Gambogic Acid Inhibits STAT3 Phosphorylation Through Activation of Protein Tyrosine Phosphatase SHP-1: Potential Role in Proliferation and Apoptosis

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Abstract

The transcription factor, signal transducer and activator of transcription 3 (STAT3), is associated with proliferation, survival, and metastasis of cancer cells. We investigated whether gambogic acid (GA), a xanthone derived from the resin of traditional Chinese medicine, *Gamboge hanburyi* (mangosteen), can regulate the STAT3 pathway, leading to suppression of growth and sensitization of cancer cells. We found that GA induced apoptosis in human multiple myeloma cells that correlated with the inhibition of both constitutive and inducible STAT3 activation. STAT3 phosphorylation at both tyrosine residue 705 and serine residue 727 was inhibited by GA. STAT3 suppression was mediated through the inhibition of activation of the protein tyrosine kinases Janus-activated kinase (JAK) 1, and JAK2. Treatment with the protein tyrosine phosphatase (PTP) inhibitor pervanadate reversed the GA-induced down-regulation of STAT3, suggesting the involvement of a PTP. We also found that GA induced the expression of the PTP SHP-1. Deletion of the SHP-1 gene by small interfering RNA suppressed the ability of GA to inhibit STAT3 activation and to induce apoptosis, suggesting the critical role of SHP-1 in its action. Moreover, GA down-regulated the expression of STAT3-regulated antiapoptotic (Bcl-2, Bcl-xL, and Mcl-1), proliferative (cyclin D1), and angiogenic (VEGF) proteins, and this correlated with suppression of proliferation and induction of apoptosis. Overall, these results suggest that GA blocks STAT3 activation, leading to suppression of tumor cell proliferation and induction of apoptosis.
Introduction

The successes of several recent clinical trials in preventing cancer in high-risk populations suggest that chemoprevention is a rationale and appealing strategy. Chemoprevention includes the use of natural or synthetic substances to reverse, suppress or prevent the initiation, promotion, or progression of cancer. In particular, natural compounds, which include fruits and vegetables, are important in the treatment of life-threatening conditions. As many as 70% of all drugs discovered within the past 25 years have their roots in natural products (1). Thus, there is growing interest in the possible therapeutic potential of natural products against a variety of ailments. Moreover, almost 80% of the world population relies on natural products for their medical needs. Because natural compounds are considered to be affordable and safe, many potential compounds are now in different phases of clinical trials. Moreover, because cancer is a complex and multigenic disease, agents that modulate multiple targets are preferred. Natural products, because of their ability to bind multiple targets, thus have an advantage over rationally designed mono-targeted agents.

One potentially useful natural compound is gambogic acid (GA; C$_{38}$H$_{44}$O$_{8}$, Fig. 1A), a xanthone derived from the resin of *Garcinia hanburyi* (also called mangosteen), a plant that mainly grows in South China, Cambodia, Vietnam, and Thailand (2). It has been shown that GA can suppress the growth of various cancer cells such as non–small cell lung cancer cells (3), human hepatocellular carcinoma (4), oral squamous cell carcinoma (5), human breast cancer cells (6), human malignant melanoma (7), human gastric carcinoma (8), and human leukemia cancer cells (9). GA has also been shown in animal models to inhibit the development of tumors (10, 11). A variety of
mechanisms have been proposed by which GA inhibits the proliferation of cancer cells
and induces apoptosis. These include inhibition of antiapoptotic proteins bcl-2 (6, 12) and
survivin (13); induction of apoptosis-associated proteins p53 (14), bax, and pro-caspase-3
(7); activation of c-Jun–N-terminal protein kinase (JNK), p38 (15) and glycogen synthase
kinase 3beta (GSK3beta) (16); inhibition of topoisomerase II by binding to its ATPase
domain (17), and NF-κB and its regulated gene products (9); down-regulation of the
MDM2 oncogene and subsequent induction of p21 (14); and down-regulation of hTERT
(10). It has also been shown to directly bind to c-myc (18), and transferrin receptors (19)
and to block vascular endothelial growth factor (VEGF) signaling (20, 21). Recently, a
proteomic approach revealed suppression of expression of 14-3-3 protein sigma and
stathmin by the GA (8).

