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14. ABSTRACT

Betulinic acid (BA) is relatively non-toxic in rodent studies and highly effective against melanoma in both in vivo and in vitro assays. Subsequent research in several laboratories indicates that BA inhibits growth of multiple tumor types including breast cancer. Studies in this laboratory show that BA inhibits prostate cancer cell and tumor growth in a xenograft model, and one of the underlying mechanisms of action is due to BA-induced proteasome-dependent degradation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4. These proteins are highly expressed in several different cancer cell lines and tumors, whereas Sp1 levels in non-tumor tissue of rodents and humans is relatively low and decreases with age. In this study, we used BA as a model to investigate the effect of Sp1, Sp3 and Sp4 downregulation on BT474 and MDA-MD-453 breast cancer cells that express the oncogene EGFR2 (ErbB2, HER2). The proposed research focused on the role of BA-induced Sp downregulation on cell and tumor growth, ErbB2 expression and the overall mechanisms associated with the anticancer activity of BA. We also investigated the role of BA in triple-negative MDA-MB-231 cells which do not express ErbB2.

15. SUBJECT TERMS
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INTRODUCTION

Betulinic acid (BA) is relatively non-toxic in rodent studies and highly effective against melanoma in both *in vivo* and *in vitro* assays. Subsequent research in several laboratories indicates that BA inhibits growth of multiple tumor types including breast cancer. Studies in this laboratory show that BA inhibits prostate cancer cell and tumor growth in a xenograft model, and one of the underlying mechanisms of action is due to BA-induced proteasome-dependent degradation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4. These proteins are highly expressed in several different cancer cell lines and tumors, whereas Sp1 levels in non-tumor tissue of rodents and humans is relatively low and decreases with age. In this study supported by the DOD grant, we have used BA as a model to investigate the effect of Sp1, Sp3 and Sp4 downregulation on BT474 and MDA-MD-453 breast cancer cells that express the oncogene EGFR2 (ErbB2, HER2). The proposed research has focused on the role of BA-induced Sp downregulation on cell and tumor growth, ErbB2 expression and the overall mechanisms associated with the anticancer activity of BA. In addition, we also investigated the role of BA in triple-negative MDA-MB-231 cells which do not express ErbB2. The studies were carried out prior to our discovery that BA binds and activates cannabinoid receptors.

BODY

1. Betulinic Acid Targets YY1 and ErbB2 through Cannabinoid Receptor-dependent Disruption of MicroRNA-27a:ZBTB10 in Breast Cancer

Introduction

Betulinic acid (BA) is a naturally occurring triterpenoid found in bark extracts, and BA and synthetic analogs exhibit a broad spectrum of pharmacological properties including antiviral, antibacterial, anti-inflammatory, antimalarial and anticancer activities (1, 2). BA also inhibits growth of multiple tumors, and the large difference between the doses required for tumor growth inhibition and toxic side-effects in animal models has generated interest in clinical development of this compound for cancer chemotherapy (2, 3). The overall effectiveness of BA as an anticancer drug has been linked to the mitochondriotoxicity of BA and induction of reactive oxygen species (ROS) (4-6). Research in this laboratory has shown that BA inhibits growth and induces apoptosis in prostate, bladder and colon cancer cells and tumors, and this is accompanied by downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 and Sp-regulated genes (6-8). Similar effects have been observed for several anticancer drugs including curcumin, arsenic trioxide, non-steroidal anti-inflammatory drugs, and triterpenoids such as celastrol, 2-cyano-1,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid (CDODA) and their corresponding esters (6-15). The importance of BA and other agents that target Sp proteins is due to (a) the overexpression of Sp1, Sp3 and Sp4 in tumor vs. non-tumor tissue and (b) the critical roles for Sp-regulated genes in mediating cancer cell growth [epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-MET), cyclin D1], survival (bcl-2 and survivin), angiogenesis [vascular endothelial growth factor (VEGF) and VEGF receptors 1 and 2 (VEGFR1 and VEGFR2)], and inflammation (p65NFκB) (8-12, 16-18).

This study investigated the anticancer activity of BA in BT474 and MDA-MB-453 breast cancer cells which overexpress ErbB2, an important oncogenic growth factor receptor that is not an Sp-regulated gene. BA inhibited cell and tumor growth, downregulated Sp1, Sp3 and Sp4 and, surprisingly, decreased ErbB2 expression; however, this effect was due to downregulation

YY-1, an Sp-regulated gene that activates ErbB2 expression (19, 20). The mechanism of Sp downregulation by BA was due to disruption of microRNA-27a (miR-27a):ZBTB10 which was cannabinoid (CB) receptor dependent, and BA directly bound to both CB1 and CB2 receptors. This represents a novel mechanism of action of BA and highlights the clinical potential of BA and related compounds that downregulate Sp transcription factors as a new class of mechanism-based agents for treating ErbB2-overexpressing breast tumors.

Results

(i) BA inhibits growth, induces apoptosis and downregulates Sp1, Sp3 and Sp4 in BT474 and MDA-MB-453 cells: BT474 and MDA-MB-453 cells overexpress ErbB2, and 1-10 μM BA (Fig. 1A) inhibited proliferation of both BT474 and MDA-MB-453 cells. The overall decrease in cell number was both concentration- and time (2 or 4 days)-dependent, and MDA-MB-453 cells were less responsive to BA than BT474 cells (Fig. 1B). The growth inhibitory effects of BA were accompanied by induction of cleaved PARP, a marker of apoptosis, and decreased expression of survivin, an inhibitor of apoptosis, was also observed (Fig. 1C). Induction of apoptosis was also observed in a TUNEL assay in which BA increased TUNEL staining in both cell lines (Fig. 1D).

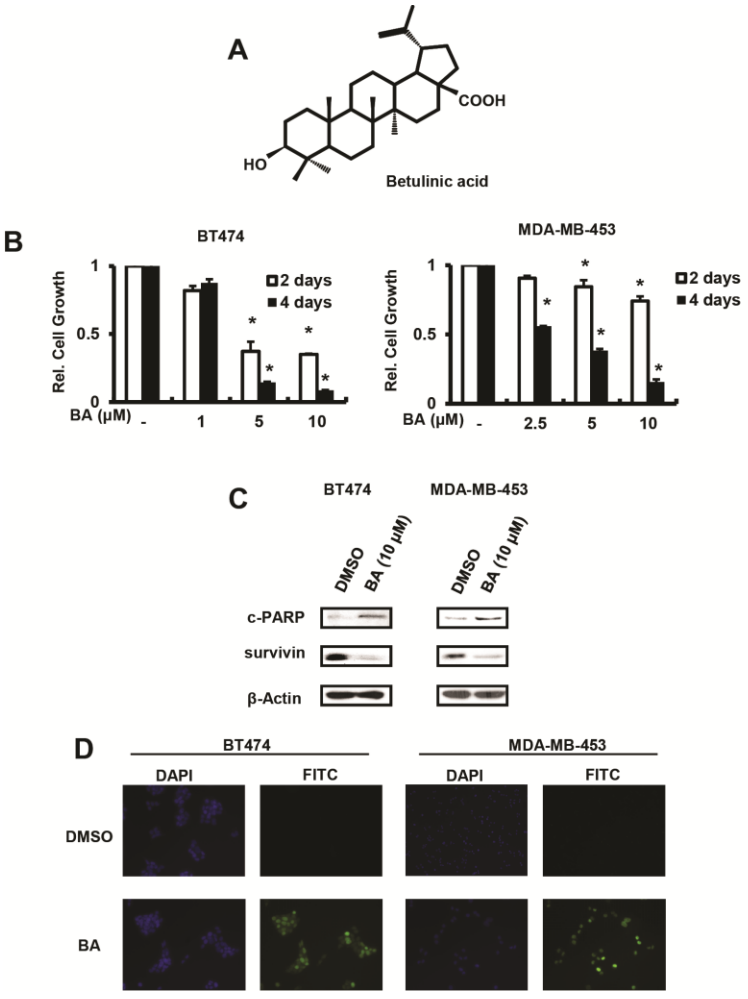

Figure 1. Effects of BA on cell proliferation and apoptosis. (A) Structure of BA. (B) BA-mediated inhibition of BT474 and MDA-MB-453 cell growth. Cells were treated with different concentrations of BA for up to 4 days and the number of cells in each treatment group was determined. Significant ($p < 0.05$) growth inhibition is indicated (*). Results are expressed as means ± SE for at least 3 replicate determinations for each treatment group. (C) Effects of BA on cleaved-PARP and survivin. Cells were treated with 10 μM BA for 48 hr and whole cell lysates were analyzed by western blots. (D) BA induces apoptosis in cancer cells. Cells were treated with DMSO or 10 μM BA for 24 hr and analyzed with a TUNEL assay.

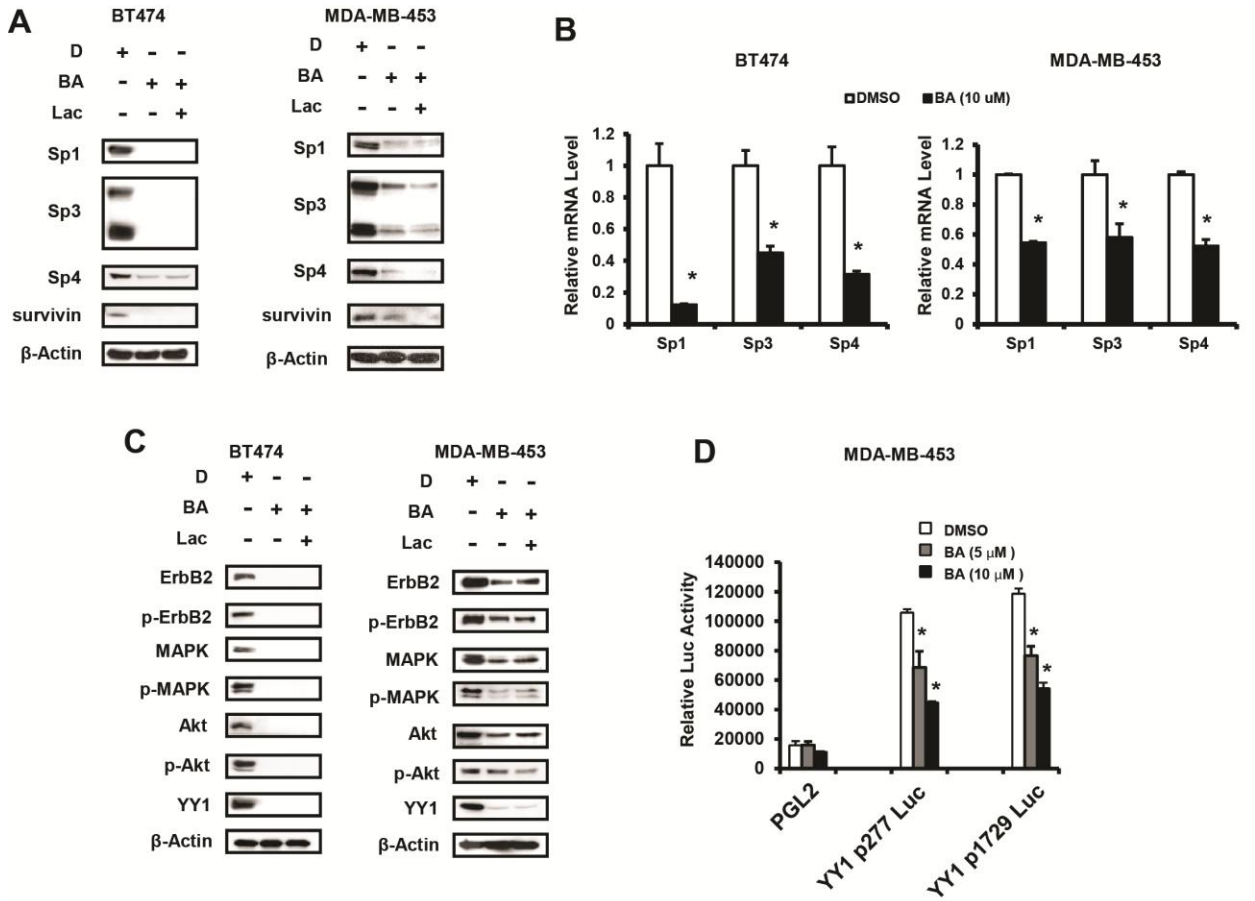

Figure 2. Effects of BA on Sp1, Sp3, Sp4, YY1, ErbB2 and ErbB2 dependent proteins. (A) BA decreases Sp protein and survivin levels in BT474 and MDA-MB-453 cells. Cells were treated with DMSO (D), 10 μM BA alone or in combination with 1 μM lactacystin for 48 hr, and whole cell lysates were analyzed by western blots. (B) BA decreases mRNA levels of Sp proteins. Cells were treated with 10 μM BA for 16 hr, and mRNA levels were determined. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) decreases are indicated (*). (C) BA decreases protein levels of ErbB2 and ErbB2 dependent proteins. Cells were treated with DMSO (D), 10 μM BA alone or in combination with 1 μM lactacystin for 48 hr, and whole cell lysates were analyzed by western blots. (D) BA decreases YY1 promoter activity. MDA-MB-453 cells were transfected with empty vector (PGL2), the YY1 p-277-luc or the YY1 p-1729-Luc construct. Cells were then treated with 5 or 10 μM BA for 24 hr. Luciferase activity was determined. Results are means ± SE for 3 separate determinations and significant ($p < 0.05$) induction of luciferase activity by AP-2 is indicated (*).

BA inhibits LNCaP prostate cancer cell growth and this is due, in part, to activation of proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins (7). Treatment of BT474 and MDA-MB-453 cells with 10 μM BA for 48 hr decreased in expression of Sp1, Sp3, Sp4 and survivin (an Sp-regulated gene) proteins (Fig. 2A) and mRNA (Fig. 2B) in both cell lines, and BT474 cells were more responsive than MDA-MB-453 cells. The proteasome inhibitor MG132 alone was cytotoxic to BT474 and MDA-MB-453 cells (data not shown), whereas lactacystin was not toxic. Treatment of BT474 and MDA-MB-453 cells with 10 μM BA alone or in combination with 1 μM lactacystin for 48 hr showed that lactacystin did not affect BA-induced downregulation of Sp proteins (Fig. 2A) and this contrasted to results for BA in prostate cancer cells (7).

(ii) *BA-induced downregulation of YY1, ErbB2 and ErbB2-regulated genes is due to decreased Sp1, Sp3 and Sp4 expression:* ErbB2 plays a major role in the proliferation of BT474

and MDA-MB-453 cells. BA alone decreased ErbB2, p-ErbB2, and downstream kinases MAPK, p-MAPK, Akt and p-Akt expression (Fig. 2C), and these effects were not reversed after co-incubation with the proteasome inhibitor lactacystin. BA-mediated downregulation of MAPK and Akt total proteins has previously been observed in bladder cancer cells (8), and results of Sp knockdown suggest that these effects are Sp-independent and are currently being investigated. YY1 is a key upstream regulator of ErbB2 in breast cancer cells (19), and BA decreased expression of YY1 in both cell lines in the presence or absence of lactacystin (Fig. 2C). Since the YY1 promoter contains multiple GC-rich Sp binding sites (20), we investigated the effects of BA on YY1 promoter activity and, in MDA-MB-453 cells transfected with GC-rich YY1 p-277 Luc or p-1729 Luc constructs, treatment with BA for 24 hr decreased luciferase activity (Fig. 2D). Figures 3A and 3B show that transfection of siRNAs against Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4) and their combination (iSp1/3/4) resulted in specific knockdown of the target Sp proteins and also decreased expression of YY1 and ErbB2 proteins. iSp1-1 and iSp1-2 were targeted against Sp1 but did not affect Sp3 or Sp4 expression (data not shown). In a second set of experiments in MDA-MB-453 cells (Fig. 3B), the siRNAs for Sp1 and Sp4 were highly specific; however, iSp3 also decreased expression of Sp3 and Sp4 proteins. iSp1, iSp4 and iSp1/3/4 decreased levels of both YY1 and ErbB2, whereas Sp3 knockdown had minimal effects on either protein. Previous RNA interference studies showed that knockdown of YY1 decreases expression of ErbB2 (19, 20), and we also observed that YY1 knockdown decreased ErbB2 levels in both cell lines (Fig. 4).

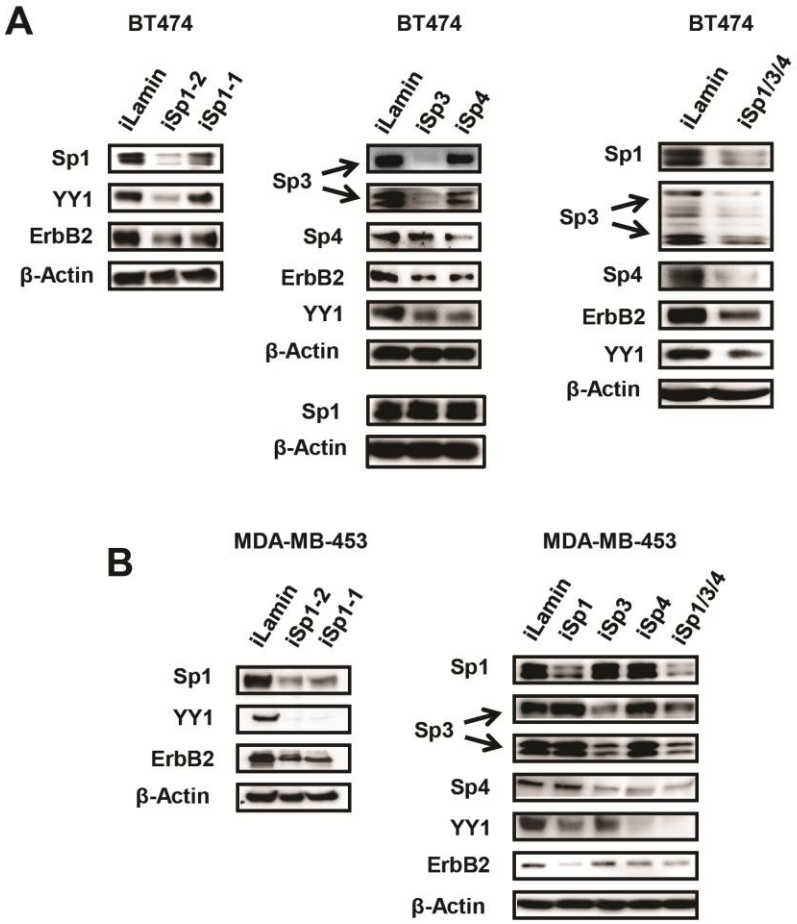

Figure 3. Role of Sp proteins in regulating level of YY1 protein. Knockdown of Sp1, Sp3, Sp4 or Sp1/Sp3/Sp4 in combination decreases protein levels of YY1 and ErbB2 in BT474 (A) and MDA-MB-453 (B) cells. Cells were transfected with siRNAs for Sp1, Sp3, Sp4 or Sp1/Sp3/Sp4 in combination for 72 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Two different oligonucleotides were used for Sp1 knockdown (iSp1-2 and iSp1-1 (A), and iSp1-2 was used in (B) (right hand panel). iLamin was used as the control oligonucleotide in the knockdown studies.

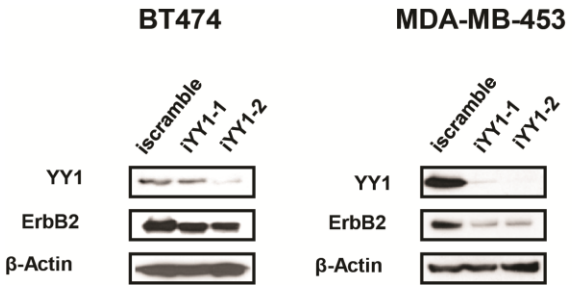

Figure 4. Knockdown of YY1 decreases protein levels of ErbB2 in BT474 and MDA-MB-453 cells. Cells were transfected with siRNA for YY1 for 72 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods.

