Combining Betulinic Acid and Mithramycin A Effectively Suppresses Pancreatic Cancer by Inhibiting Proliferation, Invasion, and Angiogenesis

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Abstract
Both betulinic acid (BA) and mithramycin A (MIT) exhibit potent antitumor activity through distinct mechanisms of Sp1 inhibition. However, it is unknown whether a combination of these two compounds results in a synergistic inhibitory effect on pancreatic cancer growth and/or has a therapeutic advantage over gemcitabine. In xenograft mouse models of human pancreatic cancer, treatment with either BA or MIT alone showed dose-dependent antitumor activity but led to systemic side effects as measured by overall weight loss. Treatment with a nontoxic dose of either compound alone had only marginal antitumor effects. Importantly, combination treatment with nontoxic doses of BA and MIT produced synergistic antitumor activity, including inhibitory effects on cell proliferation, invasion, and angiogenesis. The treatment combination also produced less discernible side effects than therapeutic doses of gemcitabine. Moreover, combined treatment of BA and MIT resulted in drastic inhibition of Sp1 recruitment onto Sp1 and VEGF promoters, leading to transcriptional inhibition of both Sp1 and VEGF and downregulation of Sp1 and VEGF protein expression. Ectopic overexpression of Sp1 rendered tumor cells resistant to BA, MIT, and the combination of the two. Overall, our findings argue that Sp1 is an important target of BA and MIT and that their combination can produce an enhanced therapeutic response in human pancreatic cancer. Cancer Res; 71(15): 5182–93. ©2011 AACR.

Introduction
Pancreatic cancer is currently the fourth leading cause of cancer-related deaths worldwide. The median survival duration from diagnosis to death is about 6 months, and the overall 5-year survival rate is less than 5% (1–3). Pancreatic tumor is highly resistant to chemotherapy and radiation therapy. Surgery resection is still the primary choice when it is feasible (4, 5). A full understanding of the cellular and molecular mechanisms of the development and progression of pancreatic cancer is crucial for identifying new targets of effective treatment modalities for this deadly disease (6–19). Previous studies have shown that Sp1 overexpression plays an important role in regulating the expression of VEGF and angiogenesis in pancreatic cancer (11–19). Also, we have shown that neutralization of VEGF by treatment with bevacizumab (Avastin) leads to feedback activation of Sp1 and subsequent upregulation of expression of VEGF and other factors, leading to Avastin resistance, whereas blockade of Sp1 expression and function sensitizes tumors to Avastin and/or reverses Avastin resistance (20). The synergistic downregulation of Sp1 by tolenamic acid and mithramycin A (MIT) produced significant antitumor activity (21).

Sp1 is a zinc finger transcription factor that is important to the transcription of many cellular and viral genes containing GC boxes in their promoters. Although Sp1 has been perceived to be a basal transcription factor since its discovery, increasing evidence suggests that it regulates a variety of biological functions, including cell survival, growth, and differentiation and tumor development and progression (19, 22–24). Recently it was reported that Sp1 is essential for the epithelial to mesenchymal transformation induced by TGF-β1 in pancreatic cancer cells (25). The epithelial to mesenchymal transformation plays an important role in pancreatic cancer resistance to chemotherapy and other tumor types (26, 27).
A recent study has shown that betulinic acid (BA) inhibits prostate cancer growth through inhibition of specificity protein transcription factors (28). BA is a naturally occurring pentacyclic triterpene that exhibits potent antitumor properties. This anticancer activity has been linked to its ability to directly trigger mitochondrial membrane permeabilization. In contrast to the potent cytotoxicity of BA against a variety of cancer types, nonneoplastic cells and normal tissue remain relatively resistant to BA, thus pointing to a therapeutic window. Because agents that exert a direct action on mitochondria may bypass resistance to conventional chemotherapeutics, there is increasing interest to develop such compounds as experimental cancer therapeutics. Thus, mitochondrion-targeted agents such as BA hold great promise as a novel approach to overcome certain forms of drug resistance in human cancers (29–31). Interestingly, MIT inhibits Sp1 activity and has antitumor activities (32, 33). Its major underlying mechanism of action includes a reversible interaction with double-stranded DNA with GC-base specificity and selective regulation of transcription of genes having GC-rich promoter sequences (34–37).

