

## Therapeutics, Targets, and Chemical Biology

**Combined Treatment of Pancreatic Cancer with Mithramycin A and Tolfenamic Acid Promotes Sp1 Degradation and Synergistic Antitumor Activity**Zhiliang Jia<sup>1</sup>, Yong Gao<sup>2</sup>, Liwei Wang<sup>3</sup>, Qiang Li<sup>1</sup>, Jun Zhang<sup>4</sup>, Xiangdong Le<sup>1</sup>, Daoyan Wei<sup>1</sup>, James C. Yao<sup>1</sup>, David Z. Chang<sup>1</sup>, Suyun Huang<sup>2</sup>, and Keping Xie<sup>1</sup>**Abstract**

Mithramycin (MIT) and tolfenamic acid (TA) inhibit the activity of the transcription factor Sp1. In the present study, we investigated whether pancreatic cancer treatment with a combination of these compounds has a synergistic effect on Sp1 activity, tumor growth, and their underlying response mechanisms. Treatment of pancreatic tumor xenografts with MIT and TA produced dose-dependent antitumor activity, and significant antitumor activity of either compound alone was directly associated with systemic side effects. Combination treatment with nontoxic doses of both compounds produced synergistic antitumor activity, whereas treatment with a nontoxic dose of either compound alone lacked a discernible antitumor effect. Synergistic therapeutic effects correlated directly with synergistic antiproliferation and antiangiogenesis *in vitro*. Moreover, combination treatment resulted in Sp1 protein degradation, drastically downregulating expression of Sp1 and vascular endothelial growth factor. Our findings established that Sp1 is a critical target of TA and MIT in human pancreatic cancer therapy, rationalizing clinical studies to determine the effect of existing pancreatic cancer therapy regimens on Sp1 signaling in tumors and normal pancreatic tissue, and the ability of Sp1-targeting strategies to modify cancer responses. *Cancer Res*; 70(3); 1111–9. ©2010 AACR.

**Introduction**

Pancreatic cancer is currently the fourth leading cause of cancer-related deaths worldwide. The median survival duration from diagnosis to death is ~6 months, and the overall 5-year survival rate is <5% (1–3). 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. Among the various potential targets are numerous proangiogenic and antiangiogenic factors released by tumor and host cells (4–6). These factors regulate angiogenesis, which determines the growth and metastasis of pancreatic tumors (6–8). Of the numerous angiogenic factors discovered thus far, studies have identified vascular endothelial growth factor

(VEGF) as a key mediator of tumor angiogenesis (9–11). Authors have reported elevated expression of VEGF in human pancreatic tumor specimens (12, 13), that its expression level correlates with microvessel density (MVD; refs. 4, 6, 14–16), and that VEGF-targeted therapy significantly inhibits angiogenesis in and growth of pancreatic cancer in animal models (4, 6, 17).

Previous studies showed that Sp1 overexpression plays an important role in regulating the expression of VEGF and angiogenesis in pancreatic tumors and is directly correlated with poor prognoses for human pancreatic cancer (18–20). In addition, 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 (21). Therefore, Sp1 seems to be a critical target for antiangiogenic therapy for pancreatic cancer.

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. Researchers have cloned transcription factors similar to Sp1 in their structural and transcriptional properties (Sp2, Sp3, and Sp4), thus identifying the Sp1 multigene family (22). 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 (20, 22–25). Consistently, mithramycin A (MIT) and tolfenamic

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org>).

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doi: 10.1158/0008-5472.CAN-09-3282

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acid (TA) inhibit Sp1 activity and have antitumor effects in various tumor models.

The antitumor activity of MIT, an aureolic acid-type polyketide produced by various soil bacteria of the genus *Streptomyces*, inhibits Sp1 activity (26–30). Its major underlying mechanism of action includes a reversible interaction with dsDNA with GC base specificity and selective regulation of transcription of genes having GC-rich promoter sequences (31–35). In comparison, TA, a potent inhibitor of prostaglandin biosynthesis and an inhibitor of leukotriene synthesis, is an effective, well-documented nonsteroidal anti-inflammatory drug used to treat migraines and was recently shown to facilitate Sp1 protein degradation (36–40). Therefore, the two compounds have distinct mechanisms of regulating Sp1 activity. In the present study, we sought to determine whether treatment with a combination of these two compounds has a synergistic effect on Sp1 activity and tumor growth in an animal model of pancreatic cancer. We also explored their underlying mechanisms.

