

# Betulinic Acid Inhibits Prostate Cancer Growth through Inhibition of Specificity Protein Transcription Factors

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## Abstract

**Betulinic acid is a pentacyclic triterpene natural product initially identified as a melanoma-specific cytotoxic agent that exhibits low toxicity in animal models. Subsequent studies show that betulinic acid induces apoptosis and antiangiogenic responses in tumors derived from multiple tissues; however, the underlying mechanism of action is unknown. Using LNCaP prostate cancer cells as a model, we now show that betulinic acid decreases expression of vascular endothelial growth (VEGF) and the antiapoptotic protein survivin. The mechanism of these betulinic acid-induced antiangiogenic and proapoptotic responses in both LNCaP cells and in tumors is due to activation of selective proteasome-dependent degradation of the transcription factors specificity protein 1 (Sp1), Sp3, and Sp4, which regulate VEGF and survivin expression. Thus, betulinic acid acts as a novel anticancer agent through targeted degradation of Sp proteins that are highly overexpressed in tumors.** [Cancer Res 2007;67(6):2816–23]

## Introduction

Natural products derived from plant sources have been used extensively in traditional medicine for treatment of a myriad of diseases including various types of cancers (1–3). Several individual phytochemicals or their synthetic analogues are among the most widely used drugs for cancer chemotherapy, and these include taxane microtubule inhibitors, such as paclitaxel, and synthetic analogues that are now widely used in cancer chemotherapy (4, 5). Phytochemical and microbial extracts from various sources are routinely screened for biological activities, and it is estimated that 20% to 25% of new drugs are derived from natural products or their synthetic analogues (1, 6, 7). The triterpenoids oleanolic acid, ursolic acid, and their derivatives exhibit anti-inflammatory and anticarcinogenic activities (8–10). Structure-activity studies among several oleanolic and ursolic acid derivatives (11–13) have identified 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and related compounds as highly potent anti-inflammatory compounds that inhibit growth and induce apoptosis in several cancer cells and tumor types (8, 14–18). CDDO-like compounds act through multiple pathways and are currently undergoing clinical trials for treatment of leukemia.

Betulin is a lupane-derived triterpene that is present in high concentrations in birch bark and betulinic acid; an oxidation product of betulin has also been detected in bark extracts (19).

Betulinic acid was initially characterized as a highly selective inhibitor of human melanoma cell and tumor growth through induction of apoptosis (20). Subsequent research has shown that betulinic acid and other derivatives are effective inhibitors of cell proliferation and induce apoptosis in many different cancer cells. However, the underlying mechanisms for these responses are not well understood.

In this study, we show that betulinic acid decreased proliferation of several cancer cell lines, including SK-MEL2 melanoma cells, and using LNCaP prostate cancer cells as a model, we determined a unique mechanism of action of this compound. Betulinic acid modulates expression of several growth-related proteins and decreases expression of the proangiogenic and proapoptotic proteins vascular endothelial growth factor (VEGF) and survivin, respectively. Previous reports show that in some cancer cell lines, VEGF and survivin expression is dependent, in part, on specificity protein (Sp) proteins (21–29), and Sp1 is overexpressed in cancer cells and tumors (30–34). Results of this study now show that betulinic acid induces proteasome-dependent degradation of Sp1, Sp3, and Sp4 in LNCaP cells. The proteasome inhibitor MG132 not only blocked betulinic acid-induced degradation of Sp proteins but also inhibited the decreased expression of VEGF and survivin in cells treated with betulinic acid. *In vivo* studies showed that betulinic acid inhibited tumor growth in athymic nude mice bearing LNCaP cell xenografts, and this was accompanied by decreased expression of Sp1, Sp3, and Sp4 proteins and VEGF and increased apoptosis in tumors from betulinic acid-treated mice. The results indicate that the antitumorigenic effects of betulinic acid are associated with targeted degradation of Sp transcription factors that are overexpressed in many tumors, and this results in activation of proapoptotic and antiangiogenic responses in tumor but not in non-target tissues (e.g., liver) that exhibit low Sp protein expression.

## Materials and Methods

**Chemicals, antibodies, plasmids, and reagents.** Betulinic acid and  $\beta$ -actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO), and proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA). Antibodies against Sp1, Sp4, Sp3, VEGF, CD1, AR, KLF6, survivin, and poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and CD31 antibody was obtained from DakoCytomation (Glostrup, Denmark). The pVEGF-2018 and pVEGF-133 constructs contain VEGF promoter inserts (positions –2018 to +50 and positions –131 to +54, respectively) linked to luciferase reporter gene (22). The pSurvivin-269 and pSurvivin-150 were kindly provided by Dr. M. Zhou (Emory University, Atlanta, GA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI).  $\beta$ -Galactosidase ( $\beta$ -gal) reagent was obtained from Tropix (Bedford, MA). LipofectAMINE reagent was supplied by Invitrogen (Carlsbad, CA). Western lighting chemiluminescence reagent was from Perkin-Elmer Life Sciences (Boston, MA).

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**Cell lines.** Human carcinoma cell lines LNCaP (prostate) and SK-MEL2 (melanoma) were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% fetal bovine serum, and 10 mL/L of 100× antibiotic antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO<sub>2</sub>.

**Cell proliferation assay.** Prostate and melanoma cancer cells ( $2 \times 10^4$  per well) were plated in 12-well plates and allowed to attach for 24 h. The medium was then changed to DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different concentrations of the compound were added. Fresh medium and compounds were added every 48 h, and cells were then trypsinized and counted after 48 and 96 h using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means  $\pm$  SE for each set of experiments.

