ARTICLE

Journal of Cellular Biochemistry 112:1552–1562 (2011)

Journal of Cellular Biochemistry

Signal Transducer and Activator of Transcription 3 Pathway Mediates Genipin-Induced Apoptosis in U266 Multiple Myeloma Cells

Jang Choon Lee,[1] Kwang Seok Ahn,[1] Soo-Jin Jeong,[1] Ji Hoon Jung,[1] Tae-Rin Kwon,[1] Yun-Hee Rhee,[1] Sun-Hee Kim,[1] Si-Young Kim,[2] Hwi-Joong Yoon,[2] Shudong Zhu,[3] Chang-Yan Chen,[3] and Sung-Hoon Kim[1*]

[1]*College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea*

[2]*Department of Hematology and Oncology, School of Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea*

[3]*Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215*

ABSTRACT

It has drawn a lot of attention to target signal transducer and activator of transcription 3 (STAT3) as a potential strategy for cancer therapeutics. Using several myelogenous cell lines, the effect of genipin (an active compound of Gardenia fruit) on the STAT3 pathway and apoptosis was investigated. Genipin suppressed the constitutive STAT3 activation in U266 and U937 cells and stimulated Src homology 2 domain-containing phosphatase 1 (SHP-1), which dephosphorylates and inactivates STAT3. Specifically, genipin blocked STAT3 activation via repressing the activation of c-Src, but not Janus kinase 1 (JAK1). Genipin also downregulated the expression of STAT3 target genes including Bcl-2, Bcl-x_L, Survivin, *Cyclin D1*, and VEGF. Conversely, protein tyrosine phosphatase inhibitor pervanadate blocked genipin induced STAT3 inactivation. Using DNA fragmentation or TUNEL assays, we demonstrated the apoptotic effect of genipin on U266, MM.1S, and U937 cells. Furthermore, genipin effectively potentiated the cytotoxic effect of chemotherapeutic agents, such as bortezomib, thalidomide, and paclitaxel in U266 cells. Our data suggest that through regulation of Src and SHP-1, genipin antagonizes STAT3 for the induction of apoptosis in myeloma cells. J. Cell. Biochem. 112: 1552–1562, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: GENIPIN; MULTIPLE MYELOMA; PACLITAXEL; STAT3; BORTEZOMIB; THALIDOMIDE

Multiple myeloma (MM) is a neoplasm of terminally differentiated neoplastic B cells, accounting for about 10% of all hematologic malignancies [Feinman et al., 1999; Haematology, 2001; Brenner et al., 2008]. The clonal B-cell neoplasm proceeds through different phases: an inactive phase in which tumor cells are nonproliferating mature plasma cells, an active phase with a small percentage (1%) of proliferating plasmablastic cells, and a fulminant phase with the frequent occurrence of extramedullary proliferation and an increase in plasmablastic cells [Hallek et al., 1998].

Nowadays the introduction of thalidomide, bortezomib, and lenalidomide has dramatically changed the treatment paradigm of multiple myeloma. In patients eligible for autologous stem cell transplant (ASCT), combinations including thalidomide/dexamethasone (Thal/Dex) bortezomib/dexamethasone (Bort/Dex) or lenalidomide/dexamethasone (Rev/Dex) have been introduced as induction regimens [Cavo, 2006]. Melphalan has also been used in younger patients as a conventional therapy [Minnema et al., 2010]. However, these treatments are associated with side effects to various extents. To this end, herbal drugs or natural compounds are being tested recently [Ma et al., 2005; Mitsiades et al., 2008; Gay and Palumbo, 2010; Ocio et al., 2009].

The Gardenia fruit (*Gardenia jasminoides*) has been used in traditional oriental medicine for the treatment of various diseases including irritability in febrile diseases, jaundice, acute conjunctivitis, epistaxis, hematemesis, hematuria, pyogenic infections, and

Jang Choon Lee, Kwang Seok Ahn, Soo-Jin Jeong-These authors contributed equally to this study.

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Sung-Hoon Kim, MD, PhD, Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Korea.
E-mail: sungkim7@khu.ac.kr

Received 19 May 2010; Accepted 3 February 2011 • DOI 10.1002/jcb.23077 • © 2011 Wiley-Liss, Inc.
Published online 22 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

ulcers of the skin [Woetmann et al., 1999]. Some active compounds such as genipin, geniposide, gardenone, crocetin, and gardendiol were identified in the fruit [Chang and But, 1987; Chang et al., 1996; Ono et al., 2007; Zhou et al., 2007]. Although genipin displayed anti-inflammatory, anti-diabetic, and proapoptotic activities in liver and prostate cancers [Kim et al., 2005; Koo et al., 2006; Zhang et al., 2006; Hong and Kim, 2007; Lima et al., 2009; Nam et al., 2010], the roles of genipin in multiple myeloma have not been reported. In the present study, using the multiple myeloma cell lines as a model system, we studied whether genipin could affect multiple myeloma cells in the cell proliferation and survival. We found that genipin suppressed constitutive STAT3 activation and its downstream signaling involved in cell survival, proliferation and angiogenesis, and potentiated apoptototic effects of anticancer agents including paclitaxel, bortezomib, and thalidomide in multiple myeloma cells.

MATERIALS AND METHODS

REAGENTS

Genipin (Fig. 1A) was purchased from Sigma–Aldrich. Stock solution of genipin (100 mM) was prepared in dimethyl sulfoxide, stored at −80°C, and diluted in cell culture medium for use. RPMI 1640, fetal bovine serum (FBS) and antibiotic–antimycotic mixture were obtained from Welgene. Trypan blue was obtained from GIBCO. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich. SDS, Tris, Glycine, and NaCl were obtained from Amresco, mouse anti-rabbit-horse-radish peroxidase (HRP) conjugate, and bovine serum albumin were purchased from Bio-Rad. Rabbit polyclonal antibody against STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr^{705}), Bcl-2, Bcl-x_L, Mcl-1, SHP-1, SHP-2, SOCS-1, PTEN, procaspase-3, cyclin D1, survivin, VEGF, and poly (ADP) ribose polymerase (PARP)