(iii) <u>*Role of cannabinoid receptors*</u>*:* BA-induced downregulation of Sp transcription factors was proteasome-independent (Fig. 2) and was not reversed by ROS inhibitors (data not shown) as previously reported for other compounds (11, 12, 14, 15). Preliminary studies in other cancer cell lines show that CBs decrease Sp proteins (data not shown); therefore, the effects of CB1 and CB2 receptor antagonists AM251 and AM630, respectively, and capsazepine (vanilloid receptor antagonist) on BA-mediated repression of Sp1, Sp3 and Sp4 and survivin were also investigated. The vanilloid receptor antagonist was included since this receptor also binds some CBs (21). The CB receptors are expressed in BT474 and MDA-MB-453 cells, and cotreatment with BA and either AM251 or AM630 attenuated the effects of BA-induced downregulation of Sp1, Sp3, Sp4 and survivin, whereas capsazepine inhibited the effects of BA only in MDA-MB-453 cells (Figs. 5A and 5B). Figure 5B also shows that the antagonists alone had minimal effects on ErbB2, Sp1, Sp3 and YY1. The CB1 and CB2

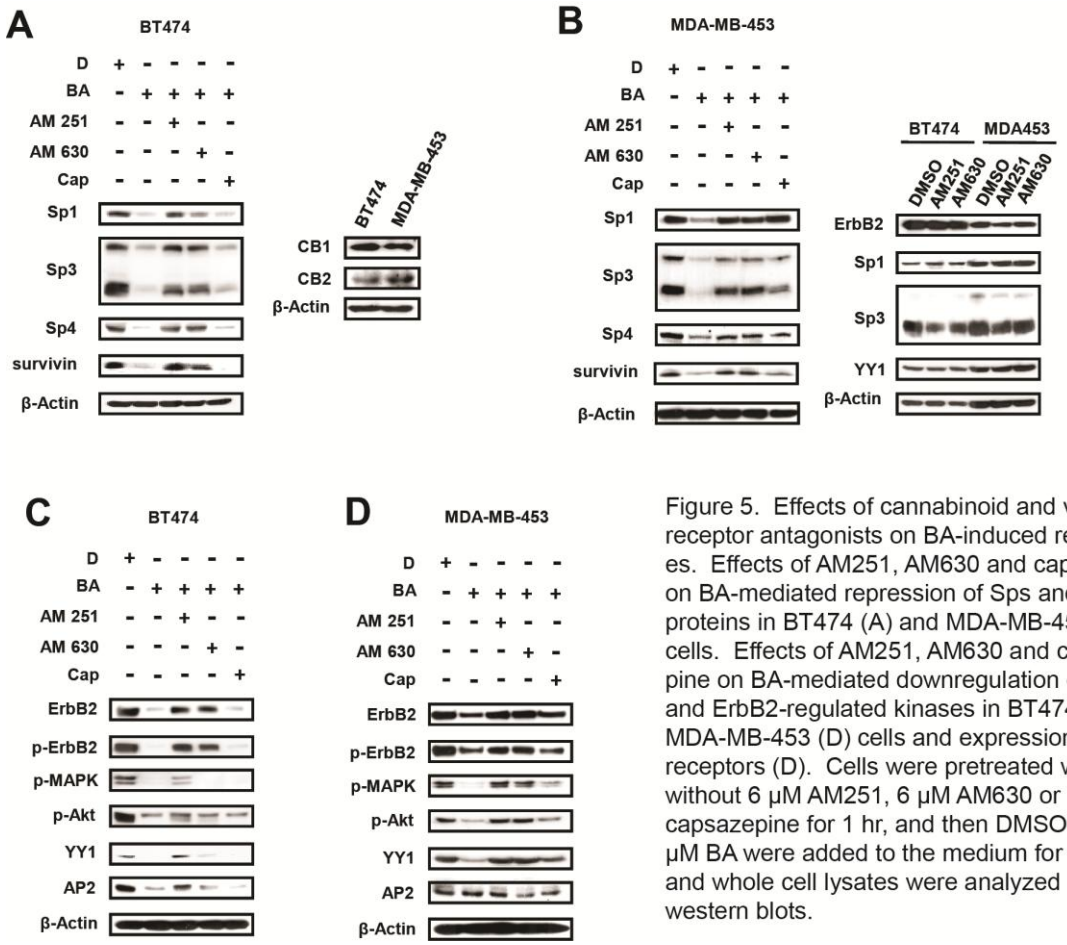

Figure 5. Effects of cannabinoid and vanilloid receptor antagonists on BA-induced responses. Effects of AM251, AM630 and capsazepine on BA-mediated repression of Sps and survivin proteins in BT474 (A) and MDA-MB-453 (B) cells. Effects of AM251, AM630 and capsazepine on BA-mediated downregulation of ErbB2 and ErbB2-regulated kinases in BT474 (C) and MDA-MB-453 (D) cells and expression of CB receptors (D). Cells were pretreated with or without 6 μM AM251, 6 μM AM630 or 2 μM capsazepine for 1 hr, and then DMSO (D) or 10 μM BA were added to the medium for 48 hr, and whole cell lysates were analyzed by western blots.

receptor antagonists inhibited BA-mediated downregulation of ErbB2, p-ErbB2, p-MAPK, p-Akt and YY1 in BT474 and MDA-MB-453 cells, whereas capsazepine was active as an inhibitor only in the latter cell line (Figs. 5C and 5D). Expression of AP2 was highly variable in both cell lines and was not further investigated. These results indicated that the CB1 and CB2 receptors mediated BA-induced effects on Sp1, Sp3 and Sp4, ErbB2 and YY1 in both cell lines. In contrast, the effects of BA on MAPK and Akt (total and phospho proteins) were CB receptor-independent and also Sp-independent in bladder cancer cells (8) and are currently being investigated.

Based on these results, the direct binding of BA to the CB receptors was investigated in a competitive binding assay using [^{3}H]CP-55,940 as the radioligand. Preliminary studies showed that high concentrations of BA (> 10^{-5} M) enhanced binding of [^{3}H]CP-55,940; however, this was due to a concentration-dependent increase in both total and non-specific binding (data not shown). Therefore, the [^{3}H]CP-55,940 specific binding to the mCB1 and hCB2 receptor was determined by subtracting the non-specific binding from the total binding as outlined in the Experimental Procedures (Fig. 6A). BA competitively bound to both receptors, and the K_i values over 5 separate determinations (Fig. 6B) were 36.7 $\pm$ 4.1 and 41.2 $\pm$ 12.1 μM for mCB1 and hCB2 receptors, respectively. As a positive control, Figure 6B shows the competitive displacement curves using the cannabinoid WIN-55,212-2 which binds both receptors with anticipated low nM affinity. These results demonstrate that BA directly binds the CB receptors. We also show that knockdown of CB1 or CB2 receptors by RNA interference partially reversed

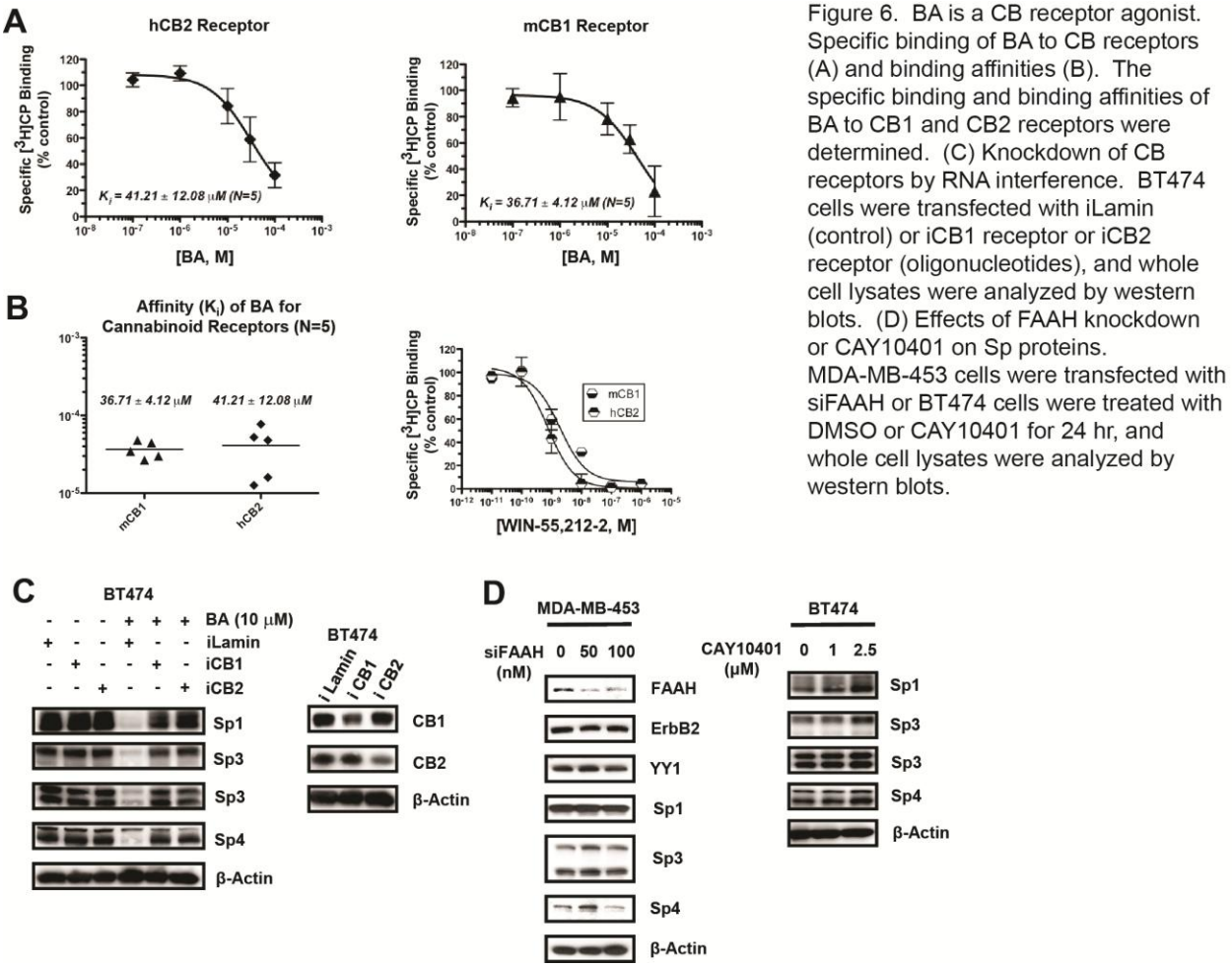

Figure 6. BA is a CB receptor agonist. Specific binding of BA to CB receptors (A) and binding affinities (B). The specific binding and binding affinities of BA to CB1 and CB2 receptors were determined. (C) Knockdown of CB receptors by RNA interference. BT474 cells were transfected with iLamin (control) or iCB1 receptor or iCB2 receptor (oligonucleotides), and whole cell lysates were analyzed by western blots. (D) Effects of FAAH knockdown or CAY10401 on Sp proteins. MDA-MB-453 cells were transfected with siFAAH or BT474 cells were treated with DMSO or CAY10401 for 24 hr, and whole cell lysates were analyzed by western blots.

BA-induced downregulation of Sp1, Sp3 and Sp4 (Fig. 6C), confirming a role for both receptors in mediating the effects of BA. A potential indirect effect of BA on cannabinoid-induced downregulation of Sp proteins could be due to inhibition of FAAH which could increase endocannabinoid levels (22); however, knockdown of FAAH by RNA interference (in MDA-MB-453 cells) or inhibition of FAAH by the specific FAAH inhibitor CAY10401 (in BT474 cells) did not affect levels of Sp1, Sp3 or Sp4 proteins (Fig. 6D), indicating that this pathway is not involved in downregulation of Sp proteins.

(iv) *BA disrupts miR-27a regulation of ZBTB10 and inhibits tumor growth:* Proteasome-independent downregulation of Sp1, Sp3 and Sp4 by BA and other anticancer agents has been linked to downregulation of miR-27a and induction of the transcriptional repressor ZBTB10 (6, 10, 15). Treatment of BT474 and MDA-MB-453 cells with 5 or 10 μM BA resulted in significant downregulation of miR-27a in both cell lines, and cotreatment with AM251 or AM630 inhibited this response (Fig. 7A) which was most pronounced in BT474 cells. Downregulation of miR-27a

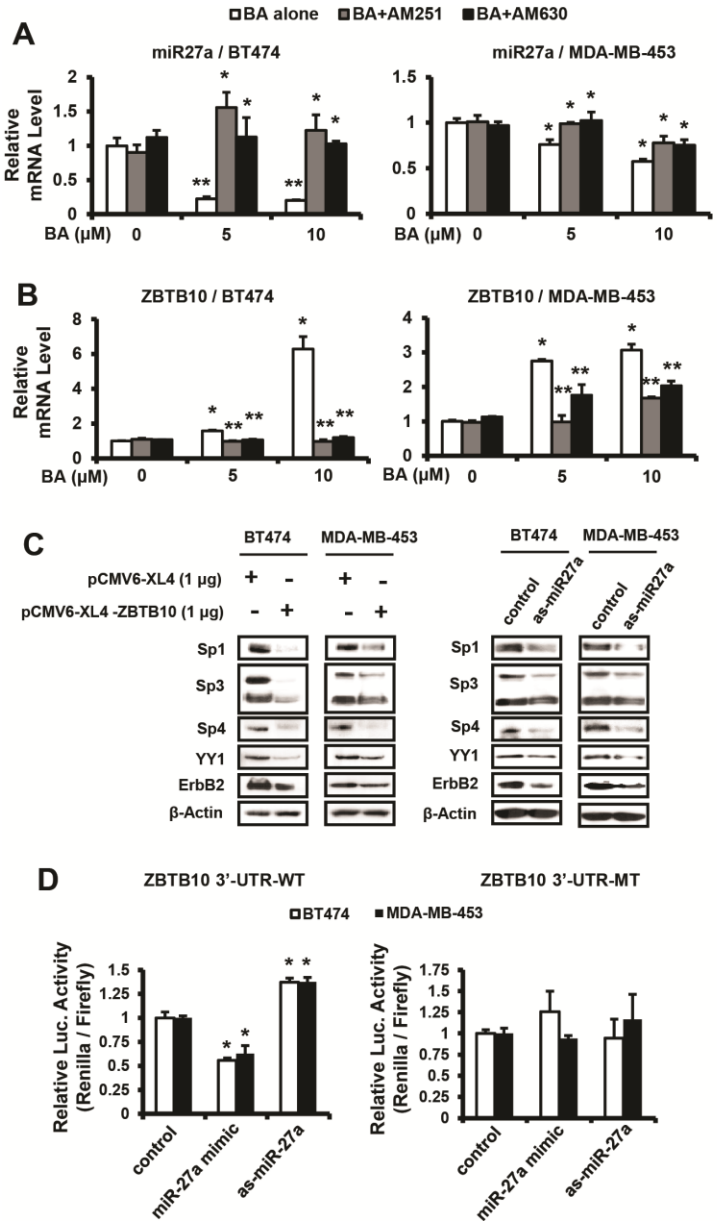

Figure 7. Effects of BA on miR-27a and ZBTB10, and the role of cannabinoid receptors on BA-mediated effects. (A) Downregulation of miR-27a. Cells were pretreated with or without 6 μM AM251 or 6 μM AM630 for 1 hr, DMSO or 5 μM or 10 μM BA were added to the medium for 24 hr, and miR-27a levels were determined. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) inhibition of miR-27a (**) and inhibition by the antagonists are indicated (*). (B) Induction of ZBTB10. Cells were treated and processed as described in (A), and significant ($p < 0.05$) induction by BA (*) and inhibition by the antagonists (**) are indicated. (C) Effects of ZBTB10 overexpression and antisense miR-27a on Sp protein levels, YY1 and ErbB2 proteins. Cells were transfected with 1 μg pCMV6-XL4-ZBTB10 plasmid or empty vector, 50 nM antisense miR-27a (as-miR-27a), or control, and whole cell lysates were analyzed by western blots. (D) Effects of miR-27a mimic or as-miR-27a on luciferase activity in ZBTB10 3'UTR-luc construct transfected cells. MiR-27a mimic (50 nM) or as-miR-27a were transfected into BT474 and MDA-MB-453 cells , and a dual luciferase reporter assay was performed according to the manufacturer's instructions. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) decreases or inductions are indicated (*).

in cells treated with BA was accompanied by induction of ZBTB10 mRNA levels in both cell lines, and cotreatment with AM251 or AM630 inhibited the induction response (Fig. 7B). A >6-fold induction of ZBTB10 was observed in BT474 cells, whereas ZBTB10 was induced >2.5-fold in MDA-MB-453 cells treated with 5 or 10 μM BA for 24 hr. The effects of antisense-miR-27a (as-miR-27a) and ZBTB10 overexpression on levels of Sp1, Sp3, Sp4, YY1 and ErbB2 proteins were also determined in BT474 and MDA-MB-453 cells (Figs. 7C and 7D), and both treatments decreased expression of Sp and Sp-regulated gene products. The effects of a miR-27a mimic and as-miR-27a on luciferase activity in BT474 and MDA-MB-453 cells transfected with ZBTB10 (UTR)-luc construct containing a miR-27a binding site resulted in decreased (miR-27a mimic) and increased (as-miR-27a) luciferase activity. In contrast, the mimic or antisense oligonucleotide did not affect luciferase activity in cells transfected with a construct [ZBTB10 (mUTR)-luc] containing a mutation in the miR-27a binding sites, confirming interactions of miR-27a with the target sequence in the 3'-UTR of ZBTB10.

The *in vivo* effects of BA on tumor growth were also investigated in athymic nude mice bearing BT474 cells as xenografts. BA was administered over a period of 28 days at a dose of 20 mg/kg/d. Tumor volumes and tumor weight were significantly inhibited, and BA decreased expression of Sp1, Sp3 and Sp4 proteins in tumors (Figs. 8A, 8B and 8C). Figure 8D illustrates that immunostaining of ErbB2 and Sp1 proteins were decreased in fixed tumor tissue from BA-treated mice compared to control (corn oil)-treated animals, and these *in vivo* data complement the results of *in vitro* studies.

