TW-37, a small-molecule inhibitor of Bcl-2, inhibits cell growth and invasion in pancreatic cancer

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Bcl-2 family of proteins plays critical roles in human cancers, including pancreatic cancer, suggesting that the discovery of specific agents targeting Bcl-2 family proteins would be extremely valuable for pancreatic cancer therapy. We have previously reported the synthesis and characterization of TW-37, which seems to be a negative regulator of Bcl-2. In this investigation, we tested our hypothesis whether TW-37 could be an effective inhibitor of cell growth, invasion and angiogenesis in pancreatic cancer cells. Using multiple cellular and molecular approaches such as MTT assay, apoptosis enzyme-linked immunosorbent assay, real-time reverse transcription-polymerase chain reaction, Western blotting, electrophoretic mobility shift assay for measuring DNA binding activity of NF-κB, migration, invasion and angiogenesis assays, we found that TW-37, in nanomolar concentrations, inhibited cell growth in a dose- and time-dependent manner. This was accompanied by increased apoptosis and concomitant attenuation of NF-κB, and downregulation of NF-κB downstream genes such as *MMP-9* and *VEGF*, resulting in the inhibition of pancreatic cancer cell migration, invasion and angiogenesis *in vitro* and caused antitumor activity *in vivo*. From these results, we conclude that TW-37 is a potent inhibitor of progression of pancreatic cancer cells, which could be due to attenuation of Bcl-2 cellular signaling processes. Our findings provide evidence showing that TW-37 could act as a small-molecule Bcl-2 inhibitor on well-characterized pancreatic cancer cells in culture as well as when grown as tumor in a xenograft model. We also suggest that TW-37 could be further developed as a potential therapeutic agent for the treatment of pancreatic cancer.
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Pancreatic cancer (PC) is one of the most common cancers and is the fourth leading cause of cancer-related death in the United States. It is estimated that 37,000 new PC patients would be diagnosed and 33,370 deaths are expected in 2007.[1] This could be due to its late diagnosis and a lack of effective treatment options. Presently, for all stages combined, the 1-year survival rate is only 20%, and the 5-year survival rate is less than 5%.[1] This disappointing outcome strongly suggests that there is a dire need for innovative research that could lead to a dramatic improvement in the survival of patients diagnosed with this deadly disease.

PC like many other tumors has been shown to overexpress the Bcl-2 and/or its family members.[2,3] Therefore, novel avenues by which Bcl-2 could be inactivated represent a promising strategy for the development of novel and selective anticancer therapies. The Bcl-2 family members are important proteins that regulate the program cell death in cancer cell lines. It includes both death antagonists such as Bcl-2, Bcl-X_L and Mcl-1 as well as death agonists such as Bax, Bak, Bid and Bad.[4] An imbalance between antiapoptotic proteins (such as Bcl-2, Bcl-X_L and Mcl-1) and proapoptotic proteins (such as Bax and Bcl-xs) is involved in the distinctive biological features of adenocarcinomas.[3] In PC, anti-apoptosis proteins Bcl-2, Bcl-X_L and Mcl-1 are highly over-expressed.[5] Clinical data showed that the enhanced expression of Bcl-2 and Bcl-X_L is related to a shorter patient survival, whereas the upregulation of Bax is associated with longer survival and

these findings suggest that the modulation of apoptotic pathways might be one of the reasons why PC shows only limited sensitivity to anticancer treatment.[3,6] Therefore, blockade of Bcl-2 activity should result in the suppression of tumor progression and should become a novel therapeutic strategy for PC.

TW-37, a recently developed small-molecular inhibitor of Bcl-2, appears to attenuate Bcl-2 activation.[7–9] We have found that TW-37 inhibits the growth of a variety of cancer cells, including breast, prostate and lymphoma *in vitro* and tumor growth *in vivo*.[7–9] Although there has been rapid progress for elucidating the mechanism of action of TW-37 as an antitumor agent, the exact mechanism has not yet been fully established. We investigated whether TW-37-induced inhibition of PC cell growth could be attributed to Bcl-2 activity and its associated signaling, especially inactivation of nuclear factor-κB (NF-κB) activity.[10,11] Moreover, since cell migration, invasion and angiogenesis are important processes that are involved in tumor development and metastasis and because Bcl-2 signaling is known to control these processes,[8,10,11] we also examined the effect of TW-37 on the processes of migration, invasion and angiogenesis of PC cells.

Overall in this study, we investigated the role and mechanism(s) by which TW-37 may lead to the attenuation of NF-κB, thereby inhibiting the growth of PC cells *in vitro* and in SCID xenograft model. We found that the inactivation of Bcl-2 by TW-37 down-regulated the NF-κB DNA-binding activity and its downstream genes, including Cyclin D1, COX-2, Survivin, MMP-9 and VEGF, resulting in the inhibition of PC cell growth, migration, invasion and angiogenesis.