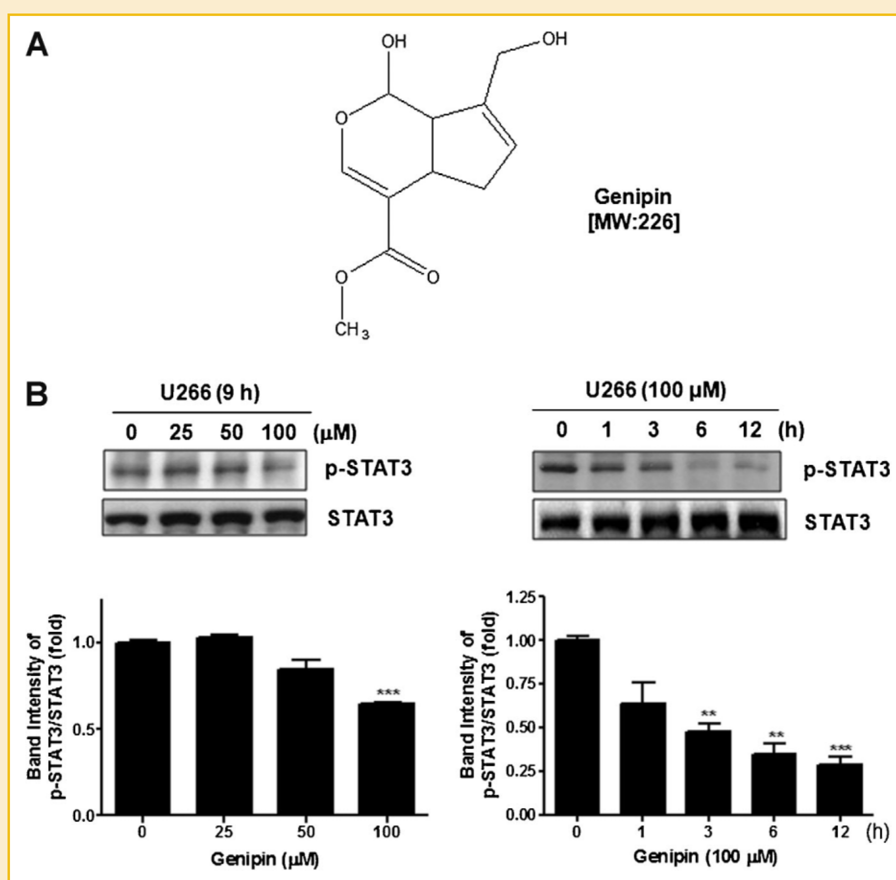

Fig. 1. Genipin inhibits STAT3 activation. (A) The chemical structure of genipin. (B) U266 cells (1×10^6 cells /ml) were incubated with the indicated concentrations of genipin for 9 h (upper) or with 100 μM genipin for the indicated time (lower). Proteins in the cell lysates were resolved by SDS–PAGE and immunoblotted with antibodies for phospho-STAT3 (p-STAT3) or STAT3. Graphs represent band intensities of indicated proteins. (C) U937 cells were incubated with genipin and analyzed as in (B). Graphs represent band intensities of indicated proteins. (D–F) Nuclear extracts were prepared from U266 cells treated with genipin as described in (B) and analyzed by EMSA using the radiolabeled oliogonucleotide containing hSIE. (G) Cellular localization of STAT3 was analyzed by confocal microscopy. Cells were treated with 100 μM genipin for 12 h on glass coverslips and incubated with anti-STAT3 and Alexa488-conjugated secondary antibodies. The immunostained cells were mounted with medium containing DAPI and visualized by use of Olympus FLUOVIEW FV10i confocal microscope (x 400). (H) U266 cells (1×10^6 cells) were treated with 100 μM genipin for up to 12 h (left), or treated with 100 μM genipin for 1 h followed by PBS washing twice to remove genipin and resuspension in fresh medium (right). Cells were lyzed at the indicated times, and same amount of lysate proteins analyzed by immunoblotting with the antibodies. Graphs represent band intensities of indicated proteins. All data were expressed as mean ± SD *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ vs. control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

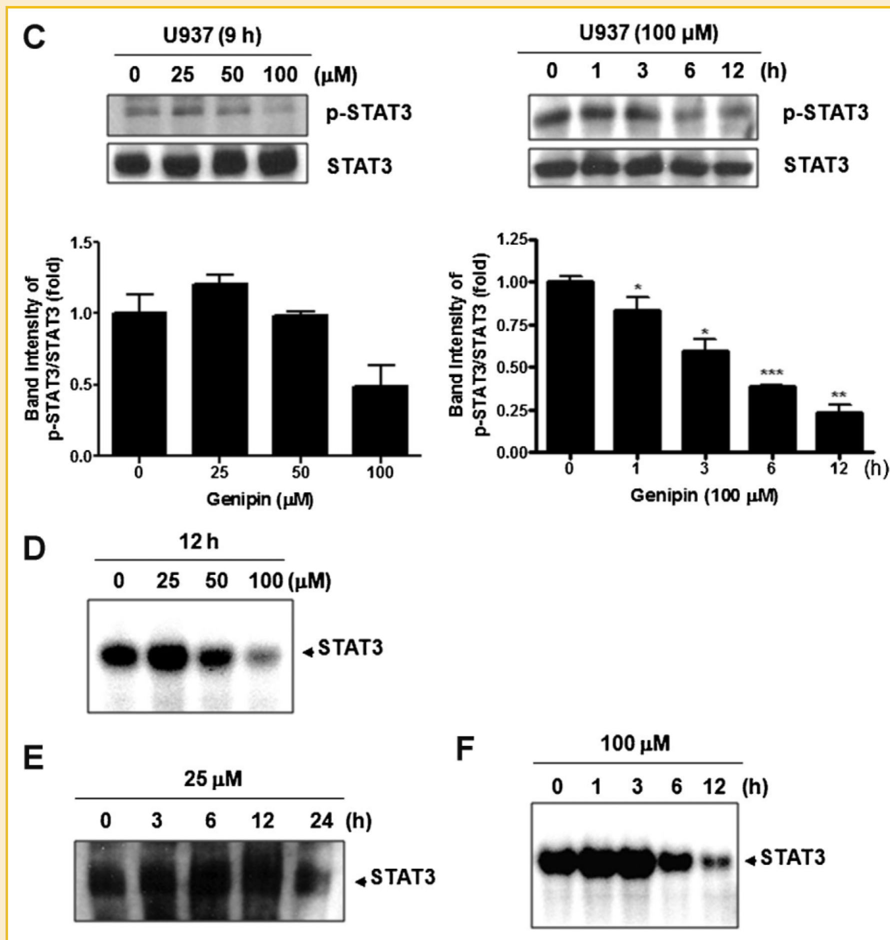

Fig. 1. (*Continued*)

were purchased from Santa Cruz Biotechnology. Antibodies against phospho-Src (Tyr^{416}), Src, phospho-JAK1 ($Tyr^{1022/1023}$), and JAK1 were purchased from Cell signaling Technology. Goat anti-mouse HRP was purchased from Bio-Rad. Bacteria-derived recombinant human IL-6 was obtained from R&D systems. Thalidomide and paclitaxel were obtained from Sigma and VELCADE® (Bortezomib) was obtained from Millennium Pharmaceuticals, Inc.

CELL LINES

Human multiple myelomas (U266 and MM.1S) and leukemia cells (U937, HL-60 and K562) were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 containing antibiotic-antimyotic with 10% FBS.

WESTERN BLOTTING

Cells were lyzed in lysis buffer [20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.003 mg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 4 mM $NaVO_4$]. Lysates were then spun at $14\,000 \times g$ at 4°C for 10 min and supernatants were in parallel lanes on 10% sodium dodecyl sulfacte (SDS) gels. After electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies (1:1000) for STAT3, Bcl-2, Bcl-x_L, survivin, cyclin D1, VEGF, PARP, and caspase-3 overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 2 h, and examined by enhanced chemiluminescence (ECL, Amersham).

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Cells were washed and suspended in Trizol reagent. Total RNA was extracted according to the manufacturer's instructions (Invitrogen, Life Technologies). One microgram of total RNA was converted to cDNA by superscript reverse transcriptase and then amplified by Platinum Taq polymerase using superscript one step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The relative expressions of *SHP-1*, *cyclin D1*, and *Bcl-2* were analyzed using PCR with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control. The reaction was performed at 50°C for 30 min, 94°C for 2 min, and 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min, with extension at 72°C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