**Transfection and luciferase assay.** Prostate cancer cells were plated in 12-well plates at  $1 \times 10^5$  per well in DMEM/Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After growth for 16 to 20 h, various amounts of reporter gene constructs [i.e., pVEGF-2018 (0.4  $\mu$ g), pVEGF-133 (0.04  $\mu$ g), pSurvivin-269 (0.04  $\mu$ g), pSurvivin-150 (0.04  $\mu$ g), and  $\beta$ -gal (0.04  $\mu$ g)] were transfected by LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After 5 h of transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated compound for 20 to 22 h. Cells were then lysed with 100  $\mu$ L of 1× reporter lysis buffer, and 30  $\mu$ L of cell extract were used for luciferase and  $\beta$ -gal assays. Lumicount was used to quantitate luciferase and  $\beta$ -gal activities, and the luciferase activities were normalized to  $\beta$ -gal activity.

**Western blotting.** An equal amount of cell lysate (60  $\mu$ g per well) was separated by 7.5% to 12% SDS-PAGE, which was followed by immunoblotting onto polyvinylidene difluoride (Bio-Rad, Hercules, CA). After blocking in TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8), 0.05% Triton X-100, 5% nonfat dry milk] for 30 min, the membranes were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Proteins were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) for 1 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

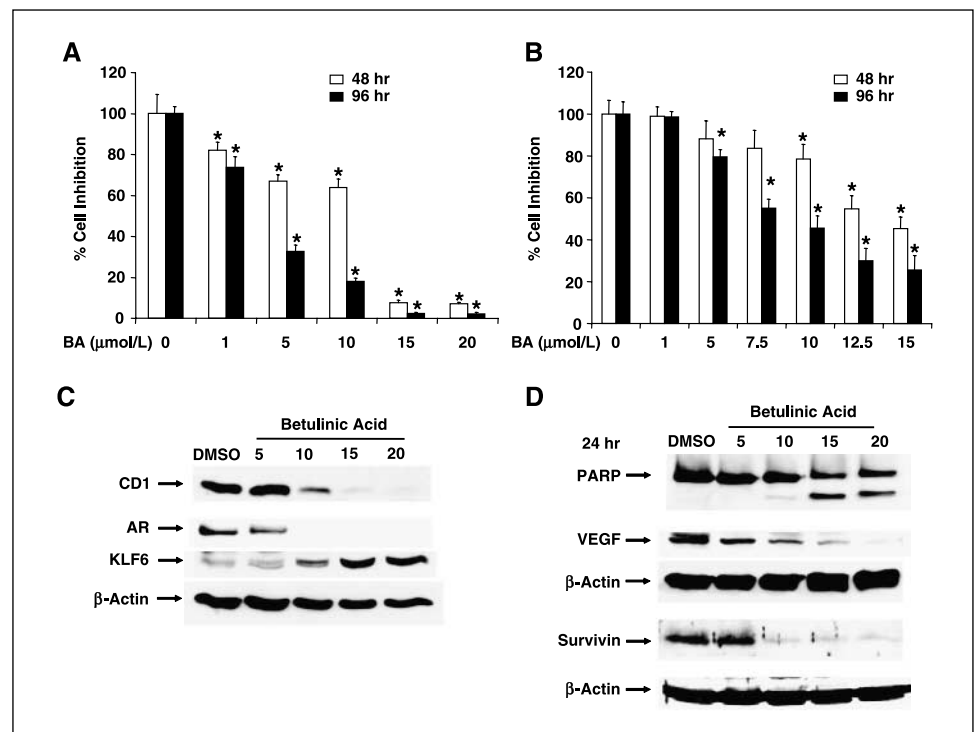
**Xenograft study.** Male athymic BALB/c nude mice (age 4–6 weeks) were purchased from Harlan (Indianapolis, IN). LNCaP cells ( $1 \times 10^6$ ) were implanted with Matrigel (BD Biosciences, San Jose, CA) s.c. into the flank of each mouse. Ten days after cell inoculation, animals were divided into three equal groups of 10 mice each. The first group received 100  $\mu$ L vehicle (1% DMSO in corn oil) by oral gavage, and the second and third groups of animals received 10 and 20 mg/kg/d doses of betulinic acid in vehicle every second day for 14 days (seven doses). The mice were weighed, and tumor areas were measured throughout the study. After 22 days, the animals were sacrificed; final body and tumor weights were determined; and selected tissues were further examined by routine H&E staining and immunohistochemical analysis.

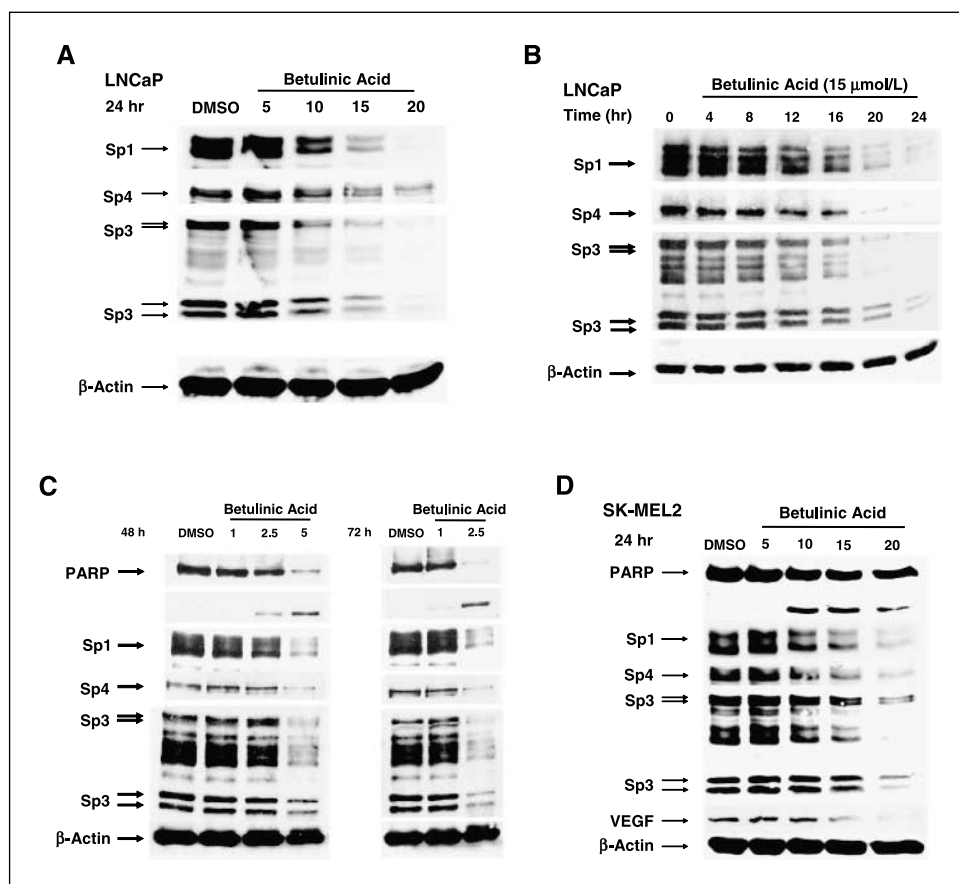
**Immunohistochemistry.** Tissue sections (4–5  $\mu$ m thick) mounted on poly-L-lysine-coated slide were deparaffinized by standard methods. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 10 min. Antigen retrieval for VEGF and CD31 staining was done for 10 min in 10 mmol/L sodium citrate buffer (pH 6) heated at 95°C in a steamer followed by cooling for 15 min. The slides were washed with PBS and incubated for 30 min at room temperature with a protein blocking solution (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA). Excess blocking solution was drained, and the samples were incubated overnight at 4°C with one of the following: a 1:100 dilution of VEGF antibody or a 1:40 dilution of CD31 antibody. Sections were then incubated with biotinylated secondary antibody followed by streptavidin (VECTASTAIN Elite ABC kit). The color was developed by exposing the peroxidase to diaminobenzidine reagent (Vector Laboratories), which forms a brown reaction product. The sections were then counterstained with Gill's hematoxylin. VEGF and CD31 expression was identified by the brown cytoplasmic staining. H&E staining was determined as previously described (26).