Extensive research in the past few decades has revealed that most chronic
illnesses, including cancer, exhibit dysregulation of multiple cell signaling pathways. One
of the major signaling molecules in this regard is signal transducer and activator of
transcription (STAT)–3. The latter is transcription factor involved in cell proliferation,
differentiation, and apoptosis (22-24). STAT3 is constitutively active in many human
cancer cells, including multiple myeloma, leukemia, lymphoma, and solid tumors (25,
26). STAT3 can also be activated by certain interleukins (e.g., IL-6), growth factors (e.g.,
epidermal growth factor [EGF]), carcinogens and tumor promoters (27). The activation of
STAT3 is regulated by phosphorylation of tyrosine 705 by receptor and nonreceptor
protein tyrosine kinases such as EGF receptor (EGFR) kinase (28), Src (29), Janus-
activated kinase (JAK) (30, 31), and extracellular signal–regulated kinase (32). Upon
activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and subsequent gene transcription.

Because of the critical role of STAT3 activation in tumor cell survival, proliferation, and chemoresistance, we hypothesized that GA may mediate its effects through suppression of the STAT3 pathway. We found that GA indeed suppressed both constitutive and inducible STAT3 activation and down-regulated the expression of cell survival, proliferative, and angiogenic gene products, leading to suppression of proliferation and induction of apoptosis.

Material and Methods

Cell Lines. Human multiple myeloma lines U266, MM.1S (melphalan-sensitive) and MM.1R (melphalan-resistant), head and neck squamous cell carcinoma SCC4, prostate cancer PC-3 and DU145, and breast cancer MCF-7 cells were obtained from the American Type Culture Collection. MCF-10A cells were kindly provided by Dr. Kapil Mehta from our Institute. U266 (ATCC TIB-196) is a plasmacytoma of B-cell origin and is known to produce monoclonal antibodies and IL-6. MM.1S cell line, established from the peripheral blood cells of a patient with IgA myeloma, secretes EL chain, is negative for the presence of the Epstein-Barr virus genome, and expresses leukocyte antigen DR, plasma cell Ag-1, and T9 and T10 antigens. U266, MM.1S, and MM.1R cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). SCC4 cells were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 units/mL of penicillin and 100 μg/mL of streptomycin. The above-mentioned cell lines were procured more than 6
months ago and have not been tested recently for authentication in our laboratory.

**Reagents.** GA was obtained from Alexis Laboratories (San Diego, CA). A 10 mM solution of GA was prepared in dimethyl sulfoxide, stored as small aliquots at -20°C, and then diluted as needed in cell culture medium. Hoechst 33342, MTT, Tris, glycine, NaCl, SDS, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, FBS, 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr705), phospho-STAT3 (Ser727), phospho-JAK1 (Tyr1022/1023), JAK1, phospho-JAK2 (Tyr1007/1008), and JAK2, Bcl-2, Bcl-xL, Mcl-1, SHP-1, cyclin D1, procaspase-3, -9, and poly(ADP-ribose) polymerase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse horseradish peroxidase was purchased from Transduction Laboratories (Lexington, KY), and goat anti-rabbit Alexa Fluor 594 was purchased from Invitrogen. Bacteria-derived recombinant human IL-6 was kindly provided by Novartis Pharmaceuticals (East Hanover, NJ). The siRNA for SHP-1, and the scrambled control were obtained from Ambion (Austin, TX). GST-JAK2 substrate was kindly provided by Dr. Zhizhuang Joe Zhao (Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK).

**Propidium Iodide (PI) Staining for Apoptosis.** Cells were pretreated with GA (2.5 μM) for different times. PI staining for cell distribution across the cell cycle was performed with a FACSCalibur device (Becton Dickinson, Bedford, MA) as described elsewhere.
A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and emission set at 610 nm.