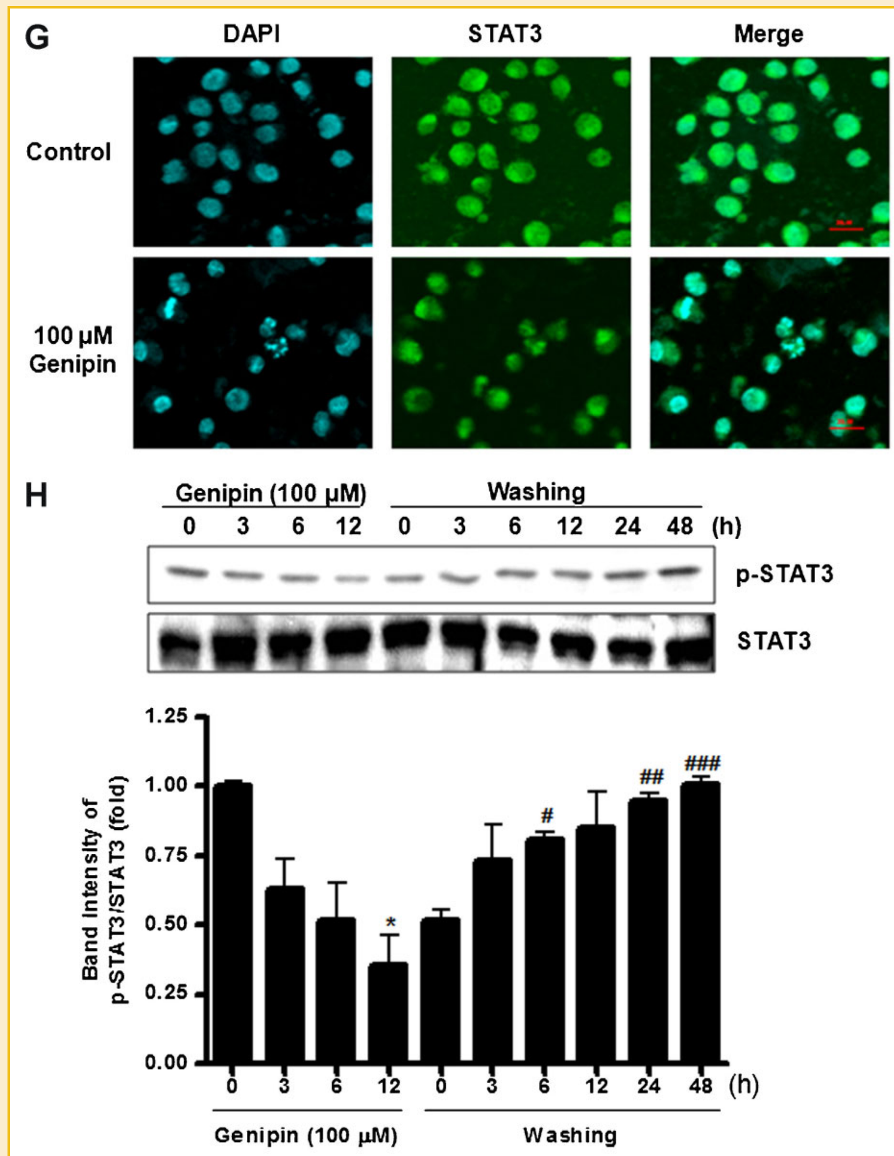

Fig. 1. (*Continued*)

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

STAT3-DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled high-affinity sis-inducible element (hSIE) probe (5'- CTTCATTTCCCGTAAATCCCTAAAGCT-3′ and 5′-AGCTTTAGGG ATTTACGGGAAATGA-3′) as previously described [Yu et al., 1995]. Briefly, nuclear extracts were prepared from genipin-treated cells and incubated with the labeled 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 Universial hood II (Bio-Rad).

IMMUNOFLUORESCENCE ASSAY

Cells were seeded onto glass coverslips at a density of 1×10^5 cells/ml and incubated in the absence or presence of 100 µM genipin for 12 h. The cells were fixed in chilled methanol, blocked in 0.5% bovine serum albumin and 3% glycerol in PBS for 1 h, and incubated with anti-STAT3 antibody (Cell signaling) for 1 h at room temperature. Then, Alexa488-conjugated anti-rabbit immunoglobulin G (IgG) antibody (Molecular probes Inc.) was used as a secondary antibody. The immunostained cells were mounted with medium containing DAPI and visualized by use of Olympus FLUOVIEW FV10i confocal microscope (x 400).

SIRNA TRANSFECTION

SiRNA oligonucleotides for SHP-1 (Santa Cruz biotechnology) were transfected by using LipofectAMINE transfection reagent (Invitrogen) according to manufacturer's protocols.

CYTOTOXICITY ASSAY

U266, U937, and MM.1S cells were seeded at a density of 5×10^3 cells/well in 96-well plates. The cells were incubated with

25, 50, and 100 μM genipin. After 24, 48, and 72 h incubation, 50 μl of MTT was added to each well. After incubation at 37°C for 2 h, extraction buffer (20% SDS and 50% dimethylformamide) was added to the cells. The cells were incubated overnight at 37°C, and the absorbance was then measured at 570 nm by a microplate reader (TECAN).

CELL CYCLE ANALYSIS

To determine apoptosis, cell cycle analysis was performed using propidium iodide staining. After treatment with genipin, the cells were collected, washed with cold PBS, fixed in 70% ethanol, and incubated at 37°C with 0.1% RNase A in PBS for 30 min. Cells were then washed, and suspended in PBS containing 25 μg/ml propidium iodide for 30 min at room temperature. Cell cycle distribution was analyzed with a FACS Vantange flow cytometry (Becton-Dickinson).

TERMINAL DEOXYNUCLEOTIDY DUTP NICK END LABELING (TUNEL) ASSAY

Individual apoptotic cell death was observed using Dead End TM fluorometric TUNEL assay kit according to the manufacturer's instructions. Briefly, U266 cells treated with genipin were washed with cold PBS. Cells were plated onto the poly-L-lysine-coated slide and fixed in 4% paraformaldehyde for 30 min and washed twice with PBS for 2 min. Cells suspended in permeabiliztion solution (0.1%Triton X-100 and 0.1% Sodium citrate) for 4°C overnight were washed with PBS twice. Then cells were incubated in 25 μl of TUNEL assay mixture (sigma TUNEL assay kit) for 60 min at 37°C in a humidified chamber in dark. The slides were mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (VECTOR, Burlingame, CA, USA) and visualized under an Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc.).

STATISTICAL ANALYSIS

All data were presented as mean ± standard deviation (SD). The statistically significant differences between control and genipin treated groups were calculated by Student's *t*-test. All experiments were carried out at least thrice.

RESULTS

GENIPIN INHIBITS STAT3 ACTIVATION

As human multiple myeloma and lymphoma cells are known to express constitutively activated STAT3 and STAT3 plays an important role in cell survival of cancer [Yu and Jove, 2004], we examined whether genipin modulates STAT3 activity, employing human multiple myeloma U266 and U937 cells. As shown in Figure 1B and C, genipin decreased the phosphorylation of STAT3 at tyrosine 705 in a dose- and time-dependent manner in U266 and U937 cells.

Additionally, genipin suppressed the binding of STAT3 to DNA at 50 or 100 μM treatment, but not 25 μM, in U266 cells by EMSA (Fig. 1D). To verify whether genipin has the duality to regulate STAT3 activation, parallel assays were performed in cells treated with 25 or 100 μM genipin as indicated time periods. As shown in Figure 1E, genipin at lower concentration weakly increased the binding of STAT3 to DNA by 12 h treatment. In contrast, 25 μM genipin suppressed the binding activity at 24 h treatment (Fig. 1E). 100 μM genipin reduced the STAT3 and DNA binding in a time-dependent manner (Fig. 1F). Furthermore, immunostaining was performed to detect cellular localization of STAT3 in the absence or presence of genipin. As shown in Figure 1G, STAT3 is clearly localized in the nucleus in untreated U266 cells. In contrast, the nuclear translocalization of STAT3 was blocked and localized in the cytoplasm by genipin treatment in the cells.