## Materials and Methods

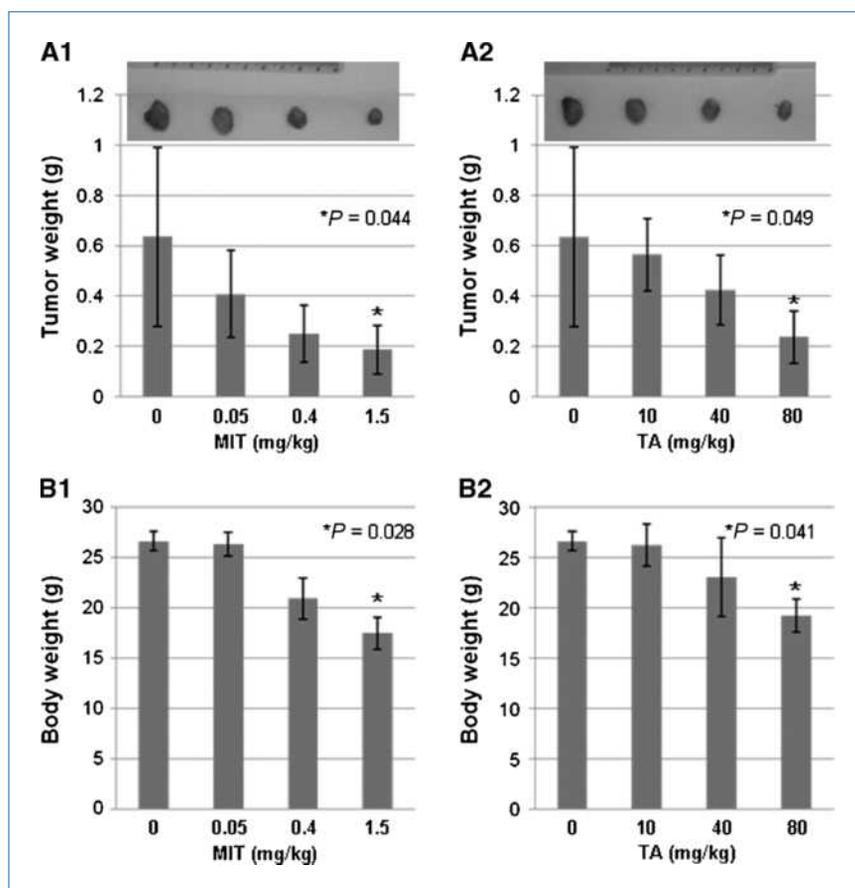
**Chemicals and reagents.** MIT (1 mg/vial crystal powder; lot 055K4011) was purchased from Sigma Chemical Co. and diluted in sterile water. TA (powder; lot 110H0469) also was

purchased from Sigma Chemical and mixed with corn oil. In our animal experiments, MIT (0.05–1.50 mg/kg body weight) was administered via i.p. injection twice a week or as indicated, and TA (10–80  $\mu$ g/kg) was administered via oral gavage twice a week.

**Cell lines and culture conditions.** The human pancreatic adenocarcinoma cell lines BxPC3 and PANC-1 were purchased from the American Type Culture Collection. FG human pancreatic adenocarcinoma cells were also used as reported previously (18). The cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories).

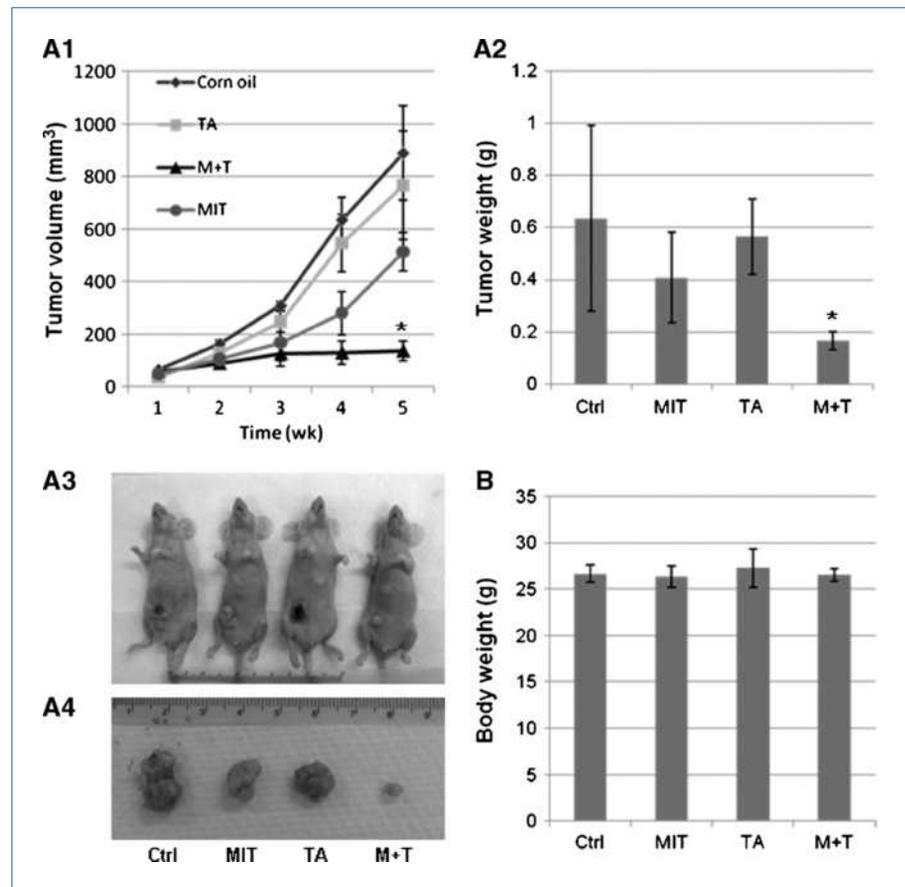
**Animals.** Female athymic BALB/c nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 wk old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation 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 performed essentially as described previously (41, 42).



