Published in final edited form as:

Mol Carcinog. 2013 August ; 52(8): 591–602. doi:10.1002/mc.21893.

Betulinic acid decreases ER-negative breast cancer cell growth *in vitro* and *in vivo*: role of Sp transcription factors and microRNA-27a:ZBTB10

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Abstract

Betulinic acid (BA), a pentacyclic triterpenoid isolated from tree bark is cytotoxic to cancer cells. There is evidence that specificity proteins (Sp), such as Sp1, Sp3 and Sp4, are overexpressed in tumors and contribute to the proliferative and angiogenic phenotype associated with cancer cells. The objective of this study was to determine the efficacy of BA in decreasing the Sp expression and underlying mechanisms. Results show that BA decreased proliferation and induced apoptosis of estrogen-receptor-negative breast cancer MDA-MB-231 cells. The BA-induced Sp1, Sp3, and Sp4 downregulation was accompanied by increased zinc finger ZBTB10 expression, a putative Sp-repressor (ZBTB10) and decreased microRNA-27a levels, a microRNA involved in the regulation of ZBTB10. Similar results were observed in MDA-MB-231 cells transfected with ZBTB10 expression plasmid. BA induced cell cycle arrest in the G2/M phase and increased Myt-1 mRNA (a microRNA-27a target gene), which causes inhibition in G2/M by phosphorylation of cdc2. The effects of BA were reversed by transient transfection with a mimic of microRNA-27a. In nude mice with xenografted MDA-MB-231 cells, tumor size and weight were significantly decreased by BA treatment. In tumor tissue, ZBTB10 mRNA was increased while mRNA and protein of Sp1, Sp3 and Sp4, as well as mRNA of vascular endothelial growth factor receptor (VEGFR), survivin and microRNA-27a were decreased by BA. In lungs of xenografted mice, human β 2-microglobulin mRNA was decreased in BA-treated animals. These results show that the anti-cancer effects of BA are at least in part based on interactions with the microRNA-27a-ZBTB10-Sp1-axis causing increased cell death.

Keywords

MDA-MB-231-breast cancer; ZBTB10; Sp-transcription factors

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Conflict of Interest: none

Introduction

Betulinic acid (BA) is a triterpenoid acid found in various bark extracts. It is readily synthesized from betulin by oxidation to betulonic acid and reduction of the 3-keto group of betulonic acid to give BA [1-4]. The bark of birch trees can contain up to 30% (by weight) of betulin [1]. BA and its derivatives have been used for treatment of several diseases and BA is a highly effective anticancer agent against numerous tumor types and is currently being explored in clinical trials [5-10].

BA induces apoptosis, inhibits growth and exhibits antiangiogenic and antimetastatic activity in cancer cell lines and in *in vivo* studies. The proapoptotic effects of BA have been reported in several different cell lines and are characterized by several markers of apoptosis including cleavage of various caspases and the nuclear protein poly (ADP-ribose) polymerase (PARP) [5-10]. BA also activates the stress kinases p38 and JNK, decreases mitochondrial membrane potential, induces reactive oxygen species (ROS) production, and acts as potent inhibitor of mammalian type 1 DNA topoisomerase [2-4,7,11-12]. Reports that BA decreased expression of genes associated with cancer cell proliferation (cyclin D1), survival (bcl-2 and survivin), and exhibited antiangiogenic activity in ECV304 cells [13] suggested that one of the underlying mechanisms of action of BA in cancer cells may involve repression of Sp transcription factors Sp1, Sp3 and Sp4. RNA interference studies which decrease expression of all three Sp proteins show that genes such as cyclin D1, bcl-2, survivin, VEGF, and VEGF receptors are all Sp-regulated genes [14-22]. Moreover, we have also shown that, in LNCaP prostate cancer cells, BA induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 accompanied by decreased expression of VEGF, survivin, and cyclin D1 and induction of PARP cleavage [23]. In this study, we show that BA and related compounds inhibit growth of ER-negative breast cancer cells. Also, using the highly metastatic MDA-MB-231 cell line as a model, we show that BA decreases cell growth, expression of Sp1, Sp3 and Sp4 and Sp-regulated gene products (survivin, VEGF and VEGFR1). Moreover, these responses are linked to a BA-dependent decrease in microRNA-27a (miR-27a) and induction of ZBTB10 and Myt-1 which are in turn, are responsible for repression of Sp proteins and growth arrest of cells in the G2/M phase of the cell cycle. These results illustrate a novel pathway and drug for treatment for triple negative (ER, progesterone receptor and HER2-negative) breast cancer which is highly resistant, even to cytotoxic drug therapy.