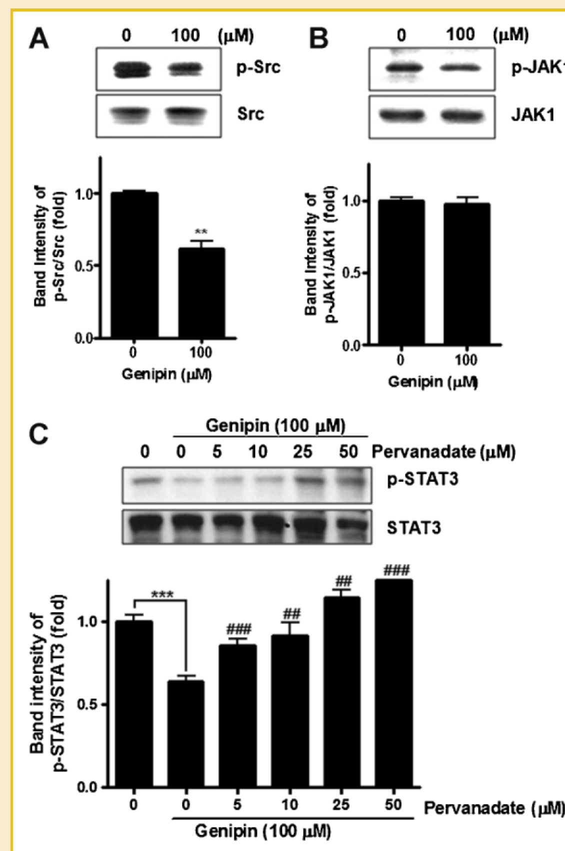

Fig. 2. Genipin suppresses the Src activation. U266 cells (1×10^6 cells/ml) were treated with 100 μM of genipin for 12 h, and same amount of lysate proteins immublotted with antibodies for phospho-Src (p-Src) and Src (A), phospho-JAK1 (p-JAK1) and JAK1 (B). Graphs represent band intensities of indicated proteins. (C) Whole cell extracts from U266 cells (1×10^6 cells/ml) treated with pervanadate and genipin for 12 h were prepared and immunoblotted with antibodies. Graphs represent band intensities of indicated proteins. (D) Whole cell extracts from U266 cells (1×10^6 cells/ml) treated with 100 μM genipin for the indicated durations were prepared and immunoblotted for SHP-1 (left) and SOCS-1, PTEN, and SHP-2 (right). The β-actin was used as an internal control. Graphs represent band intensities of indicated proteins. (E) Total RNA from U266 cells (1×10^6 cells/ml) treated with 100 μM genipin for the indicated durations was extracted, and the mRNA level of SHP-1 was analyzed by RT-PCR. GAPDH was used as an internal control. (F) Cells were transiently transfected with either SHP-1 or scrambled siRNA (50 nM) for 48 h and then treated with 100 μM genipin for 8 h. Western blotting was performed for phospho-STAT3, STAT3, and SHP-1All data were expressed as mean ± SD *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ vs. control, and $^{\#\#}$, $P < 0.01$, and $^{\#\#\#}$, $P < 0.001$.

Conversely, although genipin inhibited STAT3 phosphorylation, the STAT3 inactivation was recovered in a time course by the removal of genipin containing media, indicating that STAT3 inactivation by genipin is reversible (Fig. 1H).

GENIPIN SUPPRESSES SRC ACTIVATION

Tyrosine kinases of the Janus family and Src have been associated with STAT3 activation [Ihle, 1996]. In order to find out what has mediated the genipin-initiated STAT3 inactivation, we examined the effects of genipin on the phosphorylation of c-Src and JAK1 in U266 cells. The results showed that genipin led to reduced autophosphorylation of c-Src (Tyr^{416}), an indication of c-Src inactivation; Genipin did not reduce the phosphorylation of JAK1 at $Tyr^{1022/1023}$, which is important for JAK1 activity (Fig. 2A and B). Besides, sodium pervanadate, a tyrosine phosphatase inhibitor, reversed the genipin-initiated inhibition of STAT3 activation in the multiple myeloma U266 cells (Fig. 2C). Furthermore, genipin led to an increased expression of SHP-1, but not SOCS-1, PTEN, and SHP-2, at the protein level (Fig. 2D). Genipin also enhanced mRNA level of SHP-1 in a time-dependent manner in U266 cells (Fig. 2E).

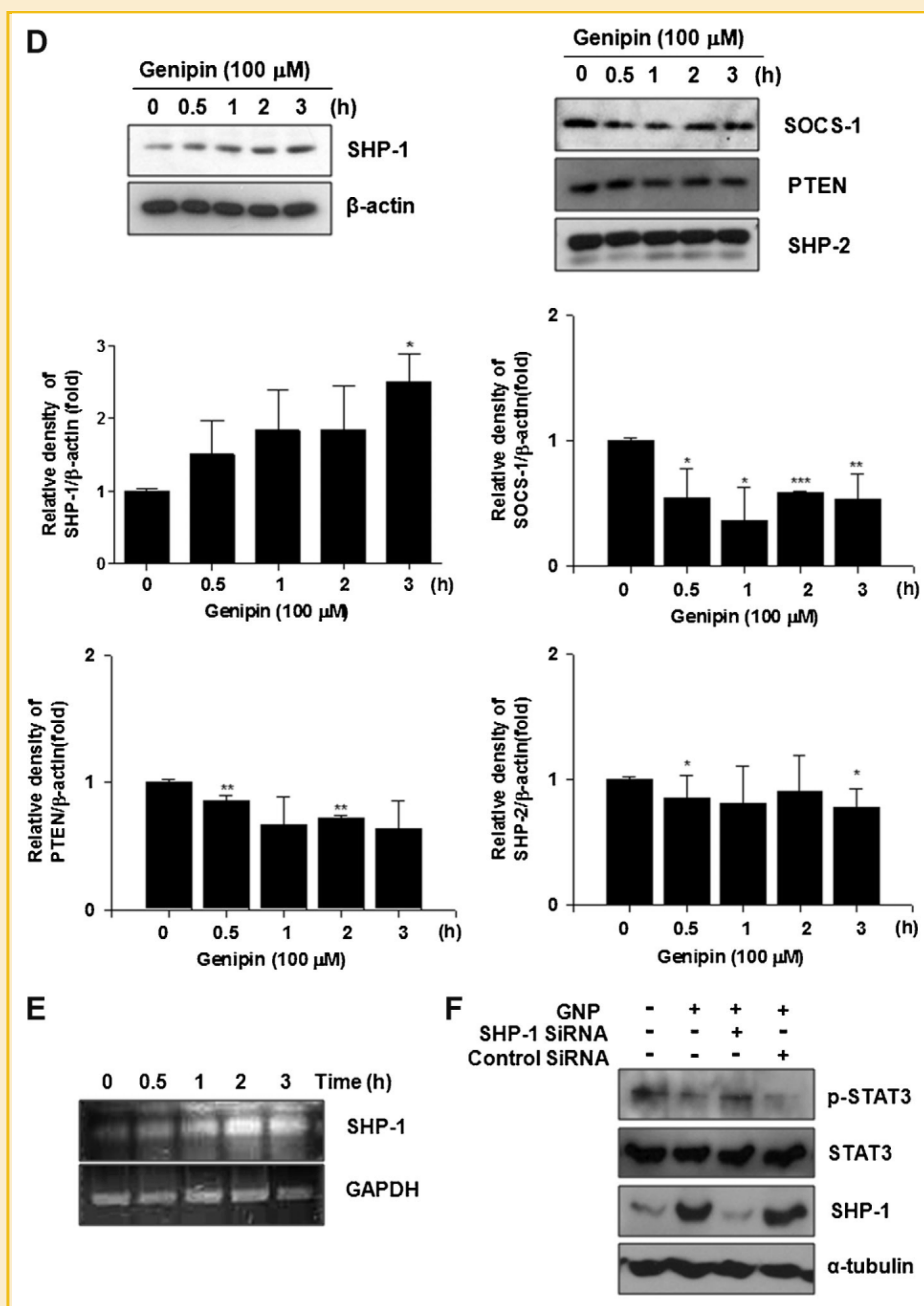

Fig. 2. (*Continued*)

Moreover, the importance of SHP-1 in genipin-mediated inhibition of STAT3 was confirmed using siRNA specific for SHP-1. As expected, SHP-1 siRNA effectively abolished genipin-induced SHP-1 expression while control siRNA did not change the expression (Fig. 2F). Taken together, these results suggested that genipin modulated the upstream signaling regulators of the STAT3, including c-Src kinase and tyrosine phosphatases SHP-1.

