European Journal of Pharmacology 654 (2011) 10–16

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Janus activated kinase 2/signal transducer and activator of transcription 3 pathway mediates icariside II-induced apoptosis in U266 multiple myeloma cells

Sun-Hee Kim [a,1], Kwang Seok Ahn [a,1], Soo-Jin Jeong [a,1], Tae-Rin Kwon [a], Ji Hoon Jung [a], Sun-Mi Yun [a], Ihn Han [a], Seok-Geun Lee [a], Dae Keun Kim [b], Minkyung Kang [c,d], Chang-Yan Chen [e], Jung Weon Lee [d,*], Sung-Hoon Kim [a,*]

[a] College of Oriental Medicine, Kyung Hee University, Seoul 130-701, South Korea
[b] College of Pharmacy, Woosuk University, Wanju, 565-701, South Korea
[c] College of Medicine, Seoul National University, Seoul 110-799, South Korea
[d] College of Pharmacy, Seoul National University, Seoul 151-742, South Korea
[e] Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 19 November 2010
Accepted 26 November 2010
Available online 21 December 2010

Keywords:
Icariside II
Multiple myeloma
STAT3
Thalidomide
Bortezomib

ABSTRACT

Although the flavonoid icariside II exhibits anti-inflammatory and anti-cancer activities, its molecular targets/pathways in human multiple myeloma cells are poorly understood. To analyze the effects on signal transducer and activator of transcription 3 (STAT3) signaling and apoptosis, U266 multiple myeloma cells were treated with icariside II and performed Western blotting, electrophoretic mobility gel shift assay (EMSA), RT-PCR, proliferation assay, cell cycle analysis and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Icariside II inhibited STAT3 activation and enhanced the expression of SHP-1 and PTEN through inhibiting Janus activated kinase 2 (JAK2) and c-Src. Icariside II down-regulated the expression of STAT3 target genes Bcl-2, Bcl-x_L, survivin, cyclin D_1, COX-2 and vascular endothelial growth factor (VEGF). Also, icariside II enhanced poly (ADP-ribose) polymerase (PARP) cleavage and caspase-3 activation. Pervanadate reversed the icariside II-mediated STAT3 inactivation and also blocked the cleavages of caspase-3 and PARP, suggesting involvement of STAT3 pathway in icariside II-induced apoptosis. Furthermore, icariside II enhanced the apoptotic effects of clinically used drugs thalidomide and bortezomib in U266 cells. Icariside II could be a potential therapeutic intervention agent alone or in combination with current drugs for multiple myeloma as a novel blocker of STAT3 signaling cascades at multiple levels, contributing to its anti-proliferative and anti-apoptosis.

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1. Introduction

The flavonoid compound icariside II is derived from the stems and leaves of *Epimedium koreanum* Nakai (Berberidaceae). This class of compounds has been used as a traditional medicine for neurasthenia, amnesia and impotence (Wang and Huang, 2005; Xu and Huang, 2007). Icariside II has shown potential anti-cancer activity as indicated by its ability to suppress the proliferation and to induce apoptosis of various human cancer cells including osteosarcoma, breast carcinoma and prostate carcinoma (Choi et al., 2008; Lee et al., 2009). However, the molecular targets and the mechanisms of action of icariside II to mediate the anti-proliferative and -apoptotic activities are poorly defined and need further clarification. We have recently shown that icariside II inhibits the cyclooxygenease (COX-2)/prostaglandin E2 signaling cascade as a primary mechanism for its induction of mitochondria-mediated apoptosis in human PC-3 prostate cancer cells Bittorf et al., 1999. Since signal transducer and activator of transcription (STAT) pathways have been closely associated with cancer cell proliferation and anti-apoptosis and that COX-2 is a downstream target of STAT3, we hypothesize that icariside II may inhibit the STAT3 signaling pathway to mediate its effects on cancer cell proliferation and apoptosis.

The STAT proteins were identified in the last decade as transcription factors which were critical in mediating all cytokine driven signaling (Buettner et al., 2002). There are seven known mammalian STAT proteins, STAT1, 2, 3, 4, 5a, 5b and 6, which are involved in cell proliferation, differentiation, angiogenesis, and apoptosis (Zhang et al., 2008). In particular, STAT3 is activated in many human cancers, including prostate cancers (Mora et al., 2002), breast cancers (Dolled-Filhart et al., 2003), squamous cell carcinoma

* Corresponding authors. Kim is to be contacted at Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea. Tel.: +82 2 961 9233; fax: +82 2 964 1074. Lee, Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 151-742, South Korea. Tel.: +82 2 880 2495; fax :+82 2 872 1795.
E-mail addresses: jwl@snu.ac.kr (J.W. Lee), sungkim7@khu.ac.kr (S.-H. Kim).
[1] These authors contributed equally to this study.

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doi:10.1016/j.ejphar.2010.11.032

of the head and neck (SCCHN) (Nagpal et al., 2002), nasopharyngeal carcinoma and multiple myelomas (Hsiao et al., 2003). Thus, STAT3 has been implicated as a potential therapeutic target for multiple human cancers (Aggarwal et al., 2006).