Therefore, MIT and BA seem to have distinct mechanisms of inhibiting Sp1 activity (20, 28). However, it is unknown whether Sp1 is a critical target for the observed antitumor activities of those drugs. Also, it is significant to investigate whether a combination of these 2 compounds has a synergistic inhibitory effect on Sp1 activity and consequent suppression of pancreatic cancer growth and whether this antitumor activity has any advantages over gemcitabine. In this study, we sought to address these issues by using cell cultures and animal models of pancreatic cancer and also explored their underlying mechanisms.

Materials and Methods

Chemicals and reagents

MIT (1 mg/vial crystal powder; lot 098K4043) was purchased from Sigma Chemical Co. and diluted in sterile water. BA (powder; lot S43559) also was purchased from Sigma Chemical Co. and was mixed with corn oil. In our animal experiments, MIT (0.05–1.50 mg/kg body weight) was administered intraperitoneally twice a week (10). –

Cell lines and culture conditions

The human pancreatic adenocarcinoma cell lines BxPC-3 and PANC-1 were purchased from the American Type Culture Collection. FG human pancreatic adenocarcinoma cells were used as reported previously (17). The cell lines were maintained in plastic flasks as adherent monolayers in minimal essential medium supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories).

Animals

Female athymic BALB/c nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH.

Matrigel plug assay

A Matrigel plug angiogenesis assay was carried out essentially as described previously (38). Matrigel (200 μL) containing 2 × 10⁶ cells was injected subcutaneously into nude mice (2 injection sites per mouse). The Matrigel plugs were recovered from the mice 8 days after injection and carefully stripped of host tissues. After photomicrography, the Matrigel plugs were weighed and homogenized in 1 mL of distilled water and then centrifuged at 10,000 rpm for 5 minutes. The supernatants were collected for hemoglobin concentration measurement by using Drabkin solution (Sigma Chemical Co.) and a Microplate Manager ELISA reader at 540 nm according to the manufacturer’s instructions. The relative hemoglobin concentrations were calculated and further normalized according to the weights of the plugs.

Tumor cell invasion/migration assay

BxPC-3, FG, or PANC-1 cells were pretreated for 12 hours with 2.5 to 10 μmol/L of BA or dimethyl sulfoxide (ctrl). Cells from each group were trypsinized, and 2 × 10⁶ to 5 × 10⁶ cells of each group were resuspended and seeded in the upper part of modified Boyden chambers with Matrigel-coated membrane. Dulbecco’s Modified Eagle Medium with 10% FBS was used as chemoattractant. For each cell line, 750 μL of respective conditioned media was added into the lower chamber. After 24 to 48 hours incubation, invasive cells which had moved through the Matrigel membrane were stained, counted, and photographed under a microscope (×200 magnification).

Gene expression analyses

For Western blot, whole-cell lysates were prepared from human pancreatic cancer cell lines and tumor tissue specimens (17). Standard Western blotting was carried out using polyclonal rabbit antibodies against human Sp1, VEGF, survivin, uPAR, and Ki67 (Santa Cruz Biotechnology) and an anti-rabbit IgG antibody, which was a horseradish peroxidase–linked F(ab)’₂ fragment obtained from a donkey (Amersham). Equal protein specimen loading was monitored by probing the same membrane filter with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ref. 17). The probe proteins were detected by using the Amersham enhanced chemiluminescence system according to the manufacturer’s instructions. For quantitative real-time PCR (qPCR), total RNA was reversely transcribed into cDNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The cDNA products were used in qPCR analyses of gene expression by using PCR primer and probe sets custom-designed or purchased from Applied Biosystems (Carlsbad) and relative RNA expression calculations were done by using a commercially available...
software programs (SDS version 1.2; Applied Biosystems; ref. 18).