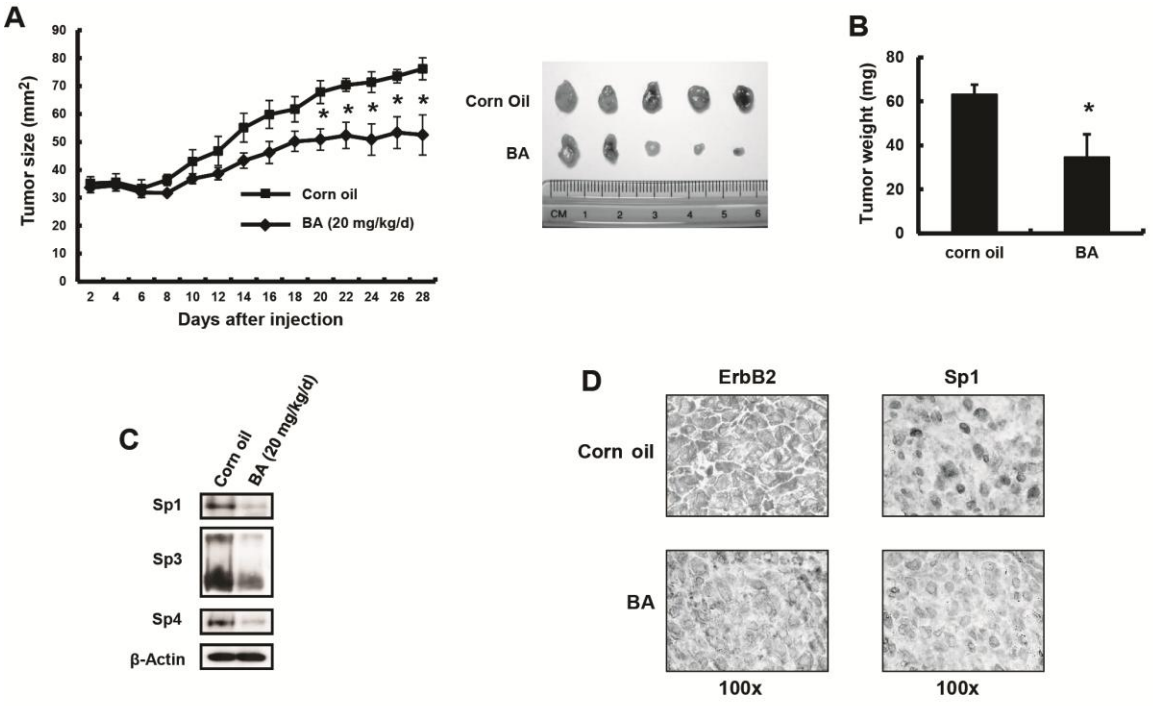

Figure 8. BA inhibits tumor growth in BT474 xenografts. Inhibition of tumor size (A) and weight (B). Athymic nude mice bearing BT474 cells as xenografts were treated with BA (20 mg/kg/d), and tumor sizes and weights were determined. Significantly ($p < 0.05$) decreased tumor sizes and weights are indicated (*). (C) BA decreases expression of Sp1, Sp3 and Sp4 proteins in tumors. Whole cell lysates from corn oil and BA-treated tumors were analyzed by western blot. (D) Immunostaining for ErbB2 and Sp1. Fixed tumor tissue from corn oil- and BA-treated mice were stained with ErbB2 and Sp1 antibodies.

Discussion

Endocrine therapies with antiestrogens and aromatase inhibitors have been successful for treating patients with early stage ER-positive breast cancer (23-25), whereas patients with ER-negative or ErbB2-overexpressing tumors must undergo more aggressive treatment and their overall prognosis and survival are much lower than patients with early stage breast cancer (26-28). The development of the monoclonal antibody Herceptin that binds the extracellular domain of ErbB2 has provided significant treatment benefits for patients with these aggressive ErbB2-overexpressing tumors (29, 30). Although Herceptin has been successfully used alone and in combination therapy, there is concern regarding cardiotoxic side-effects of this antibody, and development of other agents including tyrosine kinase inhibitors is being actively pursued for treatment of tumors which overexpress ErbB2 and other growth factor receptors (31).

BA inhibited growth and induced apoptosis in BT474 and MDA-MB-453 breast cancer cell lines (Fig. 1) and this was accompanied by downregulation of Sp1, Sp3 and Sp4 protein and mRNA levels (Fig. 2). Moreover, BA also inhibited tumor growth and downregulated Sp1, Sp3 and Sp4 in tumors from athymic nude mice bearing BT474 cells as xenografts (Fig. 8). In BT474 and MDA-MB-453 breast cancer cells, BA decreased expression of ErbB2, p-ErbB2 and downstream ErbB2-dependent kinases p-MAPK/MAPK and p-Akt/Akt (Figs. 2C and 2D), suggesting that ErbB2 downregulation may also be due repression of Sp proteins. However, unlike the EGFR which is an Sp-regulated gene (8), ErbB2 expression is dependent on other transcription factors including YY1 which contains multiple GC-rich promoter sites that bind Sp proteins (20). BA decreased YY1 protein expression in BT474 and MDA-MB-453 cells (Figs. 2C and 2D) and, not surprisingly, knockdown of Sp proteins (individually and combined) decreased expression of YY1 and ErbB2. Moreover, YY1 knockdown by RNA interference also decreased ErbB2 (Fig. 4), demonstrating that BA-mediated downregulation of ErbB2 is linked to decreased expression of Sp1, Sp3, Sp4 and Sp-regulated YY1.

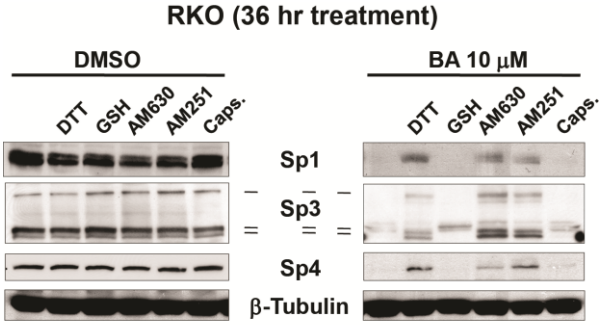

Figure 9. BA-mediated downregulation of Sp1, Sp3 and Sp4 partially blocked by CB receptor antagonists. RKO colon cancer cells were treated with DMSO or 10 μM BA alone or in combination with DTT, glutathione (GSH), 5 μM AM630, 5 μM AM251, and 2 μM capsazepine. Whole cell lysates were analyzed by western blots.

The mechanisms of BA-induced downregulation of Sp1, Sp3 and Sp4 were dependent on tumor type and cell context. BA induced proteasome-dependent downregulation in LNCaP prostate and SW480 colon cancer cells (6, 7), whereas in RKO colon cancer cells, this response was primarily ROS-dependent (7) and was due to repression of miR-27a and induction of ZBTB10, a transcriptional repressor that binds GC-rich promoter sites and downregulates Sp transcription factors and Sp-regulated genes (7, 10, 12, 15, 32). However, even in RKO cells, the CB receptor antagonists partially blocked the effects of BA on Sp proteins (Fig. 9). BA-induced repression of Sp1, Sp3 and Sp4 was proteasome-independent and not affected by lactacystin (Fig. 2A), and similar results were observed for ROS inhibitors such as glutathione (data not shown). Results summarized in Figure 7 show that BA also decreased miR-27a and induced ZBTB10 in BT474 and MDA-MB-453 cells, suggesting that the critical downstream effects of BA on the miR-27a:ZBTB10 complex are similar to those observed in previous studies with the synthetic triterpenoids CDDO-Me and CDODA-Me (10, 12). CDDO-Me-mediated downregulation of miR-27 in

pancreatic cancer cells was dependent on upstream disruption of mitochondria and induction of ROS; however, in contrast to these results, BA did not induce ROS in BT474 and MDA-MB-453 cells and the antioxidant glutathione did not modulate effects of BA on Sp1, Sp3 and Sp4 (data not shown).

Ongoing studies in this laboratory show that, like BA, cannabinoids downregulate Sp1, Sp3 and Sp4 in cancer cell lines (data not shown), and we demonstrate for the first time that BA binds directly to CB1 and CB2 receptors (Fig. 6A). The competitive binding assay was slightly modified to determine total and non-specific binding at all concentrations of BA, and K_i values were 36.7 and 41.2 μM for the CB1 and CB2 receptors (Fig. 6B). The CB receptor antagonists AM251 and AM630 inhibited BA-induced miR-27a (downregulation), ZBTB10 (induction), Sp1, Sp3, Sp4, YY1 and ErbB2 (downregulation), and downregulation of Sp proteins by BA were also blocked by knockdown of CB1 and CB2 receptors by RNA interference (Fig. 6C). These results demonstrate that the effects of BA on BT474 and MDA-MB-453 cells on Sp transcription factors and ErbB2 are mediated through activation of the CB1 and CB2 receptors which subsequently modulate the miR-27a:ZBTB10-Sp axis. CB1 and CB2 receptors are expressed in both cell lines and in human breast cancer cells and tumors, and one study showed a correlation between CB2 receptor and ErbB2 expression in human mammary tumors (33).

In summary, this study demonstrates that BA inhibits ErbB2-overexpressing breast cancer cell and tumor growth, and this is accompanied by a cascade of events involving activation of the CB1 and CB2 receptors, resulting in modulation of the miR-27a:ZBTB10-Sp transcription factor axis and downregulation of the Sp-dependent gene YY1 and the YY1-dependent gene ErbB2. This cannabinoid receptor-dependent pathway significantly contributes to the effects of this compound as an inhibitor of ErbB2-overexpressing breast cancer cell and tumor growth and is consistent with the well-known anticancer activities of cannabinoids (34, 35). Moreover, ongoing studies with cannabinoids also show that they target Sp transcription factors (unpublished observations). Current studies are focused on the mechanistic link between activation of the CB receptors and modulation of miR-27a:ZBTB10 and the efficacies and mechanism of action of other agents that repress Sp transcription factors. We are also investigating the effects of BA as an inducer of the newly identified Sp-repressor ZBTB4 which is regulated by miR-17-92 cluster miRs (36). These data demonstrate a novel pathway for targeting ErbB2 and identifies a new therapeutic approach for treating breast cancer patients that overexpress this oncogene.

2. Betulinic Acid Decreases ER-Negative Breast Cancer Cell Growth *In Vitro* and *In Vivo*: Role of Sp Transcription Factors and MicroRNA-27a:ZBTB10

Introduction

Betulinic acid (BA) is a triterpenoid acid found in various bark extracts. It is readily synthesized from betulin by oxidation to betulonic acid (BO) and reduction of the 3-keto group of BO to give BA (1, 2, 4, 37). 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; BA is a highly effective anticancer agent against numerous tumor types and is currently being explored in clinical trials (3, 5, 38-41).

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) (3, 5, 38-41). 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, 37, 38, 42, 43). 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 (44) suggested that one of the underlying mechanisms of action of BA in cancer cells may involve repression of specificity protein (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, vascular endothelial growth factor (VEGF), and VEGF receptors are all Sp-regulated genes (8-10, 13, 16-18, 45, 46). 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 (47). 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.

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 are summarized in Figures 10A and 10B. At concentrations ≤10 μM, 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 BO (Fig. 10C) and their corresponding methylesters (methyl BA and methyl BO) (Fig. 10D). 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 24 hr induced a concentration-dependent decrease in expression of Sp1, Sp3, and Sp4 proteins (Fig. 11A) confirming results of previous studies which showed that BA decreased Sp1, Sp3, and Sp4 protein levels in prostate and bladder cancer cells (8, 47). Gel mobility shift assays showed that BA also decreased formation of a retarded band containing Sp proteins bound to a GC-rich oligonucleotide (Fig. 11B, lanes 3–5) compared to DMSO (control); the retarded band intensity was also decreased after coincubation 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. 11C). 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. 11D). Similar results were observed in MDA-MB-231 cells transfected with Sp3-for 5, a construct containing the -417 to -38 region of the Sp3 promoter. Promoter constructs for Sp4 have not been characterized.

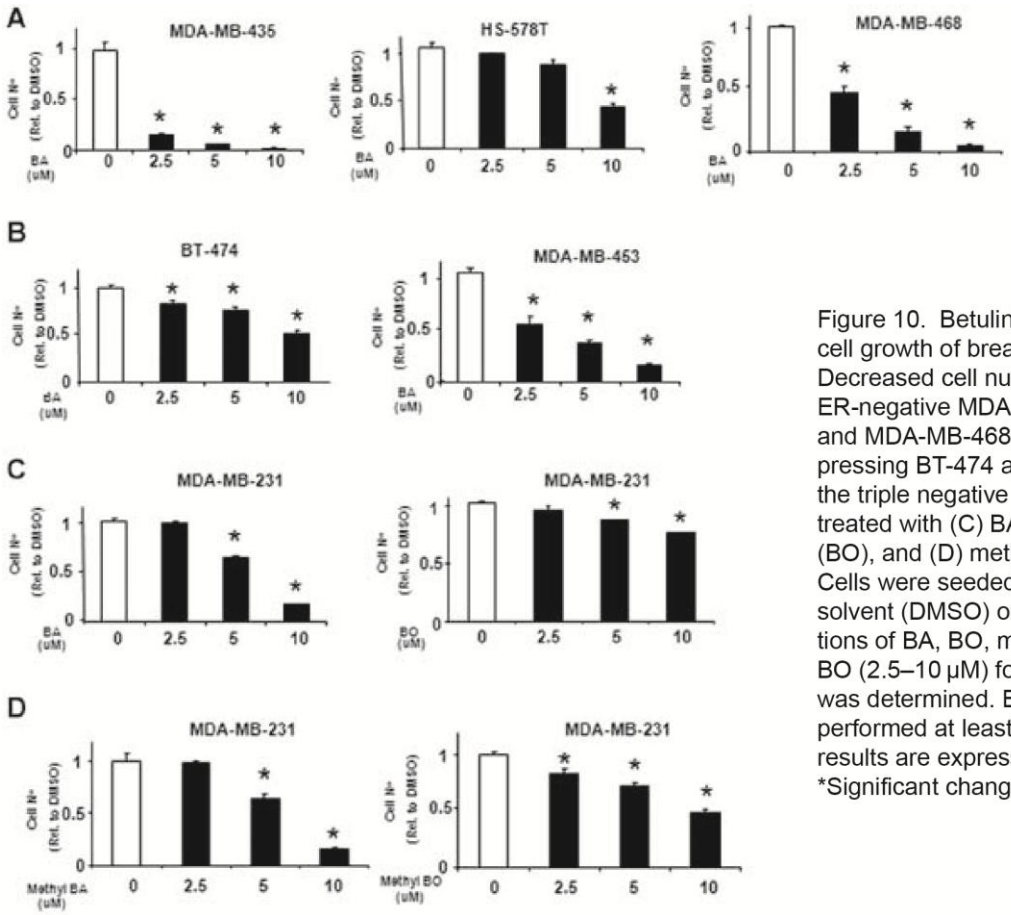

Figure 10. Betulinic acid (BA) inhibits cell growth of breast cancer cells lines. Decreased cell number of the (A) ER-negative MDA-MB-435, HS-578T, and MDA-MB-468; (B) HER2-overexpressing BT-474 and MDA-MB-453; the triple negative MDA-MB-231 cells treated with (C) BA or betulonic acid (BO), and (D) methyl 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 μM) for 48 hr; cell number was determined. Each experiment was performed at least three times, and results are expressed as means ± SD. *Significant changes at $P < 0.05$.

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. 12A). 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. 12B). 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 (8, 11, 12, 16). In addition, BA also decreased survivin, another Sp-regulated gene (Fig. 12C). This decrease was accompanied by increased PARP cleavage, and apoptosis was confirmed in a TUNEL assay showing that BA also increased TUNEL staining (Fig. 12D).

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 (7, 10, 12, 32, 48). Treatment of MDA-MB-231 cells with 2.5–10 μM BA decreased miR-27a (Fig. 13A) and this was accompanied by induction of ZBTB10 mRNA levels (Fig. 13A). 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. 13B). 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. 13C); in addition, BA also increased the luciferase activity in MDA-MB-231 cells transfected with pZBTBT-3′-UTR which contains a miR-27a binding site in the 3′-UTR of ZBTB10 as described (Fig. 13D) (32).

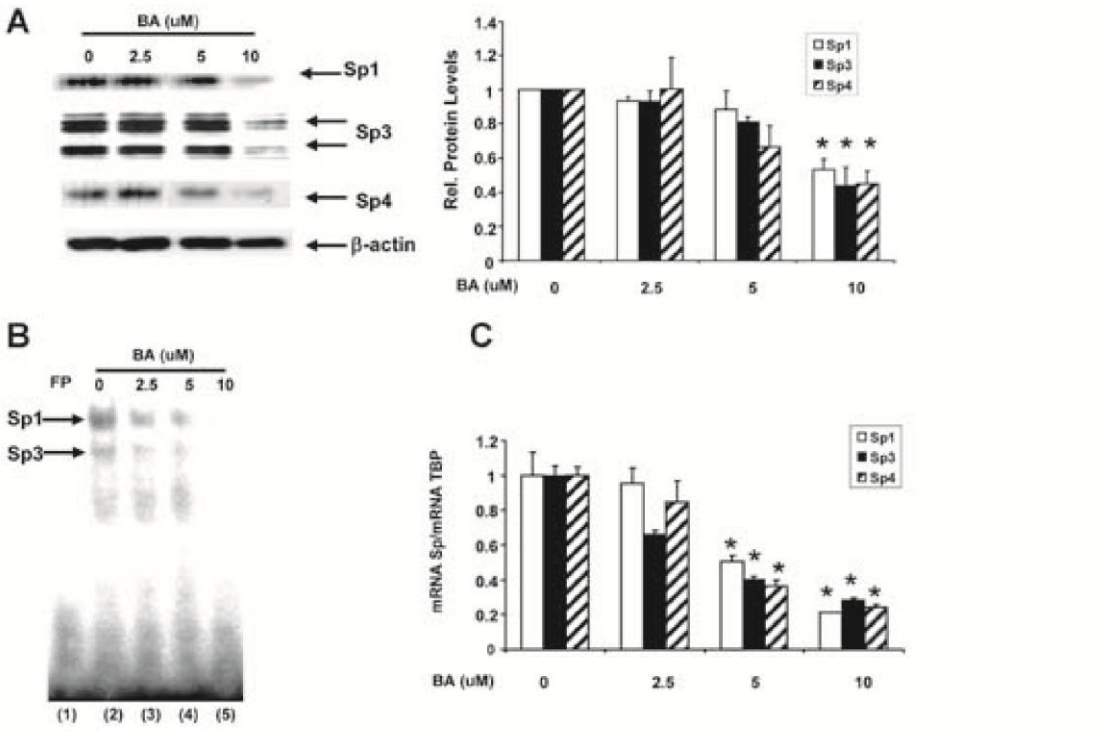

Figure 11. BA decreases Sp protein and mRNA levels in MDA-MB-231 breast cancer cells. (A) Decreased protein expression of Sp transcription factors accessed by western blots. Cells were treated with solvent (DMSO) or different concentrations of BA (2.5–10 μM) for 24 hr and expression of Sp proteins on whole-cell lysates was determined by immunoblot analysis. (B) Gel mobility shift assay. Cells were treated with BA for 24 hr. (C) mRNA levels of Sp. Relative mRNA levels were determined by qRT-PCR. (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 μM) for 24 hr was determined. Each experiment was performed at least three times. Values are mean (n = 3) ± SE for mRNA and ± SD for luciferase activity. *Significant changes at $P < 0.05$.

Previous studies (7) 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 14 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. 14A) and this is not due to induction of Wee1 (another miR-27a target) (Fig. 14B), which inactivates cdc2, but to induction of Myt-1 (Fig. 14C). This arrest was accompanied by enhanced phosphorylation of cdc2 at tyrosine-15 (Fig. 14D). The Myt-1-dependent phosphorylation of cdc2 reached a peak between 2 and 12 hr and this was consistent with BA-induced arrest of MDA-MB-231 cells in G2/M.

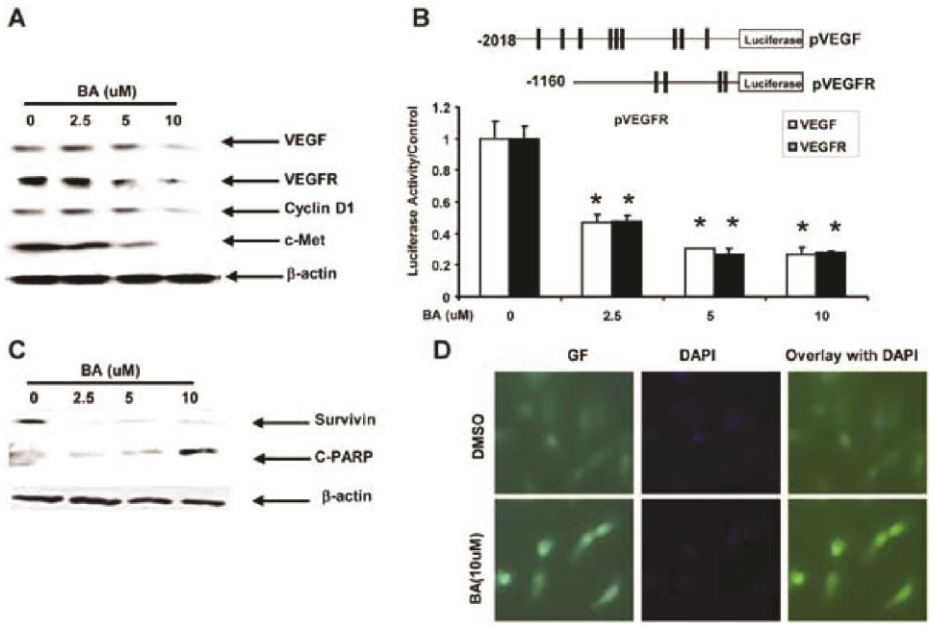

Figure 12. Betulinic acid (BA) decreases expression of the Sp-regulated genes and induces apoptosis in MDA-MB-231 cells. (A) Effects of BA on VEGF, VEGFR, cyclin D1, and c-Met proteins. (B) Luciferase activity (relative to protein content) in transfected cells with VEGF and VEGFR constructs and treated with DMSO or BA (2.5–10 μM) for 24 hr was determined. (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 24 hr and expression of proteins was determined in whole-cell lysates by immunoblot analysis. (D) In situ cell death detection. Cells were treated with DMSO or BA (10 μM) for 16 hr before cell fixation with 4% parafolmaldehyde in PBS. Cleavage of DNA during apoptosis was identified by the TUNEL assay. Values are means (n = 3) ± SD. *Significant changes at $P < 0.05$.

