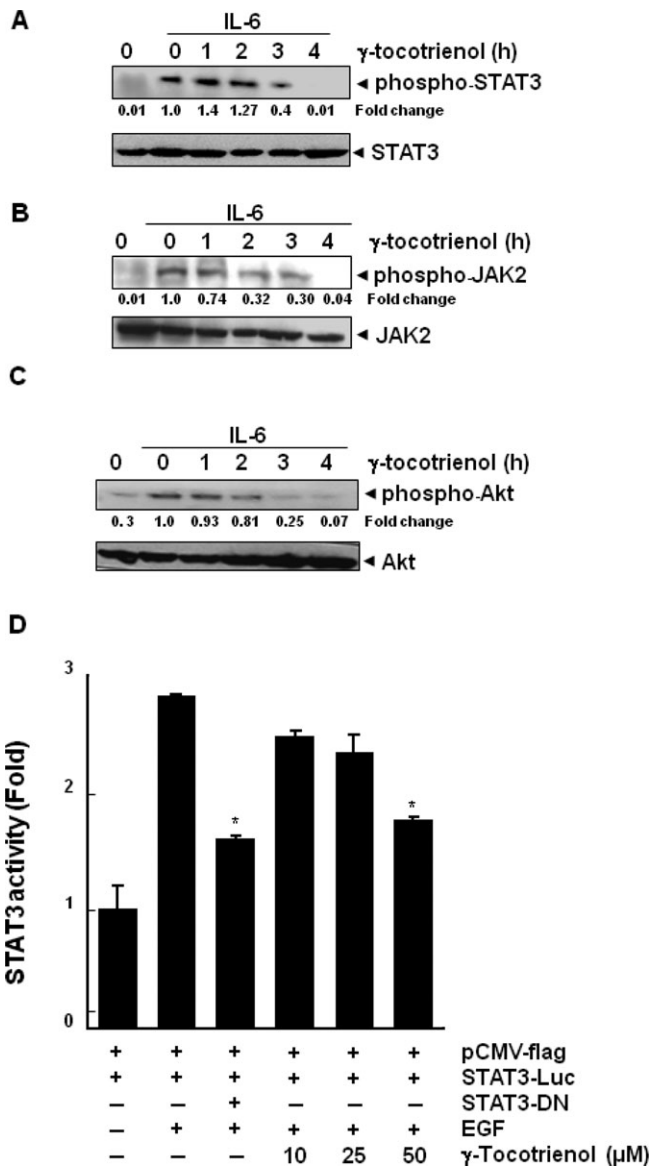


Figure 1

γ-Tocotrienol inhibits constitutively active signal transducer and activator of transcription 3 (STAT3) in HepG2 cells. (A) The structure of γ-tocotrienol. (B) γ-Tocotrienol suppresses phospho-STAT3 levels in a dose-dependent manner. HepG2 cells (2×10^6 mL^{-1}) were treated with the indicated concentrations of γ-tocotrienol for 4 h, after which whole-cell extracts were prepared, and 30 μg of protein was resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. (C) γ-Tocotrienol suppresses phospho-STAT3 levels in a time-dependent manner. HepG2 cells were treated with the 50 μM γ-tocotrienol for the indicated times, after which Western blotting was performed as described for (B). (D) HepG2 cells were treated with 50 μM γ-tocotrienol for the indicated times. Whole-cell extracts were prepared, fractionated on SDS-PAGE, and examined by Western blotting. (E) γ-Tocotrienol causes inhibition of translocation of STAT3 to the nucleus. HepG2 cells (1×10^5 mL^{-1}) were incubated with or without 50 μM γ-tocotrienol for 6 h and then analysed for the intracelullar distribution of STAT3 by immunocytochemistry. The same slides were counterstained for nuclei with Hoechst (50 ng·mL^{-1}) for 5 min. Each panel is representative of three independent experiments. (F) γ-Tocotrienol suppresses NF-κB activation in HepG2 cells. HepG2 cells were treated with indicated concentrations of γ-tocotrienol for 6 h; nuclear extracts were prepared, and 20 μg of the nuclear extract protein was used for ELISA-based DNA binding assay as described in *Methods*. The results shown are representative of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.



whether γ-tocotrienol-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 γ-tocotrienol-induced inhibition of STAT3 activation (Figure 3D). This suggests that tyrosine phosphatases are involved in γ-tocotrienol-induced inhibition of STAT3 activation.