**Figure 1.** Dose-dependent antitumor effects of MIT and TA in xenograft models of human pancreatic cancer. PANC-1 cells were injected into the subcutis of nude mice ( $n = 5$ ). A, when tumors reached ~4 mm in diameter, the animals received different doses of MIT (0.05, 0.40, and 1.50 mg/kg) via i.p. injection twice a week and TA (10, 40, and 80 mg/kg) via oral gavage thrice a week. The tumors were weighted 45 d after tumor cell injection. Insets, representative photographs of tumors, respectively, in each group. B, the mice were weighted at the same time. Columns, mean weights; bars, SD. \*,  $P < 0.05$ , compared with respective controls (Student's  $t$  test).

**Figure 2.** Synergistic antitumor effect of MIT and TA *in vivo*. A1, mice with PANC-1 tumors were given 0.05 mg/kg MIT and 10 mg/kg TA. Tumor volumes were measured every week until the mice were killed 45 d after tumor cell injection. The tumors (A2) and mice (B) were weighed at the end of experiment. Tumors in all four groups of mice were measured once a week. Points and columns, mean tumor volume in the five mice in each group calculated at each measurement; bars, SD. This was one representative experiment of two with similar results. A3 and A4, representative photographs of mice and tumors, respectively, in each group at the end of experiment. *Ctrl*, control; *M+T*, MIT and TA. \*,  $P < 0.01$ , compared with respective controls (Student's *t* test).



Matrigel (200  $\mu$ L) containing  $2 \times 10^6$  cells was injected s.c. into nude mice (two injection sites per mouse). The Matrigel plugs were recovered from the mice 8 d after injection and carefully stripped of host tissues. After photography, the Matrigel plugs were weighed and homogenized in 1 mL of distilled water and then centrifuged at 10,000 rpm for 5 min. The supernatants were collected for hemoglobin concentration measurement using Drabkin solution (Sigma Chemical) 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.

**Western blot analysis.** Whole-cell lysates were prepared from human pancreatic cancer cell lines and tumor tissue specimens (18). Standard Western blotting was performed using polyclonal rabbit antibodies against human and murine Sp1 and VEGF (Santa Cruz Biotechnology) and an anti-rabbit IgG antibody, which was a horseradish peroxidase-linked F(ab')<sub>2</sub> fragment obtained from a donkey (Amersham). Equal protein specimen loading was monitored by probing the same membrane filter with antibodies against  $\beta$ -actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ref. 18). The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

**Immunohistochemical analysis and quantification of tumor MVD.** Tissue sections were prepared and processed for immunostaining using specific antibodies against CD31 Sp1, VEGF, and proliferating cell nuclear antigen (PCNA) and appropriate secondary antibodies. The levels of gene expression and quantification of tumor MVD were evaluated as described previously (21).

**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 (18, 21). To examine transcriptional regulation of the Sp1 and VEGF promoters by TA and MIT, PANC-1 cells were seeded to ~80% confluence in six-well plates (in triplicate) and transiently transfected with 0.6  $\mu$ g of minimal Sp1 or VEGF reporter plasmids and 0.3  $\mu$ g of effector expression plasmids as indicated in each experiment using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The reporter luciferase activity was measured 48 h later using a luciferase assay kit (Promega). Promoter activity was normalized according to the protein concentration as described previously (18, 21).

**Chromatin immunoprecipitation.** Chromatin was prepared from pancreatic cancer cells and pancreatic tumors as described previously (21). A chromatin immunoprecipitation (ChIP) assay was performed 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 preclearing with a salmon sperm DNA/protein A agarose 50% slurry for 30 min 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 using the following primers: sense, 5'-caggcagcaactagtc-3'; antisense, 5'-gtaaggaggaggagcagc-3'. The region from -272 to +18 bp of the VEGF promoter was amplified using the following primers: sense, 5'-ccggggcgcgtgtctctgg-3'; antisense, 5'-tgccccaagcctccgcgacctc-3'. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

**Statistical analysis.** All *in vivo* experiments used five mice per group and were repeated at least once with similar results; one representative experiment is presented. The *in vitro* cytotoxicity experiments have been performed in triplicate for each and every time points and concentrations. The significance of the *in vitro* data was determined using the Student's *t* test (two-tailed), whereas the significance of the *in vivo* data was determined using the two-tailed Mann-Whitney *U* test. *P* levels of  $\leq 0.05$  were deemed statistically significant.