MDA-MB-231 cells were also used in an orthotopic model in athymic female nude mice. After 31 d of tumor implantation and 21 d of BA (20 mg/kg/d) treatment, tumor growth rates were significantly different from control group (Fig. 15A). 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. 15B), downregulation of miR-27a, and increased mRNA levels of ZBTB10 (Fig. 15C). 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. 15D). Thus, BA decreased proliferation of MDA-MB-231 cells and tumors through perturbing the miR-27a:ZBTB10–Sp transcription factor axis. Moreover, micrometastases in the lung were visible but not characterized. We used β2-microglobulin mRNA as the major indicator of metastasis (49, 50). Results showed that expression of the human-specific β2-microglobulin (hβ2G) gene in the lungs of xenografted mice after 35 d of tumor implantation was significantly lower in the BA treated group, and similar to levels detected as background in no-xenografted controls (Fig. 15E).

Discussion

Sp1, Sp3, and Sp4 transcription factors are overexpressed in multiple cancer cell lines and tumors (7-13, 16, 17, 45-47), and according to *in vivo* studies, there is minimal expression of these proteins in nontumor tissues (8, 11, 12, 47). The results are consistent with reports

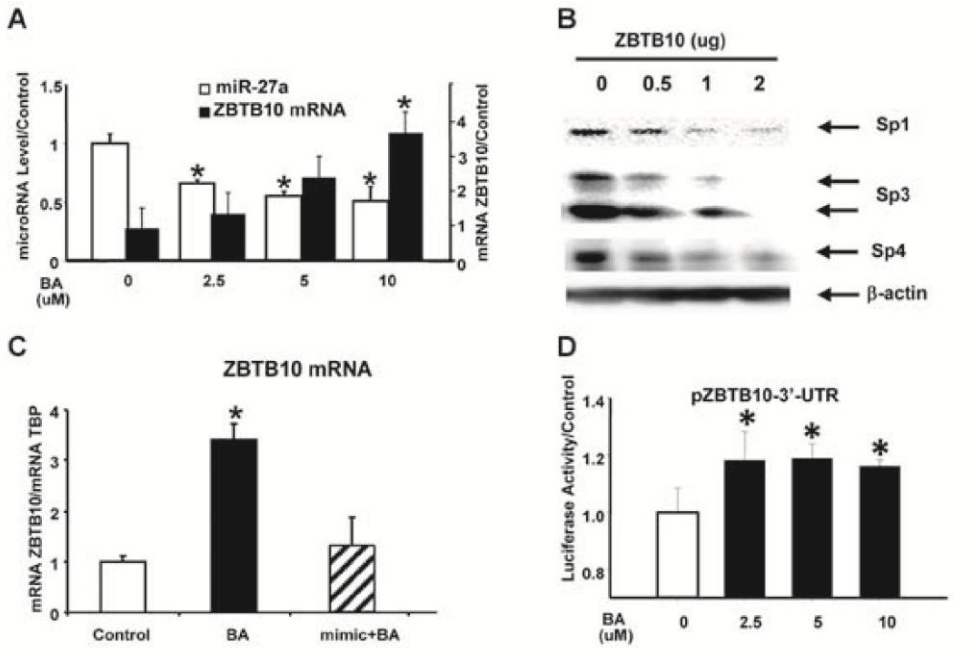

Figure 13. The betulinic acid (BA) repression of miR-27a expression causes ZBTB10-Sp regulation in MDA-MB-231 cells. (A) MicroRNA levels of miR-27a and mRNA levels of ZBTB10 relative to DMSO treated cells. Cells were treated with solvent (DMSO) or different concentrations of BA (2.5–10 μM) for 24 hr. mRNA and microRNA analyses were performed by qRT-PCR. (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 24 hr transfection by immunoblot analysis. (C) mRNA levels of ZBTB10 in cells transfected with the mimic of miR-27a or nonspecific oligonucleotide and treated with BA (5 μM). 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 16 hr. Each experiment was performed at least three times, and results are expressed as means ± SD. *Significant changes at $P < 0.05$.

showing that in rodents and humans Sp1 expression decreases with age (51-53). 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 (54). Sp1 is also a negative prognostic factor for survival of pancreatic and gastric cancer patients (55, 56). Studies in this laboratory have used RNA interference and knockdown of Sp1, Sp3, and Sp4 (individually and combined) to identify Sp-regulated genes (8-13, 16-18, 45, 46), 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-kB), survivin, bcl-2, 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 nonsteroidal anti-inflammatory drugs, BA, and synthetic triterpenoids (8-13, 16-18, 45-47, 57). Results obtained in this study in ER-negative breast cancer cells show that

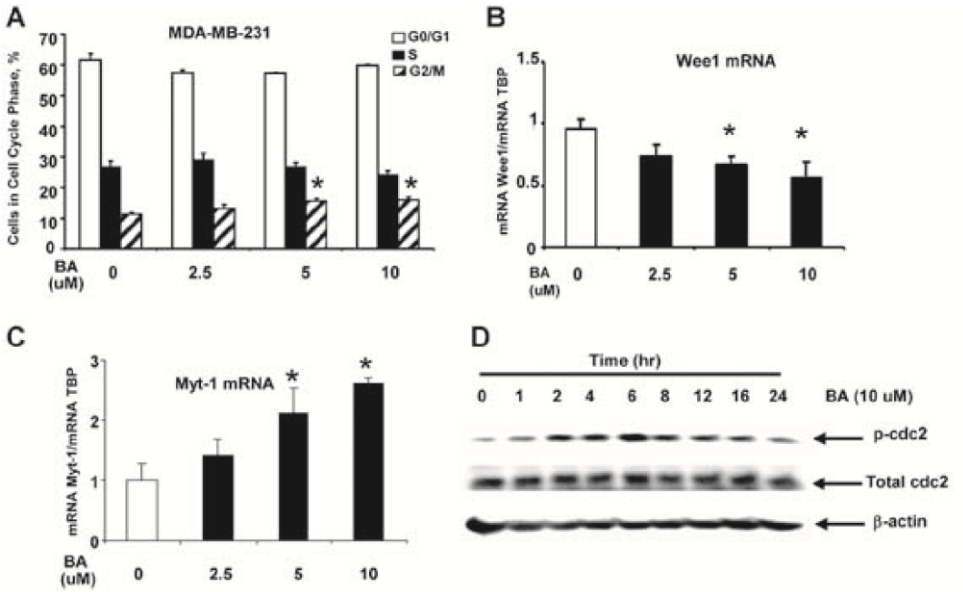

Figure 14. Betulinic acid (BA) induces cell cycle arrest at G2/M trough induction of Myt-1. (A) Flow cytometry analysis of MDA-MB-231 cells treated with solvent (DMSO) or different concentrations of BA (2.5–10 μM) for 48 hr. The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined. mRNA levels of (B) Wee1 and (C) Myt-1. Cells treated with BA or DMSO for 24 hr were analyzed by qRT-PCR. (D) Phosphor- and total cdc2 protein levels. Whole-cell lysates of cells treated with BA (10 μM) for 1–24 hr were analyzed by immunoblot analysis. Each experiment was performed at least three times, and results are expressed as means ± SE. *Significant changes at $P < 0.05$.

BA inhibited cell growth and tumor growth in athymic nude mice bearing MDA-MB-231 cells (orthotopically) (Figs. 10 and 15A). 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. 11A, 12A and 12C), and these results are similar to the effects of BA in LNCaP prostate cancer cells (47).

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 (47, 57). 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-18β-olean-1,12-dien-30-oate (CDODA-Me) decrease miR-27a and induce 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 (10, 12). 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. 11C) and tumors (Fig. 15B), 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. 13A and 15B). The Sp-repressor activity reported for ZBTB10 (32, 48) was also confirmed by overexpression of ZBTB10 in MDA-MB-231 cells (Fig. 13B). We have previously demonstrated that as-miR-27a also decreases Sp1, Sp3, and Sp4 expression in MDA-MB-231 cells (7). Moreover, overexpression of miR-27a reverses BA-induction of ZBTB10 (Fig. 13C). 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. 13) (32).

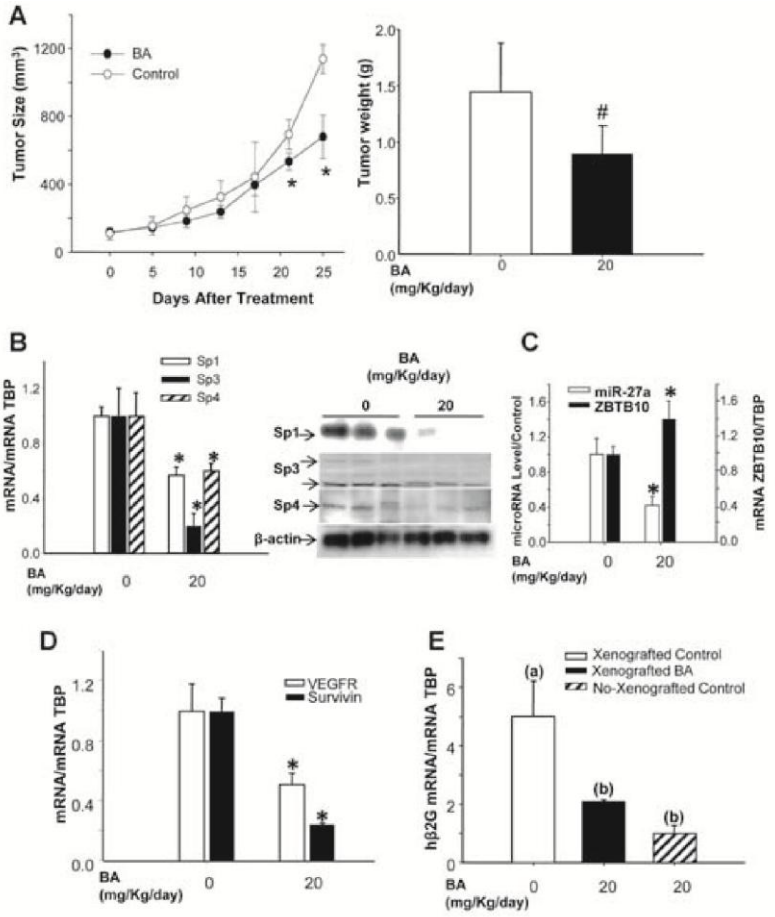

Figure 15. Antitumorigenic and antimetastatic activity of BA in xenografted nude mice. (A) Effects on tumor volume and tumor weight. Athymic nude mice (7 per group) bearing MDA-MB-231 cells as xenografts were treated with corn oil (control) or BA in corn oil (20 mg/kg/d) every second day. Treatments with BA started after implanted tumors reached a minimum volume. (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 ± 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 ± SE (n ≥ 4). Different letters indicate significant changes. *P < 0.05. #P < 0.1.

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 (58-60). 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 (7). Similar results were observed in this study, which shows that BA decreases miR-27a and this also results in induction of Myt-1 (Fig. 14C), activation of cdc2 (Fig. 14D) and an increase in the percentage of cells in G2/M (Fig. 14A). 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 (10).

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 (49). Accordingly, BA decreased the expression of hβ2G in lungs. The gene expression of hβ2G in mouse lung tissues has been previously used as a marker of metastasis and was significantly influenced by the size of tumors (49). Overall, results presented in Figure 15D and 15E 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.

3. Other studies

Ongoing studies with BA in ErbB2-overexpressing SKBR3 breast cancer cells gave results similar to that observed in BT474 and MDA-MB-453 cells, namely downregulation of Sp proteins, YY1 and ErbB2. However, the mechanisms involved in SKBR3 cells are different and are currently being investigated. In addition, ongoing studies with the synthetic triterpenoid methyl 2-cyano-3,11-dioxo-18olean-1,12-dien-30-oate (CDODA-Me) also gave results similar to that observed for BA. However, the mechanisms of Sp/YY1/ErbB2 downregulation vary with cell context and are currently being investigated.

KEY RESEARCH ACCOMPLISHMENTS

- BA decreased expression of Sp1, Sp3, Sp4 and ErbB2 in BT474 and MDA-MB-453 cells.
- BA also decreased expression of YY1 and YY1 regulates ErbB2 expression in these cells.
- YY1 was characterized as another Sp-regulated.
- BA-induced Sp downregulation was proteasome-independent and due to miR-27a downregulation and induction of the Sp repressor ZBTB10.
- The upstream targets of BA are the cannabinoid (CB) receptors, and we have shown for the first time that BA is a CB1 and CB2 receptor agonist. BA also decreases Sp proteins and Sp-regulated gene products in triple negative MDA-MB-231 cells (ErbB2-negative).
- Other compounds such as CDODA-Me give results similar to that observed for BA; however, mechanisms of Sp/YY1/ErbB2 downregulation are variable and cell context-dependent.

REPORTABLE OUTCOMES

- Liu, Xinyi. Doctoral Thesis, Texas A&M University, College Station, TX, 2011.

- Abstract: "Betulinic Acid Inhibits BT474 and SKBR3 Breast Cancer Cell Growth by Targeting Sp Proteins and ErbB2", Annual Meeting, Society of Toxicology, Salt Lake City, UT, March, 2010.
- Abstract: "Betulinic Acid Downregulates ErbB2 in Breast Cancer Cells and Tumors", Era of Hope, Breast Cancer Research Conference, Orlando, FL, 2011.
- Liu, X., Jutooru, I., Lei, P., Kim, K., Lee, S.O., Brents, L.K., Prather, P.L. and Safe, S.H. Betulinic acid targets YY1 and ErbB2 through cannabinoid receptor-dependent disruption of microRNA-27a:ZBTB10 in breast cancer. *Mol. Cancer Ther.* 11:1421-1431, 2012.
- Mertens-Talcott, S.U., Noratto, G.D., Li, X., Anglel-Morales, G., Bertoldi, M.C. and Safe, S. Betulinic acid decreases ER-negative breast cancer cell growth *in vitro* and *in vivo*: role of Sp transcription factors and microRNA-27a:ZBTB10. *Mol. Carcinog.* In press, 2012.

CONCLUSIONS

Betulinic acid is a triterpenoid anticancer agent, and treatment of epidermal growth factor 2 (EGFR2, HER2 / ErbB2)-overexpressing BT474 and MDA-MB-453 cells with 1-10 μM BA inhibited cell growth and induced apoptosis. BA also induced proteasome-independent downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 and survivin, a Sp-regulated gene, and decreased expression of ErbB2, ErbB2-regulated kinases and YY1, a transcription factor that regulates ErbB2 expression in these cells. Knockdown of Sp1, Sp3, Sp4 and their combination by RNA interference was accompanied by decreased expression of ErbB2, YY1 and luciferase activity in cells transfected with a construct containing the GC-rich YY1 promoter linked to a luciferase reporter gene. BA-dependent repression of Sp1, Sp3, Sp4 and Sp regulated genes was due, in part, to induction of the Sp repressor ZBTB10 and downregulation of microRNA-27a (miR-27a) which constitutively inhibits ZBTB10 expression. The effects of BA on the miR-27a:zBTB10-Sp transcription factor axis were inhibited in cells cotreated with the cannabinoid 1 (CB1) and CB2 receptor antagonists AM251 and AM630 respectively, or by knockdown of CB receptors by RNA interference. BA is clearly a CB receptor agonist and this novel observation will require re-interpretation of previous studies with this compound. We also found that BA and CDODA-Me exhibit similar effects in multiple breast cancer cell lines and decrease Sp1, Sp3, Sp4 and Sp-regulated genes (including YY1); however, their mechanisms are cell context-dependent and are currently being investigated.

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APPENDIX

1. Liu, X., Jutooru, I., Lei, P., Kim, K., Lee, S.O., Brents, L.K., Prather, P.L. and Safe, S.H. Betulinic acid targets YY1 and ErbB2 through cannabinoid receptor-dependent disruption of microRNA-27a:ZBTB10 in breast cancer. *Mol. Cancer Ther.* 11:1421-1431, 2012.

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Therapeutic Discovery

Molecular Cancer Therapeutics

Betulinic Acid Targets YY1 and ErbB2 through Cannabinoid Receptor-Dependent Disruption of MicroRNA-27a:ZBTB10 in Breast Cancer

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Abstract

Treatment of ErbB2-overexpressing BT474 and MDA-MB-453 breast cancer cells with 1 to 10 μmol/L betulinic acid inhibited cell growth, induced apoptosis, downregulated specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4, and decreased expression of ErbB2. Individual or combined knockdown of Sp1, Sp3, Sp4 by RNA interference also decreased expression of ErbB2 and this response was because of repression of *YY1*, an Sp-regulated gene. Betulinic acid–dependent repression of Sp1, Sp3, Sp4, and Sp-regulated genes was due, in part, to induction of the Sp repressor ZBTB10 and downregulation of microRNA-27a (miR-27a), which constitutively inhibits ZBTB10 expression, and we show for the first time that the effects of betulinic acid on the miR-27a:ZBTB10-Sp transcription factor axis were cannabinoid 1 (CB1) and CB2 receptor–dependent, thus identifying a new cellular target for this anticancer agent. *Mol Cancer Ther; 11(7); 1421–31. ©2012 AACR.*

Introduction

Betulinic acid is a naturally occurring triterpenoid found in bark extracts, and betulinic acid and synthetic analogues exhibit a broad spectrum of pharmacologic properties including antiviral, antibacterial, anti-inflammatory, antimalarial, and anticancer activities (1, 2). Betulinic acid also inhibits growth of multiple tumors, and the large difference between the doses required for tumor growth inhibition and toxic side-effects in animal models has generated interest in clinical development of this compound for cancer chemotherapy (2, 3). The overall effectiveness of betulinic acid as an anticancer drug has been linked to the mitochondriotoxicity of betulinic acid and induction of reactive oxygen species (ROS; refs. 4–6). Research in this laboratory has shown that betulinic acid inhibits growth and induces apoptosis in prostate, bladder, and colon cancer cells and tumors, and this is accompanied by downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4, and Sp-regulated genes (6–8). Similar effects have been observed for several anticancer drugs including curcumin, arsenic trioxide, nonsteroidal anti-inflammatory drugs, and triterpenoids such as celastrol, 2-cyano-1,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid (CDODA), and their corresponding esters (6–15). The importance of betulinic acid and other agents that target Sp proteins is because of (i) the overexpression of Sp1, Sp3, and Sp4 in tumor versus nontumor tissue and (ii) the critical roles for Sp-regulated genes in mediating cancer cell growth [EGF receptor (EGFR), hepatocyte growth factor receptor (c-MET), and cyclin D1], survival (bcl-2 and survivin), angiogenesis [vascular endothelial growth factor (VEGF) and VEGF receptors 1 and 2 (VEGFR1 and VEGFR2)], and inflammation (p65NFκB; refs. 8–12, 16–18).