Figure 2

γ-Tocotrienol down-regulates IL-6-induced phospho-STAT3 (signal transducer and activator of transcription 3) activation in hepatocellular carcinoma cells. (A) SNU-387 (2×10^6 mL^{-1}) were treated with 50 μM γ-tocotrienol for the indicated times and then stimulated with IL-6 (10 ng·mL^{-1}) for 15 min. Whole-cell extracts were then prepared and analysed 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. (B) SNU-387 (2×10^6 mL^{-1}) were treated with 50 μM γ-tocotrienol for the indicated times and then stimulated with IL-6 (10 ng·mL^{-1}) for 15 min. Whole-cell extracts were then prepared and analysed for phospho-JAK2 by Western blotting. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. (C) SNU-387 (2×10^6 mL^{-1}) were treated with 50 μM γ-tocotrienol for the indicated times and then stimulated with IL-6 (10 ng·mL^{-1}) for 15 min. Whole-cell extracts were then prepared and analysed for phospho-Akt by Western blotting. The same blots were stripped and reprobed with Akt antibody to verify equal protein loading. (D) PLC/PRF-5 cells (5×10^5 mL^{-1}) were transfected with STAT3-luciferase (STAT3-Luc) plasmid, incubated for 24 h, and treated with 10, 25 and 50 μM γ-tocotrienol for 6 h and then stimulated with epidermal growth factor (EGF) (100 ng·mL^{-1}) for 2 h. Whole-cell extracts were then prepared and analysed for luciferase activity. The results shown are representative of three independent experiments. * Indicates $P < 0.05$, comparison between EGF- and γ-tocotrienol-treated groups by Student's *t*-test.

γ-Tocotrienol induces the expression of SHP-1 in HCC cells

SHP-1 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 γ-tocotrienol can induce the expression of SHP-1 in HepG2 cells. Cells were incubated with different concentrations of γ-tocotrienol for 6 h, whole-cell extracts were prepared and examined for SHP-1 protein by Western blot analysis. As shown in Figure 3E, γ-tocotrienol induced the expression of SHP-1 protein in HepG2 cells in a dose-dependent manner, with maximum expression at 25–50 μM. This stimulation of