## Results

**Antiproliferative, proapoptotic, and antiangiogenic effects of betulinic acid.** The antiproliferative effects of betulinic acid were initially investigated in SK-MEL2 melanoma and LNCaP prostate cancer cells, and 6-day IC<sub>50</sub> values for growth inhibition were 5 to 10  $\mu$ mol/L and 1 to 5  $\mu$ mol/L, respectively. Similar results

**Figure 1.** Betulinic acid (BA) inhibits growth and induces apoptosis in cancer cells. Decreased cell survival in LNCaP (A) and SK-MEL2 (B) cancer cells. Cells were seeded and treated with solvent (DMSO) or different concentrations of betulinic acid (1–20  $\mu$ mol/L) for 6 d as described in Materials and Methods. Cell survival is expressed as the percentage of betulinic acid–treated cells remaining compared to DMSO (set at 100%). \*,  $P < 0.05$ , significantly decreased survival. C and D, Western blot analysis for modulation of protein expression by betulinic acid. LNCaP cells were treated with DMSO or betulinic acid (5–20  $\mu$ mol/L) for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods.





**Figure 2.** Betulinic acid induces degradation of Sp and other proteins. Decreased expression of Sp1, Sp3, Sp4, and VEGF in LNCaP (A–C) and SK-MEL2 (D) cells. LNCaP or SK-MEL2 cells were treated with DMSO or betulinic acid (1–20  $\mu\text{mol/L}$ ) for 24 h (A and D), 4 to 24 h (B), 48 or 72 h (C), and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods.

were observed for pancreatic (Panc28 and L3.6pl), bladder (KU7), and colon (SW480), cancer cells (data not shown). Cell survival curves for LNCaP and SK-MEL2 cells after treatment for 48 or 96 h are illustrated in Fig. 1A and B. At higher concentrations of betulinic acid ( $\geq 10 \mu\text{mol/L}$ ), there was an overall decrease in the number of cells remaining compared with the number of initially seeded cells, which was consistent with the cytotoxicity of betulinic acid.

We further investigated the effects of betulinic acid on growth-inhibitory and proapoptotic proteins using LNCaP cells as a model. Results illustrated in Fig. 1C show that relatively short-term exposure (24 h) to betulinic acid ( $\geq 10 \mu\text{mol/L}$ ) induced down-regulation of cyclin D1, whereas the cyclin-dependent kinase inhibitors p21 and p27 were expressed at low levels in these cells and were not affected by the treatment (data not shown). AR expression in LNCaP cells was decreased after treatment with  $5 \mu\text{mol/L}$  betulinic acid, and this protein was almost completely absent in cells treated with  $10 \mu\text{mol/L}$  concentrations. Recent studies have shown that AR knockdown by RNA interference in LNCaP cells resulted in induction of apoptosis (35), suggesting that the proapoptotic effects of betulinic acid in LNCaP cells may be due, in part, to the effects on AR expression. However, this mechanism would not be applicable to melanoma and other cancer cell lines that are AR independent. The tumor suppressor gene *KLF6* was also induced by betulinic acid at concentrations between 10 and  $15 \mu\text{mol/L}$ . Betulinic acid also induced caspase-dependent PARP cleavage in LNCaP cells, and this was accompanied by decreased expression of the antiapoptotic protein survivin (Fig. 1D) and induction of DNA laddering (data not shown)

typically observed in cells undergoing apoptosis. In addition, betulinic acid also decreased expression of the angiogenic protein VEGF.