In the present study, we investigated whether icariside II inhibits the STAT3 signaling pathway. We demonstrate that icariside II inhibits constitutive STAT3 activation in cancers of multiple organ sites and down-regulated the expression of STAT3 target genes involved in cell survival, proliferation and angiogenesis. We also found that icariside II induces apoptosis in multiple myeloma cells via STAT3 inactivation, and further potentiates apoptosis induced by bortezomib (a proteasome inhibitor) and thalidomide (an inhibitor of tumor necrosis factor (TNF) expression and angiogenesis), clinical drugs used for treating multiple myeloma patients.

2. Materials and methods

2.1. Isolation of icariside II

Icariside II (Fig. 1A) was isolated from *E. koreanum* through successive fractionation with ethyl acetate, n-hexane, chloroform, ethyl acetate, and n-butanol as described previously (Lee et al., 2009). Each fraction was evaporated and dried under reduced pressure, and further separation was performed using silica gel column chromatography (Merck Kieselgel 60, 70–230 mesh) with chloroform: ethyl acetate: methanol solution (2:1:1) as an eluent. Among seven fractions (E1–E7), the first EA1 was chromatographed on a silica gel column using chloroform: ethyl acetate: methanol (10:2:1) as an eluent to yield eight subfractions (E11–E18). Subfraction E16 was further separated through a sephadex LH-20 (ethyl acetate: methanol = 10:1) and purified by an HPLC JAI-ODS column using methanol as an eluent to give a compound. The purity of icariside II is >95% (HPLC/UV/k. max.). A 50 mM stock solution of icariside II was prepared in dimethyl sulfoxide (DMSO).

2.2. Cell lines

All cell lines used in the present study were purchased from American Type Culture Collection (ATCC) (Rockville, MD). U266 (multiple myeloma), U937 (histiocytic leukemia) and DU145 (prostate carcinoma) cells were cultured in RPMI 1640 containing antibiotic and antimycotic solution with 10% fetal bovine serum. MDA-MB-231 (breast adenocarcinoma) cells were maintained in DMEM culture medium (Welgene, Daegu, South Korea), supplemented with 10% fetal bovine serum and antibiotics.

2.3. Electrophoretic mobility shift assay (EMSA)

The STAT3-DNA binding was analyzed by electrophoretic mobility shift assay using a [γ^{32}P]-labeled high-affinity sis-inducible element (hSIE) probe (5′-CTTCATTTCCCGTAAATCCCTAAAGCT-3′ and 5′-AGCTTTAGGGATTTACGGGAAATGA-3′) as previously described (Ahn et al., 2008). Briefly, nuclear extracts were prepared from icariside II-treated cells and incubated with 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 (GE Health Care Bio-Sciences, Piscataway, NJ).

2.4. Western blotting

Whole cell extracts were lysed in lysis buffer [20 mM tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.003 mg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 4 mM $NaVO_4$]. Lysates were then spun at 13,000×*g* for 15 min to remove insoluble material and resolved on a 10% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against STAT3 (Cell Signaling, Danvers, MA), Bcl-2, Bcl-xl, Cyclin D_1, vascular endothelial growth factor (VEGF),