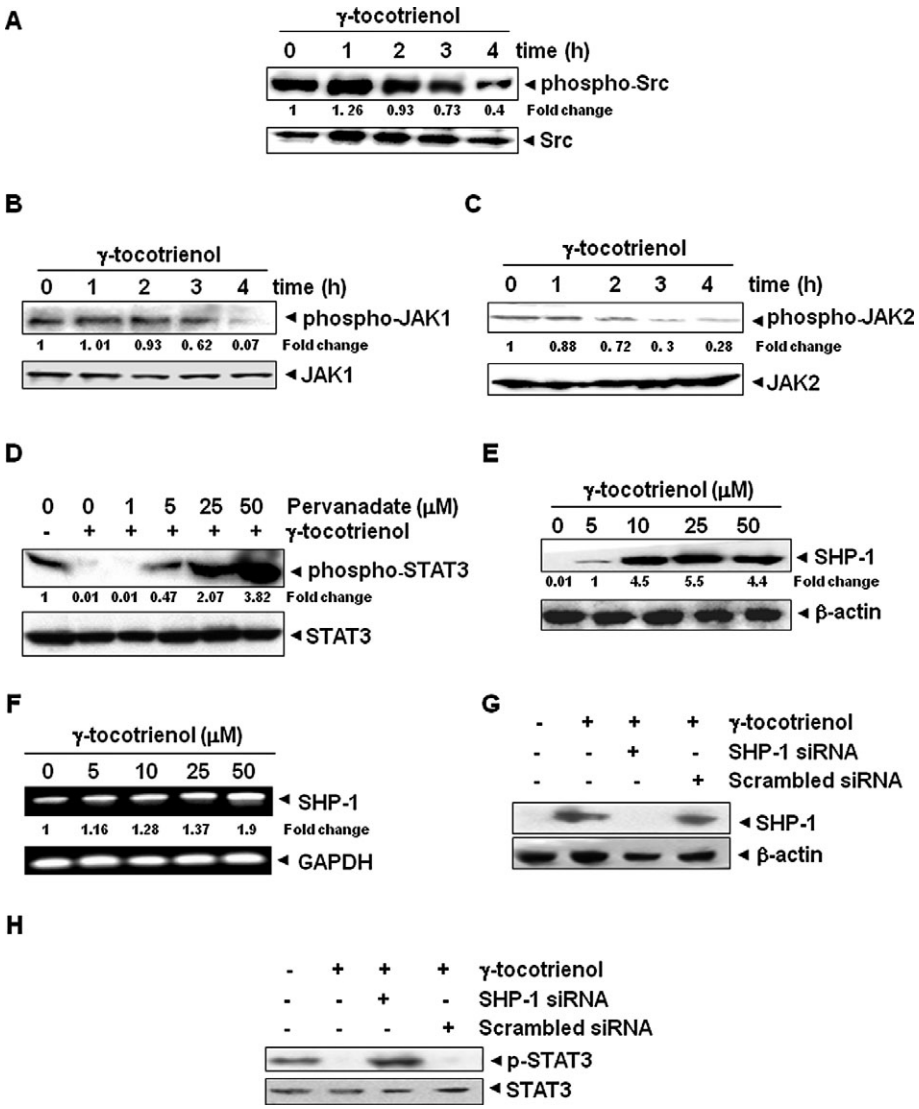


Figure 3

(A) γ-Tocotrienol suppresses phospho-Src levels. HepG2 cells (2×10^6 mL^{-1}) were treated with 50 μM γ-tocotrienol, after which whole-cell extracts were prepared and Western blotting was performed. (B) γ-Tocotrienol suppresses phospho-JAK1 levels. HepG2 cells were treated with 50 μM γ-tocotrienol for indicated time intervals, after which whole-cell extracts were prepared and Western blotting was performed. (C) γ-Tocotrienol suppresses phospho-JAK2 levels. HepG2 cells were treated with 50 μM γ-tocotrienol for indicated time intervals, after which whole-cell extracts and Western blotting was performed. (D) Pervanadate reverses the phospho-STAT3 (signal transducer and activator of transcription 3) inhibitory effect of γ-tocotrienol. HepG2 cells were treated with the indicated concentrations of pervanadate and 50 μM γ-tocotrienol for 6 h, after which Western blotting was performed. (E) γ-Tocotrienol induces the expression of SHP-1 protein in HepG2 cells. HepG2 cells were treated with indicated concentrations of γ-tocotrienol for 6 h, after which Western blotting was performed. (F) Effect of γ-tocotrienol on SHP-1 mRNA expression in HepG2 cells. HepG2 cells were treated with indicated concentrations of γ-tocotrienol for 6 h, after which RNA samples were subjected to RT-PCR with SHP-1 and GAPDH specific primers. PCR products were run on 1% agarose gel containing GelRed. Stained bands were visualized under UV light and photographed. (G) Effect of SHP-1 knockdown on γ-tocotrienol induced expression of SHP-1. HepG2 cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 24 h, cells were treated with 50 μM γ-tocotrienol for 6 h and whole-cell extracts were subjected to Western blot analysis. (H) HepG2 cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 24 h, cells were treated with 50 μM γ-tocotrienol for 6 h and whole-cell extracts were subjected to Western blot analysis for phosphorylated STAT3. The results shown are representative of three independent experiments.

SHP-1 expression by γ-tocotrienol correlated with the down-regulation of constitutive STAT3 activation in HepG2 cells (Figure 1B). Whether the modulation of SHP-1 by γ-tocotrienol is regulated at the transcriptional level was examined. We found that treatment of γ-tocotrienol induces the expression of the mRNA for SHP-1 in a dose-dependent manner (Figure 3F). Therefore, these results suggest that stimulation of SHP-1 expression by γ-tocotrienol may mediate the down-regulation of constitutive STAT3 activation in HepG2 cells.

SHP-1 siRNA down-regulated the expression of SHP-1 induced by γ-tocotrienol

Whether the suppression of SHP-1 expression by siRNA attenuates the γ-tocotrienol-induced SHP-1 expression, was also investigated. As observed by Western blot analysis, γ-tocotrienol-induced SHP-1 expression was effectively abolished in the cells transfected with SHP-1 siRNA but not in those treated with the scrambled siRNA (Figure 3G).

SHP-1 siRNA reversed the inhibition of STAT3 activation by γ-tocotrienol

We next determined whether the suppression of SHP-1 expression by siRNA prevents the inhibitory effect of γ-tocotrienol on STAT3 activation. We found that γ-tocotrienol failed to suppress STAT3 activation in the cells transfected with SHP-1 siRNA (Figure 3H). However, in cells transfected with scrambled siRNA, γ-tocotrienol caused a down-regulation of STAT3 activation. Thus these results with siRNA demonstrate the critical role of SHP-1 in the suppression of STAT3 phosphorylation by γ-tocotrienol.

γ-Tocotrienol down-regulates the expression of cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1 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.*, 2009b). We found that expression of the cell cycle regulator cyclin D1, the anti-apoptotic proteins Bcl-2, Bcl-xL, survivin, Mcl-1 and the angiogenic gene product VEGF reported to be regulated by STAT3 were modulated by γ-tocotrienol treatment. Their expression decreased in a time-dependent manner, with maximum suppression observed at around 24 h (Figure 4A). We also found that mRNA expression of cyclin D1, Bcl-xL, and Mcl-1 were modulated by γ-tocotrienol treatment in a time-dependent manner with maximum reduction observed at around 12 h of treatment (Figure 4B).