**Annexin V/PI Assay.** An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of annexin V. To identify apoptosis, we used an annexin V antibody, which was conjugated with a fluorescein isothiocyanate (FITC) fluorescent dye. Briefly, $2 \times 10^6$ cells were pretreated with GA for different times at 37°C and subjected to annexin V staining. The cells were washed in PBS, resuspended in 100 µL of binding buffer containing an FITC-conjugated anti–annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

**Live/Dead Assay.** To measure apoptosis, we used the Live/Dead Assay (Invitrogen), which assesses intracellular esterase activity and plasma membrane integrity. This assay was performed as described previously (33).

**Cytotoxicity assay.** The cytotoxic effects of gambogic acid were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method (9).

**Western Blot Analysis.** To detect various proteins, cells treated with GA were washed with PBS and protein extracted by incubation for 30 min on ice in lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM ethylenediaminetetraacetic acid, 250 mM NaCl, 0.1%
NP-40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL benzamidine, 1 mM dithiothreitol (DTT), and 1 mM sodium vanadate. The lysate was centrifuged, and the supernatant was collected. Whole-cell extract protein (40 μg) was resolved on 7.5%–12% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto a nitrocellulose membrane, blotted with antibodies, and then detected by electrochemiluminescence (Amersham Biosciences).

**Immunocytochemistry for STAT3 Localization.** GA-treated cells were plated on a glass slide by centrifugation using a Cytospin 4 (Thermoshendon, Pittsburg, PA), air-dried for 1 h at room temperature, and fixed in 4% formaldehyde. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1:100). After overnight incubation, the slides 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 slides were mounted with mounting medium and analyzed under an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Molecular Devices, Sunnyvale, CA).

**Electrophoretic Mobility Shift Assay for STAT3-DNA Binding.** STAT3-DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) using a 32P-labeled high-affinity sis-inducible element (hSIE) probe (forward, 5’-CTTCATTTCGGTAAATCCCTAAGCT-3’ and reverse 5’-CTTCATTTCGGTAAATCCCTAAGCT-3’ and reverse 5’-
AGCTTTAGGGATTTACGGGAAATGA-3’) as previously described (33). Briefly, nuclear extracts were prepared from GA-treated cells and incubated with the hSIE probe. The DNA-protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gels. The dried gels were visualized, and the radioactive bands were quantitated with a Storm 820 and Imagequant software (Amersham).

Transfection with siRNA. SCC4 cells were plated in each well of 6-well plates and allowed to adhere for 24 h. On the day of transfection, 12 µL of HiPerFect transfection reagent (QIAGEN) 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 GA for 24 h. Cells were used for the live/dead assay and Western blotting of SHP-1.

Kinase Assay. To determine the effect of GA on JAK2 activation, we performed an immunocomplex kinase assay using GST-JAK2 as the substrate, as described previously (33). In brief, the JAK complex from whole-cell extracts was precipitated with antibody against JAK2 and treated with protein A/G-agarose beads (Pierce, Rockford, IL). After 2 h, the beads were washed with whole-cell extract buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM dithiothreitol, 20 µCi [γ-32P]ATP, 10 µM unlabeled ATP, and 2 µg of substrate GST-JAK2. After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with the Storm 820 imaging system. To determine the total amounts of JAK2 in each sample, 40 µg of whole-cell proteins was
resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with anti-JAK2 antibody.

Results
The goal of this study was to determine whether GA can inhibit the STAT3 cell signaling pathway, leading to suppression of proliferation and induce apoptosis. We investigated the effect of GA on both constitutive and IL-6-inducible STAT3 activation. Whether GA affects STAT3-regulated gene products involved in cellular proliferation, survival, and apoptosis was also investigated.

GA Induces Apoptosis in multiple myeloma cells. We first examined the apoptosis-inducing effects of GA using the annexin V/PI assay, which detects phosphatidylserine externalization. For this, human multiple myeloma U266 cells were exposed to a 2.5µM concentration of GA for different times. GA significantly induced apoptosis in time-dependent manner (Fig. 1B, left panel).

To confirm the GA-induced cell death, we also measured apoptosis by propidium iodide staining of DNA. We found that GA induced apoptosis from 1% in control cells to 30% in GA-treated cells within 24 h (Fig. 1B, right panel).