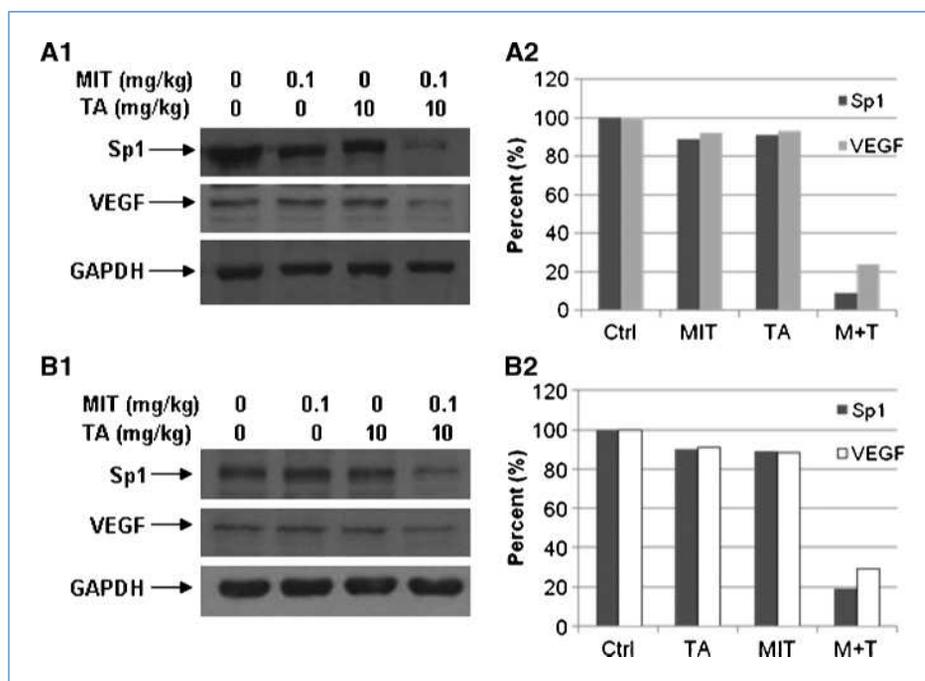
## Results

**Antitumor effects of MIT and TA in xenograft mouse models of human pancreatic cancer.** Previous studies

showed that Sp1 activity is essential for VEGF expression and that VEGF plays a major role in pancreatic tumor angiogenesis (18, 43, 44). Treatment with both MIT and TA can downregulate Sp1, VEGF, and VEGF receptor expression (39, 40). However, whether these two drugs interact synergistically in regulating Sp1 activity and pancreatic tumor growth is unknown. We treated PANC-1 xenograft tumors in nude mice with different doses of MIT (0, 0.05, 0.40, and 1.50 mg/kg) or TA (0, 10, 40, and 80 mg/kg) twice a week (Fig. 1A). Both MIT and TA had dose-dependent antitumor activity. However, the body weights of the mice decreased in a dose-dependent manner (Fig. 1B), which indicated systemic cytotoxicity.

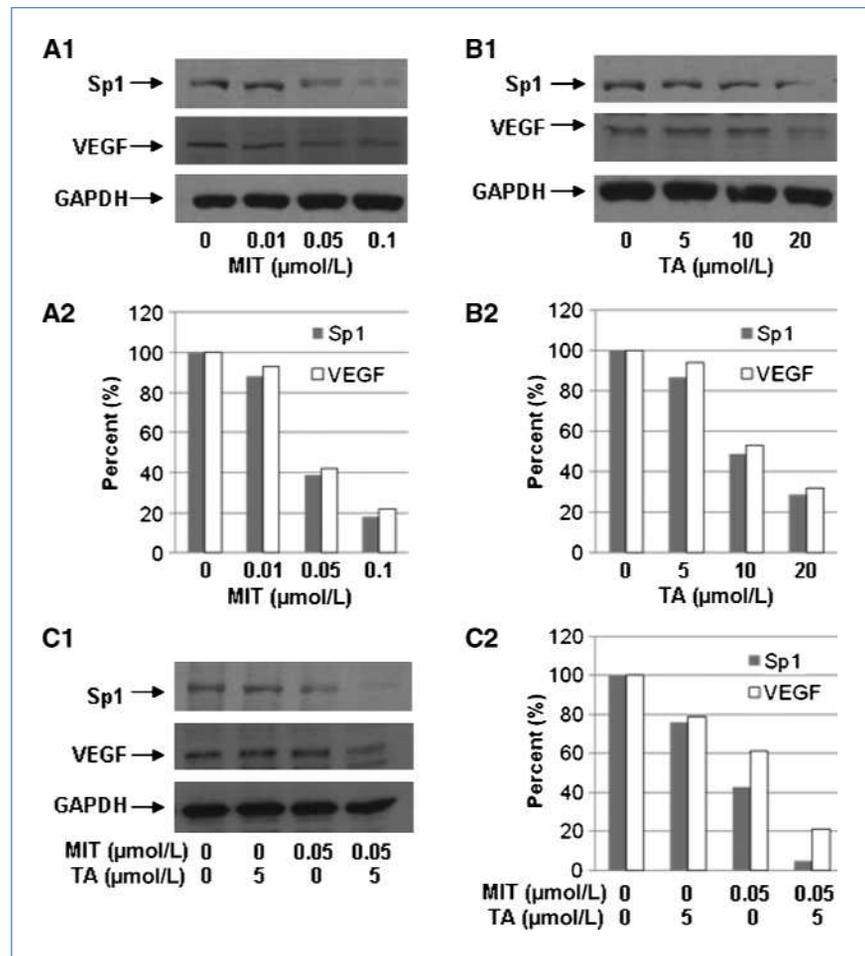
Next, we treated PANC-1 xenograft tumors in nude mice with nontoxic doses of MIT (0.05 mg/kg), TA (10 mg/kg), or both. We found that TA and MIT alone had marginal antitumor activity. In contrast, the combination of MIT and TA had significant antitumor activity (Fig. 2A). Furthermore, treatment with low doses of TA and MIT produced synergistic antitumor activity without any significant systemic side effects as indicated by a lack of significant weight loss (Fig. 2B). Therefore, combination administration of low doses of MIT and TA has a significant therapeutic benefit for pancreatic cancer.