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

Because γ-tocotrienol down-regulated the expression of cyclin D1, the gene critical for cell proliferation, we investigated whether γ-tocotrienol inhibits the proliferation of HCC cells by using the MTT method. γ-Tocotrienol inhibited the proliferation of HepG2, C3A and SNU-387 cells in a dose- and time-dependent manner (Figure 5A).

γ-Tocotrienol activates caspase-3 and causes PARP cleavage

Whether suppression of constitutively active STAT3 in HepG2 cells by γ-tocotrienol leads to apoptosis was also investigated. In HepG2 cells treated with γ-tocotrienol there was a time-dependent activation of procaspase-3 (Figure 5B). Activation of downstream caspases led to the cleavage of a 116 kDa PARP protein into an 85 kDa fragment (Figure 5C). These results clearly show that γ-tocotrienol induces caspase-3-dependent apoptosis in HepG2 cells.

Overexpression of constitutively active STAT3 rescues γ-tocotrienol-induced apoptosis

We assessed whether the overexpression of constitutive active STAT3 can reduce the apoptotic effect of γ-tocotrienol on cells. Hep3B cells were transfected with constitutively active STAT3 plasmid, incubated for 24 h, and cells were thereafter treated with γ-tocotrienol for 24 h and examined for apoptosis by esterase staining assay. The results show that the forced expression of STAT3 reduces the γ-tocotrienol-induced apoptosis significantly from 24% to 10% (Figure 6A).

γ-Tocotrienol potentiates the apoptotic effect of doxorubicin and paclitaxel in HepG2 cells

The chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, and paclitaxel, a mitotic inhibitor, have been used to treat HCC (Jin *et al.*, 2009). We examined whether γ-tocotrienol can potentiate the effect of these drugs. HepG2 cells were treated with γ-tocotrienol together with either doxorubicin or paclitaxel, and then apoptosis was measured by the live/dead assay. As shown in Figure 6B, γ-tocotrienol significantly enhanced the apoptotic effects of doxorubicin from 14% to 48% and of paclitaxel from 12% to 42%

Discussion

The aim of this study was to determine whether γ-tocotrienol exerts its anticancer effects through the attenuation of the STAT3 signalling pathway in HCC cells. We found that both constitutive and inducible STAT3 activation in human HCC cells were suppressed by γ-tocotrienol concomitant with the inhibition of Src, JAK1 and JAK2 activation. γ-Tocotrienol also down-regulated the expression of STAT3-regulated gene products, caused the inhibition of proliferation, induced apoptosis, and significantly enhanced the apoptotic effects of doxorubicin and paclitaxel in HCC cells.

We report for the first time that γ-tocotrienol can suppress both constitutive and inducible STAT3 activation in HCC cells and that these effects are specific to STAT3, as γ-tocotrienol had minimal effect on STAT5 phosphorylation. The dose and duration of γ-tocotrienol used to modulate STAT3 activation did not affect cell viability, indicating that down-regulation of STAT3 was not due to cell killing (data not shown). The effects of γ-tocotrienol on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases Src, JAK1 and JAK2. Src and JAKs act in conjunction to mediate constitutive activation of STAT3 (Campbell *et al.*, 1997; Garcia *et al.*, 2001) and interestingly γ-tocotrienol has been previously reported to suppress glutamate-induced c-Src kinase activation in neuronal cells and mesothelioma cells (Sen *et al.*, 2000; Kashiwagi *et al.*, 2009), although its effect on JAK kinases has not been elucidated before. Thus, our findings suggest that γ-tocotrienol blocks the cooperation of Src and JAKs involved in tyrosine phosphorylation of STAT3.

How γ-tocotrienol inhibits IL-6 inducible STAT3 activation was also investigated. The roles of JAK2, and Akt have been implicated in IL-6-induced STAT3 activation (Ihle, 1996; Chen *et al.*, 1999). We found that IL-6-induced Akt activation