We also measured apoptosis by intracellular esterase activity and plasma membrane integrity using the live/dead assay. The results indicated that GA treatment induced apoptosis from 2% in control cells to 65% in GA-treated cells within 24 h (Fig. 1C).
Next, we examined the effect of GA on the activation of caspase-9, caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage. We found that GA cleaved pro-caspase-9 and pro-caspase-3, leading to the appearance of caspase-9 and caspase-3 respectively, in a time-dependent manner. We also found that GA induced PARP cleavage in time-dependent manner (Fig. 1D, left panel). Taken together, all these results suggest that GA can induce apoptosis in human multiple myeloma cells.

To determine whether GA is selectively more cytotoxic to tumor cells than normal cells, we employed human breast cancer MCF-7 and human normal counterpart MCF-10A cells. Under the conditions when GA induced 75% cytotoxicity in MCF-7 cells, only 13% cytotoxicity was observed in MCF-10A cells. These results thus indicate that GA is highly cytotoxic to tumor cells (Fig. 1D, right panel).

**GA Inhibits Constitutive STAT3 Phosphorylation in Multiple Myeloma Cells.** We investigated whether GA modulates constitutive STAT3 activation in multiple myeloma cells. We incubated U266 cells with different concentrations of GA for 6 h and examined them for phosphorylated STAT3 by Western blot analysis using an antibody that recognizes STAT3 phosphorylated at the tyrosine 705 site. As shown in Figure 2A), GA inhibited constitutive STAT3 activation in the U266 cells, with maximum inhibition occurring at 2.5 μM GA. GA had no effect on STAT3 protein expression.

We also determined the effect of GA incubation time required to suppress STAT3 activation in U266 cells. As shown in Figure 2B, STAT3 inhibition was time-dependent, with maximum inhibition occurring 6 h after the beginning of GA treatment. Interestingly,
GA also inhibited STAT3 phosphorylated at serine 727 site (Fig. 2B). Under these conditions, GA had no significant effects on cell viability.

**GA Suppresses the Nuclear Translocation of STAT3.** Because tyrosine phosphorylation causes dimerization of STATs and then nuclear translocation, whether GA inhibited nuclear translocation of STAT3 was examined in U266 cells by immunocytochemistry. Our results showed that GA was able to inhibit the nuclear translocation of STAT3 (Fig. 2C).

**GA Inhibits Binding of STAT3 to the DNA.** When STAT3 is translocated to the nucleus, it binds to the DNA, an event that in turn regulates gene transcription. Whether GA inhibits DNA binding activity of STAT3 was examined by EMSA. Nuclear extracts prepared from U266 cells showed STAT3 DNA-binding activity and that GA inhibited this binding in a dose-dependent (Fig. 2D, *left panel*) and time-dependent (Fig. 2D, *right panel*) manner. No loss of cell viability was noted under these conditions.

**GA Inhibits IL-6–Induced STAT3 Phosphorylation.** Because IL-6 is a growth factor for multiple myeloma cells and induces STAT3 phosphorylation, we determined whether GA could inhibit IL-6–induced STAT3 phosphorylation. Multiple myeloma cells, which lack constitutively active STAT3, were treated with IL-6 for different times and then examined for phosphorylated STAT3. IL-6 induced phosphorylation of STAT3 as early as 15 min, but phosphorylation began to decline at 60 min (Fig. 3A). In multiple
myeloma cells pretreated with GA for 6 h, IL-6–induced STAT3 phosphorylation was suppressed (Fig. 3B).

**GA Suppresses the Constitutive Activation of JAK1 and JAK2.** STAT3 has been reported to be activated by soluble tyrosine kinases of the JAK family; thus, we sought to determine whether GA affects the constitutive activation of JAK1 in U266 cells. We found that GA suppressed the constitutive phosphorylation of JAK1 (Fig. 4A). Levels of nonphosphorylated JAK1 remained unchanged under the same conditions.

To determine the effect of GA on JAK2 activation, GA-treated cells were used for Western blot with the anti–phospho-JAK2 antibody. As shown in Fig. 4B, JAK2 was constitutively active in U266 cells and pretreatment with GA suppressed this phosphorylation in a time-dependent manner.