GENIPIN SUPPRESSES THE STAT3 TARGET GENES

STAT3 has been known to be able to regulate the genes involved in cell proliferation, survival, and angiogenesis [Bromberg et al., 1999; Epling-Burnette et al., 2001; Yu and Jove, 2004; Aggarwal et al., 2006]. To find out if this also occurs in multiple myeloma, we examined the effect of genipin on the expression of some STAT3 target genes in U266 cells, including Bcl-x_L Cyclin D1, VEGF, and Survivin. Genipin repressed the gene expressions (protein levels) of all these targets in a time-dependent manner (Fig. 3A). To further verify this, we chose to examine the gene expression of two important STAT3 targets *Bcl-*x_L and *Cyclin D1* at transcript level: genipin also decreased the mRNA levels of *Bcl-*x_L and *Cyclin D1* in a time-dependent manner in U266 cells (Fig. 3B). These data suggested that genipin can regulate proliferation, survival, and angiogenesis in multiple myeloma cells. In addition, genipin activated caspase-3 and led to the cleavage of PARP in U266 cells, suggesting that genipin induced caspase-3-dependent apoptosis in U266 cells (Fig. 3C).

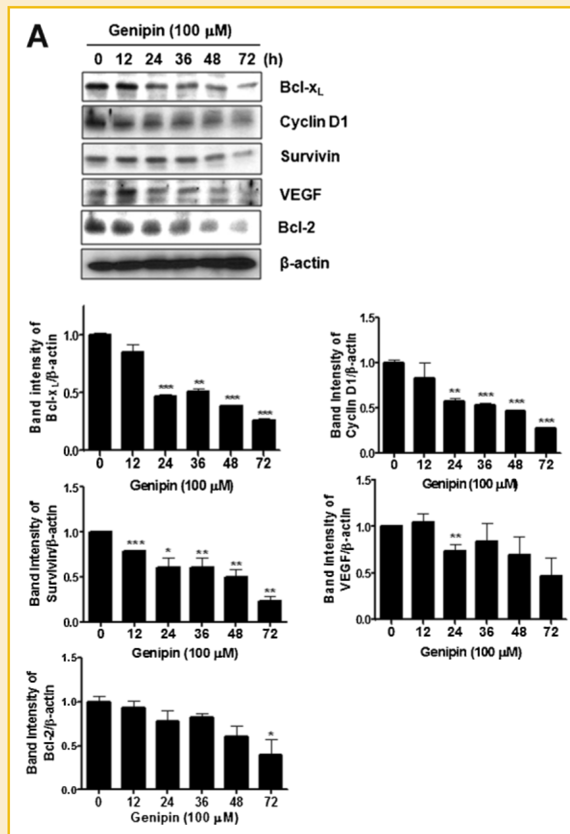

Fig. 3. Genipin suppresses the STAT3 target genes. (A) U266 cells (1×10^6 cells /ml) were treated with 100 μM genipin as indicated. Same amount of proteins from lysates were analyzed by Western blotting. Graphs represent band intensities of indicated proteins. (B) Total RNA from U266 cells (1×10^6 cells/ml) treated with 100 μM genipin for the indicated durations was isolated, and the mRNA levels of Bcl-x_L and cyclin D1 were analyzed by RT-PCR. GAPDH was an internal control. (C) Cell lysates were prepared as in (A) and caspase-3 and PARP proteins were analyzed by Western blotting. β-actin was an internal control. Graphs represent band intensities of indicated proteins. All data were expressed as mean ± SD *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ vs. control.

GENIPIN INIHIBITS THE PROLIFERATION OF U266, U937, AND MM.1S CELLS AND INDUCES APOPTOSIS IN U266 CELLS

To comparatively evaluate the effect of genipin on multiple myelomas and leukemia cells, MTT assay was performed against those cells. Genipin reduced the numbers of proliferating cells of human myelogenous cell lines including U266, U937, and HL-60, but not K562 cells, and this took place in time- and dose-dependent manners (Fig. 4A). Then we examined the extent of potential apoptosis involved in this process as MTT assay is in fact a measure of proliferation combining cell toxicity. Results showed that genipin caused increases of the sub-G_1 cell populations (Fig. 4B) and increases of TUNEL-positive cell populations, in a time-dependent manner (Fig. 4C), suggesting that genipin induced apoptosis in U266 cells.

GENIPIN POTENTIATES THE CYTOTOXIC AND APOPTOTIC EFFECTS OF CHEMOTHERAPEUTIC AGENTS IN U266 CELLS

Bortezomib (a proteasome inhibitor), thalidomide (an inhibitor of TNF expression), and paclitaxel (a mitotic inhibitor) have been approved for the treatment of patients with multiple myeloma [Park et al., 2008; Laubach et al., 2009; Napoleone et al., 2009]. We next evaluated whether genipin potentiates the effects of these three standard chemotherapeutic agents. At 24 h, proliferation of these cells were insignificant (Fig. 4A), hence the observed difference at 24 h in MTT reading mainly reflected the cytotoxicity (Fig. 5A). Genipin (25 or 50 μM) significantly enhanced the cytotoxic effect of anticancer agents in U266 cells (Fig. 5A and B). Among the three combinations, combination treatment of genipin with borezomib was more effective than those of other two combinations. In addition, genipin and bortezomib acted synergistically in inducing cytoxicity (Fig. 5A), as further supported by the cleavage of PARP in U266 cells compared with each individual treatment (Fig. 5C).

DISCUSSION

STAT is a family of six different transcription factors that play crucial roles in cytokine signaling [Shuai et al., 1993]. STAT3 is often constitutively activated in many human cancer, including multiple myeloma, lymphomas, leukemia, breast cancer, and head and neck squamous cell carcinoma [Aggarwal et al., 2006; Ahn et al., 2008; Pandey et al., 2009]. In the current study, genipin was found to decrease the phosphorylation of STAT3 at tyrosine 705 in U266 and U937 multiple myeloma cells in a dose- and time-dependent manners, demonstrating the ability of genipin to inhibit

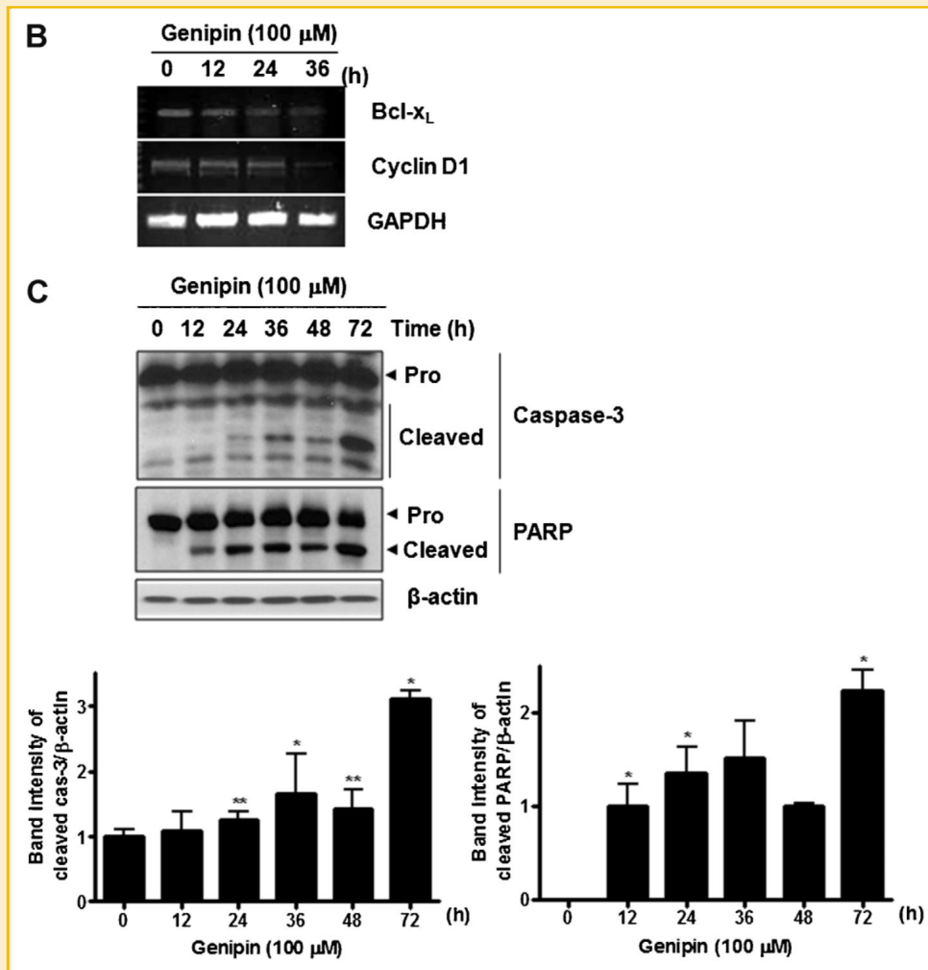

Fig. 3. (*Continued*)