Material and Methods

Chemicals, antibodies, plasmids, and reagents

Betulinic acid (BA) was purchased from Sigma Aldrich (St Louis, MO). Betulonic acid (BO), and corresponding methylesters (methyl BA and methyl BO) were prepared from betulin (Sigma-Aldrich) based on previously described methods [24]. Antibodies against Sp1, Sp4, Sp3, VEGFR, survivin, cdc2, and phosphorylated cdc2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The antibody for and poly (ADP-ribose) polymerase (PARP) was purchased from Cell Signaling Technology (Beverly, MA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). LipofectAMINE 2000 reagent was supplied by Invitrogen (Grand Island, NY). Western lightning chemiluminescence reagent was obtained from Perkin-Elmer Life Sciences (Waltham, MA). mirVana™ extraction kit and the reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). Primers for Sp1, VEGFR, survivin, ZBTB10, Myt1, and Wee were purchased from Integrated DNA Technologies Technologies. Primers for Sp3 and Sp4 were obtained from Qiagen; miR-27a mimic, and scrambled miRNA were from Dharmacon, Inc. (Lafayette, CO); and the ZBTB10 expression vector and empty vector (pCMV6-XL4) were

from Origene (Rockville, MD). Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Ciudad and Veronique Noe (University of Barcelona, Barcelona, Spain). RNase, propidium iodide, sodium citrate, and Triton X-100 were obtained from Sigma-Aldrich (St Louis, MO). The 40-bp sequence containing a miR-27a target sequence from the 3'-UTR of ZBTB10 was cloned into NotI and XhoI sites of psiCHECK2 dual luciferase reporter construct (Promega, Madison, WI). The luciferase activities were normalized and measured according to the manufacturer's protocol.

Cell lines

Human mammary carcinoma cell lines MDA-MB-231, MDA-MB-435, BT474, MDA-MB-468, MDA-MB-453, and HS-578T were obtained from the American Type Culture Collection. Cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Invitrogen, Grand Island, NY) and maintained at 37°C in the presence of 5% CO₂.

Cell proliferation

Cells (1.5×10^4) were seeded onto a 24-well plate in DMEM medium supplemented with 10% FBS and incubated for 24 h to allow cell attachment. Medium was replaced with 2.5% FBS DMEM medium containing the solvent (DMSO) or varying concentrations of BA, BO, methyl BA, and methyl BO. The number of cells relative to DMSO treated cells was quantified with an electronic cell counter (Z1™ Series, Beckman Coulter, Inc) after 48h incubation.

Western blot analysis

Cells (4×10^5) were seeded in six-well plates in DMEM medium containing 2.5% charcoal-stripped FBS and incubated 24h to allow cell attachment. After 24h BA treatment, medium was discarded and cells were washed with PBS, then removed by scraping using PBS. After centrifugation, cell pellets were lysed with non-denaturing buffer (10 mM Tris-HCl, 10 mM NaH₂PO₄, 130 mM NaCl, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, pH 7.5), and 1% proteinase inhibitor cocktail (Sigma-Aldrich) for 30min in ice. Solid cellular debris was removed by centrifugation at 10,000 rpm for 10min at 4°C. The supernatant was collected and stored at -80 °C. Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA) following the manufacturer's protocol. 60µg of protein was diluted with Laemmli's loading buffer, boiled for 5min, loaded into each lane of an acrylamide gel (10%) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100V for 2h. Proteins were transferred by wet blotting onto 0.2 µm PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked using 5% milk in 0.1% PBS-Tween (PBS-T) for 30min and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in PBS-T overnight at 4 °C with gentle shaking, followed by incubation with the secondary antibody (1:2000) in 5% milk PBS-T for 2 h. Reactive bands were visualized with a luminal reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) after 1min of reaction.

Preparation of nuclear extracts for EMSA

MDA-MB-231 cells were seeded in 100 mm tissue culture dishes (Corning, Corning, NY) in DMEM/F12 medium with 2.5% dextran/charcoal-stripped fetal bovine serum. After appropriate transfection or chemical treatments, cells were washed twice in PBS, scraped into 1 ml HEGD buffer [25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol (pH 7.6)], and homogenized. The cellular homogenate was centrifuged for 5 min at $14,000 \times g$. The supernatant was discarded, and the pellet was suspended in 200 µl HEGDK [25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 0.5 M KCl, and 10% (vol/vol) glycerol (pH 7.6)] and incubated on ice for 1 h with frequent vortexing. Samples were

centrifuged at $14,000 \times g$ for 1 min, and nuclear protein concentration in supernatant was determined using the Bradford assay. The supernatant was stored in small aliquots at -80°C for additional use.