We further investigated whether GA affects JAK2 activity in U266 cells using immunocomplex kinase assays with GST-JAK2 acting as the substrate. We found that GA suppressed the constitutive facilitation of JAK2 in a time-dependent manner (Fig. 4C).

**GA-Induced Inhibition of STAT3 Activation Involves a Protein Tyrosine Phosphatase.** Because protein tyrosine phosphatases have been implicated in STAT3 activation, we determined whether GA-induced inhibition of STAT3 tyrosine phosphorylation could be due to the activation of a protein tyrosine phosphatase (PTPase). Treatment of U266 cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate prevented the GA-induced inhibition of STAT3 activation (Fig. 5A). This
suggests that tyrosine phosphatases are involved in the GA-induced inhibition of STAT3 activation.

**GA Induces SHP-1 Expression in U266 Cells.** SHP-1 is a nontransmembrane PTPase expressed most abundantly in hematopoietic cells (34). PTPases have been shown to be involved in the negative regulation of JAK/STAT signaling in leukemia and lymphoma. Therefore, we examined whether GA modulates SHP-1 expression in U266 cells. We incubated cells with GA for various times. As shown in (Fig. 5B), GA induced SHP-1 protein expression in U266 cells. Our results suggest that the stimulation of SHP-1 expression by GA could be associated with the down-regulation of constitutive STAT3 activation in U266 cells.

**Gene Silencing of SHP-1 Reverses the Effect of GA on STAT3.** We determined whether the suppression of SHP-1 expression by siRNA would abrogate the inhibitory effect of GA on STAT3 activation. Western blotting showed that GA-induced SHP-1 expression was effectively abolished in the cells treated with SHP-1 siRNA; treatment with scrambled siRNA had no effect (Fig. 5C; *left panel*). We also found that GA failed to suppress STAT3 activation in cells treated with SHP-1 siRNA (Fig. 5C; *right panel*). These results suggest the critical role of SHP-1 in the suppression of STAT3 phosphorylation by GA.

**Gene Silencing of SHP-1 Reduces GA-Induced Apoptosis.** We showed above that SHP-1 plays a critical role in the suppression of STAT-3 phosphorylation by GA.
Whether SHP-1 siRNA also affects GA-induced apoptosis was determined. We found that knockdown of SHP-1 significantly decreased the apoptotic effects of GA (Fig. 5D). By contrast, treatment with control siRNA had no effect (Fig. 5D).

**GA Down-Regulates the Expression of Antiapoptotic Proteins.** STAT3 has been shown to regulate the expression of various gene products involved in proliferation and cell survival (22, 23); thus, whether down-regulation of STAT3 activation by GA leads to down-regulation of these gene products was examined. The results showed that GA inhibited the expression of c-IAP, survivin, Mcl-1, bcl-2 and bcl-xl in a time-dependent manner. The inhibition was less pronounced for bcl-2 than for the other gene products. Maximum suppression was observed at around 12–24 h (Fig. 6A).

**GA Suppresses the Expression of Proliferative Proteins.** Cyclin D1, which is required for cell proliferation and for transition from the G1 to S phase of the cell cycle, is also regulated by STAT3. We therefore examined the effect of GA on constitutive expression of cyclin D1 in U266 cells. Our results showed that GA treatment suppressed the expression of cyclin D1 in a time-dependent manner (Fig. 6B).

**GA Down-Regulates the Expression of Angiogenic Proteins.** VEGF, a major mediator of angiogenesis, is regulated by STAT3 activation. Therefore, we examined the effect of GA on constitutive VEGF expression in U266 cells. Our results show that GA inhibited the expression of this protein in U266 cells in a time-dependent manner (Fig. 6B).
Discussion

Because STAT3 activation has been linked with most chronic diseases, including cancer, our findings that GA modulates the STAT3 cell signaling pathway provide a rationale for its use to treat various types of cancer. We demonstrate that GA was effective in blocking the activation of the STAT3 pathway. It suppressed both constitutive and inducible activation of STAT3. This inhibition was linked to the down-regulated activation of various kinases linked to STAT3 activation and induction of phosphatases. Down-regulation of STAT3 activation led to the suppression of expression of various proteins involved in the survival and proliferation of tumor cells (Fig. 6C).