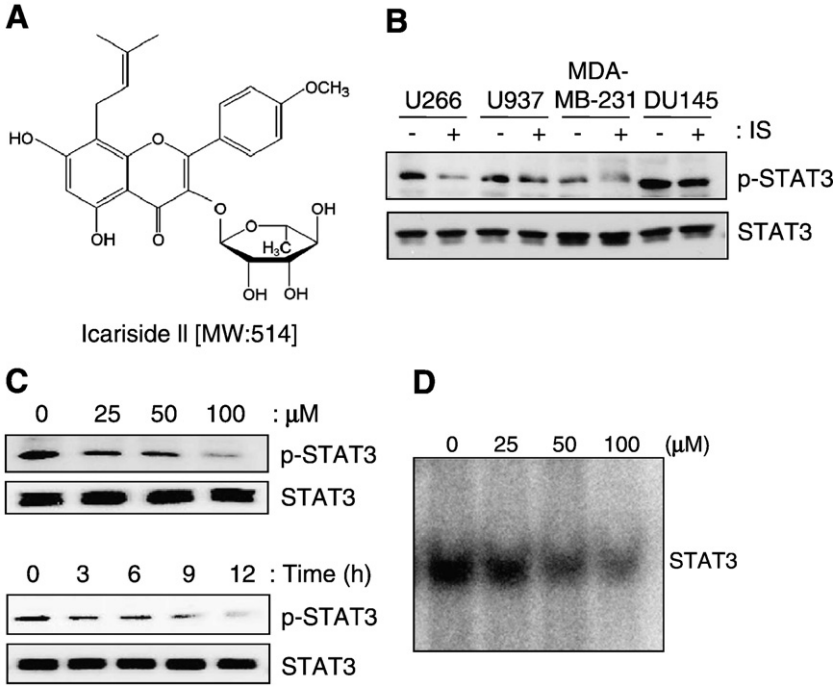

Fig. 1. Icariside II inhibited constitutive STAT3 activation in various cancer cells. (A) Chemical structure of icariside II. (B) Various cancer cell lines including U266, U937, MDA-MB-231 and DU145 were treated with icariside II (50 μM) for 9 h. (C) U266 cells (1×10^6 cells/ml) were treated with icariside II for 9 h as indicated (*upper*) or with icariside II (50 μM) for the period as indicated (*lower*). Whole cell extracts were prepared and immunoblotted with anti-STAT3 and phospho-STAT3 antibodies. (D) Nuclear extracts were prepared from the U266 cells (1×10^6 cells/ml) treated with the indicated concentrations of icariside II for 9 h and analyzed for the STAT3-DNA binding by EMSA.

COX-2, PARP or caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) for overnight. The blot was washed, exposed to HRP-conjugated secondary antibodies for 2 h, and finally examined by enhanced chemiluminescence (ECL) (GE Health Care Bio-Sciences, Piscataway, NJ). To detect STAT3-regulated proteins and caspase-3, U266 cells (1×10^6 cells/ml) were treated with icariside II as indicated.

2.5. Cytotoxicity assay

The anti-proliferative and cytotoxicity effect of icariside II in multiple myeloma cell lines was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Cells (5×10^3 cells/well) were seeded in a 96-well plate, and treated with various concentrations of icariside II as indicated. The treated cells were incubated with medium containing 5 mg/ml of MTT for 2 h at 37 °C and then solubilized by 200 μl of lysis solution. The absorbance was read on a microplate reader (Molecular Devices E-max, Sunnyvale, CA) at 570 nm.

2.6. Cell cycle analysis

U266 cells were exposed to icariside II for the indicated time intervals. Thereafter, the cells were washed and fixed in 70% cold ethanol overnight at −20 °C. Fixed cells were centrifuged, washed and resuspended in 100 μl of PBS containing 10 μl of RNase A (10 mg/ml) and incubated for 1 h at 37 °C. The cells were stained by adding 900 μl of propidium iodie (PI) (50 μg/ml) for 30 min at room temperature in dark. After filtering with nylon mesh (40 μm), the DNA contents of stained cells were analyzed using Cellquest Software with a FACSCalibur flow cytometry (BD Biosciences, San Jose, CA).

2.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Individual apoptotic cell death was observed using DeadEnd™ fluorometric TUNEL assay kit as described by manufacturer. Briefly, U266 cells were treated with 50 μM icariside II for 24 h and washed with cold PBS. Then, the cells were fixed with 4% paraformaldehyde for 30 min and washed twice with PBS for 2 min. Cells were resuspended in permeabilization solution (0.1% Triton X-100 and 0.1% Sodium citrate) for 4 °C overnight and washed twice with PBS. The cells were incubated with 25 μl of TUNEL assay mixture (Sigma, St. Louis, MO) for 60 min at 37 °C in a humidified atmosphere in dark. After washing 3 times in PBS for 2 min and filtering, the cells were analyzed by the flow cytometry.

2.8. Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA from U266 cells treated with icariside II was extracted using the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). 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, Carlsbad, CA). The relative expression of SHP-1, Cyclin D_1 and Bcl-2 was analyzed by RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The RT-PCR reaction mixture contained 12.5 μl of 2× reaction buffer, 10 μl each of RNA, 0.5 μl each of forward and reverse primers, and 0.5 μl of RT-Platinum Taq in a final volume of 24 μl. The reaction was performed at 50 °C for 30 min, 94 °C for 2 min; for 30 cycles 94 °C for 15 s each, 55 °C for 30 s; and 72 °C for 1 min with extension at 72 °C for 10 min. The 2% agarose gel electrophoresis was performed and the ethidium bromide-stained cDNAs were photographed under a UV light.

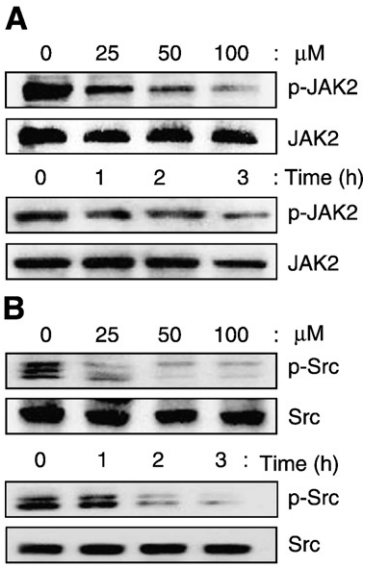

Fig. 2. Icariside II suppressed constitutive activation of JAK2 and Src in U266 cells. (A) U266 cells (1×10^6 cells/ml) were treated with icariside II for 9 h as indicated (*upper*) or with icariside II (50 μM) for the period as indicated (*lower*). Cell lysates were prepared and immunoblotted with antibodies for phospho-JAK2 (p-JAK2) and JAK2. (B) Cell lysates prepared as in (A) were immunoblotted with antibody against phospho-Src (p-Src) or Src.