This study investigated the anticancer activity of betulinic acid in BT474 and MDA-MB-453 breast cancer cells, which overexpress ErbB2, an important oncogenic growth factor receptor that is not an Sp-regulated gene. Betulinic acid–inhibited cell and tumor growth, downregulated Sp1, Sp3, and Sp4 and, surprisingly, decreased ErbB2 expression; however, this effect was because of downregulation YY-1, an Sp-regulated gene that activates ErbB2 expression (19, 20). The mechanism of Sp downregulation by betulinic acid was because of disruption of microRNA-27a (miR-27a):ZBTB10, which was cannabinoid (CB) receptor dependent, and betulinic acid directly bound to both CB1 and CB2 receptors. This represents a novel mechanism of action of betulinic acid and highlights the clinical potential of betulinic acid and related compounds that downregulate Sp transcription factors as a

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new class of mechanism-based agents for treating ErbB2-overexpressing breast tumors.

Materials and Methods

Chemicals, antibodies, plasmids, and reagents

Betulinic acid and lactacystin were purchased from Sigma-Aldrich. AM251, AM630, capsazepine, and WIN-55,212-2 were purchased from Tocris Bioscience. CAY10401 was purchased from Cayman Chemical. [^{3}H]CP-55,940 (144 Ci/mmol) was purchased from Perkin Elmer. Antibodies against ErbB2 (C-18), p-ErbB2 (Try 1248)-R, Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), Akt (H-136), p-Akt (Ser473), MAPK (C-14), p-MAPK (E-4), β-actin (C4), AP2α (C-18 and 3B5), YY1 (H-10), CB1 (H-150), and CB2 (H-60) were obtained from Santa Cruz Biotechnology. Antibodies against cleaved PARP (D214), surviving, and fatty acid amide hydrolase (FAAH; L14B8) were purchased from Cell Signaling Technology. The YY1 promoter plasmids (YY1 p-1729-luc and YY1 p-277-Luc) were kindly provided by Dr Ed Seto (University of South Florida, Tampa, FL, USA). The ZBTB10 expression vector and the 3′-untranslated region (UTR)-luc construct and other reagents have previously been described (7, 21).

Cell lines

The MDA-MB-453 and BT474 cells were purchased from the American Type Culture Collection. Cells were initially grown and multiple aliquots were frozen and stored at −80°C for future use. Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. Cell lines were cultured with 10% FBS in Dulbecco's Modified Eagles' Media and were maintained at 37°C in the presence of 5% CO_2.

Cell proliferation assay

Cells (2–3 × 10^4 per well) were plated in 12-well plates and allowed to attach for 24 hours. Then cells were treated with either vehicle (dimethyl sulfoxide, DMSO) or different concentrations of betulinic acid for up to 4 days. Fresh medium and compounds were added every 48 hours, and cells were then trypsinized and counted at the indicated time points using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means ± SE for each set of experiments.

Western blotting and TUNEL assays

Cells were rinsed with PBS and collected by scraping cells from the culture plate in 200 μL of high-salt buffer (50 mmol/L of HEPES, 0.5 mol/L of NaCl, 1.5 mmol/L of $MgCl_2$, 1 mmol/L of EGFTA, 10 glycerol, and 1% Triton X-100) and 10 μL/mL of protease inhibitor mixtures (Sigma-Aldrich). The cell lysates were incubated on ice for 1 hour with intermittent vortex mixing and then centrifuged at 40,000 × *g* for 10 minutes at 4°C. Equal amounts of protein were separated on SDS-polyacrylamide gels and processed as previously described (8, 9, 11, 12). Cells were plated in Lab-Tek II Chamber Slide System (Nalge Nunc International) and allowed to attach for 24 hours, and the effects of betulinic acid on the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay were determined as described (14).

Real-time PCR analysis of mRNAs and miRNAs

Total RNA was isolated using the RNeasy Protect Mini kit (Qiagen) and RNA was reverse transcribed using Transcription System (Promega) according to the manufacturer's protocols using primers that were previously described (8, 9, 11, 12).

DNA and siRNA transfection

Cells were plated in 12-well plates at 1 × 10^5 per well and cultured as described. After growth for 16 to 20 hours, transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol as previously described (14, 21). siRNAs for Sp1 (Sp1-1: SASI_Hs02_00363664; Sp1-2: SASI_Hs01_00070994), Sp3 (5′-GCGGCAGGUGGAGCCUUCACUTT-3′), Sp4 (5′-GCAGUGACACAUUAGUGAGCTT-3′), YY1 (YY1-1: SASI_Hs01_00155071, YY1-2: SASI_Hs01_-155072), CB1 (SASI_Hs01_00106167), and CB2 (SASI_Hs01_00041077) were purchased from Sigma-Aldrich. iLamin (5′-CUGGACUUCCAGAAGAACATT-3′), miR-27a mimic, as-miR-27a, and siRNA for FAAH were purchased from Dharmacon RNA Technologies; 100 nmol/L siRNAs were used in this study.

Luciferase assay

Transfected cells were lysed with 100 μL of 1× reporter lysis buffer as described (10–12), and 30 μL of cell extract were used for luciferase and β-gal assays. Lumicount Luminometer (Packard Instruments) was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.

Animals, xenograft study, and immunohistochemistry

Female ovariectomized athymic nu/nu mice (5–7-week-old) were purchased from the Harlan Laboratories. Under anesthetic conditions, BT474 cells (1 × 10^6) were implanted with Matrigel (BD Biosciences) subcutaneously into the flank of each mouse. Ten days later, mice were randomized into 2 groups of 6 mice/group and dosed by oral gavage with corn oil or 20 mg/kg of betulinic acid every other day for 28 days (14 doses). The mice were weighed, tumor sizes were measured at the indicated time with calipers, and immunostaining was determined as described (8, 9, 11, 12).

Competitive receptor binding

Crude mouse brain or CHO-hCB2 cell homogenates for CB receptor binding were prepared essentially as described and stored at −80°C (22). Increasing concentrations of betulinic acid (0.1–100 μmol/L) were incubated with 0.1 nmol/L of the nonselective CB1/CB2 agonist [^{3}H]CP-55,940 in a final volume of 1 mL of binding buffer

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(50 mmol/L Tris, 0.05% bovine serum albumin, 5 mmol/L of $MgCl_2$, pH 7.4) as described previously (23). Each binding assay contained 100 or 25 μg of membrane protein prepared from mouse brain or CHO-hCB2 cells, respectively. Reactions were incubated for 90 minutes at room temperature, and nonspecific binding was determined for each concentration of betulinic acid examined and was defined as binding observed in the presence of 1 μmol/L of the nonselective CB1/CB2 ligand WIN-55,212-2. Reactions were terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters and the bound radioactivity was determined as described (22, 23). Specific binding was expressed as total binding minus nonspecific binding determined for each concentration of betulinic acid examined, and is graphed for each data point as a percentage of specific binding occurring in the absence of any competitor. Analysis of the binding data was conducted using the nonlinear regression (Curve Fit) function of GraphPad Prism® v5.0b to determine the concentration of the drug that displaced 50% of [^{3}H]CP-55,940 (IC_{50}). A measure of affinity (K_i) was derived from the IC_{50} values using the Cheng–Prusoff equation (24).

Results

Betulinic acid inhibits growth, induces apoptosis, and downregulates Sp1, Sp3, and Sp4 in BT474 and MDA-MB-453 cells

BT474 and MDA-MB-453 cells overexpress ErbB2, and 1–10 μmol/L betulinic acid (Fig. 1A) inhibited proliferation of both BT474 and MDA-MB-453 cells. The overall decrease in cell number was both concentration- and time (2 or 4 days)-dependent, and MDA-MB-453 cells were less responsive to betulinic acid than BT474 cells (Fig. 1B). The growth inhibitory effects of betulinic acid were accompanied by induction of cleaved PARP, a marker of apoptosis, and decreased expression of survivin, an inhibitor of apoptosis, was also observed (Fig. 1C). Induction of apoptosis was also observed in a TUNEL assay in which betulinic acid increased TUNEL staining in both cell lines (Fig. 1D).

Betulinic acid inhibits LNCaP prostate cancer cell growth and this is due, in part, to activation of proteasome-dependent degradation of Sp1, Sp3, and Sp4 proteins (7). Treatment of BT474 and MDA-MB-453 cells with 10 μmol/L betulinic acid for 48 hours decreased expression of Sp1, Sp3, Sp4, and survivin (an Sp-regulated gene) proteins (Fig. 2A) and mRNA (Fig. 2B) in both cell lines, and BT474 cells were more responsive than MDA-MB-453 cells. The proteasome inhibitor MG132 alone was cytotoxic to BT474 and MDA-MB-453 cells (data not shown), whereas lactacystin was not toxic. Treatment of BT474 and MDA-MB-453 cells with 10 μmol/L betulinic acid alone or in combination with 1 μmol/L lactacystin for 48 hours showed that lactacystin did not affect betulinic acid–induced downregulation of Sp proteins (Fig. 2A) and this contrasted to results for betulinic acid in prostate cancer cells (7).

Betulinic acid–induced downregulation of YY1, ErbB2, and ErbB2-regulated genes is due to decreased Sp1, Sp3, and Sp4 expression

ErbB2 plays a major role in the proliferation of BT474 and MDA-MB-453 cells. Betulinic acid alone decreased ErbB2, p-ErbB2, and downstream kinases mitogen—activated protein kinase (MAPK), p-MAPK, Akt, and p-Akt expression (Fig. 2C), and these effects were not reversed after coincubation with the proteasome inhibitor lactacystin. Betulinic acid–mediated downregulation of MAPK and Akt total proteins has previously been observed in bladder cancer cells (8), and results of Sp knockdown suggest that these effects are Sp-independent and are currently being investigated. YY1 is a key upstream regulator of ErbB2 in breast cancer cells (19), and betulinic acid decreased expression of YY1 in both cell lines in the presence or absence of lactacystin (Fig. 2C). Because of the YY1 promoter contains multiple GC-rich Sp binding sites (20), we investigated the effects of betulinic acid on YY1 promoter activity and, in MDA-MB-453 cells transfected with GC-rich YY1 p-277-Luc or p-1729-luc constructs, treatment with betulinic acid for 24 hours decreased luciferase activity (Fig. 2D). Supplementary Fig. S1A and S1B shows that transfection of siRNAs against Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4), and their combination (iSp1/3/4) resulted in specific knockdown of the target Sp proteins and also decreased expression of YY1 and ErbB2 proteins. iSp1-1 and iSp1-2 were targeted against Sp1 but did not affect Sp3 or Sp4 expression (data not shown). In a second set of experiments in MDA-MB-453 cells (Supplementary Fig. S1B), the siRNAs for Sp1 and Sp4 were highly specific; however, iSp3 also decreased expression of Sp3 and Sp4 proteins. iSp1, iSp4, and iSp1/3/4 decreased levels of both YY1 and ErbB2, whereas Sp3 knockdown had minimal effects on either protein. Previous RNA interference studies showed that knockdown of YY1 decreases expression of ErbB2 (19, 20), and we also observed that YY1 knockdown decreased ErbB2 levels in both cell lines (Supplementary Fig. S2).

Role of CB receptors

Betulinic acid–induced downregulation of Sp transcription factors was proteasome-independent (Fig. 2) and was not reversed by ROS inhibitors (data not shown) as previously reported for other compounds (11, 12, 14, 15). Preliminary studies in other cancer cell lines show that CBs decrease Sp proteins (data not shown); therefore, the effects of CB1 and CB2 receptor antagonists AM251 and AM630, respectively, and capsazepine (vanilloid receptor antagonist) on betulinic acid–mediated repression of Sp1, Sp3, and Sp4 and survivin were also investigated. The vanilloid receptor antagonist was included because this receptor also binds some CBs (25). The CB receptors are expressed in BT474 and MDA-MB-453 cells, and cotreatment with betulinic acid and either AM251 or AM630 attenuated the effects of betulinic acid–induced downregulation of Sp1, Sp3, Sp4, and survivin, whereas capsazepine inhibited the effects of betulinic acid only in

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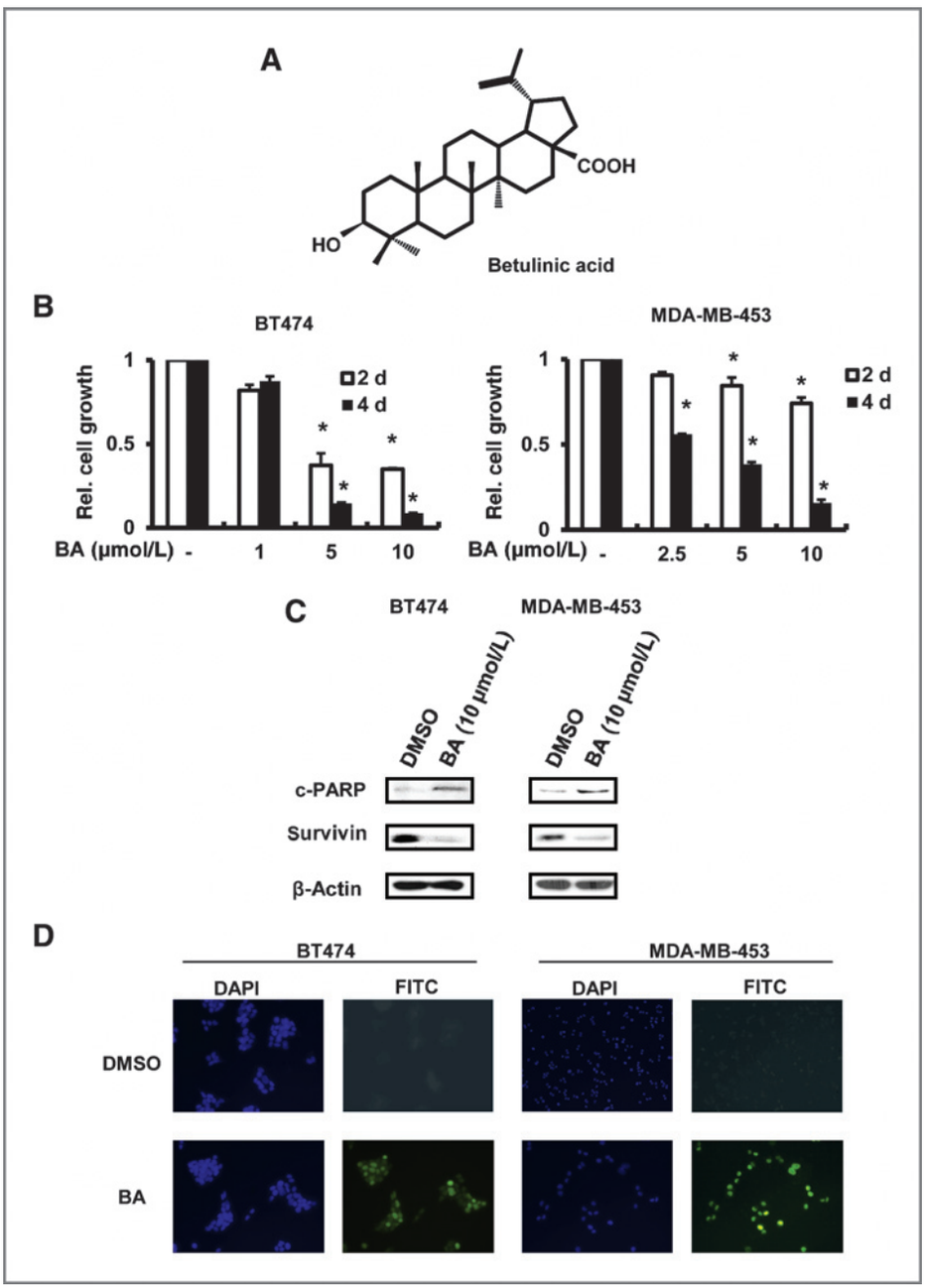

Figure 1. Effects of betulinic acid (BA) on cell proliferation and apoptosis. A, structure of BA. B, BA-mediated inhibition of BT474 and MDA-MB-453 cell growth. Cells were treated with different concentrations of BA for up to 4 days and the number of cells in each treatment group was determined as described in Materials and Methods. *, significant ($P < 0.05$) growth inhibition is indicated. Results are expressed as means ± SE for at least 3 replicate determinations for each treatment group. C, effects of BA on cleaved (c) PARP and survivin. Cells were treated with 10 μmol/L BA for 48 hours and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods. D, BA induces apoptosis in cancer cells. Cells were treated with DMSO or 10 μmol/L BA for 24 hours and analyzed with a TUNEL assay as described in Materials and Methods. DAPI, 4′,6—diamidino—2—phenylindole; FITC, fluorescein isothiocyanate.

MDA-MB-453 cells (Fig. 3A and B). Figure 3B also shows that the antagonists alone had minimal effects on ErbB2, Sp1, Sp3, and YY1. The CB1 and CB2 receptor antagonists inhibited betulinic acid–mediated downregulation of ErbB2, p-ErbB2, p-MAPK, p-Akt, and YY1 in BT474 and MDA-MB-453 cells, whereas capsazepine was active as an inhibitor only in the latter cell line (Fig. 3C and D). Expression of AP2 was highly variable in both cell lines and was not further investigated. These results indicated that the CB1 and CB2 receptors mediated betulinic acid–induced effects on Sp1, Sp3 and Sp4, ErbB2, and YY1 in both cell lines. In contrast, the effects of betulinic acid on MAPK and Akt (total and phospho proteins) were CB receptor-independent and also Sp-independent in bladder cancer cells (8) and are currently being investigated.