**Betulinic acid induces proteasome-dependent degradation of Sp1, Sp3, and Sp4 proteins.** Previous studies in this laboratory have shown that both basal and hormone-induced expression of VEGF in cancer cell lines is dependent on Sp protein expression (22–24, 26), and regulation of survivin in some cells is also dependent on these transcription factors (27–30). Moreover, because Sp proteins are up-regulated in many tumors/cancer cells (31–34) and are associated with proliferative, angiogenic, and antiapoptotic pathways, we hypothesized that the anticarcinogenic activity of betulinic acid may be due, in part, to down-regulation of Sp proteins. Results in Fig. 2A show that treatment of LNCaP cells with 5 to  $10 \mu\text{mol/L}$  betulinic acid for 24 h induced down-regulation of Sp1, Sp3, and Sp4 proteins, and this was also accompanied by decreased expression of VEGF and survivin (Fig. 1D). The time-dependent decrease of Sp proteins in LNCaP cells treated with  $15 \mu\text{mol/L}$  betulinic acid for 4, 8, 12, 16, 20, and 24 h showed that lower expression of these proteins is first observed after treatment for 12 h (Fig. 2B). Prolonged treatment of LNCaP cells with betulinic acid for 48 or 72 h showed that Sp protein degradation and PARP cleavage can be observed at doses as low as 1 to  $2.5 \mu\text{mol/L}$  (Fig. 2C). Figure 2D illustrates that betulinic acid also decreased expression of Sp1, Sp3, and Sp4 proteins in SK-MEL2 melanoma cancer cells, and similar results were obtained in other cancer lines (data not shown).

Because betulinic acid induces degradation of Sp proteins within 12 h after treatment (Fig. 2B), we investigated the possible role of

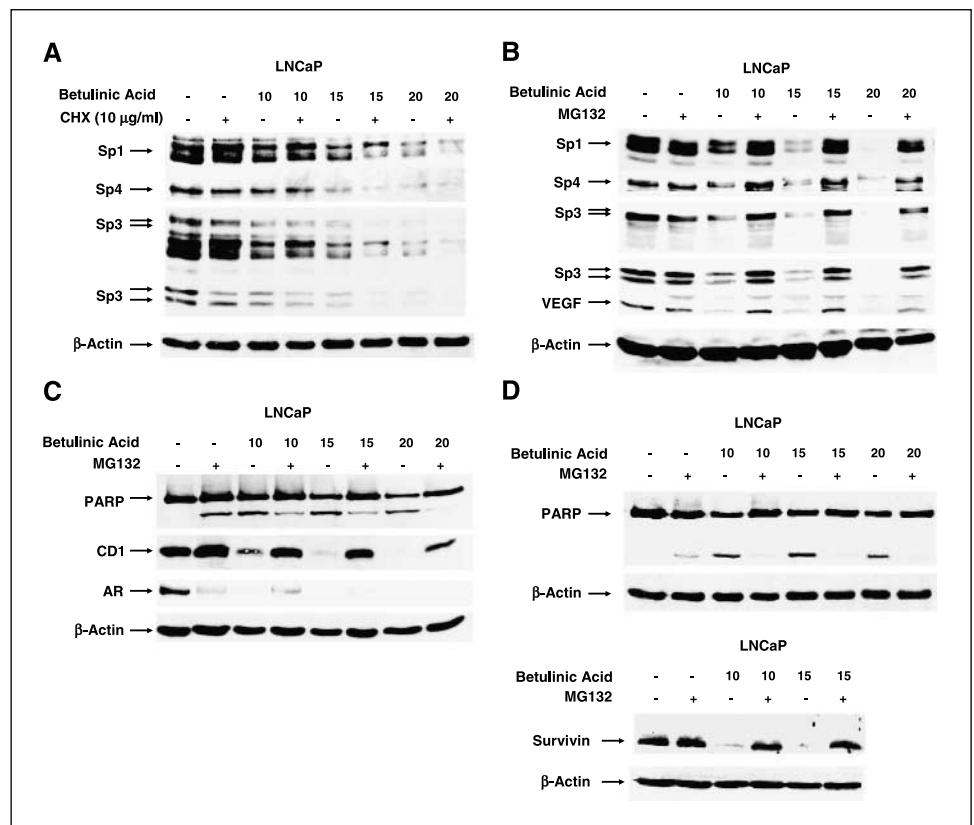
induced protein(s) in mediating this response by treating LNCaP cells with betulinic acid alone (10–20  $\mu\text{mol/L}$ ) or in the presence of 10  $\mu\text{g/mL}$  cycloheximide (Fig. 3A). The protein synthesis inhibitor did not modulate the effects of betulinic acid on Sp protein levels. Previous studies showed that cyclooxygenase-2 (COX-2) inhibitors, the nonsteroidal anti-inflammatory drug tolfenamic acid, and related compounds induced proteasome-dependent degradation of Sp proteins (23, 26); therefore, we investigated the effects of the proteasome inhibitor MG132 on betulinic acid-induced down-regulation of Sp1, Sp3, Sp4, and VEGF in LNCaP cells. MG132 reversed the effects of betulinic acid on expression of these transcription factors (Fig. 3B). Betulinic acid selectively induced proteasome-dependent degradation of Sp proteins and cyclin D1 in LNCaP cells (Fig. 3C); however, MG132 did not modulate expression of  $\beta$ -actin or reverse betulinic acid-dependent down-regulation of AR, which is due to decreased AR RNA expression (data not shown). MG132 (10  $\mu\text{mol/L}$ ) and other proteasome inhibitors induced caspase-dependent cleavage of PARP in LNCaP cells; however, MG132 also partially inhibited betulinic acid-induced apoptosis. Moreover, using a lower concentration of MG132 (5  $\mu\text{mol/L}$ ), the effects of the compound alone on PARP cleavage were decreased, but in combination with betulinic acid, there was inhibition of betulinic acid-induced PARP cleavage (Fig. 3D). MG132 also blocked betulinic acid-induced down-regulation of survivin protein (Fig. 3D), suggesting that Sp protein degradation plays a role in the apoptosis-inducing effects of betulinic acid.