Immunohistochemical analysis and quantification of tumor microvessel density

Tissue sections were prepared and processed for immunostaining by using specific antibodies against CD31, Sp1, and VEGF and appropriate secondary antibodies. The levels of gene expression and quantification of tumor microvessel density (MVD) were evaluated as described previously (20).

Sp1 and VEGF promoter constructs and analysis of Sp1 and VEGF promoter activity

The minimal Sp1 and VEGF promoter reporters in pGL3 luciferase constructs were generated and used as described previously (17, 20). To examine transcriptional regulation of the Sp1 and VEGF promoters by BA and MIT, PANC-1 cells were seeded to about 80% confluence in 6-well plates (in triplicate) and transiently transfected with 0.6 μg of minimal Sp1 or VEGF reporter plasmids and 12 ng of Renilla reporter plasmids as indicated in each experiment by using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The reporter luciferase activity was measured 48 hours later by using a luciferase assay kit (Promega). Promoter activity was normalized according to the protein concentration as described previously (17, 20).

Chromatin immunoprecipitation

Chromatin was prepared from pancreatic cancer cells as described previously (20). A chromatin immunoprecipitation (ChIP) assay was carried out by using a Chromatin Immunoprecipitation Assay Kit (Upstate) according to the manufacturer’s instructions. Briefly, DNA cross-binding proteins were cross-linked with DNA and lysed in SDS lysis buffer. The lysate was sonicated to shear DNA to 200 to 500 bp. After pre-clearing with a salmon sperm DNA/protein A agarose 50% slurry for 30 minutes at 4°C, chromatin specimens were immunoprecipitated overnight with no antibody or an anti-Sp1 antibody (PEP2). The region from −224 to −53 bp of the Sp1 promoter was amplified by using the following primers: sense, 5’-cagcagcaacacctgta-3’; antisense, 5’-gaagggaaaggagcag-3’. The region from −272 to +18 bp of the VEGF promoter was amplified by using the following primers: sense, 5’-ccggeggcggtctctg-3’; antisense, 5’-tgcccaagctcaggatatcc-3’. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Statistical analysis

All in vivo experiments used 5 mice per group and were repeated at least once with similar results; 1 representative experiment was presented. The in vitro cytotoxicity experiments have been carried out in triplicate for each and every time points and concentrations. The significance of the in vitro data was determined by using the Student’s t test (2-tailed), whereas the significance of the in vivo data was determined by using the 2-tailed Mann–Whitney U test. The values of P ≤ 0.05 and <0.01 were deemed statistically significant (*) and highly significant (**), respectively.

Results

Antitumor effects of BA and MIT in xenograft mouse models of human pancreatic cancer

Previous studies have shown that Sp1 activity is essential for VEGF expression and that VEGF plays a major role in pancreatic tumor angiogenesis (17, 39, 40). Treatment with both BA and MIT can downregulate Sp1, VEGF, and VEGF receptor expression (28, 41). However, whether these 2 drugs interact synergistically in regulating Sp1 activity and pancreatic tumor growth is unknown. In our previous studies, we have already shown a dose-dependent antitumor activity of MIT (21). In this first set of experiments, we treated FG and PANC-1 xenograft tumors in nude mice with different doses of BA 3 times a week. BA produced dose-dependent antitumor activities in both FG and PANC-1 models, whereas the body weights of mice decreased in a dose-dependent manner (Fig. 1A and B).