**Effects of treatment with MIT and TA on Sp1 and VEGF expression and recruitment of Sp1 into their promoters *in vivo*.** To determine the molecular basis for the synergistic effect of treatment of pancreatic cancer with MIT and TA, we performed Western blot analysis using total protein lysates extracted from the PANC-1 tumor specimens collected from mice that received treatment with PBS, TA, MIT, or both TA and MIT as shown in Fig. 2. We also analyzed BxPC3



**Figure 3.** Analysis of Sp1 and VEGF expression in pancreatic tumors. The tumors described in Fig. 2 were collected and processed for gene expression analysis. Results of Western blot analysis of Sp1 and VEGF expression changes and the corresponding quantities are shown for tumors induced by PANC-1 (A) and BxPC3 (B) cells.

**Figure 4.** Treatment with TA and MIT downregulates Sp1 expression *in vitro*. PANC-1 cells were incubated in a medium alone or a medium containing MIT and/or TA. Total protein lysates were harvested from the cell cultures, and the level of Sp1 and VEGF protein expression was determined using Western blot analysis. Equal protein specimen loading was monitored by probing the same membrane filter with an anti-GAPDH antibody. A, PANC-1 cells were treated with MIT (0.01, 0.05, and 0.10  $\mu\text{mol/L}$ ) and protein specimens were harvested after 24 h of treatment. B, PANC-1 cells were treated with TA (5, 10, and 20  $\mu\text{mol/L}$ ) and protein specimens were harvested after 24 h of treatment. C, PANC-1 cells were treated with MIT (0.05  $\mu\text{mol/L}$ ) and TA (5  $\mu\text{mol/L}$ ) and protein specimens were harvested after 12 h of treatment. Note that the control groups (without MIT and/or TA treatment) were set to be 100%.



tumor specimens collected from mice that received the same treatment. As shown in Fig. 3A and B, expression of both Sp1 and VEGF protein was downregulated by treatment with the combination of TA and MIT. Furthermore, immunohistochemical staining showed that treatment with TA or MIT alone decreased expression of Sp1 and its downstream molecule VEGF in PANC-1 tumors (Supplementary Fig. S1). In addition, as indicated by CD31 staining, tumor MVDs were lower than those in the control group. However, treatment with MIT and TA dramatically reduced Sp1 and VEGF expression in the pancreatic tumors, which was consistent with the reduced MVDs. Furthermore, treatment with MIT and/or TA, especially the combination treatment, decreased PCNA protein expression in the tumors. These results suggested that the synergistic antitumor activity of the combination of MIT and TA may occur through not only an antiangiogenic effect but also direct inhibition of tumor cell proliferation.

**Effects of treatment with MIT and/or TA on Sp1 and VEGF protein levels in human pancreatic cancer cells.** To further confirm the effect of treatment with MIT and TA on gene expression in pancreatic cancer cells, we incubated PANC-1 and BxPC3 cells in a medium alone or a medium containing MIT (0.01, 0.05, or 0.10  $\mu\text{mol/L}$ ) and/or TA (5, 10,

or 20  $\mu\text{mol/L}$ ). Western blot analysis showed that Sp1 protein expression in the cells was downregulated in a dose-dependent manner after 24 hours of treatment with MIT and TA as single agents *in vitro* (Fig. 4A and B). Interestingly, combined treatment with low doses of MIT and TA for 12 hours significantly downregulated Sp1 protein expression, whereas that with MIT and TA alone did not (Fig. 4C). These findings suggested that, at high doses, treatment with MIT and TA as single agents significantly decreases Sp1 protein expression after ~24 hours and that, at low doses, treatment with MIT and TA in combination synergistically downregulates Sp1 protein expression within 12 hours.