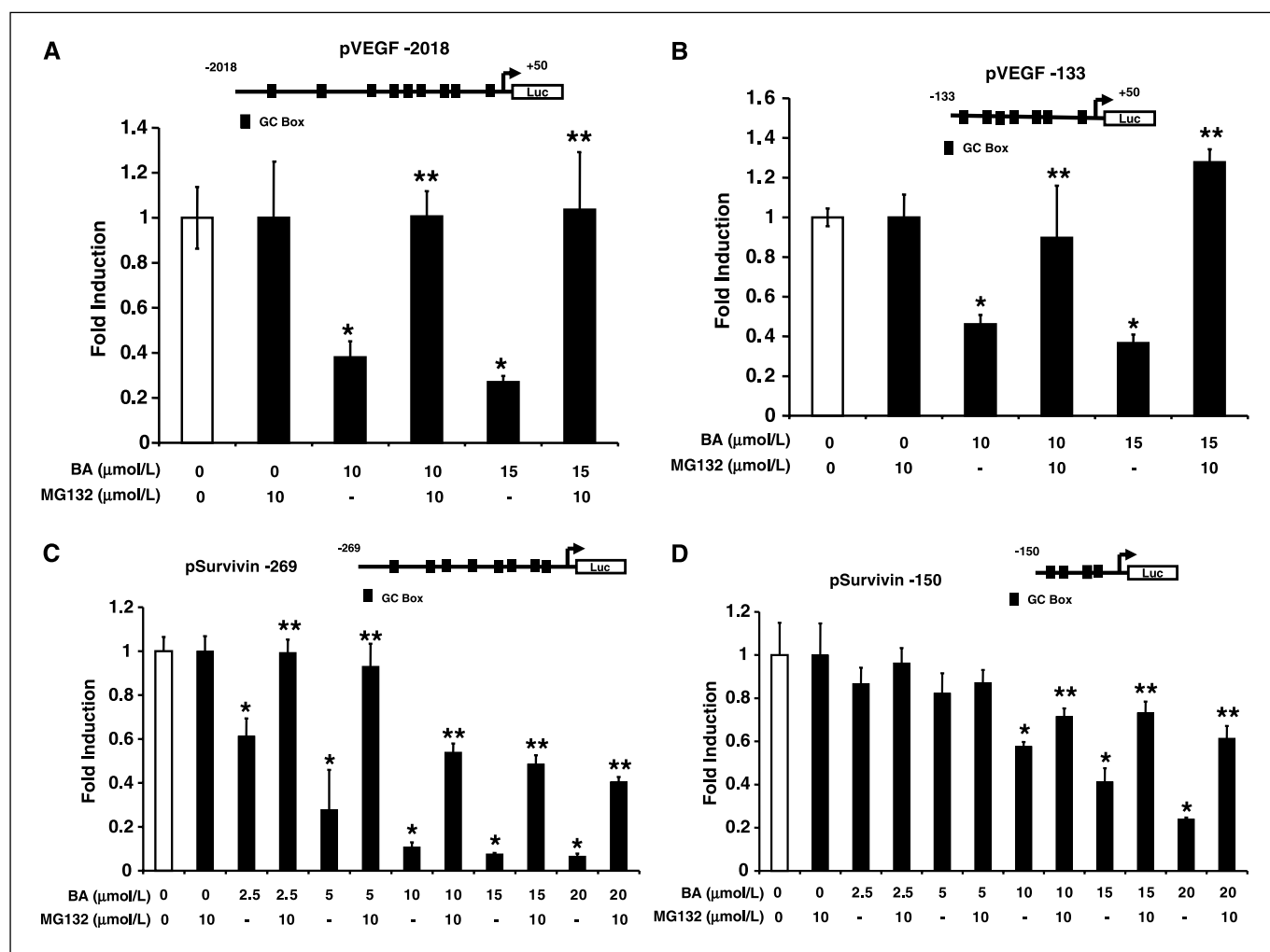
**Betulinic acid inhibits VEGF and survivin promoter expression through proteasome-dependent degradation of Sp proteins.** Expression of both VEGF and survivin in some cancer cell lines is regulated by Sp protein interactions with GC-rich promoter sites (21, 29); therefore, the effects of betulinic acid on decreased

expression of VEGF and survivin through Sp protein degradation was further investigated in transfection studies. The effects of betulinic acid on transactivation was investigated in LNCaP cells transfected with the pVEGF1 and pVEGF2 constructs containing the –2068 to +50 and –133 to +50 VEGF gene promoter inserts. Results in Fig. 4A and B show that betulinic acid decreased luciferase activity in LNCaP cells transfected with pVEGF1 and pVEGF2, and these effects were reversed by the proteasome inhibitor MG132; similar results were observed using the proteasome inhibitor lactacystin (data not shown). These observations further confirm that betulinic acid-induced degradation of Sp proteins results in decreased VEGF expression in LNCaP cells, and this is consistent with previous RNA interference studies showing that Sp1, Sp3, and Sp4 regulate VEGF expression in cancer cell lines (22).

Because the proteasome inhibitor MG132 partially blocks betulinic acid-induced PARP cleavage (Fig. 3C and D), we further investigated the role of Sp protein degradation on induction of apoptosis by examining the effects of betulinic acid on transactivation in LNCaP cells transfected with the GC-rich pSurvivin-269 and pSurvivin-150 constructs which contain the –269 to +49 and –150 to +49 survivin promoter inserts. Betulinic acid causes a concentration-dependent decrease in luciferase activity that was significantly reversed by 10  $\mu\text{mol/L}$  MG132 (Fig. 4C and D). In parallel studies, electrophoretic mobility shift assays also show that lysates from betulinic acid-treated LNCaP cells exhibited decreased binding to GC-rich survivin sequences (data not shown). These results complement a recent study showing that the DNA-binding drug hedamycin also decreases survivin expression through inhibition of Sp protein interactions with the GC-rich survivin promoter (29).

**Figure 3.** Betulinic acid induces proteasome-dependent degradation of Sp proteins in LNCaP cells. A, effects of cycloheximide. Cells were cotreated with 10 to 20  $\mu\text{mol/L}$  betulinic acid or 10  $\mu\text{g/mL}$  cycloheximide alone or in combination, and expression of Sp proteins in whole-cell lysates was determined by immunoblot analysis as described in Materials and Methods. Effects of the proteasome inhibitor MG132 on betulinic acid-induced decrease of Sp proteins/VEGF (B), PARP cleavage, cyclin D1 and AR (C), and PARP cleavage and survivin (D). LNCaP cells were treated with DMSO or betulinic acid alone or in combination with 5 or 10  $\mu\text{mol/L}$  MG132 (pretreated for 30 min) for 24 h, and protein expression in whole-cell lysates was analyzed by Western blot analysis as described in Materials and Methods.