Gel EMSA

GC-rich oligonucleotide-probes for the assessment of Sp-protein binding were synthesized by Integrated DNA Technologies. The probes were annealed and ^{32}P -labeled at the 5' end using T4 polynucleotide kinase (Promega) and [^{32}P]ATP (PerkinElmer Life and Analytical Sciences). The labeled probes were purified through Chroma Spin-TE-10 column (Clontech, Mountain View, CA). The binding reactions were performed at 4°C . For each lane, the appropriate amount of HEGDK buffer was added to 5 μg of nuclear extracts to bring the total volume to 5 μl . To dilute the salt concentration, 15 μl HEGD buffer was added to the mixture. One microgram of poly(dI-dC) (Roche, Indianapolis, IN) was added to block the nonspecific protein-oligonucleotide binding. After incubation for 10 min, 0.01 pmol radiolabeled probe, with or without 1 pmol unlabeled wild-type or mutated competing probe, was added and incubated for 10 min. The mixture was resolved in 5% nondenaturing PAGE, and protein-DNA complexes were visualized using a Storm Imager system (Molecular Dynamics, Sunnyvale, CA).

Transfection with miR-27a mimic or ZBTB10 expression vector

Cells seeded (1×10^5) into 12-well plates were incubated for 24h to allow cell attachment. Transfection with miR-27a mimic (50 nmol) and ZBTB10 expression plasmid pCMV6-XL4 vector (0.5-2 μg) was performed using LipofectAMINE 2000 according the manufacturer's protocol. The controls for the miR-27a mimic used an equal amount of a nonspecific oligonucleotide and, in the ZBTB10 overexpression experiment, the empty vector. After transfection for 5 h, the transfection mix was replaced with complete medium and incubated for different times as indicated.

Reporter gene transfection and luciferase assays

Cells were transfected with constructs essentially as previously described [20]. In brief, cells were plated in 12-well plates at 1×10^5 per well in DMEM medium supplemented with 2.5% charcoal-stripped FBS. After growth for 16 to 20 h, luciferase reporter constructs were transfected (0.4 μg) using LipofectAMINE 2000 according to the manufacturer's protocol. After transfection for 5 h, the transfection mix was replaced with complete medium and incubated for 19 h. Cells were then lysed with 100 μL of $1 \times$ reporter lysis buffer, and 30 μL of cell extract were used for luciferase assays using Promega Luciferase Assay System (Promega Corp. Madison WI) following the manufacturer's protocol. For the pZBTBT-3'-UTR containing a miR-27a binding site, luciferase activity was detected using the Dual Luciferase Assay System (Promega Corp., Madison, WI) according to the manufacturer's specifications. Lumicount was used to quantitate luciferase activity and the luciferase activities were normalized to protein concentration.

Real-time PCR analysis of mRNAs and miRNAs

For mRNA analysis, total RNA was isolated using the RNeasy (Qiagen, Valencia, CA) according to the manufacturer's protocol. Isolated RNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's protocol. qRT-PCR was carried out with the SYBR Green PCR Master Mix from Applied Biosystems (Foster City, Ca) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). TATA binding protein (TBP) was used as endogenous control to determine relative mRNA expression. microRNA was extracted using the mirVanaTM extraction kit (Applied Biosystems, Foster City, CA). Quantification

of miR-27a was performed using the Taqman®MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA) and qRT-PCR reaction using TaqMan®2X Universal PCR Master Mix (No AmpErase®UNG) (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. The small nuclear RNA miR-NU6B was used as endogenous control to determine relative microRNA expression. The comparative CT method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies Inc (San Diego, CA). Product specificity was examined by dissociation curve analysis. The following primers were used:

Myt1 (F): 5'-CCTTCCAAGAGTAGCTCCAATTC-3'.
 Myt1 (R): 5'-GCCGGTAGCTCCCATATGG-3'.
 Sp1 (F): 5'-TCACCTGCGGGCACACTT-3'.
 Sp1 (R): 5'-CCGAACGTGTGAAGCGTT-3'.
 TBP (F): 5'-TGCACAGGAGCCAAGAGTGAA-3'.
 TBP (R): 5'-CACATCACAGCTCCCCACCA-3'.
 Wee1 (F): 5'-TTGCGCCTTGCCCTCACA-3'.
 Wee1 (R): 5'-TTGATCTCCATTCTCGGAAGAG-3'.
 Survivin (F): 5'-CCA TGC AAA GGA AAC CAA CAA T-3'.
 Survivin (R): 5'-ATG GCA CGG CGC ACT T-3'.
 ZBTB10 (F): 5'-GCTGGATAGTAGTTATGTTGC-3'.
 ZBTB10 (R): 5'-CTGAGTGTTTGATGGACAGA-3'.
 VEGFR (F): 5'-AAAGGCCGTGTCATCGTTTC-3'.
 VEGFR (R): 5'-CCATATGCGGTACAAGTCAGG-3'.
 hβ2-microglobulin (hβ2G) (F) 5'-GGC TGG CAA CTT AGA G-3'.
 hβ2-microglobulin (hβ2G) (R): 5'-GCC TTA CTT TAT CAA ATG TAT-3'.
 murine actin (F) 5'-GCA ACG AGC GG T TCC G-3'.
 murine actin (R) 5'-CCC AAG AAG GAA GGC TGG A-3'.

Primers for Sp3 and Sp4 were purchased from Qiagen.

Cell cycle kinetics

Cells were treated as described for the proliferation assay. Cells were trypsinized, collected by centrifugation, resuspended in staining solution [50 µg/mL propidium iodide, 30 units/mL RNase, 4 mmol/L sodium citrate, and Triton X-100 (pH 7.8)], and incubated at 37 °C for 10 min. Sodium chloride solution was added to a final concentration of 0.15 mol/L. Stained cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) using Cell Quest acquisition software (Becton Dickinson Immunocytometry Systems, San Jose, CA) as previously described [25].

TUNEL assay

Cells (6×10^4) were seeded in four-chambered glass slides, and treated with DMSO or BA (10 µM) for 16 h. Cleavage of DNA during apoptosis was identified by the *in situ* cell death detection TUNEL assay (Roche Applied Science, Mannheim, Germany) according to the instruction manual protocol in cells fixed with 4% paraformaldehyde in PBS. The green fluorescence (GF) images were captured using an AxioCam high152 resolution digital camera 42X magnification. The green brightness provides an estimated level of DNA strand breaks.

Xenograft study

Female athymic BALB/c nude mice (age 3- 4 weeks), were purchased from Harlan Laboratories (Houston, TX) and maintained in a ventilated rack system. Irradiated food and autoclaved water were provided ad libitum. Experiments were approved by the Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX). The mice were allowed to adjust to their environment for 4 days before initiation of the experiments. MDA-MB-231 (1×10^6 cells) were implanted with matrigel (BD Bioscience, San Jose, CA) s.c. into the flank of each mouse. Ten days after tumor implantation, when tumors reached a minimum size of $117 \pm 18 \text{ mm}^3$, animals were divided into two equal groups of 7 mice each. The first group received 100 μL vehicle (1% DMSO in corn oil) by oral gavage, and the second group received 20 mg/Kg/day of BA every second day. The mice were weighed, and tumor areas were measured throughout the study using calipers. The estimated tumor volume was calculated by the formula: $a^2 \times b/2$, where “a” and “b” are the short and the long axis of the tumor, respectively. All procedures were conducted under aseptic conditions in a laminar flow hood. A no-xenografted control group received same doses of BA and was used as negative control for metastasis analysis in lungs. After 25 days of BA treatment and 35 days of tumor implantation, the animals were sacrificed; final body and tumor weights were determined. Tumors and lungs were flash-frozen in liquid nitrogen and stored at -80°C for mRNA and protein analysis.

Statistical analysis

Data were analyzed using SPSS version 15.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by pairwise comparisons was performed with posthoc Tukey-Kramer HSD ($p < 0.05$).

Results

The effects of 2.5-10 μM BA on proliferation of ER-negative mesenchymal (HS-578T and MDA-MB-435) and epithelial (MDA-MB-468) breast cancer cells as well as HER2-overexpressing MDA-MB-453 and BT474 breast cancer cells is summarized in Figures 1A and 1B. At concentrations $\geq 10 \mu\text{M}$ BA-dependent growth inhibition was observed for all these cell lines and the MDA-MB-435 cells were the most sensitive. Triple negative MDA-MB-231 cells were used to model the growth inhibitory effects of BA and betulonic acid (BO) (Fig. 1C) and their corresponding methylesters (methyl BA and methyl BO) (Fig. 1D). BA and methyl BA were equipotent whereas BO and methyl BO were less active as inhibitors of MDA-MB-231 cell proliferation.