3. Results

3.1. Icariside II inhibited constitutive STAT3 activation in various cancer cells

In order to examine whether icariside II modulates STAT3 activation, we employed four human cancer cell lines including U266 (multiple myeloma), U937 (leukemia), MDA-MB-231 (breast adenocarcinoma), and DU145 (prostate carcinoma), in which STAT3 is constitutively active. As shown in Fig. 1B, icariside II reduced the level of STAT3

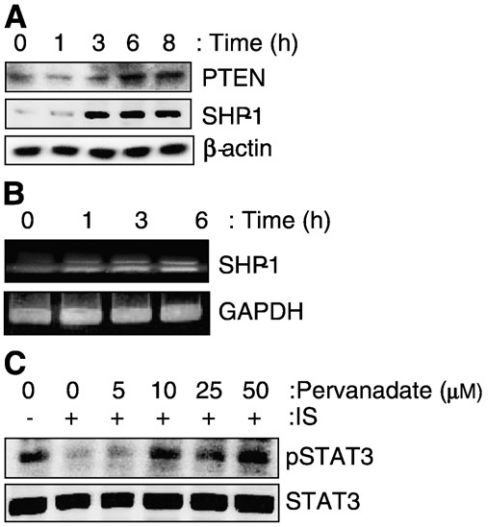

Fig. 3. Icariside II activated protein tyrosine phosphatases SHP-1 in U266 cells. (A) U266 cells (1×10^6 cells/ml) were treated with icariside II (50 μM) as indicated. Whole cell extracts were prepared and immunoblotted with anti-PTEN and SHP-1 antibodies. β-actin was used as an internal control. (B) Total RNA was extracted from the U266 cells (1×10^6 cells/ml) treated with icariside II (50 μM) as indicated, and the RNA level of SHP-1 was analyzed by RT-PCR. GAPDH was used as an internal control. (C) U266 cells (1×10^6 cells/ml) were treated with pervanadate (50 μM) and icariside II (50 μM) for 24 h as indicated. Whole cell extracts were prepared and immunoblotted with the antibodies for phospho-STAT3 (p-STAT3) and STAT3.

phosphorylation in all 4 cell lines used. Because STAT3 is constitutively active in most human multiple myeloma (Bharti et al., 2003), we further investigated the effect of icariside II on human multiple myeloma cell lines.

As shown in Fig. 1C, icariside II decreased the phosphorylation of STAT3 in dose- and time-dependent manners in U266 cells without affecting STAT3 protein expression. In contrast, icariside II had no effect on the phosphorylation of STAT5 (Supplementary Fig. 1). Because the phosphorylation at tyrosine residue 705 of STAT3 causes homo-dimerization and translocation to the nucleus, where they bind to DNA and regulate the transcription of target genes (Yu et al., 1995), we next examined whether icariside II suppresses the DNA binding activity of