Next, we treated FG and PANC-1 xenograft tumors in nude mice with nontoxic doses of MIT (0.05 mg/kg), BA (10 mg/kg), or both. We found that BA and MIT alone had marginal antitumor activity. In contrast, the combination of MIT and BA had significant antitumor activity in both FG and PANC-1 models. Furthermore, treatment with low doses of BA and MIT produced synergistic antitumor activity without any significant systemic side effects as indicated by a lack of significant weight loss (Fig. 1C and D). Similar results were obtained in orthotopic models (Fig. 1E and F). Therefore, combination administration of low doses of MIT and BA has a significant therapeutic benefit for pancreatic cancer. This notion was further confirmed by using both ectopic and orthotopic models of FG (Supplementary Fig. S1) and PANC-1 (Supplementary Fig. S2) cells. Specifically, we found that the doses of gemcitabine that produced significant antitumor activities also led to more discernible losses of animal body weights than the combination of BA and MIT.

Synergistic cytotoxicity of BA and MIT in human pancreatic cell lines in vitro

To assess the direct cytotoxicity of BA and MIT, we treated various pancreatic cancer cell lines with BA for 48 hours (Fig. 2A); and BxPC-3, FG, and PANC-1 cells with BA for 12, 24, or 48 hours (Fig. 2B). Inhibition of cell proliferation was assessed by using an MTT assay. We found that BA produced concentration-dependent cytotoxicity and FG cells exhibited the highest sensitivity to BA-mediated cytotoxicity. FG cells were then treated with different concentrations of BA or MIT. Both drugs exhibited concentration-dependent cytotoxicity and FG cells exhibited the highest sensitivity to BA-mediated cytotoxicity. FG cells were then treated with different concentrations of BA or MIT. Under this condition, the combination of BA and MIT had substantial cytotoxic effects (Fig. 2D). The combination treatment with BA and MIT revealed a synergistic effect of cytotoxicity (data not shown). Similar results were obtained from using PANC-1 cells (Supplementary Fig. S3).
Synergistic effect of treatment with BA and MIT on inhibition of pancreatic cancer cell migration and invasion

FG cell cultures in triplicate were pretreated with BA (0, 2.5, or 5 μmol/L), or pretreated with 2.5 μmol/L BA, 0.01 μmol/L MIT, or both for 24 hours, the cultures were wounded by scratching and maintained for additional 24 hours. Cell cultures were photographed and cell migration was assessed by measuring gap sizes (Fig. 3A). FG cells were treated with BA at concentrations ranging from 2.5, 5, and 10 μmol/L or MIT at concentrations ranging from 0.01, 0.05, and 0.10 μmol/L for 24 hours, or FG cells were treated with 2.5 μmol/L BA, 0.01 μmol/L MIT, or both for 24 hours. Representative photomicrographs of tumor cell invaded through Matrigel were taken, whereas the numbers of invasive cells that penetrated through Matrigel-coated filter were counted in 15 random fields identified within the lower surface of the filters and expressed as percentage of control.

Figure 1. Dose-dependent antitumor effects of BA and MIT in xenograft models of human pancreatic cancer. Dose response: FG (A) and PANC-1 (B) cells were injected into the pancreases of nude mice (n = 5). Ten days after tumor injections, the mice were treated with different doses of BA (10, 20, and 40 mg/kg) via oral gavages 3 times a week. The tumors were weighed 45 days after tumor cell injection (left); the mice were weighed at the same time and columns, mean weights; bars, SD (right). Synergistic antitumor effect in ectopic models: FG (C) and PANC-1 (D) cells were injected into the subcutis of nude mice (n = 5). When tumors reached around 4 mm in diameter, the animals received MIT (0.05 mg/kg) via intraperitoneal injection twice a week and BA (10 mg/kg). Tumor volumes were measured every week until the mice were killed 45 days after tumor cell injection (left); the mice were weighed at the time of termination of experiments (right). Synergistic antitumor effect in orthotopic models: FG (E) and PANC-1 (F) cells were injected into the pancreases of nude mice (n = 5). Ten days after tumor cell injections, the animals received MIT (0.05 mg/kg) via intraperitoneal injection twice a week and BA (10 mg/kg) via oral gavage 3 times a week. The mice were killed 45 days after tumor cell injection; both tumors (left) and the mice (top right) were weighed, and hepatic metastases were determined (bottom right); *, P < 0.05 and **, P < 0.01 as compared with respective controls (2-tailed Student’s t or Fisher exact test). C, control; O, corn oil; B, BA; M, MIT; MB, MIT + BA.