STAT3 activation. This was further verified by the ability of genipin to suppress the STAT3 binding to hSIE by EMSA and immunostaining for STAT3, indicating genipin inhibits the nuclear translocation and DNA binding of STAT3, the processes activated STAT3 goes through to regulate the transcription of its target genes [Yu et al., 1995]. The fact that the inhibition of genipin on STAT3 activation is reversible suggested flexibility in potential cancer therapeutics based on genipin.

With herbal drug or natural compounds, frequently known are their biological effects, but usually little is known about the underlying mechanisms of how they exert their biological effects. In this report, we not only found an herbal drug that displayed novel potential in treatment of multiple myeloma, we also found that it targets STAT3 and its effectors, which has laid a solid foundation for future therapeutics interventions.

Activation of STAT3 is often linked to JAKs, which phosphorylates STAT3 followed by phosphorylation-dependent homodimerization of STAT3, nuclear translocation, DNA binding, and subsequent gene transcription [Ahn et al., 2008; Pandey et al., 2009]. It is interesting to note that STAT3 inhibition by genipin in U266 cells was mediated by inhibition of c-Src activity, but not that of JAK1. However, this is not totally surprising as studies have shown that Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks the transformation [Bowman et al., 2000; Brierley and Fish, 2005]. It would be interesting to find out whether or not genipin is a direct inhibitor for Src kinase activity. More interestingly, SHP-1 has been suggested to work as a positive regulator of Src kinase activity via dephosphorylation of Src at the C-terminus [Somani et al., 1997]. Src is frequently activated in cancers and regulates cancer cell apoptosis, proliferation and angiogenesis [Kim et al., 2009]; SHP-1, on the contrary, is often silenced in leukemias and lymphomas [Oka et al., 2002; Han et al., 2006] but also involved in apoptosis and cell survival [Inoue et al., 2009]. The Src was inhibited despite of presence of an increased level of positive regulator SHP-1 upon genipin treatment, which was supported by Aggarwal's recent works [Kannappan et al., 2010; Sandur et al., 2010]. It is also possible that other JAKs are involved in regulating STAT3 activation. Although the change in STAT3 phosphorylation at tyrosine 705 was observed as early as 3 h after genipin treatment, suggesting involvement of

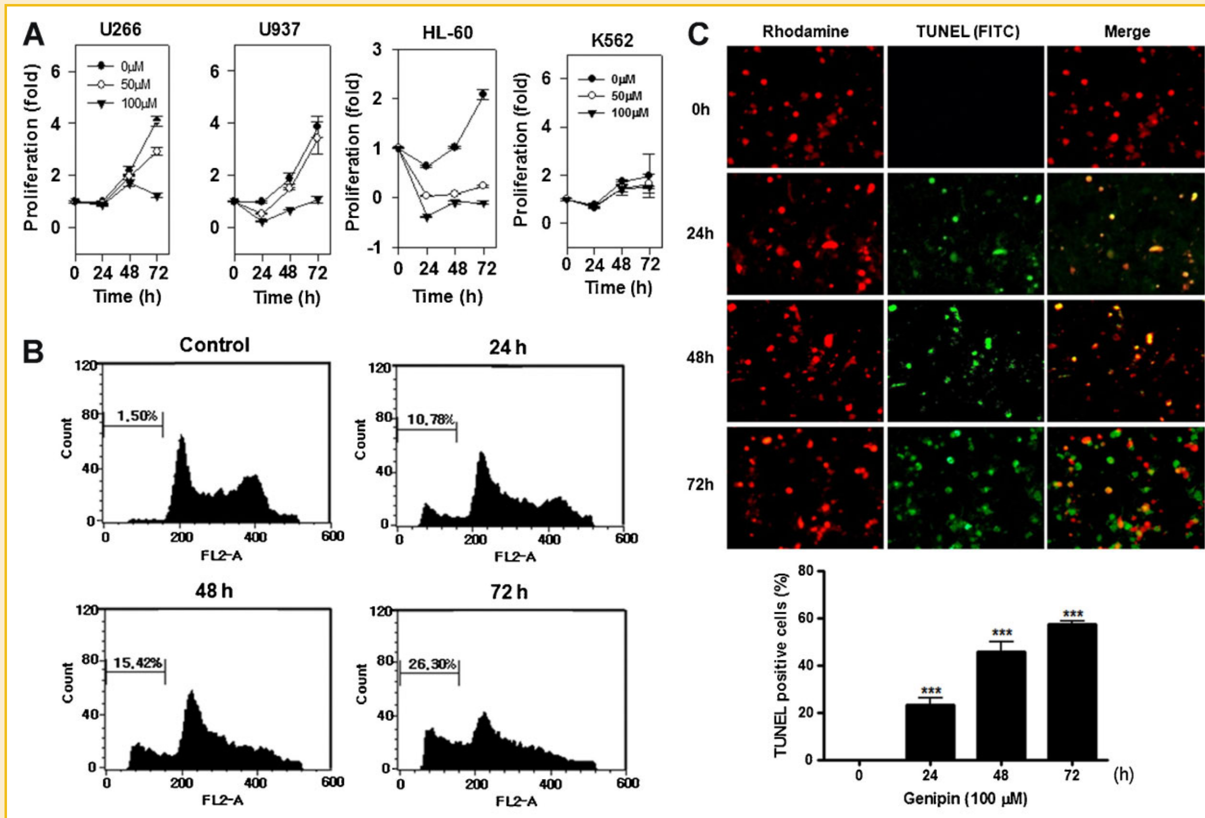

Fig. 4. Genipin and induces apoptosis in multiple myeloma cells. (A) U266, U937, HL-60, and K562 cells were plated in triplicate, treated with genipin, and then analyzed by MTT assays. (B) U266 cells (2×10^6 cells /ml) were treated with 100 μM genipin for 0, 24, 48, or 72 h. The treated cells were harvested, fixed with 70% ethanol, and stained with propidium iodide for flow cytometric analysis. The percentage of cells in sub-G_1 fraction was calculated using Cellquest software. (C) TUNEL staining was performed for the U266 cells treated with 100 μM genipin for 0, 24, 48 or 72 h. Graphs represent the percentages of TUNEL-positive cells from three randomly selected fields. All data were expressed as mean ± SD ***, $P < 0.001$ vs. control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

enzymatic reaction(s) (Src phosphorylation on STAT3 in this case), the most significant change was observed at/after 12 h, consistent with the involvement of a transcriptional regulation (changes in SHP-1 mRNA and protein levels). These data also support that both Src and SHP-1 played a role in the activation of STAT3.

The STAT3 activation has been implicated in the resistance to apoptosis, possibly through the expression of Bcl-2, Bcl-x_L, Cyclin D1 [Catlett-Falcone et al., 1999; Nielsen et al., 1999], VEGF and Bcl-x_L [Tu et al., 1998; Buettner et al., 2002]. The fact that genipin attenuated the expression of STAT3 target genes, including Bcl-2, Bcl-x_L, Survivin, Cyclin D1, and VEGF in the multiple myeloma cells, is not only consistent with the roles of Src and SHP-1 in this matter, but is also consistent with the fact that genipin inhibits cell proliferation, promotes apoptosis and cytotoxicity as observed.