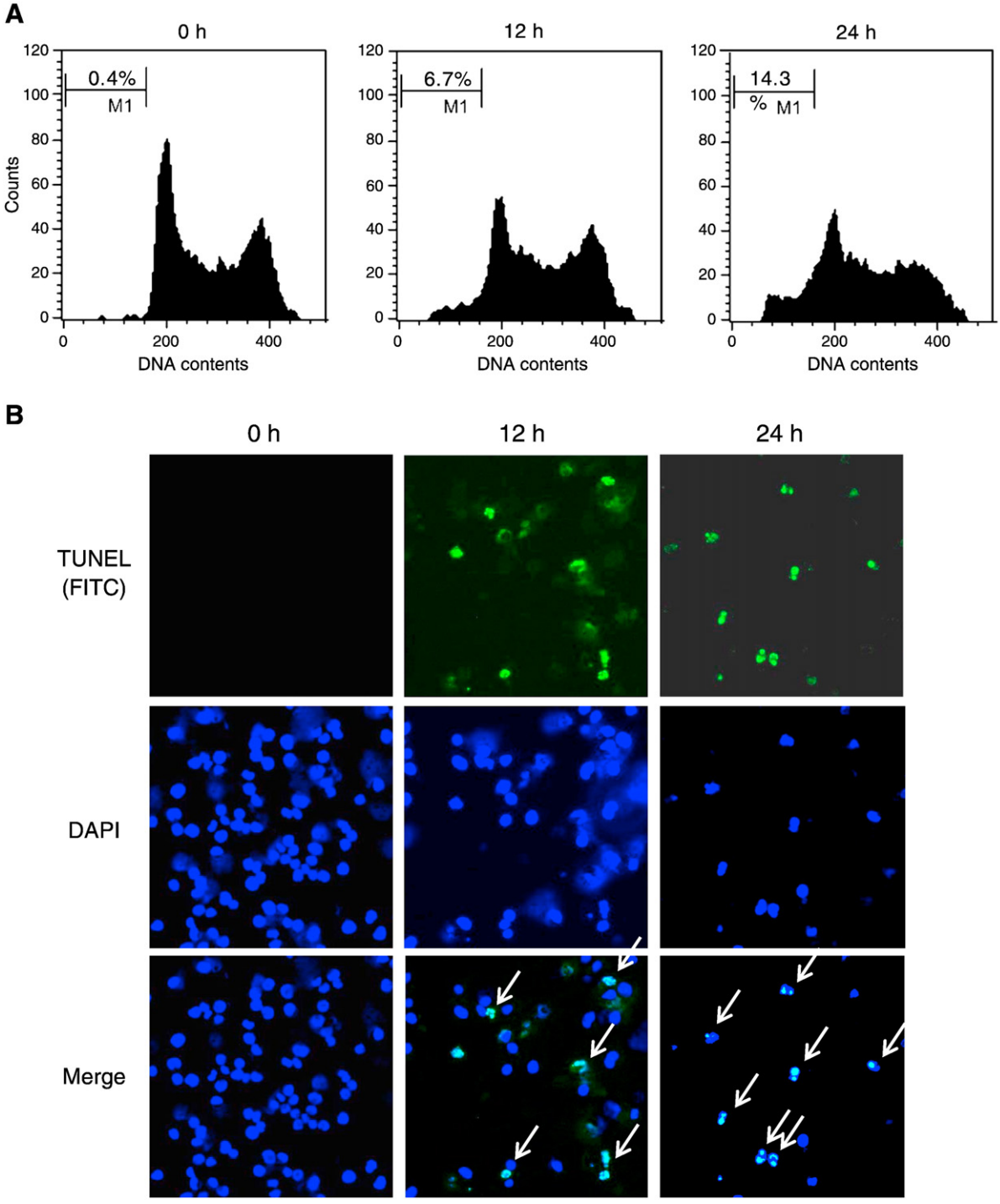

Fig. 4. Icariside II induced apoptosis, inhibited the proliferation and down-regulated STAT3 related gene products in U266 cells. (A) U266 cells (2×10^6 cells/ml) were incubated in the serum-free media overnight and treated with icariside II (50 μM) for 0, 12 or 24 h. The treated cells were harvested, fixed with 70% ethanol, and stained with propidium iodide prior to analysis by flow cytometry. The percentage of cells in sub-G_1 fraction was calculated using Cellquest software. (B) TUNEL staining was performed in the U266 cells treated with icariside II (50 μM) for 0, 12 or 24 h. (C) U266 cells were treated with icariside II as indicated, and then subjected to MTT assays. (D) U266 cells (1×10^6 cells/ml) were treated with icariside II (50 μM) as indicated. Whole cell extracts were prepared and immunoblotted with anti-Bcl-2, Bcl-x_L, Cyclin D1, survivin, VEGF and COX-2. (E) Whole cell lysates prepared as in (D) were immunoblotted with antibodies against caspase-3 and PARP. β-actin was used as an internal control. (F) U266 cells (1×10^6 cells/ml) were treated with icariside II (50 μM) in the absence or presence of pervanadate (50 μM) for 9 h as indicated. Whole cell extracts were prepared and immunoblotted with the antibodies for PARP and cleaved caspase-3.