Synergistic effect of treatment with BA and MIT on inhibition of pancreatic cancer cell migration and invasion

FG cell cultures in triplicate were pretreated with BA (0, 2.5, or 5 μmol/L), or pretreated with 2.5 μmol/L BA, 0.01 μmol/L MIT, or both for 24 hours, the cultures were wounded by scratching and maintained for additional 24 hours. Cell cultures were photographed and cell migration was assessed by measuring gap sizes (Fig. 3A). FG cells were treated with BA at concentrations ranging from 2.5, 5, and 10 μmol/L or MIT at concentrations ranging from 0.01, 0.05, and 0.10 μmol/L for 24 hours, or FG cells were treated with 2.5 μmol/L BA, 0.01 μmol/L MIT, or both for 24 hours. Representative photomicrographs of tumor cell invaded through Matrigel were taken, whereas the numbers of invasive cells that penetrated through Matrigel-coated filter were counted in 15 random fields identified within the lower surface of the filters and expressed as percentage of control.
Similar results were obtained by using PANC-1 cells (Supplementary Fig. S4).

**Antiangiogenic effects of BA and MIT in vitro**

We treated FG cells with 2.5 μmol/L BA and/or 0.01 μmol/L MIT. Once Sp1 expression downregulation was confirmed by Western blot analysis, we then used an endothelial cell tube formation assay to determine the angiogenic potential of the supernatants of the FG cells. We assessed the degree of tube formation as the percentage of cell surface area versus the total surface area. We obtained representative photomicrographs of tube formation by human umbilical vein endothelial cells in the supernatants *in situ* (Fig. 4A). Treatment with MIT and/or BA reduced the capacity of supernatants of the FG cells to stimulate tube formation by endothelial cells compared with that of supernatants of control FG cells (Fig. 4B). We confirmed this impaired angiogenic potential by using an *in vivo* Matrigel plug assay (Fig. 4C). Our data suggested that treatment with MIT and/or BA impaired the angiogenic potential of FG cells.
Effects of treatment with BA and MIT on Sp1 and VEGF expression and MVD in vivo

To determine the molecular basis for the antitumor effect of treatment of pancreatic cancer with BA, we carried out immunohistochemical staining on tissue sections harvested from FG tumors in Figure 1A. Treatment with BA decreased expression of Sp1 and its downstream molecules in FG tumors in a dose-dependent manner (Fig. 4D). Also, as indicated by CD31 staining, tumor MVDs were inhibited in a dose-dependent manner (Fig. 4E). The immunostaining results were further confirmed by using Western blot analysis (Fig. 4F).

To determine the molecular basis for the synergistic effect of treatment of pancreatic cancer with BA and MIT, we carried out Western blot analysis by using total protein lysates extracted from the FG tumor specimens collected from mice that received treatment with PBS, BA, MIT, or both BA and MIT as shown in Figure 1C. As shown in Figure 4G, expression of Sp1 and its downstream targets were downregulated by treatment with the combination of BA and MIT (Supplementary Fig. S5). Similar results were obtained from using PANC-1 cells (Supplementary Fig. S6). These results suggested that the synergistic antitumor activity of the combination of BA and MIT occur through not only an antiangiogenic effect but also direct inhibition of tumor cell proliferation.

BA and MIT inhibited the recruitment of Sp1 onto the Sp1 and VEGF promoters and suppressed Sp1 and VEGF protein expression in human pancreatic cancer cells in vitro

To further confirm the impact of treatment with BA and MIT on gene expression in pancreatic cancer cells, we incubated FG cells in a medium alone or a medium containing MIT (0, 0.01, or
0.05 μmol/L and/or BA (1, 5, or 10 μmol/L). Sp1 protein expression in the cells was downregulated in a dose-dependent manner after 24 hours of treatment with BA and MIT as single agents in vitro as determined by Western blot analysis and its respective quantitation by densitometry (Fig. 5A).