Material and methods

Cell culture and experimental reagents

Human PC cell lines AsPC-1, BxPC-3, Colo-357, HPAC, L3.6pl, MIAPaCa and PANC-1 were used in this study. BxPC-3, HPAC and PANC-1 (ATCC, Manassas, VA) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. AsPC-1, Colo-357, L3.6pl and MIAPaCa cells were gener-

FIGURE 1 – (*a*,*b*), Chemical structure of TW-37 and TW-37a. (*c*), The level of Bcl-2 expression was compared between a panel of pancreatic cancer cell lines. The expression of protein was assayed by Western blot analysis. (*d*), NF-κB activation was evaluated by the EMSA between a panel of pancreatic cancer cell lines, including AsPC-1, BxPC-3, Colo-357, HPAC, L3.6pl, MIAPaCa and PANC-1 cell lines (lanes 1–7, respectively). Retinoblastoma protein level served as the nuclear protein loading control. Supershift assay showed that NF-κB band was shifted because of the formation of a bigger complex after addition of anti- NF-κB p65 antibody. This assay confirmed the specificity of NF-κB binding to the DNA consensus sequence. Lane 1, nonspecific antibody (anti-cyclin D1); lane 2, p65 antibody.
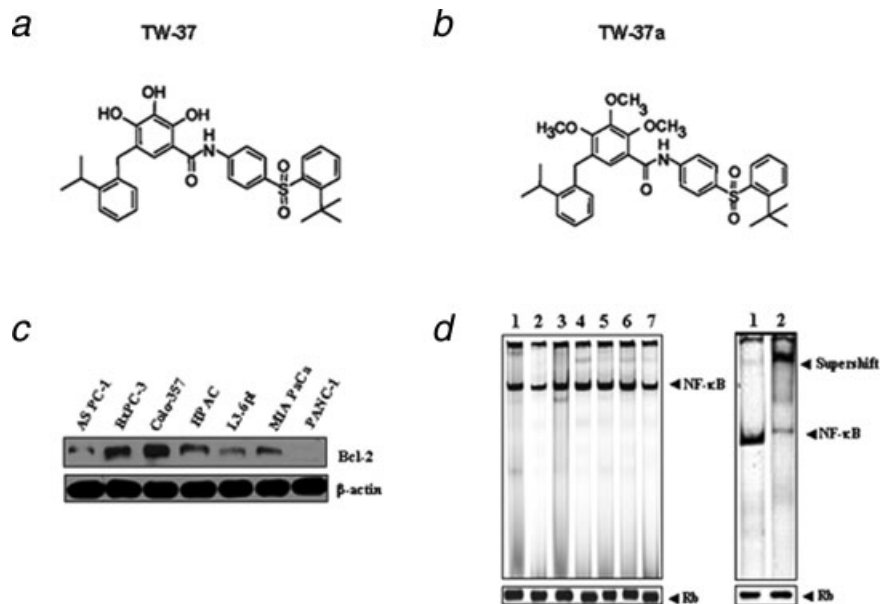
ously provided by Dr. Paul Chiao at M.D. Anderson Cancer Center (Houston, TX), and grown as a monolayer cell culture in DMEM containing 4.5 mg/ml D-glucose and L-glutamine supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs, ATCC, Manassas, VA) was cultured in F12K medium (ATCC, Manassas, VA) supplemented with 10% FBS, 0.1 mg/ml heparin sulfate, 0.05 mg/ml endothelial cell growth factor supplement (BD Bioscience, San Jose, CA), 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a 5% CO_2-humidified atmosphere at 37°C. Cell death enzyme-linked immunosorbent assay (ELISA) kit was obtained from Roche (Indianapolis, IN). Primary antibodies for Bcl-2, Cyclin D1, MMP-9, Survivin, IKKβ, COX-2 and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Pierce (Rockford, IL). Chemiluminescence detection of proteins was done with the use of a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and all other chemicals were obtained from Sigma (St. Louis, MO).

TW-37

Design, synthesis, purification and chemical characterization of *N*-[(2-tert-butyl-benzenesulfonyl)-phenyl]-2,3,4-trihydroxy-5-(2-isopropyl-benzyl)-benzamide (TW-37) is described in detail by Wang *et al.*[12]; in the inactive congener TW-37a [compound 6 in ref. 12], all 3 hydroxyl groups in the polyphenolic ring have been substituted with a methyl group, resulting in a 100-fold loss of binding (see Fig. 1*a* for structures).

Cell growth inhibition studies by MTT assay

The PC cells (5×10^3) were seeded in a 96-well culture plate and subsequently incubated with MTT reagent (0.5 mg/ml) at 37°C for 2 hr and MTT assay was performed as described earlier.[13] The results were plotted as means ± SD of 3 separate experiments having 6 determinations per experiment for each experimental condition.