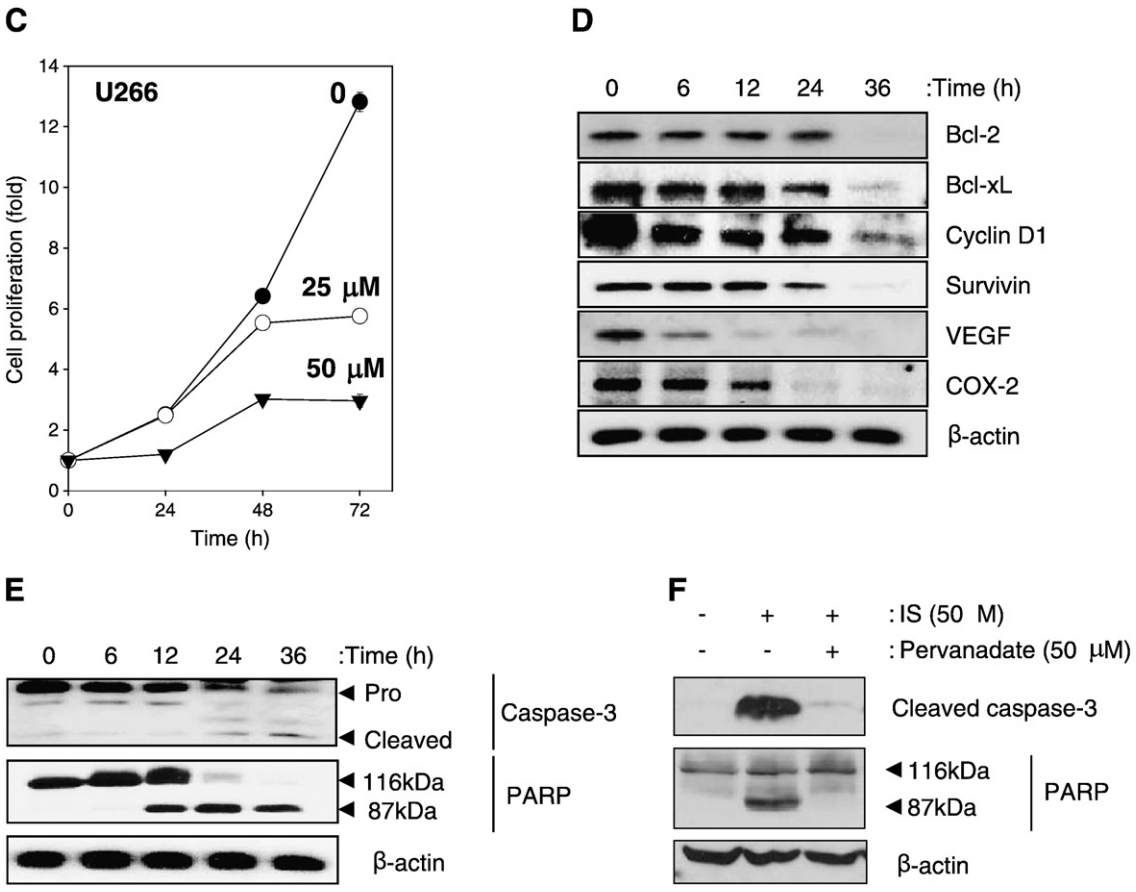

Fig. 4 (*continued*).

STAT3 of the nuclear extract of icariside II-treated cells as a functional test. The EMSA results showed that icariside II reduced the STAT3 binding on its target DNA sequence in a dose-dependent manner (Fig. 1D).

3.2. Icariside II suppressed the STAT3 signaling pathway in U266 cells

STAT3 is activated by non-receptor tyrosine kinases of the Janus family and c-Src has also been linked with the STAT3 activation (Ihle, 1996; Schreiner et al., 2002). Thus, the effect of icariside II on the activity of JAK2 and c-Src was examined using anti-phospho-JAK2 and -phospho-c-Src antibodies. As shown in Fig. 2A, icariside II reduced the phosphorylation of JAK2 in dose- and time-dependent manners with no change of the JAK2 expression in U266 cells. In addition, icariside II decreased the phosphorylation of c-Src without affecting its expression (Fig. 2B), suggesting that icariside II inhibits the activation of STAT3 upstream kinases including JAK2 and c-Src.

Protein tyrosine phosphatases have been also implicated in the STATs signaling pathways (Han et al., 2006). Thus, we examined whether icariside II regulates the expression of SHP-1 and PTEN, which are nontransmembrane PTPs expressed most abundantly in hematopoietic cells (Bittorf et al., 1999; Pandey et al., 2009; Wu et al., 2003). As shown in Fig. 3A, icariside II induced the expression of SHP-1 and PTEN in U266 cells in a time-dependent manner with expression saturating at 3 to 6. The icariside II-mediated up-regulation of SHP-1 protein was accompanied with an increased mRNA level of SHP-1 (Fig. 3B). Since SHP-1 is a known upstream PTP for JAK2, these results therefore support the induction of SHP-1 and PTEN by icariside II to inhibit STAT3 upstream kinases. Further, the broadly-acting tyrosine phosphatases inhibitor sodium pervanadate reversed the icariside II-mediated STAT3 inactivation (Fig. 3C). These results indicate that icariside II-mediated inhibition of STAT3 may be related to PTP activation by icariside II.

3.3. Icariside II inhibited proliferation, induced apoptosis and suppressed STAT3-related gene products in U266 cells

To delineate the nature of icariside II-induced and apoptosis in U266 cells, cell cycle analysis was performed. We found that icariside II significantly increased the population of U266 cells in the sub-G_1 phase from 0.4% to 14.3% in a time-dependent manner, indicating its induction of apoptosis in U266 cells (Fig. 4A). The induction of apoptosis was further confirmed by TUNEL staining. A significant increase in TUNEL-positive cells of total cells was observed in icariside II-treated U266 cells compared to the untreated group (Fig. 4B). Also, we performed the MTT assay to delineate the nature of icariside II-induced anti-proliferation. As shown in Fig. 4C, icariside II inhibited the proliferation of U266 in time- and dose-dependent manners.

STAT3 regulates the expression of various gene products involved in cell survival, proliferation, inflammation and angiogenesis (Kunnumakkara et al., 2009; Yu et al., 2009). Consistent with these results, icariside II repressed the expression of the cell cycle regulator protein cyclin D_1; anti-apoptotic proteins Bcl-2, Bcl-x_L and survivin; and angiogenesis- and inflammation-related proteins VEGF and COX-2 in U266 cells (Fig. 4D).