Next, we determined whether treatment with BA and/or MIT regulated Sp1 and VEGF expression at the transcriptional level. FG cells were treated in a medium alone or a medium containing 2.5 μmol/L BA or 0.01 μmol/L MIT, or both was used as described in Materials and Methods (Inset: recovered representative Matrigel plugs from corresponding groups). D–F, the FG tumors from mice receiving dissolve (Ctrl, corn oil) or different doses of BA treatment (described in Fig. 1A) were collected and processed for gene expression analysis by immunostaining of VEGF and Sp1 expression (D), quantitation of tumor angiogenesis by microvessel counting (E), and confirmation of gene expression by Western blot analysis (F). G, the FG tumors from mice receiving dissolve (Ctrl, corn oil) or treatment of BA, MIT, or both (described in Fig. 1C) were collected and processed for gene expression analysis by Western blot analysis. *, P < 0.05 and #, P < 0.01 as compared with respective controls (2-tailed Student’s t test).
Figure 5. Treatment with BA and MIT downregulates Sp1 expression in vitro. A, FG cells were incubated in a medium alone or a medium containing different concentrations of BA and/or MIT for 24 hours. Total protein lysates were harvested from the cell cultures, and the level of Sp1 and VEGF protein expression was determined by using Western blot analysis. Equal protein specimen loading was monitored by probing the same membrane filter with an anti-GAPDH antibody and changes in gene expression levels were quantitated (A). Total RNA was harvested for qRT-PCR analysis of both Sp1 and VEGF mRNA (B). Sp1 (C) and VEGF (D) promoter reporter constructs were transfected into PANC-1 cells in triplicate and incubated for 12 hours. The cells were then incubated for another 24 hours in a medium alone or a medium containing 2.5 µmol/L BA, 0.01 µmol/L MIT, or both. Total protein lysates were harvested from the cell cultures for measurement of Sp1 promoter activity by using a luciferase assay kit. The relative Sp1 promoter activities in treated groups were expressed as the fold changes from that in their respective control groups. FG (E and F) and PANC-1 (G and H) cells were incubated in vitro in a medium alone or a medium containing 2.5 µmol/L BA, 0.01 µmol/L MIT, or both for 24 hours and chromatin was extracted from the cells. The ChIP assay was carried out by using a specific anti-Sp1 antibody and oligonucleotides flanking the VEGF and Sp1 promoter regions containing Sp1-binding sites. Lane 1, input chromatin DNA; lane 2, chromatin DNA with a control IgG; lane 3, chromatin DNA with an anti-Sp1 antibody. Ctrl, control; M B, MIT plus BA. Quantitative data were also presented (F and H).
treatment with BA or MIT at the given dose resulted in low levels of suppression of Sp1 and VEGF promoter activity, whereas treatment with the combination of BA and MIT significantly suppressed this activity. However, further deletion of Sp1-binding sites eliminated the ability of MIT to suppress Sp1 (Fig. 5C) and VEGF promoter activity (Fig. 5D).

Finally, we carried out a ChIP assay by using chromatin extracted from FG and PANC-1 cells. Treatment with BA or MIT at the given dose had a minor effect on inhibition of Sp1 recruitment to its own promoter and the VEGF promoter, whereas treatment with BA combined with MIT at the same dose significantly decreased Sp1 recruitment to these 2 promoters in both FG (Fig. 5E and F) and PANC-1 cells (Fig. 5G and H). These results suggested that treatment with BA and MIT at low doses resulted in insignificant transcriptional suppression of Sp1 and VEGF mRNA transcription activated by Sp1, whereas treatment with BA combined with MIT at the same doses produced synergistic transcriptional suppression of Sp1 and VEGF transcription.