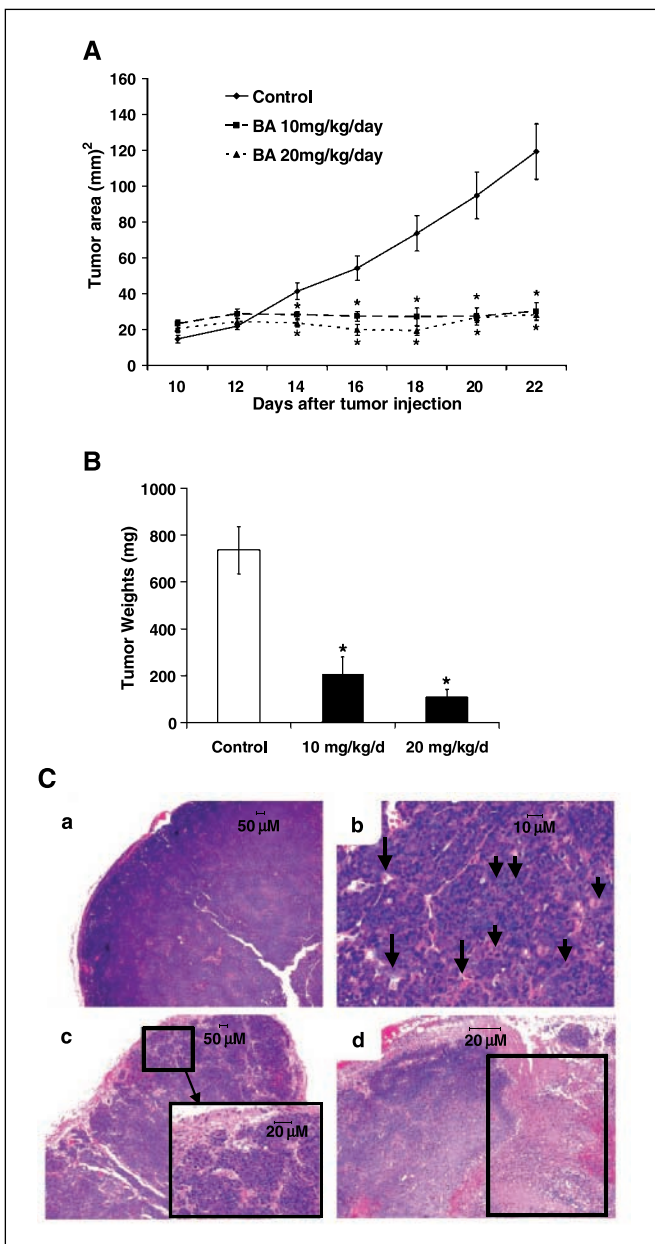


**Figure 4.** Betulinic acid decreases transactivation in LNCaP cells transfected with VEGF and survivin constructs. Transfection with pVEGF-2068 (A), pVEGF-133 (B), pSurvivin-269 (C), and pSurvivin-150 (D). LNCaP cells were transfected with the various constructs and treated with DMSO or betulinic acid (2.5–20 μmol/L) alone or in combination with 10 μmol/L MG132, and luciferase activity (relative to β-gal) was determined as described in Materials and Methods. Luciferase activity significantly ( $P < 0.05$ ) decreased by betulinic acid (\*) and inhibition of this response by cotreatment with MG132 (\*\*).

**Betulinic acid inhibits tumor growth in athymic nude mice bearing LNCaP cells as xenografts.** Pisha et al. previously reported that betulinic acid inhibits growth of tumors in athymic nude mice bearing MEL-1 melanoma cells as xenografts (20). Results in Fig. 5A show that 10 and 20 mg/kg/d betulinic acid inhibited tumor growth in mice bearing LNCaP cell xenografts, and this was accompanied by significantly decreased tumor weights in both treatment groups (Fig. 5B). Examination of the mice showed that there were no treatment-related changes in organ or body weights or in the histopathology of liver and other tissues (data not shown), and this was consistent with the reported low toxicity of this compound (20). Representative H&E-stained histopathology sections of prostate tumors from the control and treated mice were examined. Tumors from untreated mice consisted of minimally encapsulated (Fig. 5C, a), dense expansile nests of epithelial cells with marked atypical features such as anisocytosis, anisokaryosis, multiple variably sized nucleoli, nuclear molding, binucleation and multinucleation. Bizarre mitotic figures were frequently noted within the neoplastic cells (Fig. 5C, b, arrowheads). Abundant vascular channels were frequently present within neoplastic cells (Fig. 5C, b, arrows). Tumors from the treated mice consisted of

neoplastic cells similar to that noted from the untreated mice. However, the mitotic activity (1–2 microfigures per high-power field compared with 6–8 microfigures per high-power field in the corn oil group) and the epithelial atypia seemed to be decreased (Fig. 5C, c). In addition, the tumor tissue was remarkably less vascular with evidence for necrosis (Fig. 5C, d, area within box), and this is consistent with the antiangiogenic effect of betulinic acid through decreased expression of Sp proteins and VEGF.