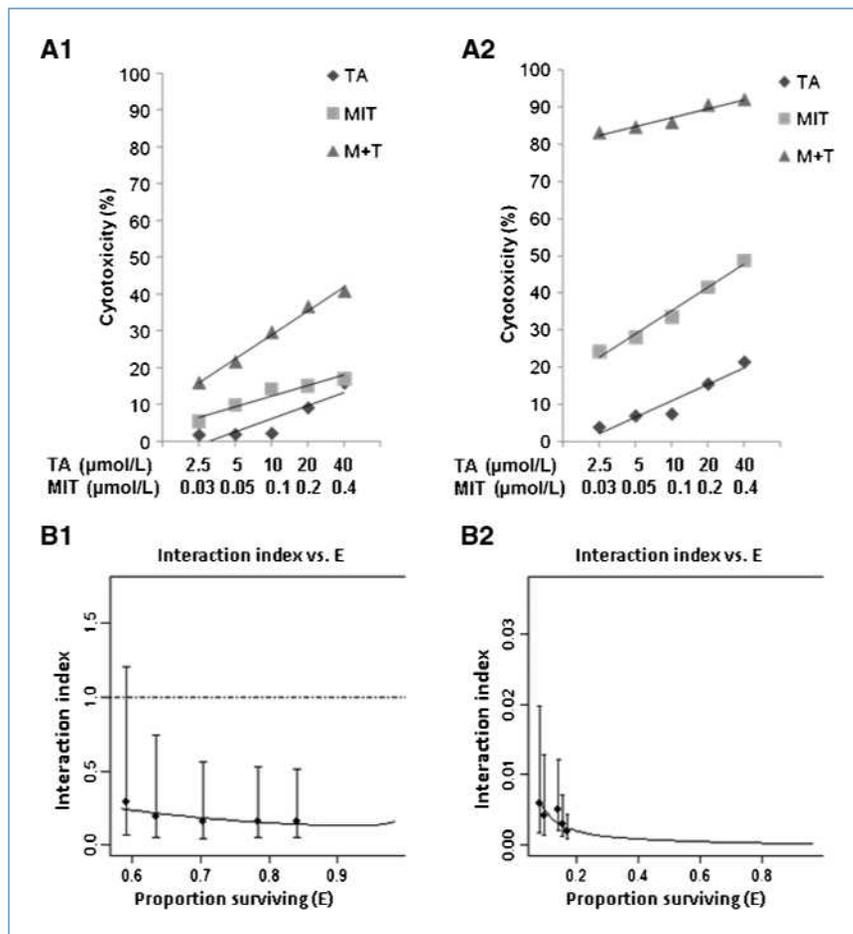
**Synergistic cytotoxicity of MIT and TA in human pancreatic cell lines *in vitro*.** To assess the direct cytotoxicity of MIT and TA, we treated FG cells with MIT (0.03, 0.050, 0.100, 0.200, or 0.400  $\mu\text{mol/L}$ ) and/or TA (2.5, 5.0, 10.0, 20.0, or 40.0  $\mu\text{mol/L}$ ) for 24 to 48 hours. Both drugs exhibited concentration-dependent cytotoxicity as determined using a MTT assay (Fig. 5A). We then optimized the drug concentrations so that neither agent alone had an extensive cytotoxic effect. Under this condition, the combination of MIT and TA had substantial cytotoxic effects. To determine the potential synergistic effect of combination treatment with MIT and

TA, we subjected the MTT cell viability data to further statistical analysis using the Loewe additivity model, which is among the best general reference models used to evaluate drug interactions (45). We used the S-PLUS/R software program to evaluate the interaction between MIT and TA in this model (Supplementary Fig. S2). We used Chou and Talalay's median-effect equation to perform the calculation (46). Supplementary Fig. S3 shows the estimated interaction indices from the corresponding fitted dose-effect curve (Fig. 5B). Synergy between MIT and TA occurs when the interaction index is  $<1$ , whereas antagonism occurs when the interaction index is  $>1$  (45). As shown in Fig. 5B, four of the five 24-hour and all five of the 48-hour data points were in the synergistic area, indicating that the combination of MIT and TA had a synergistic cytotoxic effect in FG cells. We also confirmed this synergy in BxPC3 cells (Supplementary Fig. S4).

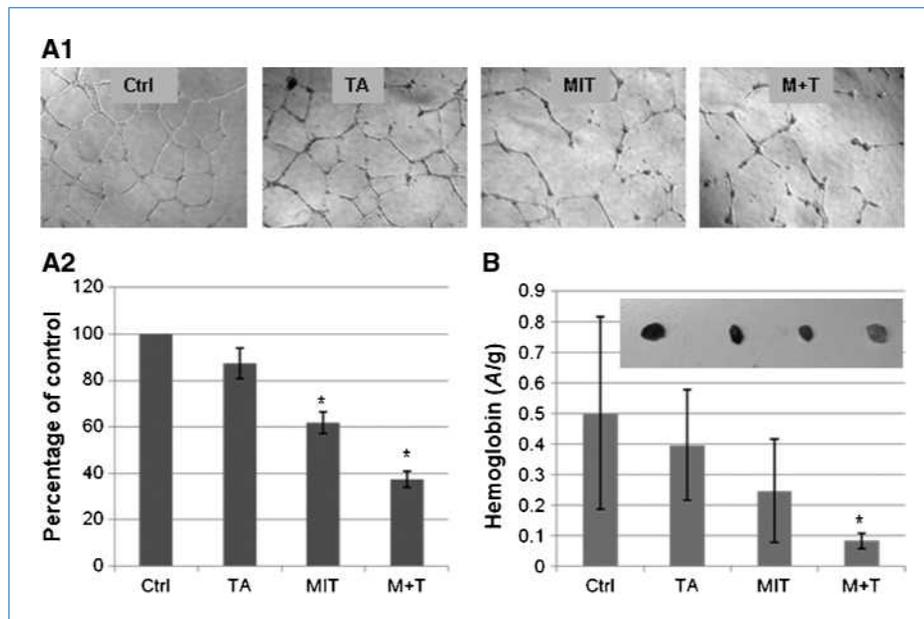
**Antiangiogenic effects of MIT and TA in vitro.** We treated PANC-1 cells with 50  $\mu\text{mol/L}$  TA and/or 0.1  $\mu\text{mol/L}$  MIT. Western blot analysis confirmed that Sp1 expression was downregulated in these cells. We then used an endothelial cell tube formation assay to determine the angiogenic potential of the supernatants of the PANC-1 cells. We assessed the degree of tube formation as the percentage of cell surface ar-