Treatment of MDA-MB-231 cells with 2.5, 5 and 10 μM BA for 24h induced a concentration-dependent decrease in expression of Sp1, Sp3 and Sp4 proteins (Fig. 2A) confirming results of previous studies which showed that BA decreased Sp1, Sp3 and Sp4 protein levels in prostate and bladder cancer cells [22-23]. Gel mobility shift assays showed that BA also decreased formation of a retarded band containing Sp proteins bound to a GC-rich oligonucleotide (Fig. 2B) (lanes 3-5) compared to DMSO (control); the retarded band intensity was also decreased after incubation with excess unlabeled GC-rich oligonucleotide (lane 1). The effects of BA on Sp1, Sp3 and Sp4 mRNA levels were also determined in MDA-MB-231 cells, and concentrations of 2.5 – 10 μM BA significantly decreased expression of these genes. The highest decrease was observed using 10 μM BA (Fig. 2C). Both the Sp1 and Sp3 gene promoters are GC-rich and BA decreased luciferase in MDA-MB-231 cells transfected with pSp1-for 4, a construct containing the -751 to -20 region of the Sp1 promoter insert linked to a luciferase reporter gene (Fig. 2D). Similar results were observed in MDA-MB-231 cells transfected with Sp3-for5, a construct

containing the –417 to –38 region of the Sp3 promoter. Promoter constructs for Sp4 have not been characterized.

Previous reports in other cancer cell lines show that c-Met, cyclin D1, VEGF, and VEGFR1 are Sp-regulated genes [16] and BA decreased expression of all gene products in MDA-MB-231 cells (Fig. 3A). Both the VEGF and VEGFR1 promoter contain GC-rich Sp binding sites and in MDA-MB-231 cells transfected with pVEGF and pVEGFR1, BA decreased luciferase activity (Fig. 3B). These findings are consistent with previous studies in other cell lines using compounds or RNA interference with small inhibitory RNAs for Sp1, Sp3 and Sp4 that decrease Sp1, Sp3 and Sp4 expression [16,22,26-27]. In addition, BA also decreased survivin, another Sp-regulated gene (Fig. 3C). This decrease was accompanied by increased PARP cleavage and apoptosis was confirmed in a TUNEL assay showing that BA also increased TUNEL staining (Fig. 3D).

BA-induced downregulation of Sp1, Sp3 and Sp4 proteins was not reversed by cotreatment with proteasome inhibitors (data not shown); however, previous studies showed that transcriptional regulation of Sp1, Sp3 and Sp4 was linked to miR-27a and chemical- or antagomir-induced downregulation of miR-27a enhanced expression of ZBTB10, an Sp repressor [19,25-26,28-29]. Treatment of MDA-MB-231 cells with 2.5-10 μ M BA decreased miR-27a (Fig. 4A) and this was accompanied by induction of ZBTB10 mRNA levels (Fig. 4A). ZBTB10 is a transcriptional repressor of Sp transcription factors and overexpression of ZBTB10 in MDA-MB-231 decreased expression of Sp1, Sp3 and Sp4 (Fig. 4B). Moreover, induction of ZBTB10 in cells treated with BA was significantly repressed in cells treated with BA and transfected with a miR-27a mimic (Fig. 4C); in addition BA also increased the luciferase activity in MDA-MB-231 cells transfected with pZBTB10-3'-UTR which contains a miR-27a binding site in the 3'-UTR of ZBTB10 as described (Fig. 4D) [28].

Previous studies [25] showed that antisense miR-27a (as-miR-27a) induced a G2/M arrest in MDA-MB-231 cells through induction of the kinase Myt-1 which catalyzes phosphorylation of cdc2 to inhibit progression of cells through G2/M phase. Results in Figure 5, showed that the effects of BA were similar to those previously reported for as-miR-27a; treatment of MDA-MB-231 cells with BA arrested cells in G2/M (Fig. 5A) and this is not due to induction of Wee1 (another miR-27a target) (Fig. 5B); which inactivates cdc2, but to induction of Myt-1 (Fig. 5C). This arrest was accompanied by enhanced phosphorylation of cdc2 at tyrosine-15 (Fig. 5D). The Myt-1-dependent phosphorylation of cdc2 reached a peak between 2-12 h, this was consistent with BA-induced arrest of MDA-MB-231 cells in G2/M.