Multiple myeloma relapsed after conventional therapy or stem cell transplantation is now treated with high-dose of thalidomide or bortezomib [Cavo, 2006]. Genipin enhancing the cytotoxic effect of the three anticancer agents tested including bortezomib, thalidomide, and paclitaxel in U266 cells, with the most efficient combination treatment of genipin plus bortezomib, renders genipin a potent sensitizer to chemotherapeutic agents and offers alternative, new, promising strategies to cancer therapy in multiple myeloma.

In summary, genipin inhibited c-Src activity and upregulated SHP-1, and led to inactivation of their downstream target STAT3, and downregulation of STAT3 target genes including Bcl-2, Bcl-x_L, Survivin, Cyclin D1, and VEGF in multiple myeloma cells. These are reflected in the increased cytotoxicity in multiple myeloma cells. In addition, genipin potentiated the chemotherapeutic agents in killing multiple myeloma cells. As Src is one of the heavily addicted oncogenes in various cancer cells which frequently involves STAT3 and protein phosphatase [Sharma et al., 2006], it is possible this current finding that genipin suppress STAT3 activation and induce apoptosis via Src and SHP-1 and the relevant sensitization to chemotherapeutic agents could be extended to other cancer types.

ACKNOWLEDGMENTS

The present study was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 2010-0063466).

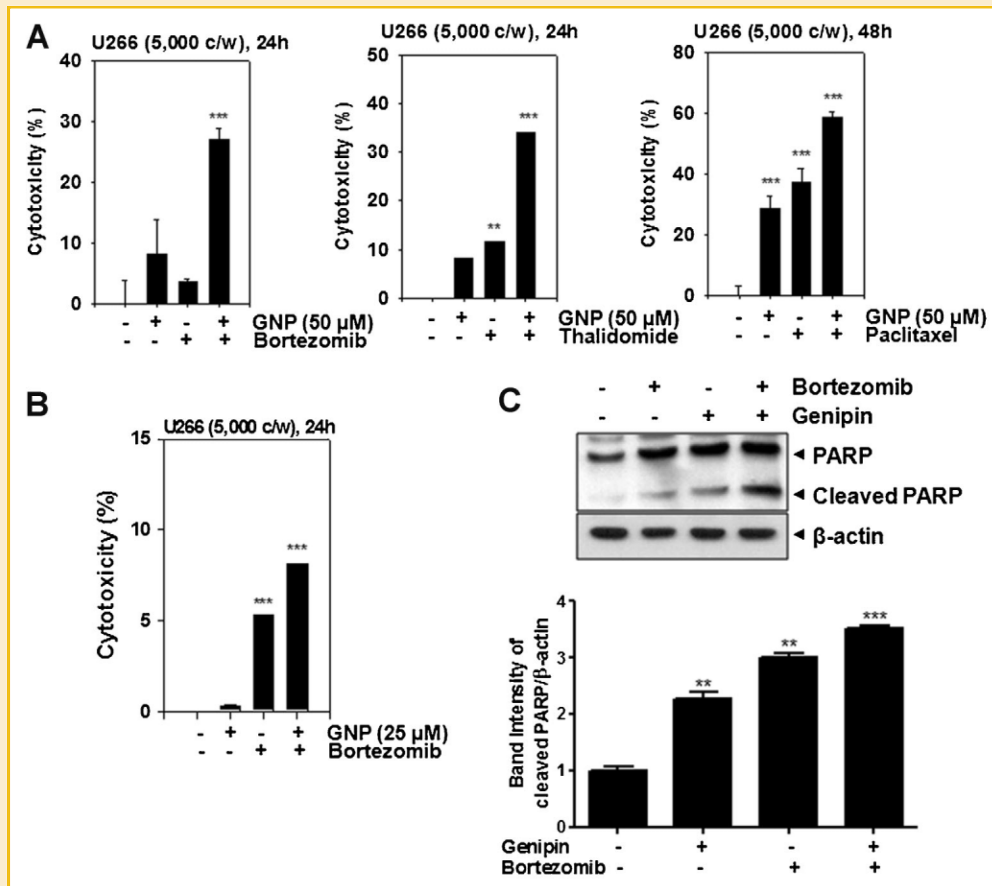

Fig. 5. Genipin potentiates the cytotoxic and apoptotic effects of chemotherapeutic agents in U266 cells. (A) U266 cells (5000 cells/well) were treated with 50 μM genipin and 20 nM bortezomib (Bor), 100 μg/ml thalidomide (Thal) or 20 nM paclitaxel (Pacl), for 24 or 48 h as indicated. The cytotoxicity was determined by MTT assays. (B) U266 cells (5000 cells/well) were treated with 25 μM genipin and 20 nM bortezomib (Bor) for 24 h. The cytotoxicity was determined by MTT assays. (C) U266 cells (1×10^6 cells/ml) were treated with 50 μM genipin or/and 20 nM bortezomib (Bor) for 36 h as indicated. PARP and cleaved PARP were analyzed by immunoblotting. β-actin was an internal control. Graphs represent band intensities of indicated proteins. All data were expressed as mean ± SD ***, $P < 0.001$ vs. control.

REFERENCES

Aggarwal BB, Sethi G, Ahn KS, Sandur SK, Pandey MK, Kunnumakkara AB, Sung B, Ichikawa H. 2006. Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. Ann N Y Acad Sci 1091:151–169.

Ahn KS, Sethi G, Sung B, Goel A, Ralhan R, Aggarwal BB. 2008. Guggulsterone, a farnesoid X receptor antagonist, inhibits constitutive and inducible STAT3 activation through induction of a protein tyrosine phosphatase SHP-1. Cancer Res 68:4406–4415.

Bowman T, Garcia R, Turkson J, Jove R. 2000. STATs in oncogenesis. Oncogene 19:2474–2488.

Brenner H, Gondos A, Pulte D. 2008. Recent major improvement in long-term survival of younger patients with multiple myeloma. Blood 111:2521–2526.

Brierley MM, Fish EN. 2005. Stats: multifaceted regulators of transcription. J Interferon Cytokine Res 25:733–744.

Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. 1999. Stat3 as an oncogene. Cell 98:295–303.

Buettner R, Mora LB, Jove R. 2002. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 8:945–954.

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, Jove R. 1999. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 10:105–115.

Cavo M. 2006. Proteasome inhibitor bortezomib for the treatment of multiple myeloma. Leukemia 20:1341–1352.

Chang HM, and But PPH. (1987) Pharmacology and applications of chinese materia medica. World Scientific Publishing, Singapore 1005.

Chang WC, Lin YL, Lee MJ, Shiow SJ, Wang CJ. 1996. Inhibitory effect of crocetin on benzo(a)pyrene genotoxicity and neoplastic transformation in C3H10T1/2 cells. Anticancer Res 16:3603–3608.

Epling-Burnette PK, Liu JH, Catlett-Falcone R, Turkson J, Oshiro M, Kothapalli R, Li Y, Wang JM, Yang-Yen HF, Karras J, Jove R, Loughran TP Jr. 2001. Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. J Clin Invest 107:351–362.

Feinman R, Koury J, Thames M, Barlogie B, Epstein J, Siegel DS. 1999. Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. Blood 93:3044–3052.

Gay F, Palumbo A. 2010. Multiple myeloma: management of adverse events. Med Oncol 27:646–653.

Haematology, UmfBCfSi. 2001. Diagnosis and management of multiple myeloma. Br J Haematol 115:522–540.

Hallek M, Bergsagel PL, Anderson KC. 1998. Multiple myeloma: increasing evidence for a multistep transformation process. Blood 91:3–21.

Han Y, Amin HM, Franko B, Frantz C, Shi X, Lai R. 2006. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteosome degradation of JAK3 and NPM-ALK in ALK+ anaplastic large-cell lymphoma. Blood 108: 2796–2803.