Histone/DNA ELISA for detection of apoptosis

The cell death detection ELISA kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, cells were treated with TW-37 for different periods of time. After treatment, the cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis as described earlier.[14]

Western blot analysis

Cells were lysed in lysis buffer [50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/l sodium orthovanadate, 10 μl/mL protease inhibitor cocktail and 1 mmol/l PMSF] by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS-PAGE and transferred onto a nitrocellulose membrane for Western blotting as described earlier.[14]

Real-time reverse transcription-PCR analysis for gene expression studies

The total RNA from treated cells was isolated by Trizol (Invitrogen, Carlsbad, CA) and purified by RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's protocols. One microgram of total RNA from each sample was subjected to first strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 50 μl, including 6.25 U Multi-Scribe reverse transcriptase and 25 pmol random hexamers. RT reaction was performed at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. Real-time polymerase chain reaction (PCR) amplications were performed as described earlier.[13]

Plasmids and transfections

Bcl-2 siRNA and p65 siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Bcl-2 cDNA plasmid was generated as described earlier.[15] Human PC cells, BxPC-3 and Colo-357 were transfected with Bcl-2 siRNA, p65 siRNA, Bcl-2 cDNA and p65 cDNA, respectively, using Lipofectamine 2000 as described earlier.[16] After 7 hr, the p65 transfected cells were treated with 500 nM TW-37 as described earlier. Then nuclear proteins were extracted. NF-κB DNA-binding activity was measured by electrophoretic mobility shift assay (EMSA). Also, the apoptotic cells in the p65 transfected cells with different treatments were detected using cell apoptosis ELISA detection kit (Roche).

Electrophoretic mobility shift assay for measuring NF-κB activity

BxPC-3 and Colo-357 PC cells exposed to TW-37 or kept as control were washed with cold phosphate-buffered saline and sus-

pended in 0.15 ml of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM ethylene-diamine-tetra-acetic acid (EDTA), 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin and 0.5 mg/ml benzamidine]. The nuclear protein was prepared and subjected to DNA binding activity of NF-κB by EMSA as described earlier.[13]

VEGF assay

Human Colo-357 PC cells were seeded in 6-well plates (1.0 × 10^5 cells per well) and incubated at 37°C. After 24 hr, the cells were incubated in medium supplemented with TW-37 for 72 hr. The cell culture supernatant was harvested and cell count was performed after trypsinization. After collection, the medium was spun at 800g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for VEGF assay later or assayed immediately using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

MMP-9 activity assay

Human Colo-357 PC cells were seeded in 6-well plates and incubated at 37°C. After 24 hr, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium supplemented with TW-37 for 72 hr. MMP-9 activity in the medium was detected using Fluorokine E Human MMP-9 Activity Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Cell migration and invasion assay

Cell migration was assessed using 24-well inserts (BD Biosciences, Bedford, MA) with 8 μm pores according to the manufacturer's protocol. The invasive activity of cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA). Briefly, human Colo-357 PC cells (5 × 10^4) with serum-free medium supplemented were seeded into the upper chamber of the system and treated with 250 nM TW-37. Bottom wells in the system were filled with complete medium. After 24 hr of incubation, the cells in the upper chamber were removed and the cells that had invaded through matrigel matrix membrane were stained with 4 μg/ml Calcein AM in Hanks buffered saline at 37°C for 1 hr. The fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

Matrigel in vitro HUVECs tube formation assay

Human Colo-357 PC cells cultured in serum-free RPMI 1640 were treated with 250 nM TW-37 for 24 hr. The conditioned media were collected, centrifuged, transferred to fresh tubes, and stored at −20°C. HUVECs were purchased from ATCC (Manassas, VA) and cultured in F12K medium supplemented with 10% FBS, 0.1 mg/ml heparin sulfate, 0.05 mg/ml endothelial cell growth factor supplement (BD Bioscience San Jose, CA), 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. Growth factor reduced Matrigel (125 μl), after being thawed on ice, was plated in 8-well chamber. The chamber was then incubated at 37°C for 30 min to allow the matrigel to polymerize. HUVECs were trypsinized and seeded (5 × 10^4 cells/well) in each well with 250 μl of conditioned medium from TW-37 treated Colo-357 cells. The chamber was incubated for 6 hr. Each well was photographed using an inverted microscope with digital camera. The vessel number and length of vessel perimeter in each of the entire field were carried out using Scion Image analysis program.

Colo-357 Xenografts

Four-week-old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice were adapted to animal housing and Colo-357 xenografts were developed as described earlier.[17] Briefly, 3 mice received 10^7 Colo-357 cells (in serum-free RPMI 1640) s.c. in each flank area. When s.c. tumors developed to about 1,500 mg, the tumors were excised, and serial propagation was accomplished by trimming extraneous material, cutting the tumors into fragments of 20–30 mg, which were then transplanted s.c. using a 12-gauge trocar into the flanks of a new group of mice for maintenance of tumors as well as for experimental purpose. For the subsequent drug efficacy trials, small fragments of the Colo-357 xenograft were implanted s.c. and bilaterally into naive, similarly adapted mice. Mice were checked 3 times per week for tumor development. Once transplanted, Colo-357 fragments developed into palpable tumors (60–100 mg); animals were removed randomly and assigned to different treatment groups. Using this model, the efficacy of TW-37 was studied. TW-37's maximum tolerated dose in SCID mice was previously determined in our laboratory.[9] Mice were injected with TW-37 at 20 mg/kg i.v., 3 consecutive days/week, for 2 weeks (as shown in Fig. 6a). Mice in the control and TW-37-treated group were followed for measurement of s.c. tumors, changes in body weight and side effects of the drugs. Tumors were measured 2 times per week. Tumor was calculated using the formula $(A \times B^2)/2$, where A and B are the tumor length and width (in mm). To avoid discomfort in the control group, animals were euthanized when their total tumor burden reached 2,000 mg. Tumor tissues were harvested for Western Blotting and NF-κB activity analysis. All studies involving mice were performed under Animal Investigation Committee-approved protocols. Tumor volumes in SCID mice were plotted against time on a semilog sheet with the growth pattern resembling an S-shape. Tumor doubling (Td) is the time (in days) required in order for the tumor to double its weight during the exponential growth phase.