MDA-MB-231 cells were also used in an orthotopic model in athymic female nude mice. After 31 days of tumor implantation and 21 days of BA (20 mg/kg/day) treatment, tumor growth rates were significantly different from control group (Fig. 6A). Accordingly, final tumor volumes and weights were suppressed in the BA group compared to the control group. This suppression was accompanied by decreased Sp1, Sp3 and Sp4 mRNA and protein levels (Fig. 6B), downregulation of miR-27a, and increased mRNA levels of ZBTB10 (Fig. 6C). BA also decreased expression of Sp-regulated VEGFR and survivin genes in the tumors of animals treated with BA compared to the control (corn oil) tumors (Fig. 6D). Thus, BA decreased proliferation of MDA-MB-231 cells and tumors through perturbing the miR-27a:ZBTB10 – Sp transcription factor axis. Moreover, micro metastases in the lung were visible but not characterized. We used β 2-microglobulin mRNA as the major indicator of metastasis [30-31]. Results showed that expression of the human-specific β 2-microglobulin (h β 2G) gene in the lungs of xenografted mice after 35 days of tumor implantation was significantly lower in the BA treated group, and similar to levels detected as background in no-xenografted controls (Fig. 6E).

Discussion

Sp1, Sp3 and Sp4 transcription factors are overexpressed in multiple cancer cell lines and tumors [15-23,25-27], and according to *in vivo* studies, there is minimal expression of these proteins in non-tumor tissues [22-23,26-27]. The results are consistent with reports showing that in rodents and humans Sp1 expression decreases with age [32-34]. The pro-oncogenic activity of Sp1 is illustrated in a report showing that carcinogen-induced transformation of mouse skin fibroblasts is accompanied by an 8- to 18-fold increase in Sp1 and the formation of tumors; whereas knockdown of Sp1 in these cells results in loss of tumorigenicity [35]. Sp1 is also a negative prognostic factor for survival of pancreatic and gastric cancer patients [36-37]. Studies in this laboratory have used RNA interference and knockdown of Sp1, Sp3 and Sp4 (individually and combined) to identify Sp-regulated genes [14-22,26-27], and results demonstrate that Sp transcription factors regulate expression of critical genes responsible for cancer cell growth, survival and angiogenesis/metastasis including cyclin D1, epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-MET), p65 subunit of nuclear factor kappa-B (NF- κ B), survivin, bcl-2, vascular endothelial growth factor (VEGF) and its receptors VEGFR1 and VEGFR2.

Based on the pro-oncogenic activities of Sp-regulated genes, several studies have now identified anticancer agents that decrease expression of Sp1, Sp3, Sp4 and Sp-regulated genes in cancer cells and tumors, these include: curcumin, arsenic trioxide, tolfenamic acid and structurally-related non-steroidal anti-inflammatory drugs, betulinic acid and synthetic triterpenoids [14-23,26-27,38]. Results obtained in this study in ER-negative breast cancer cells show that BA inhibits cell growth and tumor growth in athymic nude mice bearing MDA-MB-231 cells (orthotopically) (Figs. 1 and 6A). BA also decreased expression of Sp1, Sp3 and Sp4 proteins and Sp-regulated gene products (VEGF, VEGFR, cyclin D1, c-Met and survivin) in MDA-MB-231 cells (Figs. 2A, 3A and 3C), similar to the effects of BA in LNCaP prostate cancer cells [23].

The mechanisms associated with Sp downregulation are complex and dependent on the agent and cell context. For example, tolfenamic acid and BA induce proteasome dependent downregulation of Sp1, Sp3 and Sp4 in pancreatic and prostate cancer cells, respectively [23,38]. In contrast, the synthetic triterpenoids methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) and methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me) decreased miR-27a and induced expression of the miR-27a-regulated gene ZBTB10 which acts as an Sp repressor by competitively binding GC-rich promoter sites and decreasing gene expression [19,26]. BA did not induce proteasome-dependent degradation of Sp1, Sp3 or Sp4 in MDA-MB-231 cells (data not shown); however, BA decreased Sp1, Sp3 and Sp4 mRNA levels in both cells (Fig. 2C) and tumors (Fig. 6B) suggesting a transcriptional pathway was involved. BA-mediated inhibition of Sp1, Sp3 and Sp4 gene expression is consistent with the observed downregulation of miR-27a and induction of ZBTB10 in MDA-MB-231 cells (Figs. 4A and 6B). The Sp-repressor activity reported for ZBTB10 [28-29] was also confirmed by overexpression of ZBTB10 in MDA-MB-231 cells (Fig. 4B). We have previously demonstrated that as-miR-27a also decreases Sp1, Sp3, and Sp4 expression in MDA-MB-231 cells [25]. Moreover, overexpression of miR-27a reverses BA-induction of ZBTB10 (Fig. 4C). BA also increased luciferase activity in MDA-MB-231 cells transfected with a construct containing a miR-27a binding site in the 3'-UTR region of pZBTB10 (Fig. 4D) [28].