Hong HY, Kim BC. 2007. Mixed lineage kinase 3 connects reactive oxygen species to c-Jun NH2-terminal kinase-induced mitochondrial apoptosis in genipin-treated PC3 human prostate cancer cells. Biochem Biophys Res Commun 362:307–312.

Ihle JN. 1996. STATs: signal transducers and activators of transcription. Cell 84:331–334.

Inoue T, Suzuki Y, Mizuno K, Nakata K, Yoshimaru T, Ra C. 2009. SHP-1 exhibits a pro-apoptotic function in antigen-stimulated mast cells: positive regulation of mitochondrial death pathways and negative regulation of survival signaling pathways. Mol Immunol 47:222–232.

Kannappan R, Yadav VR, Aggarwal BB. 2010. {Gamma}-tocotrienol but not {gamma}-tocopherol blocks STAT3 cell signaling pathway through induction of protein-tyrosine phosphatase SHP-1 and sensitizes tumor cells to chemotherapeutic agents. J Biol Chem 285:33520–33528.

Kim BC, Kim HG, Lee SA, Lim S, Park EH, Kim SJ, Lim CJ. 2005. Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway. Biochem Pharmacol 70:1398–1407.

Kim LC, Song L, Haura EB. 2009. Src kinases as therapeutic targets for cancer. Nat Rev Clin Oncol 6:587–595.

Koo HJ, Lim KH, Jung HJ, Park EH. 2006. Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. J Ethnopharmacol 103:496–500.

Laubach JP, Mitsiades CS, Mahindra A, Schlossman RL, Hideshima T, Chauhan D, Carreau NA, Ghobrial IM, Raje N, Munshi NC, Anderson KC, Richardson PG. 2009. Novel therapies in the treatment of multiple myeloma. J Natl Compr Canc Netw 7:947–960.

Lima EG, Tan AR, Tai T, Marra KG, DeFail A, Ateshian GA, Hung CT. 2009. Genipin enhances the mechanical properties of tissue-engineered cartilage and protects against inflammatory degradation when used as a medium supplement. J Biomed Mater Res A 91:692–700.

Ma Z, Otsuyama K, Liu S, Abroun S, Ishikawa H, Tsuyama N, Obata M, Li FJ, Zheng X, Maki Y, Miyamoto K, Kawano MM. 2005. Baicalein, a component of Scutellaria radix from Huang-Lian-Jie-Du-Tang (HLJDT), leads to suppression of proliferation and induction of apoptosis in human myeloma cells. Blood 105:3312–3318.

Minnema MC, van der Spek E, van de Donk NW, Lokhorst HM. 2010. New developments in the treatment of patients with multiple myeloma. Neth J Med 68:24–32.

Mitsiades CS, Ocio EM, Pandiella A, Maiso P, Gajate C, Garayoa M, Vilanova D, Montero JC, Mitsiades N, McMullan CJ, Munshi NC, Hideshima T, Chauhan D, Aviles P, Otero G, Faircloth G, Mateos MV, Richardson PG, Mollinedo F, San-Miguel JF, Anderson KC. 2008. Aplidin, a marine organism-derived compound with potent antimyeloma activity in vitro and in vivo. Cancer Res 68:5216–5225.

Nam KN, Choi YS, Jung HJ, Park GH, Park JM, Moon SK, Cho KH, Kang C, Kang I, Oh MS, Lee EH. 2010. Genipin inhibits the inflammatory response of rat brain microglial cells. Int Immunopharmacol 10:493–499.

Napoleone E, Zurlo F, Latella MC, Amore C, Di Santo A, Iacoviello L, Donati MB, Lorenzet R. 2009. Paclitaxel downregulates tissue factor in cancer and host tumour-associated cells. Eur J Cancer 45:470–477.

Nielsen M, Kaestel CG, Eriksen KW, Woetmann A, Stokkedal T, Kaltoft K, Geisler C, Ropke C, Odum N. 1999. Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. Leukemia 13:735–738.

Ocio EM, Maiso P, Chen X, Garayoa M, Alvarez-Fernandez S, San-Segundo L, Vilanova D, Lopez-Corral L, Montero JC, Hernandez-Iglesias T, de Alava E, Galmarini C, Aviles P, Cuevas C, San-Miguel JF, Pandiella A. 2009. Zalypsis: a novel marine-derived compound with potent antimyeloma activity that reveals high sensitivity of malignant plasma cells to DNA double-strand breaks. Blood 113:3781–3791.

Oka T, Ouchida M, Koyama M, Ogama Y, Takada S, Nakatani Y, Tanaka T, Yoshino T, Hayashi K, Ohara N, Kondo E, Takahashi K, Tsuchiyama J, Tanimoto M, Shimizu K, Akagi T. 2002. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. Cancer Res 62:6390–6394.

Ono M, Ishimatsu N, Masuoka C, Yoshimitsu H, Tsuchihashi R, Okawa M, Kinjo J, Ikeda T, Nohara T. 2007. Three new monoterpenoids from the fruit of Genipa americana. Chem Pharm Bull (Tokyo) 55:632–634.

Pandey MK, Sung B, Ahn KS, Aggarwal BB. 2009. Butein suppresses constitutive and inducible signal transducer and activator of transcription (STAT) 3 activation and STAT3-regulated gene products through the induction of a protein tyrosine phosphatase SHP-1. Mol Pharmacol 75:525–533.

Park J, Ayyappan V, Bae EK, Lee C, Kim BS, Kim BK, Lee YY, Ahn KS, Yoon SS. 2008. Curcumin in combination with bortezomib synergistically induced apoptosis in human multiple myeloma U266 cells. Mol Oncol 2:317–326.

Sandur SK, Pandey MK, Sung B, Aggarwal BB. 2010. 5-hydroxy-2-methyl-1,4-naphthoquinone, a vitamin K3 analogue, suppresses STAT3 activation pathway through induction of protein tyrosine phosphatase, SHP-1: potential role in chemosensitization. Mol Cancer Res 8:107–118.

Sharma SV, Gajowniczek P, Way IP, Lee DY, Jiang J, Yuza Y, Classon M, Haber DA, Settleman J. 2006. A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. Cancer Cell 10:425–435.

Shuai K, Stark GR, Kerr IM, Darnell JE Jr. 1993. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science 261:1744–1746.

Somani AK, Bignon JS, Mills GB, Siminovitch KA, Branch DR. 1997. Src kinase activity is regulated by the SHP-1 protein-tyrosine phosphatase. J Biol Chem 272:21113–21119.

Tu Y, Renner S, Xu F, Fleishman A, Taylor J, Weisz J, Vescio R, Rettig M, Berenson J, Krajewski S, Reed JC, Lichtenstein A. 1998. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res 58:256–262.

Woetmann A, Nielsen M, Christensen ST, Brockdorff J, Kaltoft K, Engel AM, Skov S, Brender C, Geisler C, Svejgaard A, Rygaard J, Leick V, Odum N. 1999. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. Proc Natl Acad Sci U S A 96:10620–10625.

Yu CL, Meyer DJ, Campbell GS, Larner AC, Carter-Su C, Schwartz J, Jove R. 1995. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science 269:81–83.

Yu H, Jove R. 2004. The STATs of cancer–new molecular targets come of age. Nat Rev Cancer 4:97–105.

Zhang CY, Parton LE, Ye CP, Krauss S, Shen R, Lin CT, Porco JA Jr, Lowell BB. 2006. Genipin inhibits UCP2-mediated proton leak and acutely reverses obesity- and high glucose-induced beta cell dysfunction in isolated pancreatic islets. Cell Metab 3:417–427.

Zhou T, Zhao W, Fan G, Chai Y, Wu Y. 2007. Isolation and purification of iridoid glycosides from Gardenia jasminoides Ellis by isocratic reversed-phase two-dimensional preparative high-performance liquid chromatography with column switch technology. J Chromatogr B Analyt Technol Biomed Life Sci 858:296–301.