Densitometric and statistical analysis

The cell growth inhibition by TW-37 treatment was statistically evaluated using GraphPad StatMate software (GraphPad Software, San Diego, CA). Comparisons were made between control and TW-37 treatment. $p < 0.05$ was used to indicate statistical significance.

Results

TW-37-induced cell growth inhibition of BxPC-3, HPAC and Colo-357 cells

The baseline expression of Bcl-2 was determined in a panel of human PC cell lines that included AsPC-1, BxPC-3, Colo-357, HPAC, L3.6pl, MIAPaCa and PANC-1. The results showed that Bcl-2 was frequently but differentially expressed in different human PC cell lines (Fig. 1c). It has been reported that Bcl-2 over-expression enhanced the NF-κB activity and NF-κB is frequently constitutively activated in various types of cancer.[10,11] Therefore, we also examined the NF-κB DNA-binding activity in 7 PC cell lines. All 7 cell lines expressed different levels of NF-κB DNA-binding activity (Fig. 1d). The specificity of NF-κB DNA binding to the DNA consensus sequence was confirmed by supershift assay using p65 antibodies. Next, we examined the growth inhibitory effects of TW-37 using the MTT assay in 3 human PC cell lines such as BxPC-3, HPAC and Colo-357. The reason for choosing these 3 PC cell lines was due to the fact that these cell lines showed higher expression of Bcl-2. The treatment of PC cells for 1–3 days with 250, 500 and 750 nM of TW-37 resulted in cell growth inhibition in a dose- and time-dependent manner in all 3 PC cell lines (Fig. 2a). To confirm cell growth inhibition, we have also conducted the cell proliferation assay using the BrdU labeling and Detection Kit (Roche, Indianapolis, IN). We found similar results as MTT assay using this method (data not shown). Next, we examined whether the inhibition of cell growth was also accompanied by the induction of apoptosis induced by TW-37. DNA/histone fragmentation analysis was employed to investigate the degree of apoptosis induced by TW-37.
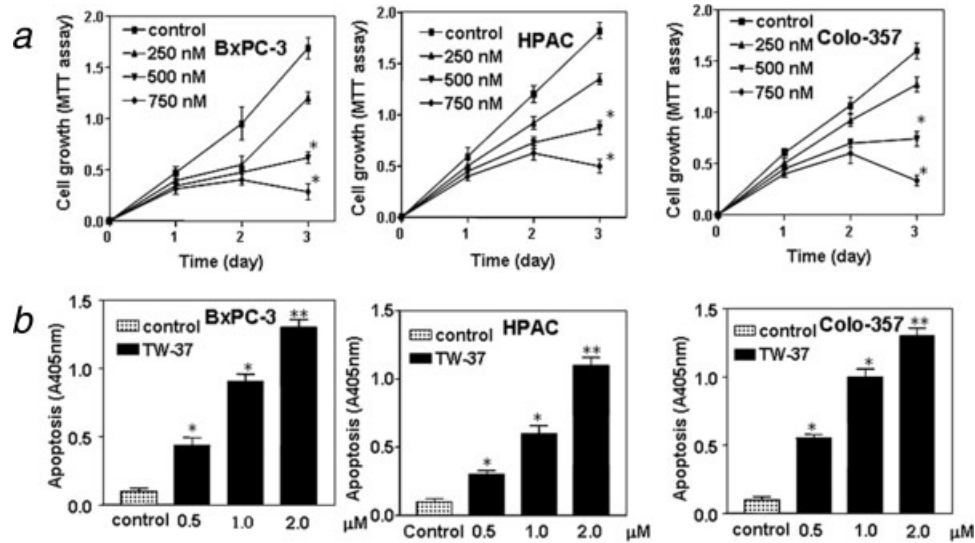
FIGURE 2 – Effect of TW-37 on pancreatic cancer cell growth and apoptosis. (*a*) Dose and time responses of TW-37 on growth of pancreatic cancer cells. Cells were seeded in 96-well plates at 5,000 cells per well and treated with varied concentrations of TW-37 or for different time periods. After treatment, cell densities were determined by MTT assay. Each value represents the mean $\pm$ SD ($n = 6$) of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, compared to the control. (*b*) Cell death assay for measuring apoptosis induced by TW-37.BxPC-3, HPAC and Colo-357 cells were cultured in RPMI containing 5% FBS and exposed to different dose TW-37 for 72 hr. Apoptosis was measured by Histone DNA ELISA. Values are reported as mean $\pm$ SD. *$p < 0.05$, **$p < 0.01$, compared to the control.