**Betulinic acid decreases Sp protein and VEGF expression in tumors but not liver.** We also compared expression of Sp proteins, AR and VEGF, as well as PARP cleavage in tumor lysates from control and betulinic acid-treated mice (five animals per group) by Western blot analysis using β-actin as a loading control. Relatively high levels of Sp1, Sp3, Sp4, and VEGF proteins were observed in the control tumors, and we did not detect PARP cleavage in protein lysates from these tumors (Fig. 6A). In contrast, expression of Sp1, Sp3, Sp4, AR, and VEGF proteins was decreased in tumors from betulinic acid-treated mice, and PARP cleavage was observed. Sp protein levels in liver from untreated or betulinic acid-treated mice could be visualized only after prolonged exposures, and the pattern of Sp1, Sp3, or Sp4 protein expression was similar in both groups



**Figure 5.** Antitumor activity by betulinic acid *in vivo*. *A*, decreased tumor area. Athymic nude mice (10 per group) bearing LNCaP cells as xenografts were treated with corn oil (control) or betulinic acid in corn oil (10 or 20 mg/kg) every second day, and tumor areas were determined as described in Materials and Methods. *B*, tumor weights. After the final treatment, animals were sacrificed, and tumor weights were determined as described in Materials and Methods. \*,  $P < 0.05$ , significantly decreased tumor areas or volumes. *C*, histopathologic evaluation of tumors. Tumors from corn oil (*a* and *b*) and betulinic acid (*c* and *d*) mice were fixed, stained with H&E, and examined histopathologically as described in Materials and Methods.

(Fig. 6B). A direct comparison of Sp protein expression in tumors and liver from untreated animals (Fig. 6C) indicates high levels in tumors, whereas in liver, only Sp3 could be detected, and levels of Sp1 and Sp4 were very low. We also examined Sp proteins in other tissues/organs from mice treated with corn oil or betulinic acid and observed uniformly very low to nondetectable Sp1, Sp3, and Sp4 in both treatment groups (data not shown). Tumors from untreated and treated mice were also stained for VEGF and CD31 (micro-

vessel density), and the staining for both factors was decreased in tumors from betulinic acid-treated mice (Fig. 6D). This study shows that betulinic acid is a potent inhibitor of prostate cancer cell and tumor growth, and this parallels results showing that betulinic acid exhibited anticancer activity in *in vitro* and *in vivo* models of melanoma (20). The underlying mechanisms of proapoptotic, antiproliferative, and antiangiogenic activities of betulinic acid are associated with induction of proteasome-dependent degradation of Sp proteins in prostate tumor cells. Moreover, Sp protein expression was decreased in SK-MEL2 (Fig. 2D) and other cancer cell lines (data not shown), suggesting that the betulinic acid-dependent down-regulation of Sp proteins that is accompanied by both proapoptotic and antiangiogenic responses is an integral part of the anticarcinogenic activity of this compound.

## Discussion

Betulinic acid is a natural product identified in various bark extracts and is readily synthesized from betulin, a major component in bark from birch trees (19). Pisha et al. (20) initially reported that this triterpenoid inhibited growth of several melanoma cell lines, and ED<sub>50</sub> values for cytotoxicity varied from 4.8 to 1.1  $\mu\text{g}/\text{mL}$ , whereas the corresponding values for a series of colon, prostate, breast, lung, squamous, and glioma cancer cells were  $>20 \mu\text{g}/\text{mL}$ . However, other reports also show that betulinic acid inhibits growth and induces apoptosis in several different cancer cell lines (19), and this corresponds to results of this study in which IC<sub>50</sub> values (growth inhibition) for melanoma and prostate cancer cells were 5 to 10  $\mu\text{mol}/\text{L}$  and 1 to 5  $\mu\text{mol}/\text{L}$ , respectively, and similar results were obtained in other cancer cell lines. It has also been reported that betulinic acid derivatives and related lupane analogues are more cytotoxic than betulinic acid to cancer cells (19); however, a major advantage in using the latter compound for cancer chemotherapy is the low toxicity. Doses as high as 500 mg/kg every 4th day ( $\times 6$ ) exhibited no detectable toxic side effects (20).

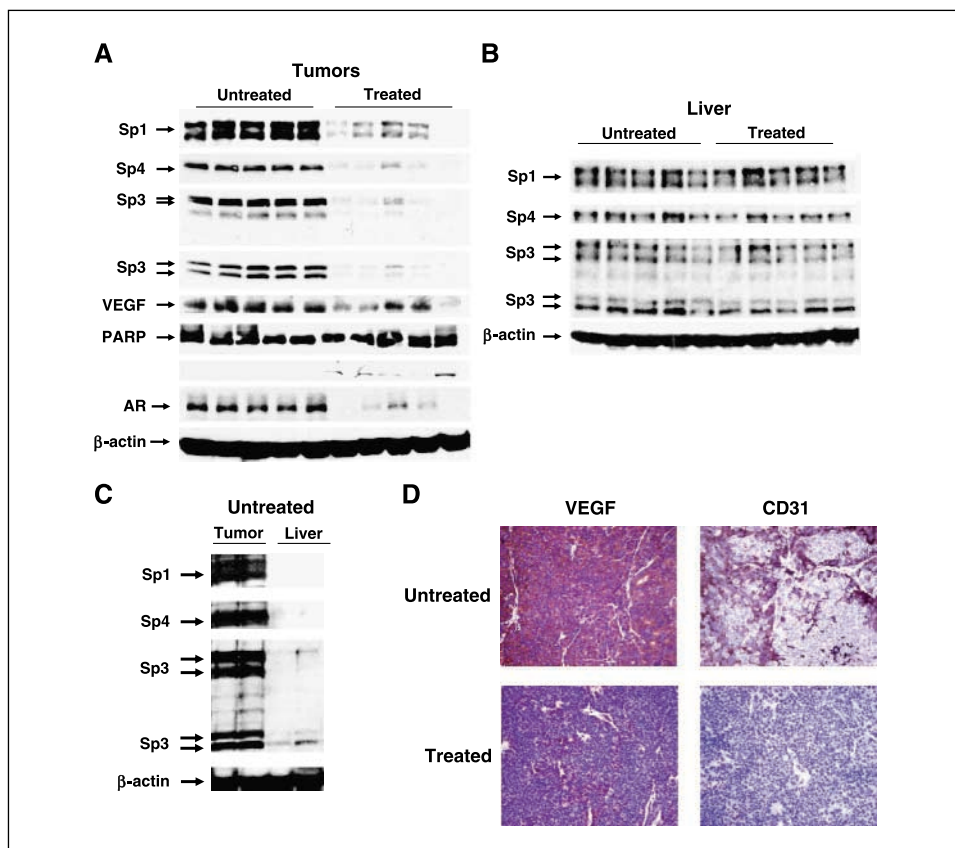
It has been reported that betulinic acid induces apoptosis in several different cancer cell lines through multiple pathways, which include direct effects on the mitochondria accompanied by decreased mitochondrial membrane potential, up-regulation of death receptors, and interactions with other agents (36–47). In addition, betulinic acid exhibits antiangiogenic activity in an *in vitro* assay for tube-like structures in ECV304 cells (48); however, the underlying mechanisms of action for these responses are unknown. In this study, betulinic acid also induces proapoptotic and antiangiogenic responses in LNCaP cells as evidenced by decreased expression of VEGF and survivin and activation of caspase-dependent PARP cleavage (Fig. 1D) and DNA laddering (data not shown). Betulinic acid decreases AR expression in LNCaP cells (Fig. 1C), and this response is in itself proapoptotic in this cell line because it has been reported that decreased AR expression via RNA interference results in increased apoptosis in LNCaP cells (35). Previous studies show that in several cancer cell lines VEGF expression is dependent, in part, on Sp1 and other Sp proteins (23, 25), and there is also evidence that survivin expression is Sp dependent (27–29). Therefore, we examined the effects of betulinic acid on expression of Sp1, Sp3, and Sp4 proteins in LNCaP cells, and the results show for the first time that betulinic acid decreases expression of Sp1, Sp3, and Sp4. Similar results are observed in SK-MEL2 and other cancer cell lines (Fig. 2A–D).