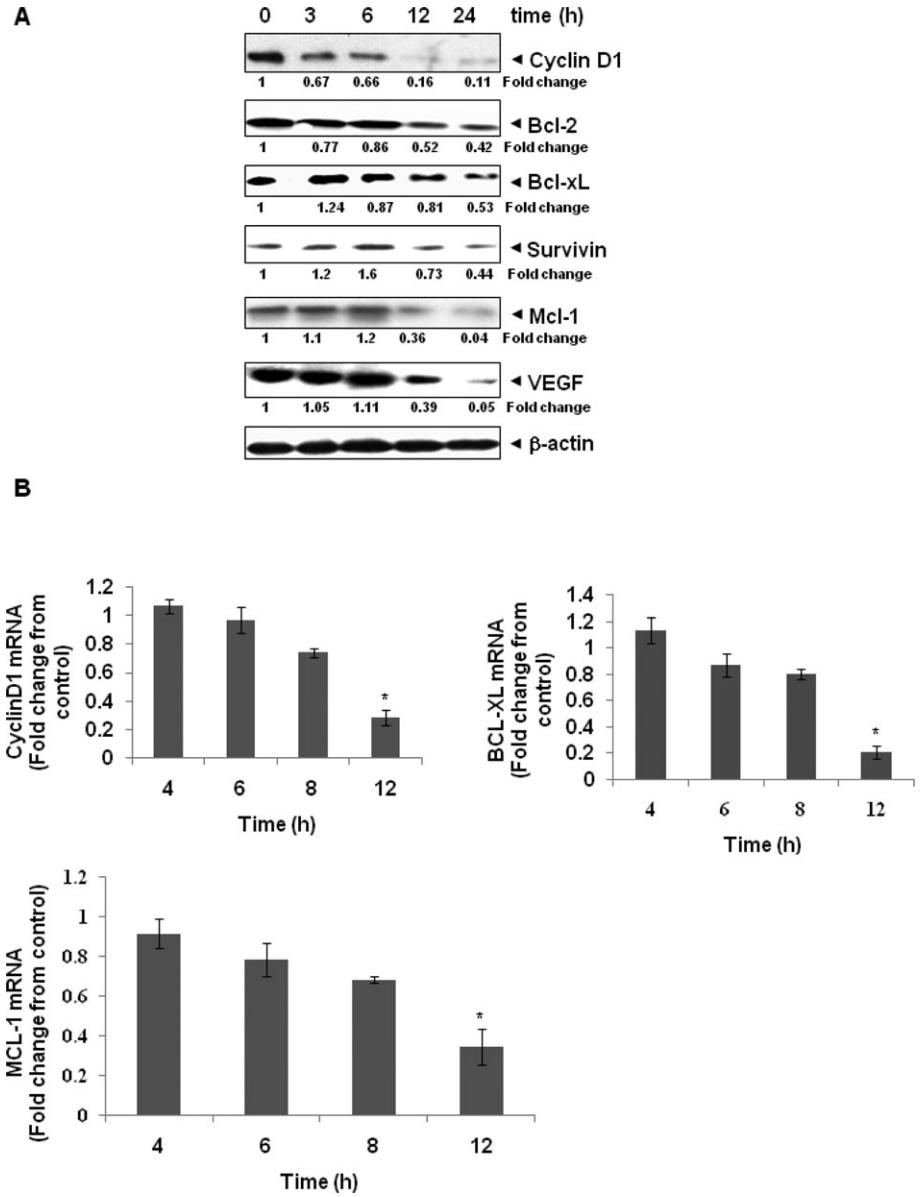


Figure 4

(A) γ-Tocotrienol suppresses signal transducer and activator of transcription 3 (STAT3) regulated gene products involved in proliferation, survival and angiogenesis. HepG2 cells (2×10^6 mL^{-1}) were treated with 25 μM γ-tocotrienol for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE, membrane sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1 and vascular endothelial growth factor (VEGF) antibodies. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. The results shown are representative of three independent experiments. (B) HepG2 cells (3×10^5 mL^{-1}) were treated with 25 μM γ-tocotrienol for the indicated time intervals, after which cells were harvested after treatment and RNA samples, were extracted; 1 μg portions of the respective RNA extracts then proceed for reverse transcription to generate corresponding cDNA. Real-time PCR was performed to measure the relative quantities of mRNA. cDNA product was targeted against cyclin D1, Bcl-xL and Mcl-1 TaqMan probes, with HuGAPDH as endogenous control for measurement of equal loading of RNA samples. Results were analysed using Sequence Detection Software version 1.3 provided by Applied Biosystems. Gene expression was normalized with endogenous HuGAPDH by determination of the difference in threshold cycle (Ct) between treated and untreated cells using 2-ΔΔCt method. Relative gene expression was expressed as fold change of treated samples against untreated. Untreated samples were set at a value of one. Values represent mean ± SEM. *$P < 0.05$, indicates comparison between untreated and γ-tocotrienol-treated groups for 4, 6, 8 and 12 h by Student's *t*-test.