**Overexpression of Sp1 renders pancreatic cancer cells resistance to BA cytotoxicity**

To determine whether Sp1 is a key target of BA, we evaluated the effects of ectopic Sp1 overexpression on BA-mediated cytotoxicity in both BxPC-3 and PANC-1 cells. The ectopic Sp1 protein expression was determined by Western blot by using anti-HA antibody (for exogenous Sp1 protein) and anti-Sp1 antibody (for total Sp1 protein; Fig. 6A). Clearly, the ectopic Sp1 overexpression led to resistance to BA in both BxPC-3 and PANC-1 cells (Fig. 6B) and also to the combination of BA and MIT (Fig. 6C).

**BA inhibited the growth of gemcitabine-resistant FG cells**

In the last set of experiments, we determined whether pancreatic cancer cells cross-resist to both BA and gemcitabine. FG, BxPC-3, and PANC-1 cells were treated with gemcitabine (0–1,000 nmol/L) and cytotoxicity was determined by MTT assay. We found the sensitivities to gemcitabine (Fig. 6D) were correlated with that to BA among the 3 cell lines (Fig. 2A). We then established a gemcitabine-resistant FG cell line (FG-GR) by incubating the FG cells with increasing concentrations of gemcitabine for a period of 3 months. As shown in Figure 6E and F, FG cells were highly sensitive to gemcitabine as compared with FG-GR cells, whereas both FG and FG-GR cells remain similarly sensitive to BA or with the combination of lower levels of both BA and MIT.

**Discussion**

BA and its derivatives have significant antitumor activities to various tumor types (28, 31, 42). Of particular interest is its direct and relatively selective cytotoxic effect on various tumor cells versus normal or nonneoplastic cells (42). Our current study showed that an increasing level of antitumor activities seem to be accompanied by increasing systemic side effects as reflected by an increasing loss of body weight. Interestingly, FG and PANC-1 tumor cells exhibited quite different sensitivities to BA-mediated cytotoxicity in vitro, whereas we observed very similar in vivo antitumor activities in the animal models at similar doses of BA. These results suggested that the mechanisms for its antitumor activities might not solely because of direct cytotoxic effects of BA. Therefore, exploration of other mechanisms underlying its antitumor activities should help enhance therapeutic index of BA-based cancer therapy, that is, increased tumor suppression and decreased systemic side effects. Indeed, BA has significant antiangiogenesis and anti-invasion activities other than antiproliferation activity. More importantly, a combination of BA and MIT has a synergistic antitumor activity and exhibit less discernible side effect than gemcitabine does, and Sp1 is an important target.

Sp1/VEGF pathway is important to pancreatic tumor angiogenesis and its targeted inhibition suppresses pancreatic tumor growth in mouse models (17, 20, 23, 43). We have shown that MIT could downregulate Sp1 expression through direct interference of Sp1 auto upregulation (21, 32). However, as the important regulator of VEGF, Sp1 protein is of high stability and abundance, which prevent MIT from rapidly downregulating Sp1 protein level in pancreatic cancer cells. A relative long treatment time and high dosage of MIT are required to downregulate Sp1 protein, thus increasing the possibility of systemic side effects (21). Interestingly, recent studies have indicated that BA and its derivatives promote the degradation of Sp1 proteins (28, 44). In this study, we showed that BA promoted Sp1 protein downregulation, which is consistent with a previous finding (28). More importantly, combined treatment with MIT and BA, neither of which has significant effects on Sp1 protein level, substantially downregulated Sp1 protein expression and suppressed angiogenesis, which was consistent with the synergistic antitumor effect in our mouse models.