ea versus the total surface area (Fig. 6A). We obtained representative photomicrographs of tube formation by human umbilical vein endothelial cells in the supernatants *in situ* (Fig. 6A). Treatment with MIT and/or TA reduced the capacity of supernatants of the PANC-1 cells to stimulate tube formation by endothelial cells compared with that of supernatants of control PANC-1 cells. We confirmed this impaired angiogenic potential using an *in vivo* Matrigel plug assay (Fig. 6B). Our data suggested that treatment with MIT and/or TA impaired the angiogenic potential of PANC-1 cells.

**Effects of treatment with MIT and TA on recruitment of Sp1 into the Sp1 and VEGF promoters in human pancreatic cancer cells in vitro.** In this set of experiments, we sought to determine whether treatment with TA and/or MIT regulated Sp1 and VEGF expression at the transcriptional level. We transfected Sp1 and VEGF promoter reporter constructs into PANC-1 cells and then incubated them in a medium alone or a medium containing 5  $\mu\text{mol/L}$  TA or 0.01  $\mu\text{mol/L}$  MIT. *In vitro*, treatment with TA or MIT at the given dose resulted in low levels of suppression of Sp1 and VEGF promoter activity, whereas treatment with the combination of TA and MIT significantly suppressed this activity. However, further deletion of Sp1-binding sites eliminated the ability of



**Figure 5.** Synergistic effect of treatment with MIT and TA on inhibition of pancreatic cancer cell proliferation. PANC-1 cells were treated with MIT at concentrations of 0.03, 0.05, 0.10, 0.20, and 0.40  $\mu\text{mol/L}$  and TA at concentrations of 2.5, 5.0, 10.0, 20.0, and 40.0  $\mu\text{mol/L}$  for 24 and 48 h. A, inhibition of cell proliferation was assessed using a MTT assay. B, results of analysis of MIT and TA cytotoxicity using the S-PLUS/R software program. B1, 24 h. B2, 48 h.



**Figure 6.** Effect of treatment with MIT and TA on the PANC-1 cell angiogenic phenotype. Culture supernatants were harvested from PANC-1 cells treated with 0.5  $\mu\text{mol/L}$  MIT, 50  $\mu\text{mol/L}$  TA, or 0.5  $\mu\text{mol/L}$  MIT + 50  $\mu\text{mol/L}$  TA. The angiogenic potential of the supernatants was determined using an endothelial cell tube formation assay. A1, representative photographs of tube formation in the supernatants taken *in situ*. A2, assessment of the degree of tube formation as the percentage of cell surface area versus total surface area. Control cell cultures were given arbitrary percentage values of 100. B, for a Matrigel plug assay, Matrigel (200  $\mu\text{L}$ ) containing  $2 \times 10^6$  untreated PANC-1 cells or PANC-1 cells treated with 0.5  $\mu\text{mol/L}$  MIT, 50  $\mu\text{mol/L}$  TA, or 0.5  $\mu\text{mol/L}$  MIT + 50  $\mu\text{mol/L}$  TA was used as described in Materials and Methods. Of note is that downregulation of Sp1 expression impaired the angiogenic potential of pancreatic cancer cells *in vitro* and *in vivo*. \*,  $P < 0.01$ , compared with respective controls (Student's *t* test).

MIT to suppress Sp1 and VEGF promoter activity (Supplementary Fig. S5). Finally, we performed a ChIP assay using pancreatic tumors formed by PANC-1 cells in nude mice that received treatment as described in Fig. 2. Treatment with TA or MIT at the given dose had a minor effect on inhibition of Sp1 recruitment to its own reporter and the VEGF promoter, whereas treatment with TA combined with MIT at the same dose significantly decreased Sp1 recruitment to these two promoters (Supplementary Fig. S6). These results suggested that treatment with TA and MIT at low doses results in insignificant transcriptional suppression of Sp1 and VEGF mRNA transcription activated by Sp1, whereas treatment with TA combined with MIT at the same doses produces synergistic transcriptional suppression of Sp1 and VEGF transcription.

## Discussion

In this study, we found that treatment with the combination of MIT and TA at low doses synergistically downregulated the expression of Sp1 and VEGF and produced synergistic antitumor effects in xenograft mouse models of human pancreatic cancer. This therapeutic effect was consistent with suppression of the activity of Sp1 and downregulation of the expression of its downstream proangiogenic molecule, VEGF. Our experimental results indicated that MIT targets Sp1 at the transcriptional level by inhibiting Sp1 recruitment into the Sp1 sites of its own promoter, whereas TA facilitates Sp1 protein degradation (Supplemen-

tary Fig. S7). This study is the first to show synergistic downregulation of expression of the transcription factor Sp1 and an enhanced therapeutic index resulting from the combined administration of two drugs having distinct mechanisms of action in pancreatic cancer.