To further confirm the connection of down-regulation of STAT3 downstream targets to apoptosis, we examined the effect of icariside II on the caspase activation. We found that icariside II increased the cleavages of caspase-3 and PARP, indicating induction of caspase-mediated apoptosis in U266 cells (Fig. 4E). Additionally, to elucidate whether icariside II suppression of STAT3 is associated with icariside II-

induced apoptosis, the cells were treated with pervanadate in icariside II-treated U266 cells and then analyzed the expressions of PARP and caspase-3. Pervanadate treatment clearly blocked icariside II-induced PARP cleavage and caspase-3 activation, suggesting that icariside II-induced apoptosis via STAT3 inactivation (Fig. 4F).

3.4. Icariside II enhanced the effect of bortezomib and thalidomide on induction of apoptosis in U266 cells

Bortezomib (a proteasome inhibitor) and thalidomide (an inhibitor of TNF expression) have been used for treating multiple myeloma patients (Cavo, 2006; Glasmacher et al., 2006; Sung et al., 2009). Since we have observed icariside II-induced suppression of survival proteins such as Bcl-x_L and survivin as well as cleavages of PARP and caspase-3, we further examined the possibility that icariside II could enhance apoptosis in multiple myeloma cells induced by these standard chemotherapeutic agents. As shown in Fig. 5A, icariside II significantly enhanced the cytotoxic effect of thalidomide from 20% to 50% and that of bortezomib from 25% to 60% in U266 cells. In addition, caspase-3 activation and PARP cleavage were further increased by co-treatment of icariside II with thalidomide or bortenzomib than monotherapy in U266 cells (Fig. 5B). These results indicate that icariside II augments the efficacy of currently-used drugs in multiple myeloma cells. Icariside II may thus be a potential candidate for treating multiple myeloma patients in combination with standard chemotherapy.

4. Discussion

Although icariside II has been known to suppress the proliferation of a wide variety of cancer cells, its molecular targets and mechanisms of action on multiple myeloma are poorly understood. Thus, in the current study, we have found that icariside II suppressed STAT3 activity. However icariside II had no effect on STAT5 expression, supporting a specificity of the suppression mechanism on STAT3. The inhibitory effect of icariside II on STAT3 activity was observed in multiple human cancer cell lines including multiple myeloma, breast cancer and prostate cancer, suggesting that its effect on STAT3 is not cell-type specific.

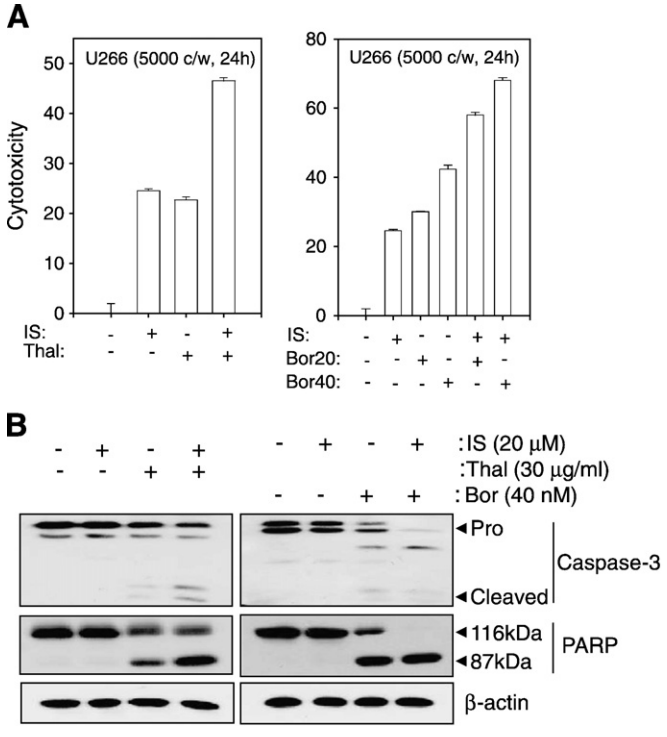

Fig. 5. Icariside II potentiated the efficacy of standard chemotherapy in multiple myeloma cells. (A) U266 cells (5000 cells/well) were treated with 20 μM icariside II, 30 μg/ml of thalidomide (Thal) and 20 or 40 nM of bortezomib (Bor20 or Bor40) for 24 h as indicated. The cytotoxicity was determined by MTT assay as described in the Materials and methods. (B) U266 cells (1×10^6 cells/ml) were treated with icariside II, thalidomide (Thal) and bortezomib (Bor) for 24 h as indicated. Whole cell extracts were prepared from the treated cells and immunoblotted with the indicated antibodies. β-actin antibody was used as an internal control.