TW-37 induced apoptosis in pancreatic cancer cell lines

BxPC-3, HPAC and Colo-357 cells were treated with 0.5, 1.0 and 2.0 μM TW-37 for 72 hr. After treatment, the degree of apoptosis was measured in all 3 cell lines. The induction of apoptosis was found to be dose-dependent (Fig. 2*b*). These results provided convincing data showing that TW-37 could induce apoptosis in PC cells. To further understand the molecular mechanism involved in TW-37-induced apoptosis of PC cells, alterations in the cell survival pathway were investigated.

Inhibition of NF-κB activation by TW-37

It has been shown that there is a cross-talk between Bcl-2 and NF-κB.[10,11] Indeed, we also found that overexpression of Bcl-2 by cDNA transfection increased NF-κB DNA-binding activity. However, downregulation of Bcl-2 by siRNA inhibited the NF-κB DNA-binding activity in Colo-357 cells (Fig. 3*a*) and we found similar results in BxPC-3 cells (data not shown). Therefore, we investigated whether the downstream effect of TW-37 induced by inhibition of Bcl-2 was mechanistically associated with NF-κB pathway. Nuclear extracts from control and TW-37-treated PC cells were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. We found that TW-37 significantly inhibited NF-κB DNA-binding activity in a dose- and time-dependent manner in Colo-357 and BxPC-3 PC cell lines compared to the control (Fig. 3*a*). These results indicated that TW-37 decreases NF-κB DNA-binding activity in PC cells.

TW-37 inhibited NF-κB-dependent reporter gene expression

Though our results have shown by EMSA that TW-37 inhibited NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription. To confirm our results, we also determined the expression of NF-κB-dependent gene products, including Cyclin D1, Survivin, VEGF, MMP-9 and COX-2. Western blot analysis showed that TW-37 inhibited the expression of these genes in Colo-357 cells (Fig. 3*b*). These results further support the role of TW-37 in blocking NF-κB-regulated gene products. To determine whether the reduction of NF-κB DNA binding activity by TW-37 was due to decreased IKK protein, we investigated whether TW-37 could affect the level of expression of the IKK protein by Western blot analysis.

TW-37 inhibited expression of IKKβ and phosphorylation of IκBβ

IKKβ, an IκBα kinase, has been identified to phosphorylate inhibitory proteins of NF-κB complex and retain NF-κB in the cytoplasm in an inactive form. In this study, IKKβ activation was inhibited by TW-37 (Fig. 3*c*). These results suggest that the functional IKK complex, which is important for IκB phosphorylation, could be efficiently inactivated by TW-37. Next, we investigated whether TW-37 blocks phosphorylation of the inhibitory protein IκB. We found that TW-37 inhibited only the phosphorylated form of IκBα (Fig. 3*c*), but there was no change in IκBα level (data not shown).

Apoptosis-enhancing effect of TW-37 is mediated through the NF-κB pathway

We transfected NF-κB p65 cDNA or siRNA into Colo-357 cells, treated the transfected cells with TW-37, and detected NF-κB DNA-binding activity and apoptosis. We found that p65 cDNA transfection enhanced the NF-κB DNA-binding activity, and inhibited apoptosis in TW-37-treated and untreated Colo-357 cells (Figs. 3*d* and 4*a*). In contrast, p65 siRNA transfection inhibited the activation of NF-κB and enhanced apoptosis induced by TW-37 (Figs. 3*d* and 4*a*). Moreover, we found that TW-37 treatment combined with p65 siRNA transfection exerted greater inhibitory effect on the activation of NF-κB, resulting in a more pronounced effect on the induction of apoptosis (Figs. 3*d* and 4*a*). These results provide mechanistic support in favor of our claim that the apoptosis-inducing effect by TW-37 is partly mediated through the inactivation of NF-κB pathway.