Both Wee-1 and Myt-1 are also putative miR-27a-regulated mRNAs, and these genes catalyze inactivating phosphorylation of cdc2 to block G2/M phase progression through the cell cycle [39-41]. Previous studies showed that as-miR-27a blocked MDA-MB-231 cells in G2/M, induced Myt-1, but did not induce Wee1 expression, and enhanced phosphorylation

of cdc2 on tyrosine-15 [25]. Similar results were observed in this study, which shows that BA decreases miR-27a and this also results in induction of Myt-1 (Fig. 5C), activation of cdc2 (Fig. 5D) and an increase in the percentage of cells in G2/M (Fig. 5A). Although the increase in the % of cells in G2/M is not large, the results are statistically significant and are consistent with our previous studies in which the increase in cells in G2/M was also not large but significant and confirmed in studies with as-mR-27a [19].

In summary, BA inhibits growth of MDA-MB-231 cells and tumors in athymic nude mice and this is due, in part, to transcriptional repression of Sp transcription factors and Sp-regulated genes. The tumor growth inhibition of BA *in vivo* was associated with the decreased expression of VEGFR in tumors, and decreased escape of tumor cells through the blood vessels [30]. Accordingly, BA decreased the expression of h β -2G in lungs. The gene expression of h β -2G in mouse lung tissues has been previously used as a marker of metastasis and was significantly influenced by the size of tumors [30]. Overall, 1 results presented in Fig. 6D and 6E support our hypothesis that BA inhibits angiogenesis and metastasis. The mechanism associated with this response involves disruption of the miR-27a:ZBTB10 circuitry resulting in the induction of Sp repressor that binds GC-boxes. The initial target(s) of BA that result in downregulation of miR-27a have not yet been defined in MDA-MB-231 cells, and these are currently being investigated.

In conclusion the molecular mechanism underlying the tumor growth inhibition exerted by BA was identified as miR-27a suppression and induced expression of the miR-27a-regulated gene “ZBTB10” which acts as an Sp repressor. These studies show, for the first time, in triple negative breast cancer cells that anticancer agents targeting Sp transcription factors are highly effective and have potential for clinical applications that would include combination therapies.

Acknowledgments

We would like to thank Dr. Robert Burghardt, Department of Veterinary Anatomy & Public Health, Texas A&M University, College Station, TX for his kind assistance with imaging technology.

Grant Support

Financial support for this research has been provided by the National Institutes of Health (KOIATOO 4597 to SM-T) and the DOD-Army Breast Cancer Research Program (BC095260 to SS).

Abbreviations

BA	betulinic acid
BO	betulonic acid
Sps	specificity proteins
VEGFR	vascular endothelial growth factor receptor
PARP	poly (ADP-ribose) polymerase
ROS	reactive oxygen species
miR-27a	microRNA-27a
BO	betulonic acid
GF	green fluorescence
hβ2G	human-specific β 2-microglobulin

EGFR	epidermal growth factor receptor
c-MET	hepatocyte growth factor receptor
NF-kB	nuclear factor kappa-B

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Figure 1.

Betulinic acid (BA) inhibits cell growth of breast cancer cells lines. Decreased cell number of the (A) ER-negative MDA-MB-435, HS-578T and MDA-MB-468; (B) HER2-overexpressing BT-474 and MDA-MB-453; the triple negative MDA-MB-231 cells treated with (C) BA or betulonic acid (BO), and (D) methyl BA or methyl BO. Cells were seeded and treated with solvent (DMSO) or different concentrations of BA, BO, methyl BA or methyl BO (2.5-10 μ M) for 48h, cell number was determined as described in materials and methods. Each experiment was performed at least three times, and results are expressed as means $\pm$ SD. * indicates significant changes at $P < 0.05$.

Figure 2.