was also suppressed by γ-tocotrienol. We also observed that γ-tocotrienol suppressed STAT3 nuclear translocation and EGF-induced reporter activity of STAT3. This suggests that γ-tocotrienol could manifest its effect on STAT3 activation through multiple mechanisms. Our results are partially in agreement with a recent report in which a novel redox-silent analogue of tocotrienol, 6-O-carboxypropyl-alpha-tocotrienol (T3E) was found to suppress STAT3 activation in

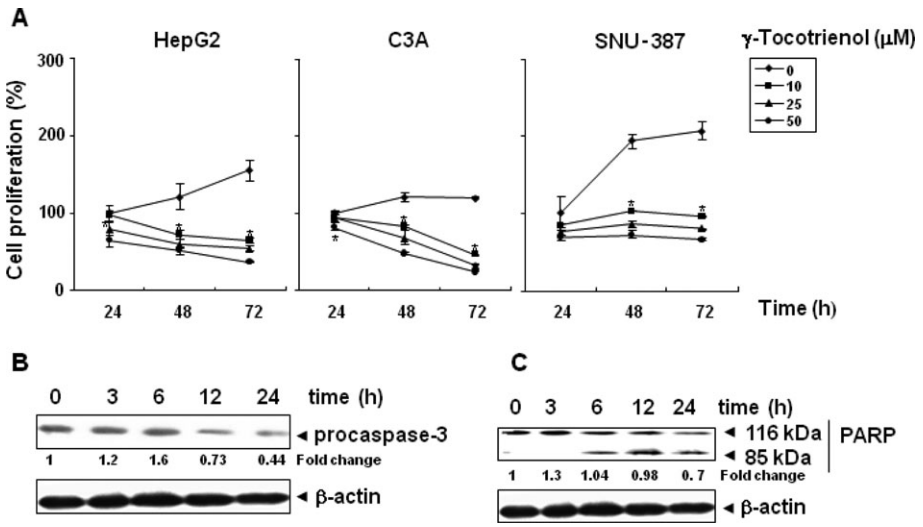


Figure 5

γ-Tocotrienol suppresses proliferation and activates caspase-3. (A) HepG2, C3A and SNU-387 cells (5×10^3 mL^{-1}) were plated in triplicate, treated with indicated concentrations of γ-tocotrienol, and then subjected to 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide assay after 24, 48 and 72 h to analyse proliferation of cells. Vertical lines indicate SD between the triplicates. *$P < 0.05$, comparison between untreated and γ-tocotrienol-treated groups for 24, 48 and 72 h by Student's *t*-test. (B) HepG2 cells were treated with 25 μM γ-tocotrienol for the indicated times, whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against procaspase-3 antibody. The same blot was stripped and reprobed with β-actin antibody to show equal protein loading. (C) HepG2 cells were treated with 25 μM γ-tocotrienol 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. Densitiometric analysis for 116 kDa band of PARP was performed using Image J software. The results shown are representative of three independent experiments.

chemoresistant mesothelioma cells, although via a Src-independent mechanism (Kashiwagi *et al.*, 2009).

STAT3 phosphorylation plays a critical role in the proliferation and survival of different tumour cells (Aggarwal *et al.*, 2006; Yue and Turkson, 2009). 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, we have reported that γ-tocotrienol can suppress NF-κB activation in tumour cells (Ahn *et al.*, 2007). We also found that γ-tocotrienol inhibits NF-κB DNA binding activity in HCC cells. Whether suppression of STAT3 activation by γ-tocotrienol is linked to inhibition of NF-κB activation is not clear. A recent study indicated that STAT3 prolongs NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export (Lee *et al.*, 2009). Moreover, erythropoietin has been shown to activate both NF-κB and STAT3 through the activation of JAK2 kinase (Digicaylioglu and Lipton, 2001). Thus, it is possible that suppression of JAK activation is the critical target for inhibition of both NF-κB and STAT3 activation by γ-tocotrienol.