On the basis of these results, the direct binding of betulinic acid to the CB receptors was investigated in a competitive binding assay using [^{3}H]CP-55,940 as the radioligand. Preliminary studies showed that high concentrations of betulinic acid (>10^{-5} mol/L) enhanced binding of [^{3}H]CP-55,940; however, this was because of a concentration-dependent increase in both total and nonspecific binding (data not shown). Therefore, the [^{3}H]CP-55,940 specific binding to the mCB1 and hCB2

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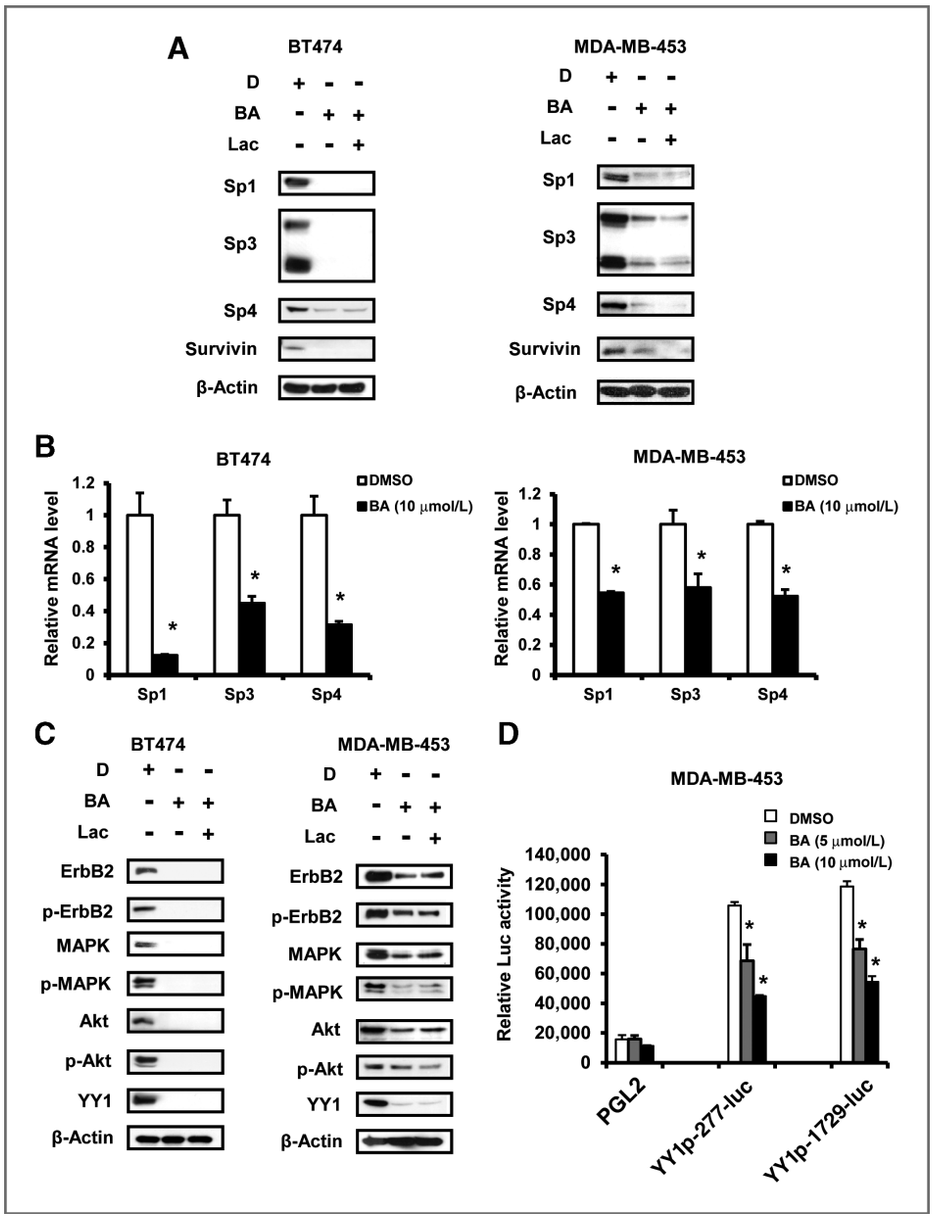

Figure 2. Effects of betulinic acid (BA) on Sp1, Sp3, Sp4, YY1, ErbB2, and ErbB2-dependent proteins. A, BA decreases Sp protein and survivin levels in BT474 and MDA-MB-453 cells. Cells were treated with DMSO (D), 10 μmol/L BA alone or in combination with 1 μmol/L lactacystin (Lac) for 48 hours, and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods. B, BA decreases mRNA levels of Sp proteins. Cells were treated with 10 μmol/L BA for 16 hours, and mRNA levels were determined as described in Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($P < 0.05$) decreases are indicated (*). C, BA decreases protein levels of ErbB2 and ErbB2-dependent proteins. Cells were treated with DMSO (D), 10 μmol/L BA alone or in combination with 1 μmol/L lactacystin for 48 hours, and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods. D, BA decreases YY1 promoter activity. MDA-MB-453 cells were transfected with empty vector (PGL2), the YY1 p-277-luc, or the YY1 p-1729-luc construct. Cells were then treated with 5 or 10 μmol/L BA for 24 hours. Luciferase activity was determined as described in Materials and Methods. Results are means ± SE for 3 separate determinations and significant ($P < 0.05$) induction of luciferase activity by BA is indicated (*).

receptor was determined by subtracting the nonspecific binding from the total binding as outlined in the Experimental Procedures (Fig. 4A). Betulinic acid competitively bound to both receptors, and the K_i values over 5 separate determinations (Fig. 4B) were 36.7 ± 4.1 and 41.2 ± 12.1 μmol/L for mCB1 and hCB2 receptors, respectively. As a positive control, Fig. 4B shows the competitive displacement curves using the CB WIN-55,212-2, which binds both receptors with anticipated low nanometers affinity. These results show that betulinic acid directly binds the CB receptors. We also show that knockdown of CB1 or CB2 receptors by RNA interference partially reversed betulinic acid–induced downregulation of Sp1, Sp3, and Sp4 (Fig. 4C), confirming a role for both receptors in mediating the effects of betulinic acid. A potential indirect effect of betulinic acid on CB-induced downregulation of Sp proteins could be due to inhibition of FAAH, which could increase endocannabinoid levels (26); however, knockdown of FAAH by RNA interference (in MDA-MB-453 cells) or inhibition of FAAH by the specific FAAH inhibitor CAY10401 (in BT474 cells) did not affect levels of Sp1, Sp3, or Sp4 proteins (Fig. 4D), indicating that this pathway is not involved in downregulation of Sp proteins.

Betulinic acid disrupts miR-27a regulation of ZBTB10 and inhibits tumor growth

Proteasome-independent downregulation of Sp1, Sp3, and Sp4 by betulinic acid and other anticancer agents has been linked to downregulation of miR-27a and induction of the transcriptional repressor ZBTB10 (6, 10, 15).

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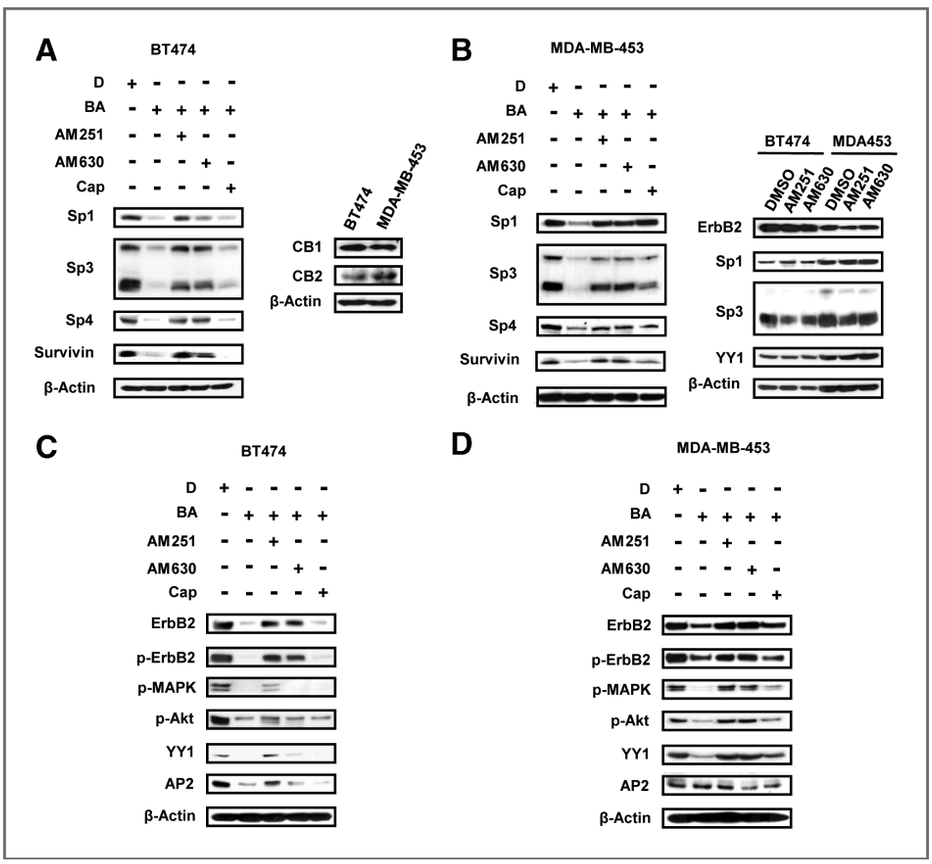

Figure 3. Effects of cannabinoid and vanilloid receptor antagonists on betulinic acid (BA)-induced responses. Effects of AM251, AM630, and capsazepine (Cap) on BA-mediated repression of Sps and survivin proteins in BT474 (A) and MDA-MB-453 (B) cells. Effects of AM251, AM630, and capsazepine on BA-mediated downregulation of ErbB2 and ErbB2-regulated kinases in BT474 (C) and MDA-MB-453 (D) cells and expression of CB receptors (D). Cells were pretreated with or without 6 μmol/L AM251, 6 μmol/L AM630, or 2 μmol/L capsazepine for 1 hour, and then DMSO (D) or 10 μmol/L BA were added to the medium for 48 hours, and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods.

Treatment of BT474 and MDA-MB-453 cells with 5 or 10 μmol/L betulinic acid resulted in significant downregulation of miR-27a in both cell lines, and cotreatment with AM251 or AM630 inhibited this response (Fig. 5A), which was most pronounced in BT474 cells. Downregulation of miR-27a in cells treated with betulinic acid was accompanied by induction of ZBTB10 mRNA levels in both cell lines, and cotreatment with AM251 or AM630 inhibited the induction response (Fig. 5B). A >6-fold induction of ZBTB10 was observed in BT474 cells, whereas ZBTB10 was induced >2.5-fold in MDA-MB-453 cells treated with 5 or 10 μmol/L betulinic acid for 24 hours. The effects of antisense-miR-27a (as-miR-27a) and ZBTB10 overexpression on levels of Sp1, Sp3, Sp4, YY1, and ErbB2 proteins were also determined in BT474 and MDA-MB-453 cells (Fig. 5C and D), and both treatments decreased expression of Sp and Sp-regulated gene products. The effects of a miR-27a mimic and as-miR-27a on luciferase activity in BT474 and MDA-MB-453 cells transfected with ZBTB10 (UTR)-luc construct containing a miR-27a binding site resulted in decreased (miR-27a mimic) and increased (as-miR-27a) luciferase activity. In contrast, the mimic or antisense oligonucleotide did not affect luciferase activity in cells transfected with a construct [ZBTB10 (mUTR)-luc] containing a mutation in the miR-27a binding sites, confirming interactions of miR-27a with the target sequence in the 3′UTR of ZBTB10.

The *in vivo* effects of betulinic acid on tumor growth were also investigated in athymic nude mice bearing BT474 cells as xenografts. Betulinic acid was administered over a period of 28 days at a dose of 20 mg/kg/d. Tumor volumes and tumor weight were significantly inhibited, and betulinic acid decreased expression of Sp1, Sp3, and Sp4 proteins in tumors (Fig. 6A–C). Figure 6D illustrates that immunostaining of ErbB2 and Sp1 proteins were decreased in fixed tumor tissue from betulinic acid–treated mice compared with control (corn oil)-treated animals, and these *in vivo* data complement the results of *in vitro* studies.

Discussion

Endocrine therapies with antiestrogens and aromatase inhibitors have been successful for treating patients with early stage ER-positive breast cancer (27–29), whereas patients with ER-negative or ErbB2-overexpressing tumors must undergo more aggressive treatment and their overall prognosis and survival are much lower than patients with early stage breast cancer (30–32). The development of the monoclonal antibody Herceptin that binds the extracellular domain of ErbB2 has provided significant treatment benefits for patients with these aggressive ErbB2-overexpressing tumors (33, 34). Although Herceptin has been successfully used

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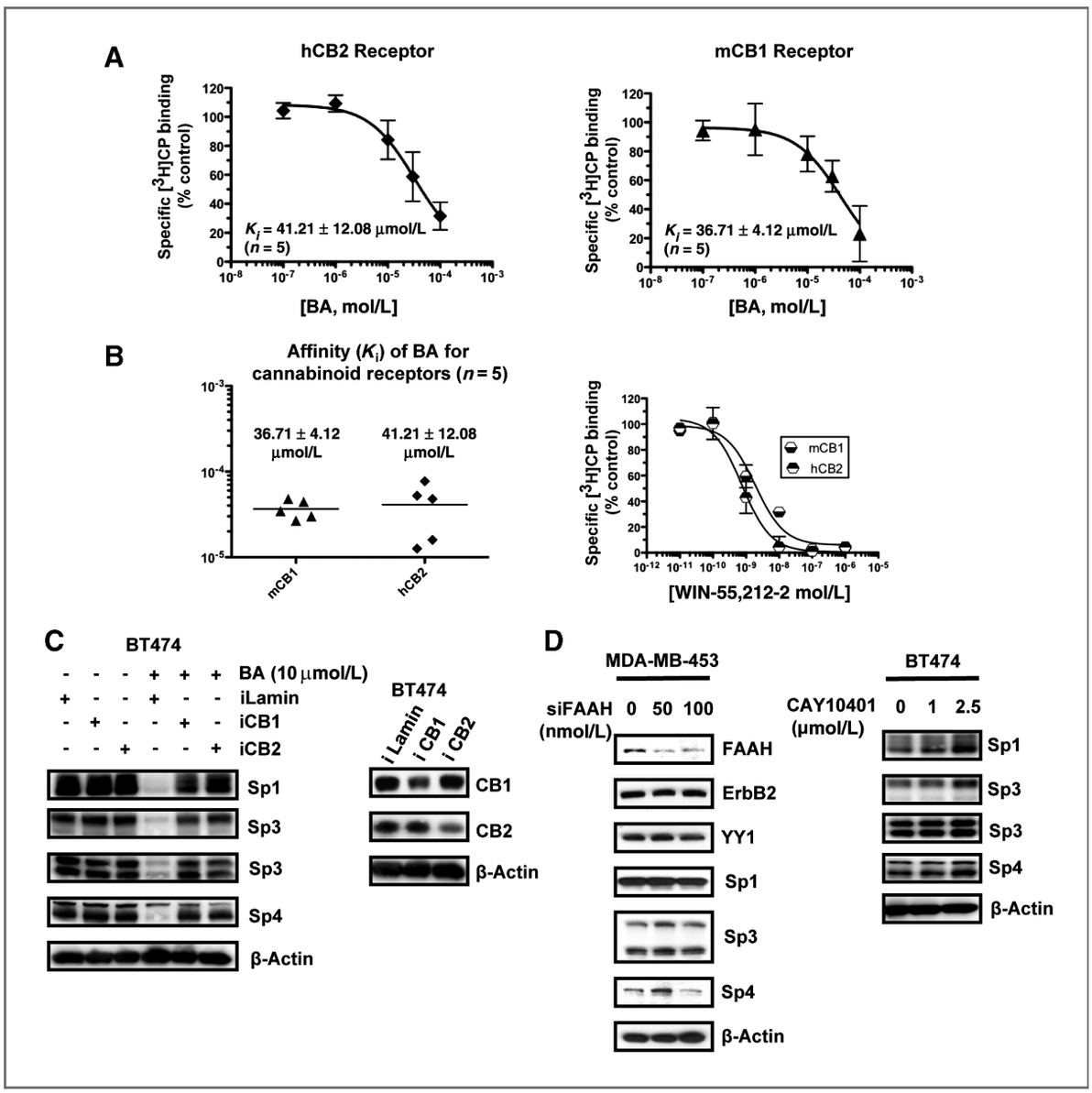

Figure 4. Betulinic acid (BA) is a CB receptor agonist. Specific binding of BA to CB receptors (A) and binding affinities (B). The specific binding and binding affinities of BA to CB1 and CB2 receptors were determined as described in Materials and Methods. C, knockdown of CB receptors by RNA interference. BT474 cells were transfected with iLamin (control) or iCB1 receptor or iCB2 receptor (oligonucleotides), and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods. D, effects of FAAH knockdown or CAY10401 on Sp proteins. MDA-MB-453 cells were transfected with siFAAH or BT474 cells were treated with DMSO or CAY10401 for 24 hours, and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods.

alone and in combination therapy, there is concern regarding cardiotoxic side-effects of this antibody, and development of other agents including tyrosine kinase inhibitors is being actively pursued for treatment of tumors that overexpress ErbB2 and other growth factor receptors (35).

Betulinic acid inhibited growth and induced apoptosis in BT474 and MDA-MB-453 breast cancer cell lines (Fig. 1) and this was accompanied by downregulation of Sp1, Sp3, and Sp4 protein and mRNA levels (Fig. 2). Moreover, betulinic acid also inhibited tumor growth and downregulated Sp1, Sp3, and Sp4 in tumors from athymic nude mice bearing BT474 cells as xenografts (Fig. 6). In BT474 and MDA-MB-453 breast cancer cells, betulinic acid decreased expression of ErbB2, p-ErbB2, and downstream ErbB2-dependent kinases p-MAPK/MAPK and p-Akt/Akt (Fig. 2C and D), suggesting that ErbB2 downregulation may also be due repression of Sp proteins. However, unlike the EGFR that is an Sp-regulated gene (8), ErbB2 expression is dependent on

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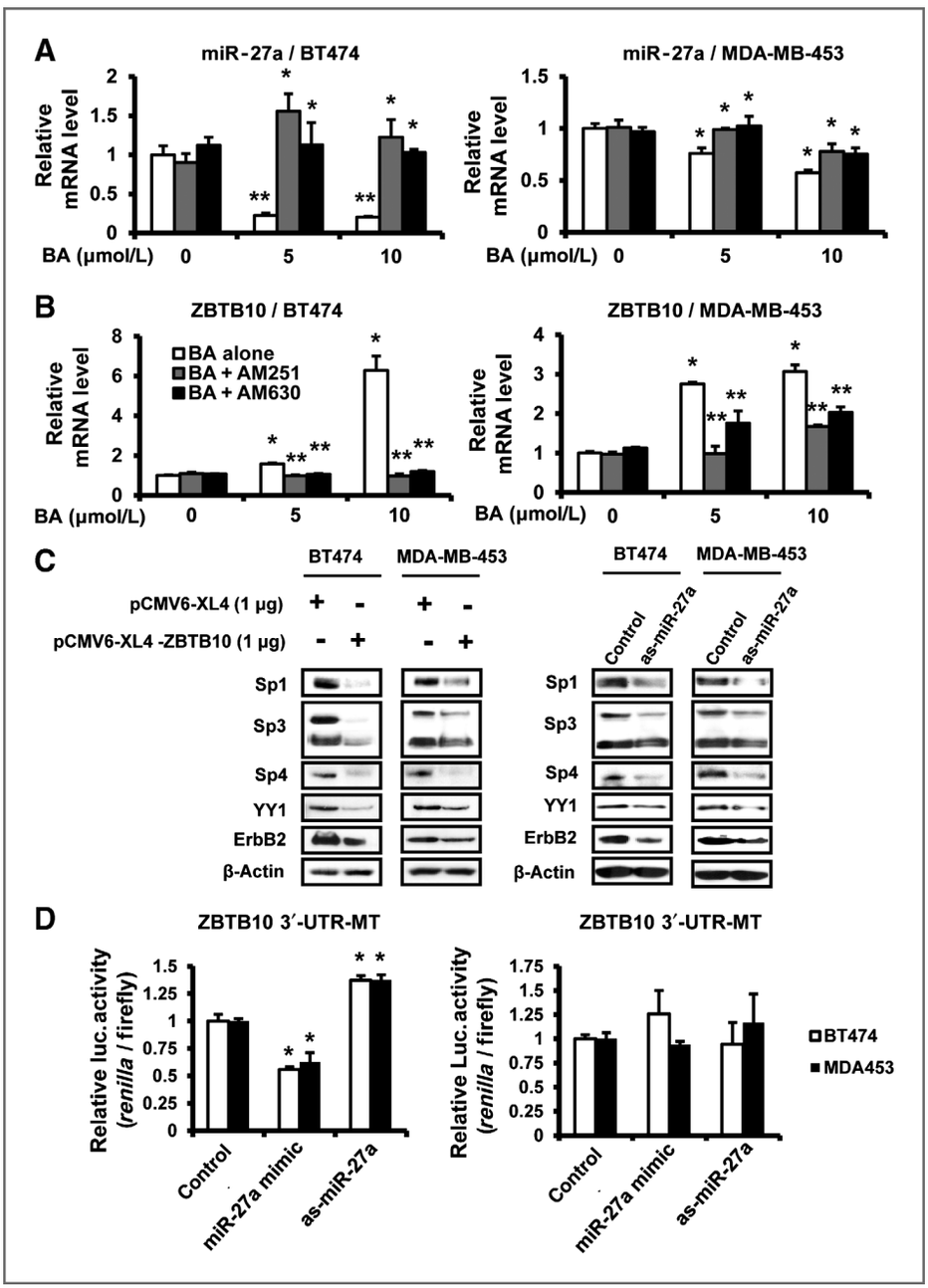

Figure 5. Effects of betulinic acid (BA) on miR-27a and ZBTB10, and the role of cannabinoid receptors on BA-mediated effects. A, downregulation of miR-27a. Cells were pretreated with or without 6 μmol/L AM251 or 6 μmol/L AM630 for 1 hour, DMSO or 5 or 10 μmol/L BA were added to the medium for 24 hours, and miR-27a levels were determined as described in Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($P < 0.05$) inhibition of miR-27a (**) and inhibition by the antagonists are indicated (*). B, induction of ZBTB10. Cells were treated and processed as described in A, and significant ($P < 0.05$) induction by BA (*) and inhibition by the antagonists (**) are indicated. C, effects of ZBTB10 overexpression and antisense miR-27a on Sp protein levels, YY1, and ErbB2 proteins. Cells were transfected with 1 μg pCMV6-XL4-ZBTB10 plasmid or empty vector, 50 nmol/L antisense miR-27a (as-miR-27a), or control, and whole-cell lysates were analyzed by Western blotting as described in Materials and Methods. D, effects of miR-27a mimic or as-miR-27a on luciferase activity in ZBTB10 3′UTR-luc construct transfected cells. MiR-27a mimic (50 nmol/L) or as-miR-27a were transfected into BT474 and MDA-MB-453 cells as described in Materials and Methods, and a dual luciferase reporter assay was conducted according to the manufacturer's instructions. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($P < 0.05$) decreases or induction are indicated (*).

other transcription factors including YY1, which contains multiple GC-rich promoter sites that bind Sp proteins (20). Betulinic acid decreased YY1 protein expression in BT474 and MDA-MB-453 cells (Fig. 2C and D) and, not surprisingly, knockdown of Sp proteins (individually and combined) decreased expression of YY1 and ErbB2 (Supplementary Fig. S1). Moreover, YY1 knockdown by RNA interference also decreased ErbB2 (Supplementary Fig. S2), showing that betulinic acid–mediated downregulation of ErbB2 is linked to decreased expression of Sp1, Sp3, Sp4, and Sp-regulated YY1.