Angiogenesis plays an important role in the growth and metastasis of pancreatic tumors. We have shown that both Sp1 and VEGF are important to pancreatic tumor angiogenesis (18, 21). Other studies have shown that VEGF-targeting antiangiogenic therapies inhibit pancreatic tumor growth in mouse models. Researchers have developed strategies targeting VEGF receptors, including the use of anti-VEGF antibodies, to directly interfere with its signal effect (47, 48). However, resistance to anti-VEGF antibodies occurs in both animal models and humans. Although the mechanisms of this resistance are not entirely clear at present, a previous study by our group suggested that upregulation of Sp1 expression may play a critical role (21). Specifically, treatment with Avastin increased Sp1 protein expression and activity in pancreatic tumors and significantly upregulated expression of VEGF. In contrast, treatment with MIT, which inhibits Sp1 expression, inhibited VEGF expression in the tumors and sensitized them to the antitumor activity of Avastin (21).

However, downregulation of Sp1 protein expression in pancreatic tumors requires prolonged treatment with MIT, increasing the occurrence of systemic side effects (21). Studies have suggested that MIT inhibits Sp1 expression via direct competition for Sp1 recruitment into Sp1 sites of the Sp1 promoter (21, 32). Although MIT can effectively block Sp1

mRNA synthesis, the abundance and strong stability of the Sp1 protein in pancreatic tumor cells prevent MIT from rapidly downregulating Sp1 protein expression. In the present study, we showed that TA can promote Sp1 protein downregulation, which is consistent with a previous finding of TA-facilitated Sp1 degradation (38). More importantly, combined treatment with MIT and TA, neither of which has significant effects on Sp1 protein expression, substantially downregulated Sp1 protein expression, which was consistent with the synergistic antitumor effect in our mouse model.

Studies have shown that several nonsteroidal anti-inflammatory drugs have antiangiogenic activity in a wide variety of xenograft models, including celecoxib (19) and TA (40). Although Sp1 is the primary target of these drugs, they clearly induce degradation of other members of the Sp1 family, such as Sp3 and Sp4 (39, 40). Experimental results showed that via activation of proteasome-dependent degradation of Sp proteins, celecoxib and TA exhibited growth-inhibitory effects via an antiangiogenic strategy (19, 39, 40). However, downregulation of Sp1 expression by MIT-based treatment is primarily involved in transcriptional repression of Sp1 expression (21). Therefore, MIT and TA have distinct mechanisms of action in regulation of Sp1 expression and activity, which is the molecular basis for their synergistic antiangiogenic and antitumor activity.

In addition to its reported antiangiogenic function that is consistent with our finding using cDNA microarray analysis (Supplementary Fig. S8), downregulation of Sp1 may alter the expression of genes important to cell survival, a mechanism that is likely responsible for the antitumor activity of TA and MIT. For example, TA-based treatment activates Sp protein degradation, decreases Sp protein binding to the survivin promoter, and inhibits survivin expression in pancreatic cancer cells and subsequently sensitizes the cells to radiotherapy (39, 49). Consistently, our results showed that TA inhibits tumor cell growth *in vitro* and that this effect is synergistic with that of MIT. Altered survivin expression may be one of the mechanisms underlying the cytotoxic effect of TA and MIT.

As our experimental results showed, single-agent MIT is significantly cytotoxic when it has a significant antitumor ef-

fect using mouse body weight change as the measurement of systemic side effect. When we administered MIT and TA together, Sp1 protein expression was synergistically downregulated through different level and tumor growth was significantly inhibited. However, we observed no detectable cytotoxic effects of MIT and TA. Our data suggested that administration of the combination of TA and MIT may achieve the highest therapeutic index.

Collectively, our results suggest that MIT competes Sp1 recruitment to Sp1 sites in both Sp1 and VEGF promoters. TA does not have this competing ability, but it downregulates Sp1 protein expression by directly targeting Sp1 at the protein level (Supplementary Fig. S9). The use of low-dose MIT in combination with low-dose TA is an important novel strategy of targeting the angiogenic molecule Sp1 at both the transcriptional and the protein degradation level. Treatment with the combination of MIT and TA in clinical studies is a rational step forward in the development of effective targeted therapies for pancreatic cancer as well as other cancers.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

We thank Don Norwood for editorial comments.

### Grant Support

National Cancer Institute, NIH, Pancreatic Cancer Specialized Program of Research Excellence grants 1P20-CA101936-01-PP4 and 5R01-CA129956 (K. Xie) and M.D. Anderson Cancer Center Institutional Start-up Funds (D.Z. Chang).

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Received 9/2/09; revised 11/23/09; accepted 11/30/09; published OnlineFirst 1/19/10.

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*Cancer Res* 2010;70:1111-1119. Published OnlineFirst January 19, 2010.

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