We also found evidence that the γ-tocotrienol-induced inhibition of STAT3 activation involves a protein tyrosine phosphatase (PTP). Numerous PTP have been implicated in STAT3 signalling, including SHP1, SH-PTP2, TC-PTP, PTEN, PTP-1D, CD45, PTP-epsilon, low molecular weight and PTP (Chiarugi *et al.*, 1998; Kim and Baumann, 1999; Woetmann *et al.*, 1999; Tanuma *et al.*, 2000; Tenev *et al.*, 2000; Gunaje and Bhat, 2001; Irie-Sasaki *et al.*, 2001; Sun and Steinberg, 2002; Yamamoto *et al.*, 2002). SHP1 is implicated in the negative regulation of JAK/STAT signalling pathways (Calvisi *et al.*, 2006) and it has been found that loss of SHP-1 may contribute to the activation of JAK or STAT proteins in cancer (Wu *et al.*, 2003). Indeed, we found that γ-tocotrienol induces the expression of SHP-1 protein in HCC cells, which correlated, with the down-regulation of STAT3 phosphorylation. Transfection with SHP-1 siRNA reversed the STAT3 inhibitory effect of γ-tocotrienol, thereby further implicating a critical role for this phosphatase in γ-tocotrienol-induced down-regulation of STAT3 activation. However, several groups have isolated putative inhibitors for the JAK/STAT pathways by functional or molecular screening of cDNA libraries (Chung *et al.*, 1997; Endo *et al.*, 1997; Starr *et al.*, 1997). Aberrant methylation of SH2 domain-containing protein known as suppressor of cytokine signalling (SOCS1), which is a negative regulator of the STAT3 pathway, has been found in 65% of human primary HCC tumour samples (Yoshikawa *et al.*, 2001). Another group identified a family of proteins with a putative zinc-binding motif that was named PIAS, protein inhibitor of activated STAT (Chung *et al.*, 1997). Although further investigation is required for elucidation of the molecular mechanism for functions of these inhibitors, it is conceivable that the JAK/STAT pathways might be regulated at multiple levels.

We further found that γ-tocotrienol suppressed the expression of several STAT3-regulated genes; including proliferative (cyclin D1) and anti-apoptotic gene products (Bcl-2, Bcl-xL, survivin and Mcl-1) and angiogenic gene product (VEGF). The down-regulation of cyclin D1 expression by γ-tocotrienol may explain its reported antiproliferative effects on various tumour cells, as cyclin D1 is required for transition from G1 to S phase of the cell cycle. Mcl-1 is