We investigated in detail how GA induces apoptosis. First, we found that GA inhibited the phosphorylation of STAT3 at both tyrosine residue 705 and serine residue 727. Although the role of tyrosine 705 in STAT3 activation is well known (22). PKC, MAPK, and CDK5 have been implicated in the phosphorylation of STAT3 at serine 727 (22). PKC-ε has been shown to interact with STAT3 directly and phosphorylate serine 727 (35). Whether GA affects any of these kinases is not clear at present. Similarly, a large number of tyrosine kinases have been linked to phosphorylation of STAT3. These include EGFR (36), JAK1 and JAK2 (30, 31), and c-Src (29). We found that GA inhibited c-Src, JAK1, and JAK2 activation. C-Src–mediated STAT3 activation has been linked to the transformation of cells. Various tumors exhibit persistently active STAT3 that is associated with activated Src, including breast cancer (37), and melanoma (38). Inhibition of Src in these tumors by GA should down-regulate STAT3 activation and suppress growth.
We also found evidence that inhibition of STAT3 activation is linked to the induction of a PTP by GA. Numerous PTPs have been implicated in STAT3 signaling, including SHP-1 (39), SHP-2 (40), TC-PTP (41), PTEN (42), PTP-1D (43), CD45 (43), and PTP-ε. We found that GA inhibits the STAT3 activation pathway through the induction of SHP1. GA was found to stimulate the expression of SHP-1 protein in U266 cells, which correlated with down-regulation of constitutive STAT3 phosphorylation in these cells. Silencing of the SHP-1 gene by siRNA reversed the STAT3 inhibitory effect of GA, thereby further implicating a critical role of this phosphatase in GA-induced down-regulation of STAT3 activation. The silencing the SHP1 also reversed GA-induced apoptosis. Loss of SHP-1 has been shown to enhance JAK3/STAT3 signaling in anaplastic lymphoma kinase–positive anaplastic large-cell lymphoma (39). SHP-1 has been shown to be inactive in various human tumors, including multiple myeloma (44) and lymphoma (39). DNA methylation has been described as one of the mechanisms for inactivation of SHP-1 in different cancers (44).

Previously, we showed that GA can also suppress NF-κB activation (9). Whether the suppression of STAT3 activation by GA is also linked to the inhibition of NF-κB activation is not clear. The p65 subunit of NF-κB has been shown to interact with STAT3 (45). STAT3 and NF-κB, however, are activated in response to different cytokines: IL-6 is a major activator of STAT3 and tumor necrosis factor is a potent activator of NF-κB. Interestingly, erythropoietin has been shown to activate NF-κB through the activation of JAK2 kinase (46). Thus, it is possible that the suppression of JAK2 kinase activation is the critical target for the inhibition of both NF-κB and STAT3 activation by GA.

We also found that GA suppresses the expression of STAT3-regulated proteins,
including cell proliferative cyclin D1, COX-2, the angiogenic protein VEGF, and antiapoptotic gene products, including c-IAP, McI-1, survivin, bcl-2, and bcl-xL. However, no appreciable change was observed in the expression of ICAM-1 by GA treatment. Amongst the many genes controlled by NF-κB and STAT3, either synergistically or individually. Some genes are prominent targets for both NF-κB and STAT3, such as Bcl-xL, Bcl-2, c-IAP, cyclin D1, VEGF, COX-2 whereas A1 and c-FLIP are mostly NF-κB-dependent and McI-1 and survivin are STAT3-dependent (22, 45, 47, 48). The down-regulation of bcl-2 and survivin by GA that we found is in agreement with previous reports (12, 13). Expression of Bcl-xL has been reported to be regulated by STAT3 (38), and it is overexpressed in multiple myeloma cells (49). Bcl-xL has also been shown to block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemoresistance. The down-regulation of Bcl-xL expression that we found is likely linked to the ability of GA to induce apoptosis in multiple myeloma cells. The down-regulation of Bcl-2, Bcl-xL, and survivin expression is likely linked to the ability of GA to induce apoptosis in multiple myeloma cells. We further observed that GA induced the down-regulation of McI-1 protein. Because VEGF expression is also regulated by STAT3, GA may mediate antiangiogenesis through the down-regulation of VEGF. We and others have indeed shown that GA can suppress angiogenesis (9, 20, 21).