Significantly, we have shown that BA has a strong inhibitory effect on pancreatic cancer cell migration and invasion. This effect might not be simply because of its cytotoxicity, because BA at nontoxic concentrations suppressed tumor cell invasion. Furthermore, BA sensitizes pancreatic cancer cells to MIT treatment. One of the potential underlying mechanisms seems to be a rapid downregulation of Sp1 protein expression. This notion is further supported by our findings, showing that BA produced dose-dependent suppression of uPAR in vitro and xenograft tumors. The uPA and uPAR are known downstream target genes of Sp1 and play an important role in adhesion, migration, and invasion of pancreatic cancer cells (45). Given that uncontrolled growth and extensive invasion and metastasis are hallmarks of pancreatic cancer (46), our results suggest a novel and significant mechanism underlying the antitumor activities of BA and its derivatives. Besides uPA and uPAR, other potential targets of BA treatment warrant further investigation.

BA produces antitumor activity in combination with other chemotherapeutic drugs or radiation therapy (47). For example, BA sensitizes drug-resistant colon cancer cells and esophageal squamous carcinoma cells to 5-fluorouracil, irinotecan, and oxaliplatin (31, 48). Although the underlying mechanisms remain to be determined, prior studies have indicated that BA induces apoptosis through intrinsic pathway independent of p53 and the Fas–FasL extrinsic pathway...
Figure 6. Influence of Sp1 expression on cytotoxicity in vitro. BxPC-3 and PANC-1 cells were treated with Ad-EGFP or Ad-Sp1 (10 MOI) for 6 hours, and cultures were incubated for additional 18 hours. The cells were either harvested for analysis of Sp1 expression by using Western blot (A) or plated in 96-well plates and treated with different concentrations of BA (B) or with 2.5 μmol/L BA, 0.01 μmol/L MIT, or both (C) for additional 24 hours before cell viability determination by MTT assay. D, BxPC-3, FG, and PANC-1 cells were treated with gemcitabine (“Gem”, 0–1,000 nmol/L) for 48 hours. Inhibition of cell proliferation was assessed by using an MTT assay. E and F, FG and FG-GR (gemcitabine-resistant variant) cells were treated with gemcitabine (0–320 nmol/L) or BA (0–40 μmol/L) for 72 hours or treated with 2.5 μmol/L BA, 0.01 μmol/L MIT, or both for 48 hours. Treatment with 10 nmol/L of gemcitabine was used as a control. Inhibition of cell proliferation was assessed by using an MTT assay. *, P < 0.05 and #, P < 0.01 as compared with respective controls (2-tailed Student’s t test).
Xie K, Wei D, Huang S. Transcriptional anti-angiogenesis therapy of feration activity, BA exhibited strong antiangiogenesis and observed. suggested that Sp1 is crucial for the antitumor activities. Finally, ectopic overexpression of Sp1 rendered tumor tant mechanism for antitumor activities of BA and its deriva-

It was reported that BA-based treatment activates Sp protein degradation and inhibits its downstream target survivin expression in cancer cells, which plays an important role in tumor cell resistance to apoptosis (28). Our results showed that BA inhibits tumor cell growth in vitro and that this effect is synergistic with that of MIT. Consistently, BA and MIT syner-

Likewise, a synergistic downregulation of Sp1 may also cause downregulation of VEGF and uPAR, hence suppression of pancreatic cancer angiogenesis and invasion. Therefore, it is likely that an accelerated downregulation of Sp1 and its major target genes including VEGF, survivin, and uPAR be an important mechanism for antitumor activities of BA and its deriva-

Finally, ectopic overexpression of Sp1 rendered tumor cells resistance to BA, MIT, and their combination, strongly suggested that Sp1 is crucial for the antitumor activities observed.

In summary, we investigated the antitumor activity of natural product BA and its underlying mechanisms of actions in pancreatic cancer models. Besides its expected antiproliferation activity, BA exhibited strong antiangiogenesis and antivinvasion abilities in pancreatic cancer. Our experimental results further indicated that Sp1 was one of the important targets for BA-mediated protein degradation and MIT-mediated transcriptional repression. Furthermore, our find-

No potential conflicts of interest were disclosed.

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