The mechanisms of betulinic acid–induced downregulation of Sp1, Sp3, and Sp4 were dependent on tumor-type and cell context. Betulinic acid induced proteasome-dependent downregulation in LNCaP prostate and SW480 colon cancer cells (6, 7), whereas in RKO colon cancer cells, this response was primarily ROS-dependent (7) and was because of repression of miR-27a and induction of ZBTB10, a transcriptional repressor that binds GC-rich promoter sites and downregulates Sp transcription factors and Sp-regulated genes (7, 10, 12, 15, 21). However, even in RKO cells, the CB receptor antagonists partially blocked the effects of

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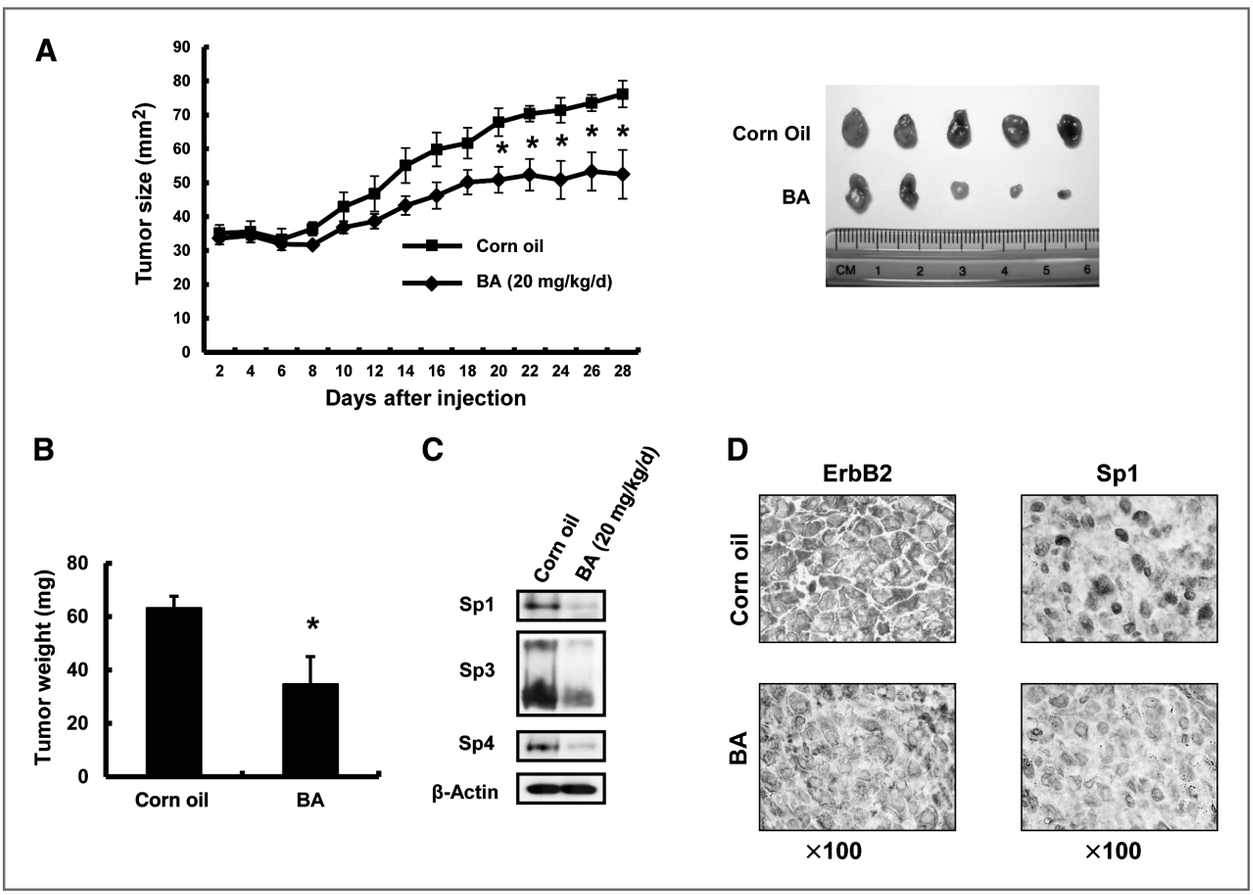

Figure 6. Betulinic acid (BA) inhibits tumor growth in BT474 xenografts. Inhibition of tumor size (A) and weight (B). Athymic nude mice bearing BT474 cells as xenografts were treated with BA (20 mg/kg/d), and tumor sizes and weights were determined as described in Materials and Methods. Significantly ($P < 0.05$) decreased tumor sizes and weights are indicated (*). C, BA decreases expression of Sp1, Sp3, and Sp4 proteins in tumors. Whole-cell lysates from corn oil and BA-treated tumors were analyzed by Western blotting as described in Materials and Methods. D, immunostaining for ErbB2 and Sp1. Fixed tumor tissue from corn oil- and BA-treated mice were stained with ErbB2 and Sp1 antibodies as described in Materials and Methods.

betulinic acid on Sp proteins (Supplementary Fig. S3). Betulinic acid–induced repression of Sp1, Sp3, and Sp4 was proteasome-independent and not affected by lactacystin (Fig. 2A), and similar results were observed for ROS inhibitors such as glutathione (data not shown). Results summarized in Fig. 5 show that betulinic acid also decreased miR-27a and induced ZBTB10 in BT474 and MDA-MB-453 cells, suggesting that the critical downstream effects of betulinic acid on the miR-27a: ZBTB10 complex are similar to those observed in previous studies with the synthetic triterpenoids CDDO-Me and CDODA-Me (10, 12). CDDO-Me–mediated downregulation of miR-27 in pancreatic cancer cells was dependent on upstream disruption of mitochondria and induction of ROS; however, in contrast to these results, betulinic acid did not induce ROS in BT474 and MDA-MB-453 cells and the antioxidant glutathione did not modulate effects of betulinic acid on Sp1, Sp3, and Sp4 (data not shown).

Ongoing studies in this laboratory show that, like betulinic acid, CBs downregulate Sp1, Sp3, and Sp4 in cancer cell lines (data not shown), and we show for the first time that betulinic acid binds directly to CB1 and CB2 receptors (Fig. 4A). The competitive binding assay was slightly modified to determine total and nonspecific binding at all concentrations of betulinic acid, and K_i values were 36.7 and 41.2 μmol/L for the CB1 and CB2 receptors (Fig. 4B). The CB receptor antagonists AM251 and AM630 inhibited betulinic acid–induced miR-27a (downregulation), ZBTB10 (induction), Sp1, Sp3, Sp4, YY1, and ErbB2 (downregulation), and downregulation of Sp proteins by betulinic acid were also blocked by knockdown of CB1 and CB2 receptors by RNA interference (Fig. 4C). These results show that the effects of betulinic acid on BT474 and MDA-MB-453 cells on Sp transcription factors and ErbB2 are mediated through activation of the CB1 and CB2 receptors, which subsequently modulate the miR-27a: ZBTB10-Sp axis. CB1 and CB2 receptors are expressed in both cell lines and in human breast cancer cells and tumors, and one study showed a correlation between CB2 receptor and ErbB2 expression in human mammary tumors (36).

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In summary, this study shows that betulinic acid inhibits ErbB2-overexpressing breast cancer cell and tumor growth, and this is accompanied by a cascade of events involving activation of the CB1 and CB2 receptors, resulting in modulation of the miR-27a:ZBTB10-Sp transcription factor axis and downregulation of the Sp-dependent gene YY1 and the YY1-dependent gene ErbB2. This CB receptor–dependent pathway significantly contributes to the effects of this compound as an inhibitor of ErbB2-overexpressing breast cancer cell and tumor growth and is consistent with the well-known anticancer activities of CBs (37, 38). Moreover, ongoing studies with CBs also show that they target Sp transcription factors (unpublished observations). Current studies are focused on the mechanistic link between activation of the CB receptors and modulation of miR-27a:ZBTB10 and the efficacies and mechanism of action of other agents that repress Sp transcription factors. We are also investigating the effects of betulinic acid as an inducer of the newly identified Sp-repressor ZBTB4, which is regulated by miR-17-92 cluster miRs (39). These data show a novel pathway for targeting ErbB2 and identify a new therapeutic approach for treating patients with breast cancer that overexpress this oncogene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Liu, K. Kim, P.L. Prather, S. Safe
Development of methodology: I. Jutooru, P. Lei, K. Kim, S. Safe
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Liu, I. Jutooru, P. Lei, S.-O. Lee, L.K. Brents, P.L. Prather, S. Safe
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Liu, I. Jutooru, P. Lei, K. Kim, S.-O. Lee, L.K. Brents, P.L. Prather, S. Safe
Writing, review, and/or revision of the manuscript: X. Liu, L.K. Brents, P.L. Prather, S. Safe
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Liu, S. Safe
Study supervision: X. Liu, S. Safe

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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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Betulinic acid (BA), a pentacyclic triterpenoid isolated from tree bark is cytotoxic to cancer cells. There is evidence that specificity proteins (Sps), 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 Sps 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 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 β2-microglobulin mRNA was decreased in BA-treated animals. These results show that the anticancer effects of BA are at least in part based on interactions with the microRNA-27a-ZBTB10-Sp-axis causing increased cell death.

Key words: MDA-MB-231-breast cancer; ZBTB10; Sp-transcription factors

INTRODUCTION

Betulinic acid (BA) is a triterpenoid acid found in various bark extracts. It is readily synthesized from betulin by oxidation to betulonic acid (BO) and reduction of the 3-keto group of BO 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; 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 specificity protein (Sp) transcription factors Sp1,

Abbreviations: BA, betulinic acid; BO, betulonic acid; Sp, specificity protein; VEGF, vascular endothelial growth factor; PARP, poly(ADP-ribose)polymerase; ROS, reactive oxygen species; miR-27a, microRNA-27a; RT, reverse transcription; FBS, fetal bovine serum; TBP, TATA binding protein; GF, green fluorescence; hβ2G, human-specific β2-microglobulin; EGFR, epidermal growth factor receptor; c-MET, hepatocyte growth factor receptor; NF-kB, nuclear factor kappa-B.

Conflict of interest: none.

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Sp3, and Sp4. RNA interference studies which decrease expression of all three Sp proteins show that genes such as cyclin D1, bcl-2, survivin, vascular endothelial growth factor (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.

MATERIALS AND METHODS

Chemicals, Antibodies, Plasmids, and Reagents

BA was purchased from Sigma–Aldrich (St. Louis, MO). 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 PARP was purchased from Cell Signaling Technology (Beverly, MA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega Corp. (Madison, WI). LipofectAMINE 2000 reagent was supplied by Invitrogen Corp. (Grand Island, NY). Western lightning chemiluminescence reagent was obtained from Perkin-Elmer Life Sciences (Waltham, MA). mirVana^{TM} 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 (San Diego, CA). Primers for Sp3 and Sp4 were obtained from Qiagen (Valencia, CA); 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 Cuidad and Veronique Noe (University of Barcelona, Barcelona, Spain). RNase, propidium iodide, sodium citrate, and Triton X-100 were obtained from Sigma–Aldrich. The 40-bp sequence containing a miR-27a target sequence from the 3′-UTR of ZBTB10 was cloned into *Not*I and *Xho*I sites of psiCHECK2 dual luciferase reporter construct (Promega Corp.). 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 (Manassas, VA). Cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Invitrogen Corp.) and maintained at 37°C in the presence of 5% CO_2.

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^{TM} Series, Beckman Coulter, Inc.) after 48 h 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 24 h to allow cell attachment. After 24 h BA treatment, medium was discarded and cells were washed with PBS, then removed by scraping using PBS. After centrifugation, cell pellets were lysed with nondenaturing buffer [10 mM Tris–HCl, 10 mM NaH_2PO_4, 130 mM NaCl, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, pH 7.5], and 1% proteinase inhibitor cocktail (Sigma–Aldrich) for 30 min in ice. Solid cellular debris was removed by centrifugation at 10,000 rpm for 10 min 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. 60 μg of protein was diluted with Laemmli's loading buffer, boiled for 5 min, loaded into each lane of an acrylamide gel (10%) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 V for ∼2 h. Proteins were transferred by wet blotting onto 0.2 μm PVDF membrane (Bio-Rad). Membranes were blocked using 5% milk in 0.1% PBS-Tween (PBS-T) for 30 min and incubated with primary antibodies (1:1,000) in 3% bovine serum albumin in PBS-T overnight at 4°C with gentle shaking, followed by incubation with the secondary antibody (1:2,000) in 5% milk PBS-T for 2 h. Reactive bands

were visualized with a luminal reagent (Santa Cruz Biotechnology, Inc.) after 1 min 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% (v/v) glycerol (pH 7.6)], and homogenized. The cellular homogenate was centrifuged for 5 min at 14,000*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% (v/v) glycerol (pH 7.6)] and incubated on ice for 1 h with frequent vortexing. Samples were centrifuged at 14,000*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 32P-labeled at the 5′ end using T4 polynucleotide kinase (Promega Corp.) and [γ-32P]ATP (Perkin-Elmer 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 24 h 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 16–20 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× reporter lysis buffer, and 30 μL of cell extract were used for luciferase assays using Promega Luciferase Assay System (Promega Corp.) 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.) 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) according to the manufacturer's protocol. Isolated RNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp.) 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). TATA binding protein (TBP) was used as endogenous control to determine relative mRNA expression. microRNA was extracted using the mirVana™ extraction kit (Applied Biosystems). Quantification of miR-27a was performed using the Taqman®MicroRNA Reverse Transcription Kit (Applied Biosystems) and qRT-PCR reaction using TaqMan®2X Universal PCR Master Mix (No AmpErase®UNG) (Applied Biosystems) 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. 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′-TTGATCTCCATTTCTCGGAAGAG-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′-CTGAGTGGTTTGATGGACAGA-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 U/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) 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% parafolmaldehyde in PBS. The green fluorescence (GF) images were captured using an Axiocam high 152 resolution digital camera 42× magnification. The green brightness provides an estimated level of DNA strand breaks.

Xenograft Study

Female athymic BALB/c nude mice (age 3–4 wk), 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 d 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 ± 18 mm^3, animals were divided into two equal groups of seven 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/d 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 d of BA treatment and 35 d 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 post hoc 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 Figure 1A and B. At concentrations ≤10 μM 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 BO (Figure 1C) and their corresponding methylesters (methyl BA and methyl BO) (Figure 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 24 h induced a concentration-dependent decrease in expression of Sp1, Sp3, and Sp4 proteins (Figure 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 (Figure 2B) (lanes 3–5) compared to DMSO (control); the retarded band intensity was also decreased after coincubation 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 (Figure 2C). Both the Sp1 and Sp3

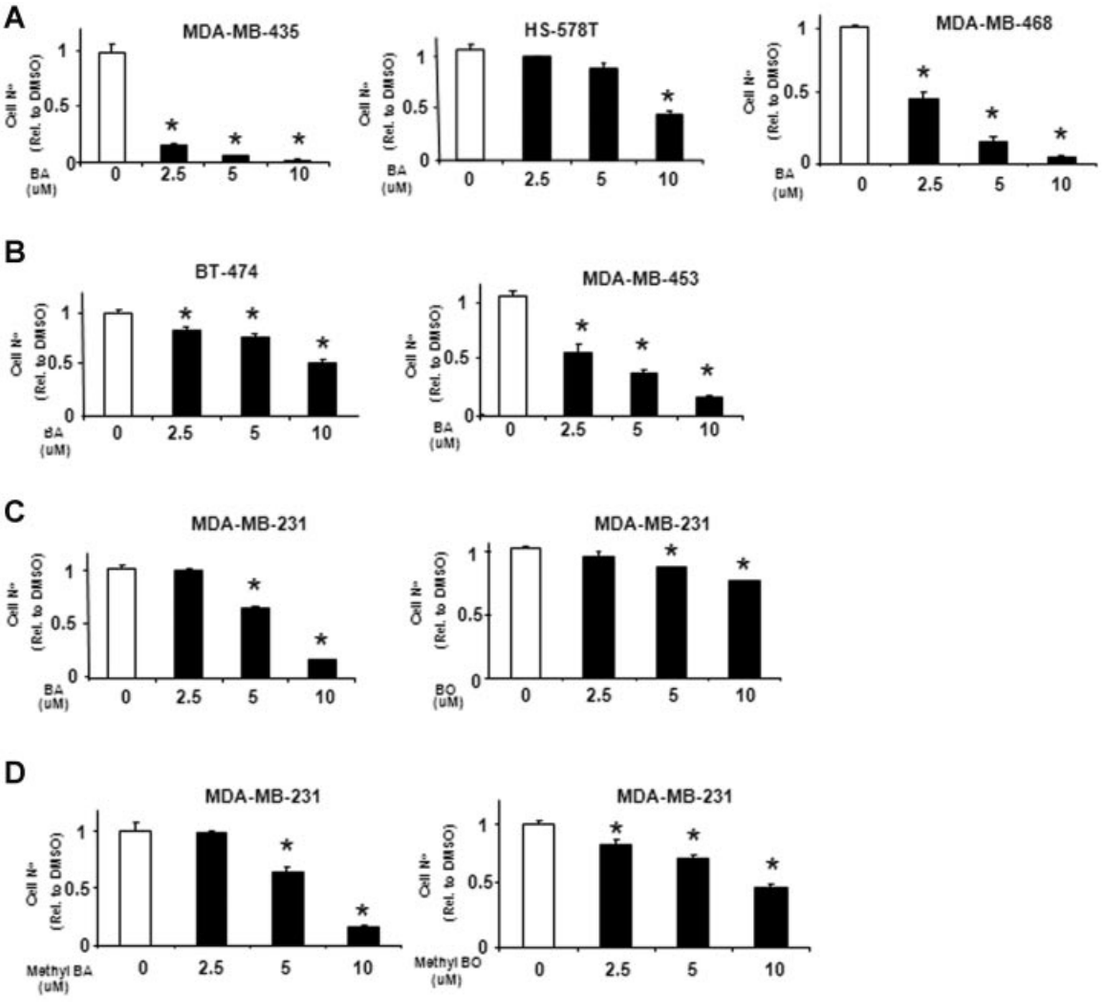

Figure 1. Betulinic acid (BA) inhibits cell growth of breast cancer cells lines. Decreased cell number of the (A) ER-negative MDA-MB-435, HS-578T, and MDA-MB-468; (B) HER2-overexpressing BT-474 and MDA-MB-453; the triple negative MDA-MB-231 cells treated with (C) BA or betulonic acid (BO), and (D) methyl 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 μM) for 48 h, cell number was determined as described in Materials and Methods Section. Each experiment was performed at least three times, and results are expressed as means ± SD. *Significant changes at $P < 0.05$.