Constitutive STAT3 activation is associated with various types of carcinoma, sarcoma, lymphoma, and leukemia (23). Thus, the suppression of constitutively active STAT3 in multiple myeloma cells raises the possibility that GA might also inhibit constitutively active STAT3 in other types of cancer cells (22). We observed that GA
inhibited the growth of head and neck cancer, breast carcinoma, and human prostate carcinoma cells.

Perhaps one of the best in vitro model of premalignancy for cancer prevention is STAT3 as suggested by the evidence, first that STAT3 plays a major role in oncogenesis and regarded as an oncogene (50-52); second, STAT3 is activated by an oncogenic Src (29, 50); third, STAT3 regulates transformation, inflammation, survival, proliferation and angiogenesis of the tumors through expression of c-myc, COX2, bcl-xl, survivin, cyclin D1 and VEGF respectively (53-56). Because our evidences indicate that gambogic acid downregulates STAT3 activation and STAT3-regulated gene expression, it suggests chemopreventive role of gambogic acid in an in vitro premalignancy model of cancer prevention. Overall, our results show that GA inhibits growth and induces apoptosis in various tumor cells through suppression of both inducible and constitutive STAT3 activation via the induction of tyrosine kinase phosphatase. Further studies in animals are needed to validate human clinical trials. Moreover, in China, this agent is already in clinical trials (13).

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Legend of figures

Figure 1. Effects of GA in induction of apoptosis in multiple myeloma cells. (A) Chemical structure of GA. (B) GA-induced apoptosis. U266 cells were treated with GA (2.5 μM) for indicated times, and cell death was determined by fluorescence-activated cell sorting using annexin V/propidium iodide staining (left panel) and propidium iodide staining (right panel). (C) GA (2.5 μM) induced cell death treated for indicated times as determined by the live/dead assay. (D) Cleavage of procaspase-9, -3 and poly(ADP ribose) polymerase as determined by Western blotting in whole-cell extracts of GA-treated cells. The same blot was stripped and reprobed with STAT3 antibody to verify equal protein loading (left panel). MCF-7 and MCF-10A cells were pretreated with indicated concentration of GA for 24 h. Cell viability was then analyzed by the MTT method (right panel).

Figure 2. (A) GA suppresses constitutive expression of phospho-STAT3. U266 (2x10^6 cells/mL) were treated with the indicated concentrations of GA for 6 h, after which whole-cell extracts were prepared, and 40 μg of protein were resolved on 10% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 (Tyr705). The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. (B) GA suppresses STAT3 phosphorylated at tyrosine705 and serine727 in a time-dependent manner. U266 cells (2 x10^6/mL) were treated with the 2.5 μM GA for the indicated times, after which Western blotting was performed for phospho-STAT3 (Tyr705) as described previously. The same blots were stripped and reprobed with phospho STAT-3 (Ser727) and then STAT3 to verify equal protein loading.
(C) GA causes inhibition of translocation of STAT3 to the nucleus. U266 cells (2x10^6/mL) were incubated with or without 2.5 µM GA for 6 h and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. (D) GA suppresses STAT3 DNA binding in a dose-dependent manner. U266 cells (2x10^6/mL) were treated with the indicated concentrations of GA for 6 h and analyzed for nuclear STAT3 levels by EMSA (left panel). GA suppresses STAT3 DNA binding in a time-dependent manner. U266 cells (2x10^6/mL) were treated with 2.5 µM GA for the indicated times and analyzed for nuclear STAT3 levels by EMSA (right panel).

Figure 3. (A) GA down-regulates IL-6–induced phospho-STAT3. MM1.S cells (2 × 10^6) were treated with IL-6 (10 ng/mL) for the indicated times. Whole-cell extracts were prepared, and phospho-STAT3 level was detected by Western blot. The same blot was stripped and reprobed with STAT3 antibody to verify equal protein loading. (B) MM1.S cells (2 × 10^6) were treated with 2.5 µM GA for the indicated times and then stimulated with IL-6 (10 ng/mL) for 15 min. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by Western blotting. The same blot was stripped and reprobed with STAT3 antibody to verify equal protein loading (lower panel).