The phosphorylation of STATs is principally mediated through the activation of non-receptor protein tyrosine kinases called as Janus-like kinase (JAK). JAK1, JAK2, JAK3 and TYK2 have been linked with the activation of STAT3 (Ihle, 1996; Ren and Schaefer, 2002). Additionally, a role of c-Src kinase in STAT3 phosphorylation has been demonstrated (Schreiner et al., 2002). Icariside II reduced the phosphorylation of JAK2 and c-Src, which are upstream activators of the STAT pathway. Furthermore, icariside II induced the expression of PTPs such as PTEN and SHP-1. Recent reports have shown that PTPs are implicated in the STAT3 signaling, including SHP-1(Tenev et al., 2000), SHP-2 (Kim and Baumann, 1999), TC-PTP (Yamamoto et al., 2002), PTEN (Sun and Steinberg, 2002), PTP-1D (Gunaje and Bhat, 2001), CD45 (Irie-Sasaki et al., 2001), and PTP-ε (Tanuma et al., 2000). Our results with pervanadate (a general PTP inhibitor) to reverse icariside II-mediated STAT3 inactivation support an important role of the SHP-1 and PTEN induction in dephoshophorylating pSTAT3.

STAT3 contributes to oncogenesis and cancer progression through up-regulation of genes encoding apoptosis inhibitors (Bcl-x_L, Mcl-1 and survivin), cell cycle regulators (cyclin D_1 and c-Myc) and inducers of angiogenesis [vascular endothelial growth factor (VEGF)] (Gao and Bromberg, 2006). Bcl-x_L is an anti-apoptotic protein within the Bcl-2 family that inhibits apoptosis by binding pro-apoptotic proteins and preventing cytochrome *c* release (Boise et al., 1993; Gonzalez-Garcia et al., 1994). High level of Bcl-x_L expression has been associated with advanced malignancies of several organ sites and poor prognosis (Cory and Adams, 2002). Suppression of the expression of Bcl-x_L and Mcl-1 may be a useful strategy to augment cancer therapy by enabling cell apoptosis (Ahn et al., 2008).

In our present study, icariside II repressed the expression of the STAT3 target genes including cyclin D_1, Bcl-2, Bcl-x_L and survivin after 24 h in U266 cells. Consistently, cell cycle analysis revealed that the sub-G1 cell populations were significantly increased after 24 h, up to 41.7% and 54.1% at 36 and 48 h, respectively (Supplementary Fig. 2). Also, these results were further confirmed by icariside II-induced cleavages of caspase-3 and PARP in U266 cells. In addition, icariside II down-regulated angiogenesis-related VEGF and COX-2 expression at early time point. The different time course of the down-regulation of apoptosis- and angiogenesis-related genes by icariside II might be explained by the relationship between angiogenesis and apoptosis. Previous report demonstrated the close relationship of angiogenesis and tumor apoptosis (Folkman, 2003). We also reported a similar paper that penta-o-galloyl-beta-D-glucose (PGG) exerted antitumor activity via a primary antiangiogenesis effect, leading to tumor cell and apoptosis (Huh et al., 2005). In the present study, we may suggest that icariside II inhibits angiogenesis and leads to apoptosis in U266 cells. Additional works are required to understand the cause-effect relationship between antiangiogenesis and apoptogenic effects of icariside in cancer cells.

These results suggest that the STAT3 pathway have a reciprocal interaction with the COX-2/PGE_2 pathway to induce a positive feedback loop, and that icariside II may have potentials to inhibit the cross-talk among these pathways that are important in tumor progression.

The constitutive STAT3 activation has been implicated in the resistance of tumor cells to apoptosis possibly through induction of Bcl-x_L, Mcl-1, survivin, Bcl-2 and cyclin D_1 (Danial et al., 1995; Kaliski et al., 2005; Nielsen et al., 1999). This implies that inhibition of STAT3 and these downstream targets by icariside II could facilitate apoptosis in tumor cells. As expected, icariside II promoted PARP cleavage and caspase-3 activation, and increased the apoptotic portion with sub-G1 DNA content in cell cycle analysis in a time-dependent manner in U266 cells. In addition, PTP inhibitor pervanadate blocked icariside II-

induced apoptosis, indicating that icariside II-induced apoptosis is regulated by STAT3 inactivation in U266 cells. Interestingly, bortezomib (a proteasome inhibitor) and thalidomide (a TNF inhibitor) were approved for the treatment of multiple myeloma (Cavo, 2006; Glasmacher et al., 2006; Sung et al., 2009). We have found that icariside II potentiated the apoptotic effect of bortezomib and thalidomide in multiple myeloma cells. These results together with the pharmacological safety of icariside II and its ability to down-regulate the expression of several genes involved in cell survival and chemoresistance may provide a rationale to further carry out preclinical studies in combination with standard chemotherapy.

Taken together, our results demonstrate that the JAK2-SHP1-STAT3 signaling pathway is a novel target pathway for icariside II, especially in multiple myeloma cells that may mediate the anti-proliferation and apoptosis action of icariside II. Thus, icariside II could be a potential therapeutic intervention agent alone or in combination with current drugs for multiple myeloma.

Supplementary materials related to this article can be found online at doi:10.1016/j.ejphar.2010.11.032.

Acknowledgement

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

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