TW-37 decreased MMP-9 gene transcription and its activity

To explore whether TW-37 decreased MMP-9 gene at the transcriptional level, Real-time RT-PCR was conducted to determine the alteration in MMP-9 mRNA. We found that MMP-9 mRNA was dramatically decreased in the TW-37-treated Colo-357 PC cells (Fig. 4*b*). Next, we examined whether TW-37 could lead to a decrease in MMP-9 activity. There was about 2–3-fold decrease in the activity of MMP-9 in 500 nM TW-37-treated PC cell lines (Fig. 4*b*). These results are consistent with our observation of down regulation of NF-κB activity that leads to transcriptional down regulation of MMP-9 and its activity.
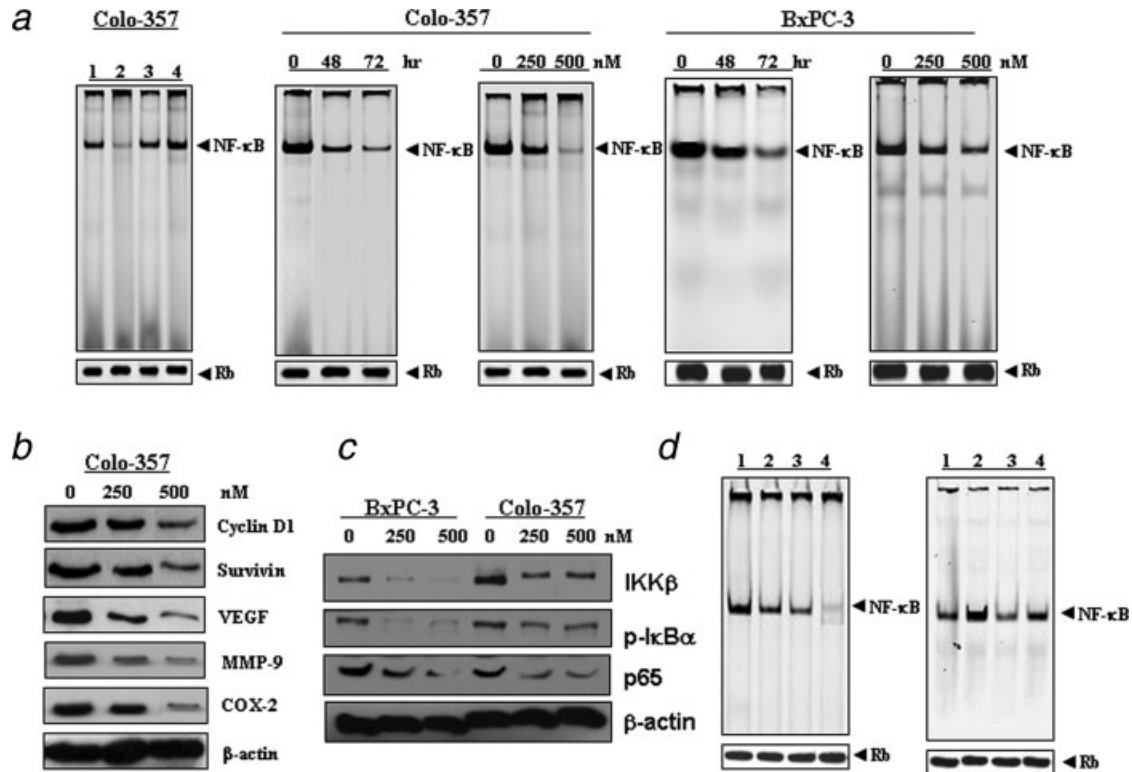
FIGURE 3 – Inhibition of NF-κB activation and the expression of its target genes by TW-37. (*a*), EMSA analysis was done for pancreatic cancer cells. Nuclear extracts were prepared from control and treated cells and subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. Retinoblastoma protein level served as the nuclear protein loading control. Left panel: over-expression of Bcl-2 by cDNA transfection increased NF-κB DNA-binding activity. However, down-regulation of Bcl-2 by siRNA inhibited the NF-κB DNA-binding activity in Colo-357 pancreatic cancer cells. 1, control siRNA; 2, Bcl-2 siRNA; 3, control plasmid; 4, Bcl-2 cDNA plasmid. Middle and right panel: inhibition of NF-κB DNA binding activity by 500 nM TW-37 in time-dependent manner and dose-dependent manner for 72 hr in Colo-357 and BxPC-3 pancreatic cancer cells, respectively. (*b*) Inhibition of NF-κB target gene expression by TW-37 treatment of Colo-357 pancreatic cancer cells for 72 hr. Western blot analysis showed that TW-37 inhibited the expression of Cyclin D1, Survivin, VEGF, MMP-9, and COX-2 genes in pancreatic cancer cells. (*c*), TW-37 inhibited the expression of IKKβ, pIκBα and p65 in BxPC-3 and Colo-357 cell lines. (*d*), Left panel: inhibition of NF-κB DNA-binding activity by p65 siRNA and TW-37 in Colo-357 cells tested by EMSA (1, control; 2, p65 siRNA; 3, 500 nM TW-37; 4, p65 siRNA and 500 nM TW-37). Right panel: the effect of p65 cDNA transfection and TW-37 on the NF-κB DNA-binding activity (1, control; 2, p65 cDNA; 3, 500 nM TW-37; 4, p65 cDNA and 500 nM TW-37).

TW-37 reduced VEGF gene transcription and its secretion

To further investigate whether TW-37 has any effect on VEGF reduction, whose expression is transcriptionally regulated by NF-κB, real-time RT-PCR was performed to examine the transcription level of VEGF. We found that VEGF mRNA level was significantly reduced in the TW-37-treated Colo-357 PC cells (Fig. 4*c*). Most importantly, we also found that TW-37 could lead to a decrease in the levels of VEGF secreted in the culture medium (Fig. 4*c*).