Figure 4. (A) GA suppresses phospho-JAK1 expression. U266 cells (2x10^6/ml) were treated with 2.5 µM GA for indicated time, after which whole-cell extracts were prepared and 40 µg protein were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-JAK1 antibody. The same blots were stripped and reprobed with JAK1 antibody to verify equal protein loading. (B) GA
suppresses expression of phospho-JAK2. U266 cells (2x10^6/ml) were treated with 2.5 μM GA for indicated time, after which whole cell extracts were prepared. Western blotting for phospho-JAK2 was performed. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. (C) To determine the activity of JAK2, a kinase assay was performed using GA (2.5 μM) treated whole-cell extract as described in ‘Materials and Methods’. Total protein level was determined by western bloting.

Figure 5. (A) Pervanadate reverses the phospho-STAT3 inhibitory effect of GA. U266 cells (2x10^6/ml) were treated with pervanadate and 2.5 μM GA for 6 h, after which whole-cell extracts were prepared, and 40 μg protein extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. (B) GA induces the expression of SHP-1 protein. U266 cells (2 x 10^6/mL) were treated with indicated concentrations of GA for 6 h, after which whole-cell extracts were prepared and 40 μg proteins were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with SHP-1 antibody. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. (C) Effect of SHP-1 knockdown on GA–induced expression of SHP-1. SCC4 cells (2 x 10^6/mL) were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 48 h, cells were treated with 2.5 μM GA for 6 h, and whole-cell extracts were subjected to Western blot analysis for SHP-1. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading (left panel). Transfection with SHP-1 siRNA reverses GA-induced suppression of STAT3 activation. SCC4 cells (2 x 10^6/mL) were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After
48 h, cells were treated with 2.5 µM GA for 6 h, and whole-cell extracts were subjected to Western blot analysis for phosphorylated STAT3. The same blots were stripped and reprobed with STAT3 antibody (right panel). (D) Knockdown of SHP-1 inhibited the apoptotic effect of GA. SCC4 cells (1 x 10^5/mL) were transfected with either scrambled or SHP-1–specific siRNA (50 nM). After 48 h, cells were treated with 50 µM GA for 24 h, and the percentage of apoptosis was analyzed by the live/dead assay.

**Figure 6.** GA suppresses STAT3-regulated proliferative, survival, and angiogenic gene products. U266 cells (2 x 10^6/mL) were treated with 2.5 µM GA for the indicated time intervals, after which whole cell extracts were prepared and 40 µg proteins resolved on 10% SDS-PAGE and probed against (A) c-IAP, survivin, Mcl-1, bcl-2, bcl-xl (B) cyclin D1, VEGF, COX-2, and ICAM-1 antibody. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. (C) Schematic representation of mechanism by which GA inhibits STAT3 activation and induces apoptosis.
F5

A

Pervanadate

- + - -

GA

- - + -

pSTAT3

- - + +

STAT3

B

GA (time, hr)

0 1 2 4 6

SHP1

- - + -

β-actin

C

SHP1 Control

Control siRNA siRNA

GA

- - + +

SHP-1

- - + +

β-actin

D

Medium

2%

GA

67%

SHP1 siRNA

1%

34%

Control siRNA

2%

72%
A 0 6 12 24 GA (Time, hr)
- c-IAP1
- Survivin
- Mcl-1
- Bcl-xL
- Bcl-2
- β-actin

B 0 6 12 24 GA (Time, hr)
- Cyclin-D1
- VEGF
- CDK-2
- ICAM1
- β-actin

C
siRNA
Gambogic acid

JAK
SNP-1
PYPase inhibitor

STAT3
Nuclear translocation of STAT3
STAT3 DNA binding
Transcription of cell survival genes
Apoptosis
Gambogic Acid Inhibits STAT3 Phosphorylation Through Activation of Protein Tyrosine Phosphatase SHP-1: Potential Role in Proliferation and Apoptosis

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