BA decreases Sp protein and mRNA levels in MDA-MB-231 breast cancer cells. (A) Decreased protein expression of Sp transcription factors accessed by western blots. Cells were treated with solvent (DMSO) or different concentrations of BA (2.5-10 μ M) for 24h and expression of Sp proteins on whole-cell lysates was determined by immunoblot analysis as described in materials and methods. (B) Gel mobility shift assay. Cells were treated with BA for 24 h and prepared as described in materials and methods. (C) mRNA levels of Sp. Relative mRNA levels were determined by qRT-PCR as described in materials and methods. (D) Luciferase activity of MDA-MB-231 cells transfected with Sp promoter constructs. Luciferase activity (relative to protein content) in transfected cells with Sp luciferase reporter constructs and treated with DMSO or BA (2.5-10 μ M) for 24 h was determined as described in materials and methods. Each experiment was performed at least three times. Values are mean (n=3) $\pm$ SE for mRNA and $\pm$ SD for luciferase activity. * indicates significant changes at $P < 0.05$.

Figure 3. Betulinic acid (BA) decreases expression of the Sp-regulated genes and induces apoptosis in MDA-MB-231 cells. (A) Effects of BA on VEGF, VEGFR, Cyclin D1 and c-Met proteins. (B) Luciferase activity (relative to protein content) in transfected cells with VEGF and VEGFR constructs and treated with DMSO or BA (2.5-10 μM) for 24 h was determined as described in materials and methods. (C) protein levels of survivin and cleaved PARP (C-PARP). Cells were treated with solvent (DMSO) or different concentrations of BA (2.5-10 μM) for 24h and expression of proteins was determined in whole-cell lysates by immunoblot analysis as described in materials and methods. (D) *in situ* cell death detection. Cells were treated with DMSO or BA (10 μM) for 16 h before cell fixation with 4% paraformaldehyde in PBS. Cleavage of DNA during apoptosis was identified by the TUNEL assay as described in materials and methods. Values are means (n=3) ± SD. * indicates significant changes at P < 0.05.

Figure 4.

The Betulinic acid (BA) repression of miR-27a expression causes ZBTB10-Sp regulation in MDA-MB-231 cells. (A) microRNA levels of miR-27a and mRNA levels of ZBTB10 relative to DMSO treated cells. Cells were treated with solvent (DMSO) or different concentrations of BA (2.5-10μM) for 24h. mRNA and microRNA analyses were performed by qRT-PCR as described in materials and methods. (B) Sp protein levels in cells transfected with ZBTB10 expression vector. Cells were transfected with ZBTB10 expression vector (0.5-2 μg) and Sp protein levels were analyzed in whole-cell lysates after 24h Transfection by immunoblot analysis as described in materials and methods. (C) mRNA levels of ZBTB10 in cells transfected with the mimic of miR-27a or non-specific oligonucleotide and treated with BA (5μM). mRNA levels of ZBTB10 were analyzed by qRT-PCR. (D) Luciferase activity in cells transfected with pZBTB10-3'-UTR construct and treated with BA. Dual luciferase activity was assessed in transfected cells and treated with BA for 16h as described on materials and methods. Each experiment was performed at least three times, and results are expressed as means ± SD. * indicates significant changes at P < 0.05.

Figure 5.

Betulinic acid (BA) induces cell cycle arrest at G2/M through induction of Myt-1. (A) Flow cytometry analysis of MDA-MB-231 cells treated with solvent (DMSO) or different concentrations of BA (2.5-10 μM) for 48h. The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined as described in materials and methods. mRNA levels of (B) Wee1 and (C) Myt-1. Cells treated with BA or DMSO for 24h were analyzed by qRT-PCR as described in materials and methods. (D) phosphor- and total cdc2 protein levels. Whole-cell lysates of cells treated with BA (10 μM) for 1-24 h were analyzed by immunoblot analysis as described in materials and methods. Each experiment was performed at least three times, and results are expressed as means ± SE. * indicates significant changes at $P < 0.05$.

Figure 6.

Antitumorigenic and antimetastatic activity of BA in xenografted nude mice. (A) Effects on tumor volume and tumor weight. Athymic nude mice (7 per group) bearing MDA-MB-231 cells as xenografts were treated with corn oil (control) or BA in corn oil (20 mg/kg/day) every second day. Treatments with BA started after implanted tumors reached a minimum volume as described in materials and methods. (B) mRNA and protein levels of Sp1, Sp3, and Sp4. (C) miR-27a and ZBTB10 gene expression. (D) mRNA levels of VEGFR and Survivin. Relative mRNA and microRNA levels in BA group were normalized to corn oil controls, values are means $\pm$ SE, (n=6). *, P < 0.05. (E) Expression of human specific hβ2G in lungs of xenografted and no-xenografted control mice. Relative hβ2G mRNA on lungs of xenografted mice were normalized to hβ2G mRNA of no-xenografted controls, values are averages $\pm$ SE (n = 4). Different letters indicate significant changes. *, P < 0.05. #, P < 0.1.