The role of betulinic acid-induced down-regulation of Sp proteins in mediating the proapoptotic and antiangiogenic activities of this compound were further investigated in LNCaP cells. Proteasome inhibitors, such as MG132, block Sp protein degradation induced by COX-2 inhibitors and tolfenamic acid in colon and pancreatic cancer cells (23, 37). The results in Fig. 2D show that MG132 inhibited betulinic acid-induced degradation of Sp proteins. Moreover, MG132 blocked degradation of VEGF and survivin proteins in LNCaP cells treated with betulinic acid, and similar results were observed for cyclin D1 but not AR (Fig. 3B–D), which is decreased through proteasome-independent pathways. Moreover, in LNCaP cells transfected with constructs containing critical GC-rich sequences from the *VEGF* and *survivin* gene promoters, betulinic acid decreased transactivation that was inhibited after cotreatment with MG132 (Fig. 4). These results confirm the linkage between the decreased expression of survivin and VEGF in LNCaP cells treated with betulinic acid with degradation of Sp1, Sp3, and Sp4 in this cell line.

Sp proteins are overexpressed in many human tumors and cell lines (30–34); therefore, these transcription factors are potential targets for development of drugs for cancer chemotherapy. Lou et al. (49) reported that transformation of fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression, and these transformed cells formed highly malignant tumors in athymic nude mouse xenograft models, whereas fibroblasts expressing low levels of Sp1 did not form tumors. In addition, ribozyme-dependent knockdown of Sp1 in the transformed cells decreased VEGF expression and increased apoptosis. Results in Fig. 5 clearly showed that betulinic acid (10 and 20 mg/kg/d) inhibited tumor growth and final tumor weights in athymic nude mice bearing LNCaP cells as xenografts.

Moreover, examination of individual tumors clearly showed that Sp1, Sp3, and Sp4 proteins were highly expressed in tumors from control animals, whereas there was a marked decreased in expression of these proteins in tumors from mice treated with betulinic acid (Fig. 6A). Decreased VEGF expression also paralleled decreased Sp protein levels in tumors from betulinic acid-treated animals, although the magnitude of decreased VEGF was lower than that observed for Sp proteins in the latter treatment group. The antiangiogenic activity of betulinic acid was confirmed by decreased VEGF and CD31 staining in tumors from betulinic acid-versus corn oil-treated mice (Fig. 6D), and this was consistent with the antiangiogenic activity previously reported for betulinic acid (48). H&E staining also showed that the vascularity of tumors in mice treated with betulinic acid was significantly lower than in tumors from corn oil-treated mice (Fig. 5C).

We also examined Sp1, Sp3, and Sp4 protein expression in non-tumor tissue from control and betulinic acid-treated mice, and levels were barely detectable to nondetectable in both treatment groups; however, when gels were overexposed, it was evident that levels of Sp protein expression were similar in liver lysates from control and betulinic acid-treated mice (Fig. 6B and C). These results suggest that activation of proteasome-dependent degradation of Sp proteins by betulinic acid may be specific for cancer cells and tumors that overexpress these transcription factors to gain a growth and metastatic advantage over normal tissue. The critical role for Sp proteins in regulating expression of antiapoptotic and proangiogenic genes/proteins in tumors is supported by results of this study that also highlights the efficacy of anticancer drugs that target specific transcription factors, such as Sp proteins, that are overexpressed in tumors. The effects of betulinic acid in this study



**Figure 6.** Sp and VEGF protein expression in tumors and liver. Whole-cell lysates from corn oil (untreated) and betulinic acid-treated tumors (A) and liver (B) were obtained from tissue from at least five rats per group and analyzed by Western blot analysis as described in Materials and Methods. C, comparative Sp protein expression in tumors and liver from corn oil (solvent)-treated animals. Tumor and liver lysates containing the same amount of protein were analyzed by electrophoresis and visualized as described in Materials and Methods. D, immunostaining for CD31 and VEGF. Fixed tumor tissue from corn oil and betulinic acid-treated mice were stained with CD31 and VEGF antibodies as described in Materials and Methods.

were also observed for the closely related derivative betulonic acid (data not shown), and we conclude that betulonic acid and similar derivatives represent a novel class of transcription factor–targeting anticancer drugs. Moreover, because betulonic acid induces many other cytotoxic and proapoptotic effects in cancer cell lines (36–47), it is likely that down-regulation of Sp proteins differentially contributes to the overall effect of this compound. Current studies with betulonic acid are investigating tumor-type similarities and differences in its mechanism of action and the development of

more potent analogues for clinical applications in the treatment of cancer.

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