TW-37 decreased pancreatic cancer cell migration and invasion

MMP-9 and VEGF are thought to be critically involved in the processes of tumor cell migration, invasion and metastasis. Because TW-37 inhibited the expression and activity of MMP-9 and VEGF, we tested the effects of TW-37 on cancer cell migration and invasion. We found that 250 nM TW-37 decreased Colo-357 PC cell migration (Fig. 5*a*). Moreover, as illustrated in Figure 5*b*, 250 nM TW-37 treated cells showed a low level of penetration through the matrigel-coated membrane compared to the control cells. The value of fluorescence from the invaded PC cells was decreased about 5–6-fold compared to that of control cells (Fig. 5*b*). We also found that 500 nM TW-37 showed a lower level of penetration through the membrane compared to the 250 nM TW-37 treated cells, suggesting that TW-37 has a dose dependency effect on cancer cell migration and invasion.

Reduced tube formation of HUVECs induced by conditioned media from TW-37 treated pancreatic cancer cells

Because TW-37 inhibited VEGF expression, we tested whether conditioned media from TW-37-treated Colo-357 cells could reduce the tube formation, an indirect measure of angiogenesis. We performed the tube formation assay in growth factor reduced matrigel *in vitro*. As shown in Figure 5*c*, conditioned media from 250 nM TW-37-treated cells were able to significantly reduce the tube formation of HUVECs in 6 hr incubation compared to the medium from control cells.

Effect of TW-37 on pancreatic tumor growth *in vivo*

To determine whether systemic therapy with TW-37 could stunt tumor growth in animals, we established Colo-357 human PC xenografts in SCID mice as described earlier.[14] We found that mice in all treatment groups developed SC tumors. As shown in Figures 6*a* and 6*b*, TW-37 treatment significantly inhibited tumor growth (*p* = 0.015 *vs.* vehicle) compared to untreated control. We weighed the mice over 20 days of treatment using the same treatment dose of TW-37. TW-37 did not cause any toxicity or loss in body weight during the course of the treatment and up to 20 days. We subsequently asked the most important question of whether the antitumor activity of TW-37 could be correlated with changes in the biological markers that are known to be altered, as shown
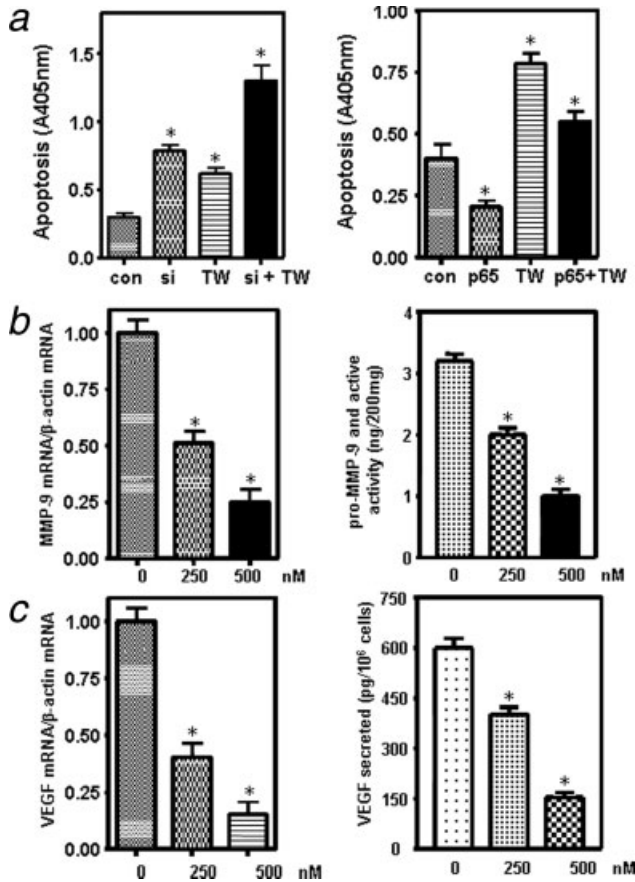
FIGURE 4 – (*a*) Left panel: Induction of apoptosis by p65 siRNA and TW-37 in Colo-357 cells tested by ELISA (con, control; si, p65 siRNA; TW, 500 nM TW-37; si + TW, p65 siRNA and 500 nM TW-37). Right panel: the effect of p65 cDNA transfection and TW-37 on the induction of apoptosis (con, control; p65, p65 cDNA; TW, 500 nM TW-37; p65 + TW, p65 cDNA and 500 nM TW-37). *$p < 0.05$, compared to the control. (*b*) TW-37 inhibited the expression and activity of MMP-9 in Colo-357 pancreatic cancer cells. Left panel: Real-time RT-PCR analysis of MMP-9 mRNA expression in pancreatic cancer cells treated with TW-37 for 72 hr. Right panel: MMP-9 activity assay showing that MMP-9 was inhibited by TW-37 treatment for 72 hr. *$p < 0.05$, compared to the control. (*c*) TW-37 inhibited the expression and activity of VEGF. Left panel: Real-time RT-PCR analysis of VEGF mRNA expression in Colo-357 pancreatic cancer cells treated with TW-37. Right panel: VEGF activity assay showing that VEGF level in the culture medium was inhibited by TW-37 treatment for 72 hours. *$p < 0.05$, compared to the control.

earlier in our *in vitro* studies. The answer to this question is presented later.