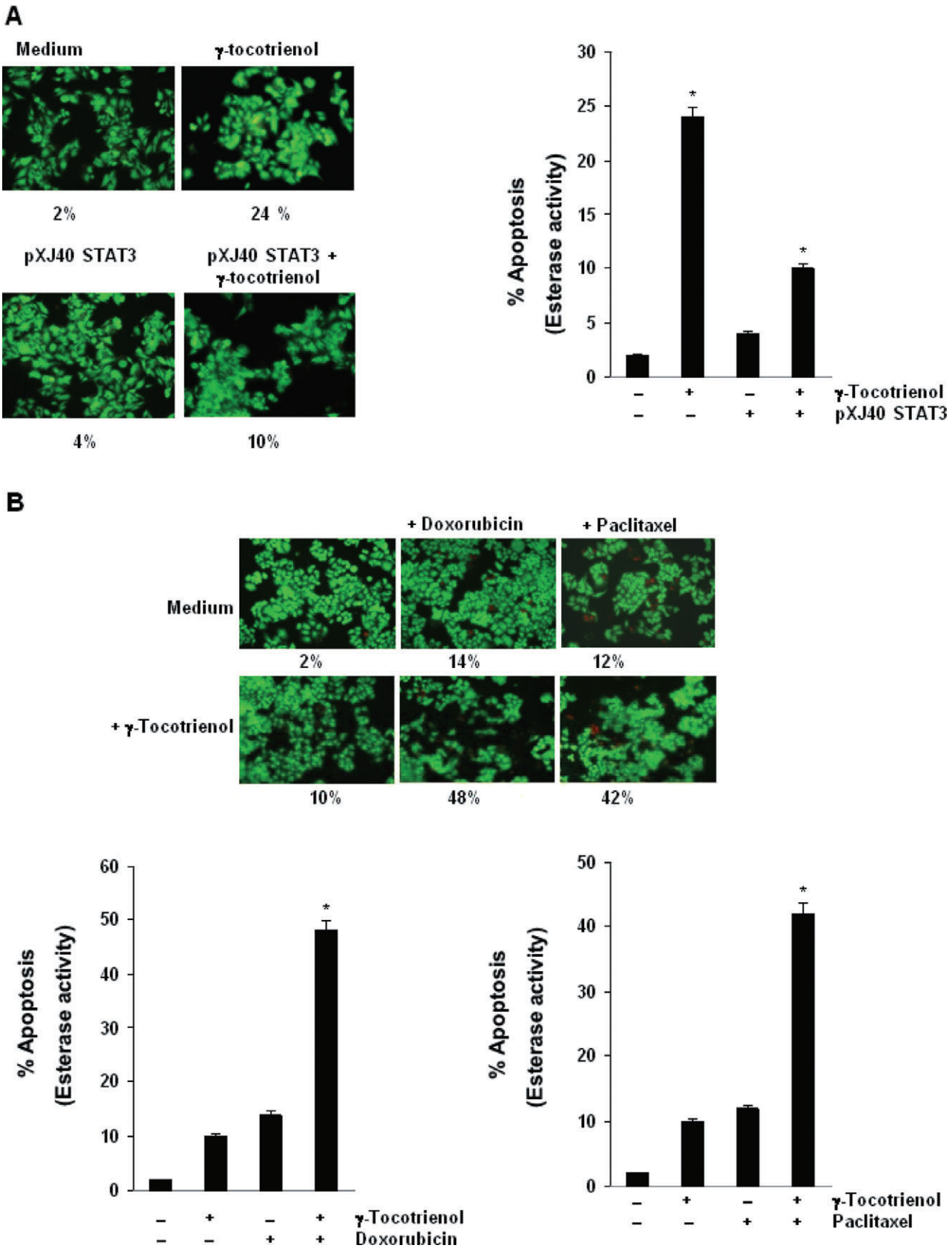

A
Medium
γ-tocotrienol
2%
24 %
pXJ40 STAT3
pXJ40 STAT3 + γ-tocotrienol
4%
10%
% Apoptosis (Esterase activity)
γ-Tocotrienol
pXJ40 STAT3
B
+ Doxorubicin
+ Paclitaxel
Medium
2%
14%
12%
+ γ-Tocotrienol
10%
48%
42%
% Apoptosis (Esterase activity)
γ-Tocotrienol
Doxorubicin
% Apoptosis (Esterase activity)
γ-Tocotrienol
Paclitaxel

Figure 6

(A) Overexpression of constitutive signal transducer and activator of transcription 3 (STAT3) protects Hep3B cells from γ-tocotrienol-induced cytotoxicity. Hep3B cells were transfected with constitutive STAT3 plasmid. After 24 h of transfection, the cells were treated with 50 μM γ-tocotrienol for 24 h, and then the cytotoxicity was determined by the live/dead assay and 20 random fields were counted. (B) γ-Tocotrienol potentiates the apoptotic effect of doxorubicin and paclitaxel. HepG2 cells (1×10^6 mL^{-1}) were treated with 10 μM γ-tocotrienol and 10 nM doxorubicin or 5 nM paclitaxel alone or in combination for 48 h at 37°C. Cells were stained with a live/dead assay reagent for 30 min and then analysed under a fluorescence microscope as described in *Methods*. The results shown are % apoptosis and are representative of three independent experiments.

highly expressed in tumour cells (Epling-Burnette *et al.*, 2001), and Niu *et al.* (2002) reported that inhibition of STAT3 by a Src inhibitor results in the down-regulation of the expression of the Mcl-1 gene in melanoma cells. In addition, activation of STAT3 signalling 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 down-regulation of the expression of Bcl-2, Bcl-xL, survivin and Mcl-1 is likely to be linked to the ability of γ-tocotrienol to induce apoptosis in HCC cells. The down-modulation of VEGF expression is in line with a recent report on the anti-angiogenic potential of tocotrienols (Weng-Yew *et al.*, 2009).

Doxorubicin and paclitaxel are commonly used chemotherapeutic drugs for the treatment of HCC (Jin *et al.*, 2009). We found that γ-tocotrienol significantly potentiated the apoptotic effect of doxorubicin and paclitaxel in HCC cells. Additionally, overexpression of STAT3 also protected the cells from the apoptotic effects of γ-tocotrienol, supporting our hypothesis that the antiproliferative effects of γ-tocotrienol are mediated through the attenuation of the STAT3 signalling pathway. Our results corroborate well with a recent report, in which combined treatment of γ-tocotrienol with EGFR blockers (erlotinib or gefitinib) suppressed STAT3 and Akt signalling in murine mammary tumour cells (Bachawal *et al.*, 2010), thereby suggesting that γ-tocotrienol can be effectively used in conjunction with existing anticancer therapies. γ-Tocotrienol has been reported to target multiple pathways of tumourigenesis, including proliferation, apoptosis, angiogenesis, invasion and metastasis in various tumour cells and *in vivo* cancer models (Ahn *et al.*, 2007; Sen *et al.*, 2007). However, no reports exist in the literature specifically elucidating the effect of γ-tocotrienol on STAT3/JAK2 signalling cascade in HCC. Further *in vivo* studies with γ-tocotrienol either alone or in conjunction with existing chemotherapeutic drugs are needed to demonstrate the potential application of γ-tocotrienol for the treatment of HCC and other cancers.

Acknowledgements

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Conflict of interest

None declared.

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