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 (Figure 2D). Similar results were observed in MDA-MB-231 cells transfected with Sp3-for 5, 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 (Figure 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 (Figure 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 (Figure 3C).This decrease was accompanied by increased PARP cleavage and apoptosis was confirmed in a TUNEL assay showing that BA also increased TUNEL staining (Figure 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

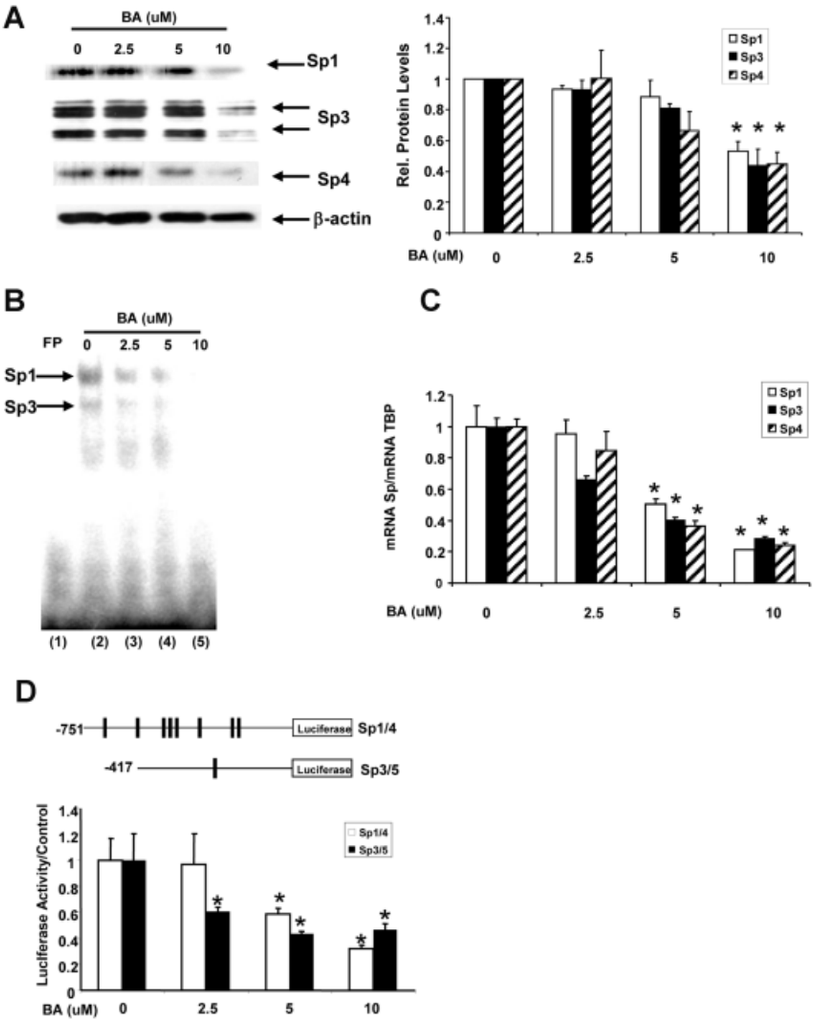

Figure 2. BA decreases Sp protein and mRNA levels in MDA-MB-231 breast cancer cells. (A) Decreased protein expression of Sp transcription factors accessed by western blots. Cells were treated with solvent (DMSO) or different concentrations of BA (2.5–10 μM) for 24 h and expression of Sp proteins on whole-cell lysates was determined by immunoblot analysis as described in Materials and Methods Section. (B) Gel mobility shift assay. Cells were treated with BA for 24 h and prepared as described in Materials and Methods Section. (C) mRNA levels of Sp. Relative mRNA levels were determined by qRT-PCR as described in Materials and Methods Section. (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 μM) for 24 h was determined as described in Materials and Methods Section. Each experiment was performed at least three times. Values are mean ($n = 3$) ± SE for mRNA and ±SD for luciferase activity. *Significant changes at $P < 0.05$.

miR-27a enhanced expression of ZBTB10, an Sp repressor [19,25–26,28–29]. Treatment of MDA-MB-231 cells with 2.5–10 μM BA decreased miR-27a (Figure 4A) and this was accompanied by induction of ZBTB10 mRNA levels (Figure 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 (Figure 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 (Figure 4C); in addition BA also increased the luciferase activity in MDA-MB-231 cells transfected with pZBTBT-3′-UTR which contains a miR-27a binding site in the 3′-UTR of ZBTB10 as described (Figure 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 (Figure 5A) and this is not due to induction of Wee1 (another miR-27a target) (Figure 5B); which

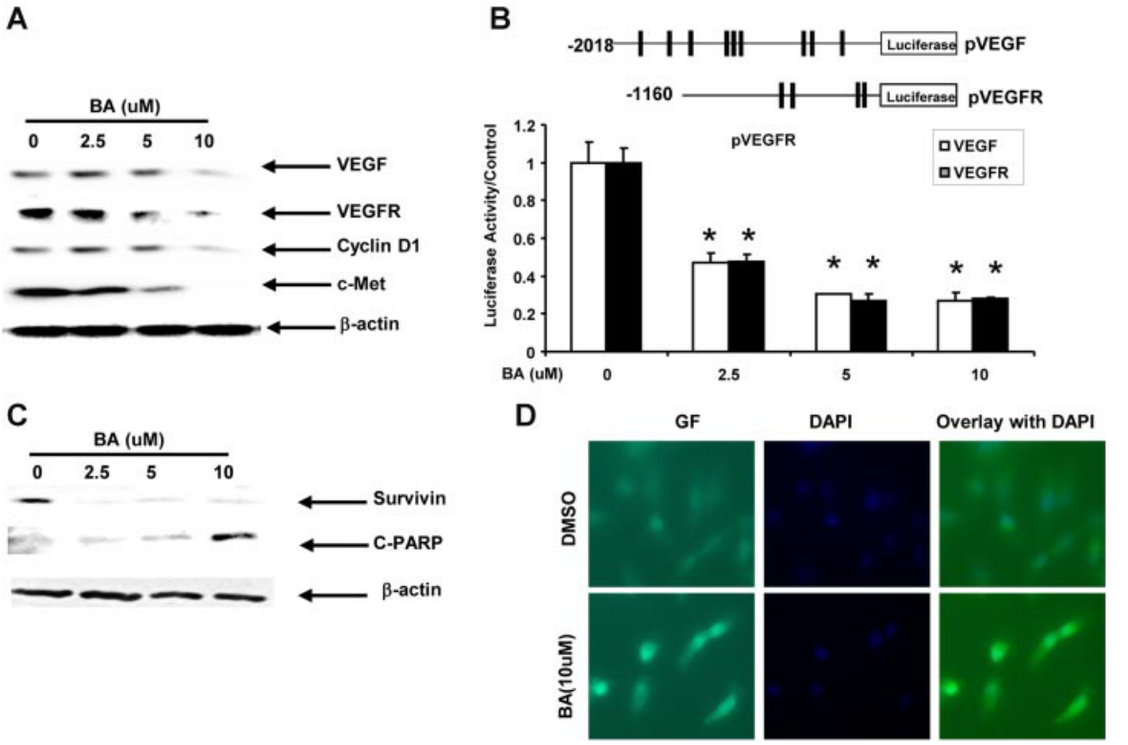

Figure 3. Betulinic acid (BA) decreases expression of the Sp-regulated genes and induces apoptosis in MDA-MB-231 cells. (A) Effects of BA on VEGF, VEGFR, Cyclin D1, and c-Met proteins. (B) Luciferase activity (relative to protein content) in transfected cells with VEGF and VEGFR constructs and treated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

inactivates cdc2, but to induction of Myt-1 (Figure 5C). This arrest was accompanied by enhanced phosphorylation of cdc2 at tyrosine-15 (Figure 5D). The Myt-1-dependent phosphorylation of cdc2 reached a peak between 2 and 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 d of tumor implantation and 21 d of BA (20 mg/kg/d) treatment, tumor growth rates were significantly different from control group (Figure 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 (Figure 6B), downregulation of miR-27a, and increased mRNA levels of ZBTB10 (Figure 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 (Figure 6D). Thus, BA decreased proliferation of MDA-MB-231 cells and tumors through perturbing the miR-27a:ZBTB10–Sp transcription factor axis. Moreover, micrometastases in the lung were visible but not characterized. We used β2-microglobulin mRNA as the major indicator of metastasis [30–31]. Results showed that expression of the human-specific β2-microglobulin (hβ2G) gene in the lungs of xenografted mice after 35 d of tumor implantation was significantly lower in the BA treated group, and similar to levels detected as background in no-xenografted controls (Figure 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 nontumor 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 prooncogenic activity of Sp1 is illustrated in a report showing that carcinogen-induced transformation of mouse skin fibroblasts is accompanied by an 8- to

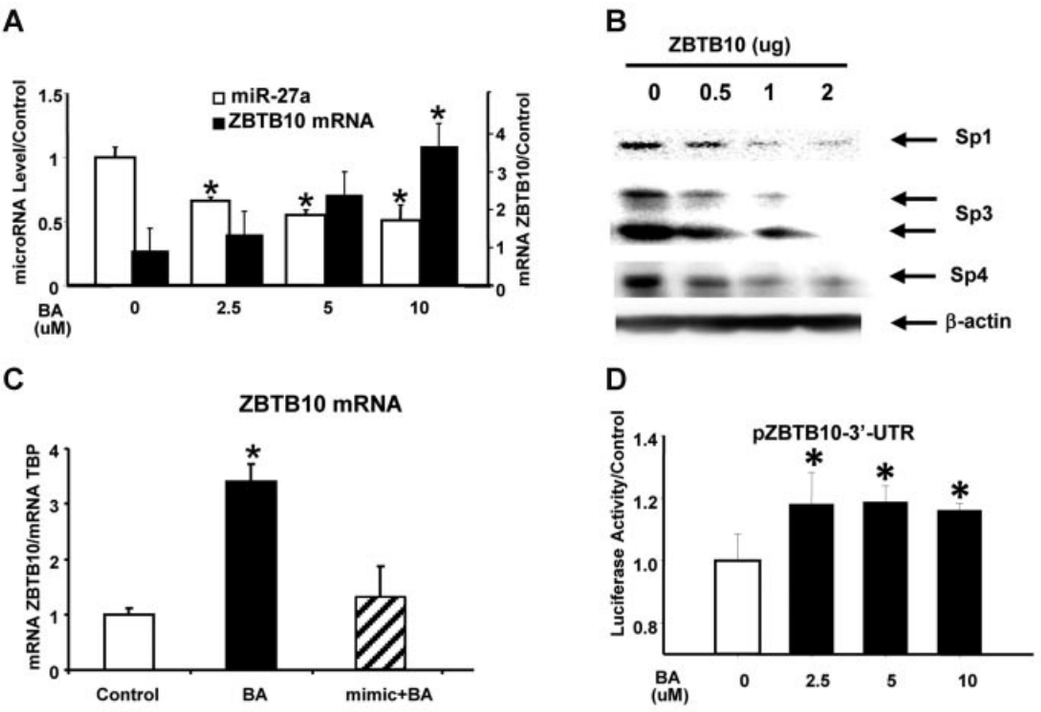

Figure 4. The Betulinic acid (BA) repression of miR-27a expression causes ZBTB10-Sp regulation in MDA-MB-231 cells. (A) MicroRNA levels of miR-27a and mRNA levels of ZBTB10 relative to DMSO treated cells. Cells were treated with solvent (DMSO) or different concentrations of BA (2.5–10 μM) for 24 h. mRNA 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 24 h 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 nonspecific oligonucleotide and treated with BA (5 μM). 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 16 h as described in Materials and Methods Section. Each experiment was performed at least three times, and results are expressed as means ± SD. *Significant changes at $P < 0.05$.

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-kB), survivin, bcl-2, 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 nonsteroidal anti-inflammatory drugs, BA, 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) (Figures 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 (Figures 2A, 3A and C), 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-18β-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

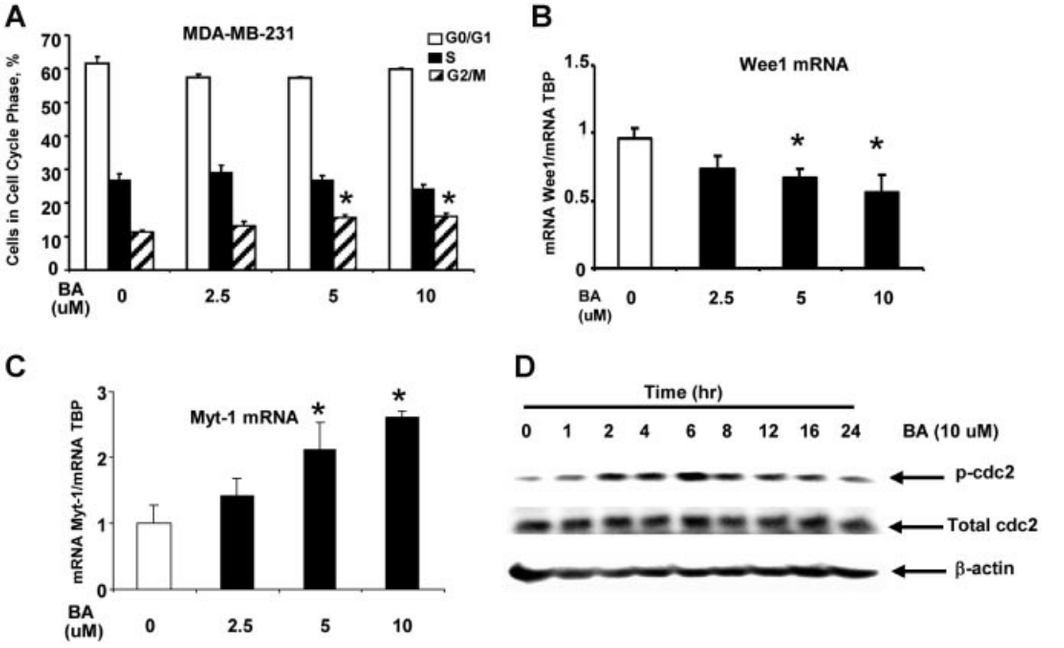

Figure 5. Betulinic acid (BA) induces cell cycle arrest at G2/M trough induction of Myt-1. (A) Flow cytometry analysis of MDA-MB-231 cells treated with solvent (DMSO) or different concentrations of BA (2.5–10 μM) for 48 h. The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined as described in Materials and Methods Section. mRNA levels of (B) Wee1 and (C) Myt-1. Cells treated with BA or DMSO for 24 h were analyzed by qRT-PCR as described in Materials and Methods Section. (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 Section. Each experiment was performed at least three times, and results are expressed as means ± SE. *Significant changes at $P < 0.05$.

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 (Figure 2C) and tumors (Figure 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 (Figures 4A and 6B). The Sp-repressor activity reported for ZBTB10 [28–29] was also confirmed by overexpression of ZBTB10 in MDA-MB-231 cells (Figure 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 (Figure 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 (Figure 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 (Figure 5C), activation of cdc2 (Figure 5D) and an increase in the percentage of cells in G2/M (Figure 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 hβ2G in lungs. The gene expression of hβ2G in mouse lung tissues has been previously used as a marker of metastasis and was significantly influenced by the size of tumors [30]. Overall, results presented in Figure 6D and E 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

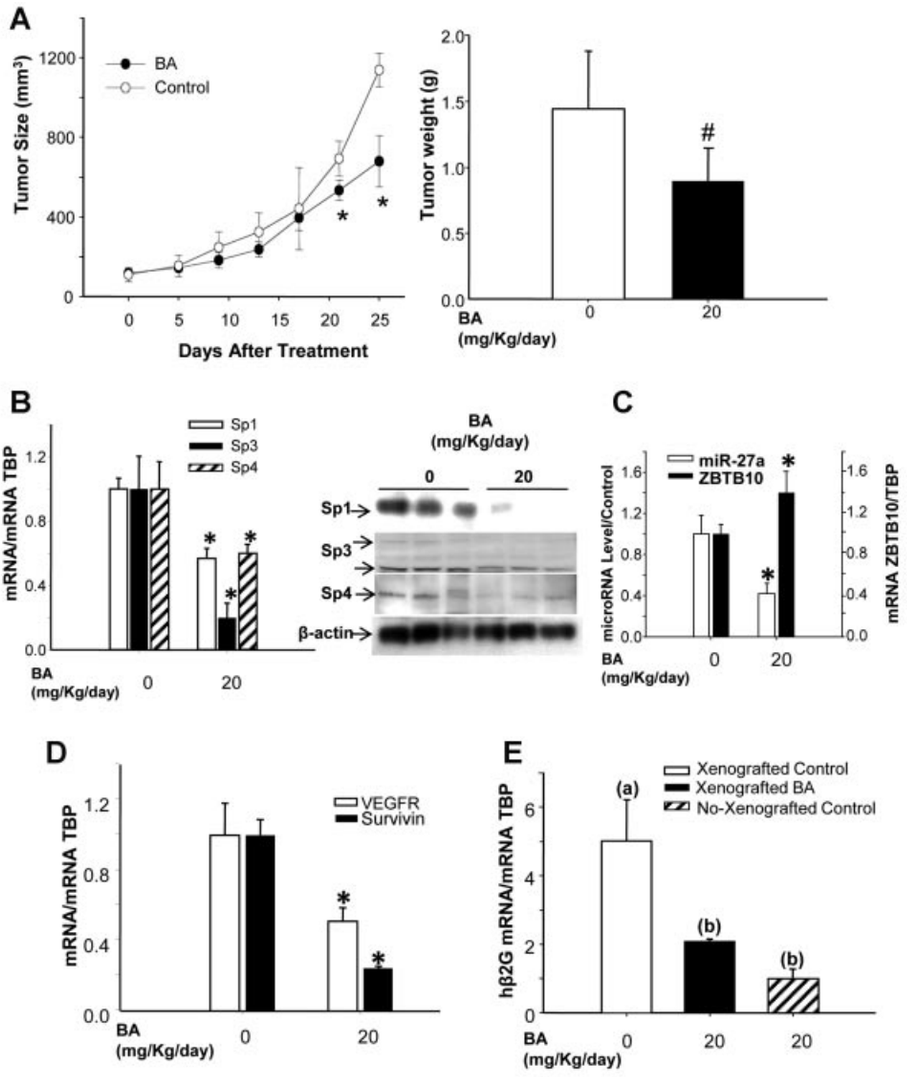

Figure 6. Antitumorigenic and antimetastatic activity of BA in xenografted nude mice. (A) Effects on tumor volume and tumor weight. Athymic nude mice (7 per group) bearing MDA-MB-231 cells as xenografts were treated with corn oil (control) or BA in corn oil (20 mg/kg/d) every second day. Treatments with BA started after implanted tumors reached a minimum volume as described in Materials and Methods Section. (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 ± 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 ± SE ($n \geq 4$). Different letters indicate significant changes. $^{*}P < 0.05$. $^{\#}P < 0.1$.

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.

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