Inhibition of Specificity Protein Transcription Factors

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Sudhakar Chintharlapalli, Sabitha Papineni, Shashi K. Ramaiah, and Stephen Safe

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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 proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA). Antibodies against 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]

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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 0.02% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% fetal bovine serum, and 10 mL/L of 100× antibiotic antifungal solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO₂.

Cell proliferation assay. Prostate and melanoma cancer cells (2 × 10⁴ 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 ± SE for each set of experiments.

Transfection and luciferase assay. Prostate cancer cells were plated in 12-well plates at 1 × 10⁵ 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 μg), pVEGF-133 (0.04 μg), pSurvivin-269 (0.04 μg), pSurvivin-150 (0.04 μg), and β-gal (0.04 μg)] 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 μL of 1× reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-gal assays. Lumicount was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.

Western blotting. An equal amount of cell lysate (60 μg 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 × 10⁶) 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 μL 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 μm thick) mounted on poly-L-lysine–coated slides 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 1:80 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 IC₅₀ values for growth inhibition were 5 to 10 μmol/L and 1 to 5 μmol/L, respectively. Similar results
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 (>10 μmol/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 (>10 μmol/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 μmol/L betulinic acid, and this protein was almost completely absent in cells treated with 10 μmol/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, 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 μmol/L betulinic acid for 24 h induced down-regulation of Sp1, Sp3, and Sp4 proteins, and this was accompanied by decreased expression of VEGF and survivin (Fig. 1D). The time-dependent decrease of Sp proteins in LNCaP cells treated with 15 μmol/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 μmol/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 betulinic acid in the degradation of Sp proteins.
Betulinic Acid Induces Sp Protein Degradation

Betulinic acid induces 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 μmol/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 μmol/L betulinic acid or 10 μg/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 μmol/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.
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 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 Induces Sp Protein Degradation

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_{50} values for cytotoxicity varied from 4.8 to 1.1 μg/mL, whereas the corresponding values for a series of colon, prostate, breast, lung, squamous, and glioma cancer cells were >20 μg/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_{50} values (growth inhibition) for melanoma and prostate cancer cells were 5 to 10 μmol/L and 1 to 5 μmol/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 (×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).

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_{50} values for cytotoxicity varied from 4.8 to 1.1 μg/mL, whereas the corresponding values for a series of colon, prostate, breast, lung, squamous, and glioma cancer cells were >20 μg/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_{50} values (growth inhibition) for melanoma and prostate cancer cells were 5 to 10 μmol/L and 1 to 5 μmol/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 (×6) exhibited no detectable toxic side effects (20).

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Figure 5. Antitumorigenic 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 ml/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 area 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 (microvessel 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.
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](cancerres.aacrjournals.org)
were also observed for the closely related derivative betulinic acid (data not shown), and we conclude that betulinic acid and similar derivatives represent a novel class of transcription factor–targeting anticancer drugs. Moreover, because betulinic acid induces many 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 betulinic 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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