Inhibition of NF-κB activity by TW-37 in vivo

NF-κB plays an important role in cancer cell growth and survival. To determine whether TW-37 could affect the function of NF-κB *in vivo*, we examined the changes in NF-κB activity in tumor tissues using EMSA. Tumors excised from vehicle or TW-37-treated mice were homogenized, and nuclear proteins were isolated. EMSA with nuclear extracts from TW-37-treated or vehicle-treated xenograft tumor tissues revealed that treatment with TW-37 strongly inhibited the DNA-binding activity of NF-κB compared to untreated controls (Fig. 6*c*). The equal protein loading was confirmed by retinoblastoma (Rb) Western blot analysis, which showed no changes in its expression. To determine whether TW-37 could affect the NF-κB-dependent gene products *in vivo*, we also examined the expression of VEGF, MMP-9, COX-2,

Cyclin D1 and Survivin in tumor tissues using Western blot analysis. We found that the expression of VEGF, MMP-9, COX-2, Cyclin D1 and Survivin was also downregulated in TW-37-treated animals (Fig. 6*d*). These results are consistent with our *in vitro* data showing that TW-37 is a powerful agent for the inhibition of PC cell growth and invasion.

Discussion

Bcl-2 signaling pathway plays important roles in human cancers, including PC.[2,4] The activation of Bcl-2 has been shown to enhance tumor growth, invasion, motility, tumor spreading and metastasis and inhibition of apoptosis.[4,18–20] The overexpression of Bcl-2 family proteins in PC has been correlated with shorter survival.[3,4] Therefore, identification of an inhibitor targeting Bcl-2 is likely to provide a therapeutic benefit for PC. TW-37, a recently discovered Bcl-2 inhibitor, has been reported to inhibit cell growth of lymphoma cells by attenuating activation of Bcl-2 *in vitro* and *in vivo*.[9] Our current data show that TW-37 not only inhibits cell growth but also induces apoptotic cell death of PC cells, a finding similar to those observed in prostate cancer.[12]

Bcl-2 has been reported to cross-talk with another major cell growth and apoptotic regulatory pathway, namely NF-κB. Specifically, Bcl-2 has been shown to strongly induce NF-κB activity in ventricular myocytes and in breast cancer cells through a mechanism that is dependent on IκB kinase β (IKKβ) activity and IκB phosphorylation.[10,21,22] Karl *et al.* have documented that Bcl-2 can function as a proangiogenic signaling molecule through its ability to activate the NF-κB signaling pathway and to induce expression of the proangiogenic CXCL8 and CXCL1 chemokines in endothelial cells.[11] Recently, it has been reported that the inhibition of Bcl-2 inhibits NF-κB activity through reactive oxygen species in PC cells.[23] Taken together, these observations have shown that NF-κB is activated as a direct consequence of Bcl-2 activation in human cancer. Indeed, we also found that overexpression of Bcl-2 by cDNA transfection increased NF-κB DNA-binding activity. However, downregulation of Bcl-2 by siRNA inhibited the NF-κB DNA-binding activity in Colo-357 and BxPC-3 cells. In an earlier study, we also observed that treatment of cancer cells with TW-37 results in attenuation of Bcl-2 activity, suggesting that TW-37 exerts its growth inhibitory effect by attenuating Bcl-2 activation.[9] On the basis of these observations, it would be logical to assume that the downstream signaling events of Bcl-2 activation, NF-κB pathway, would also be affected. It has been documented that the NF-κB is over-expressed in human PC.[24] We also found that NF-κB is differentially expressed in all 7 PC cell lines. All these reports clearly suggest a possible link between Bcl-2 over-expression, NF-κB and PC. Indeed, we found that TW-37 inhibited the activation of NF-κB *in vitro* and *in vivo* in PC. We also found that TW-37 did not inhibit the activation of NF-κB in PANC-1 PC cells, where Bcl-2 expression is undetectable, suggesting that TW-37 mediated inhibition of NF-κB activity is partly through inactivation of Bcl-2 (data not shown). Moreover, we found that TW-37 blocked NF-κB activation by inhibiting the expression of IKK, which regulates the NF-κB transcription factor. NF-κB inhibition correlated with suppression of IκBα phosphorylation by TW-37. Mortenson *et al.* also found that HA14-1, Bcl-2 inhibitor, decreased in the phosphorylated form of IκB without effect on total IκB level.[25] Recently, it has been reported that over-expression of Bcl-2 activates the NF-κB through AKT signaling pathway.[25] Indeed, we found that TW-37 inhibited AKT expression (unpublished data). We also found that TW-37 inhibited the expression of NF-κB target genes, COX-2, Survivin and Cyclin D1 *in vitro* and *in vivo*. The inhibition of NF-κB, COX-2, Survivin and Cyclin D1 is noteworthy as they play a key role in PC cell growth and apoptosis.[14,24] Therefore, inactivation of Bcl-2 mediated cell growth inhibition and induction of apoptosis by TW-37 could be partly mediated via inactivation of NF-κB activity.
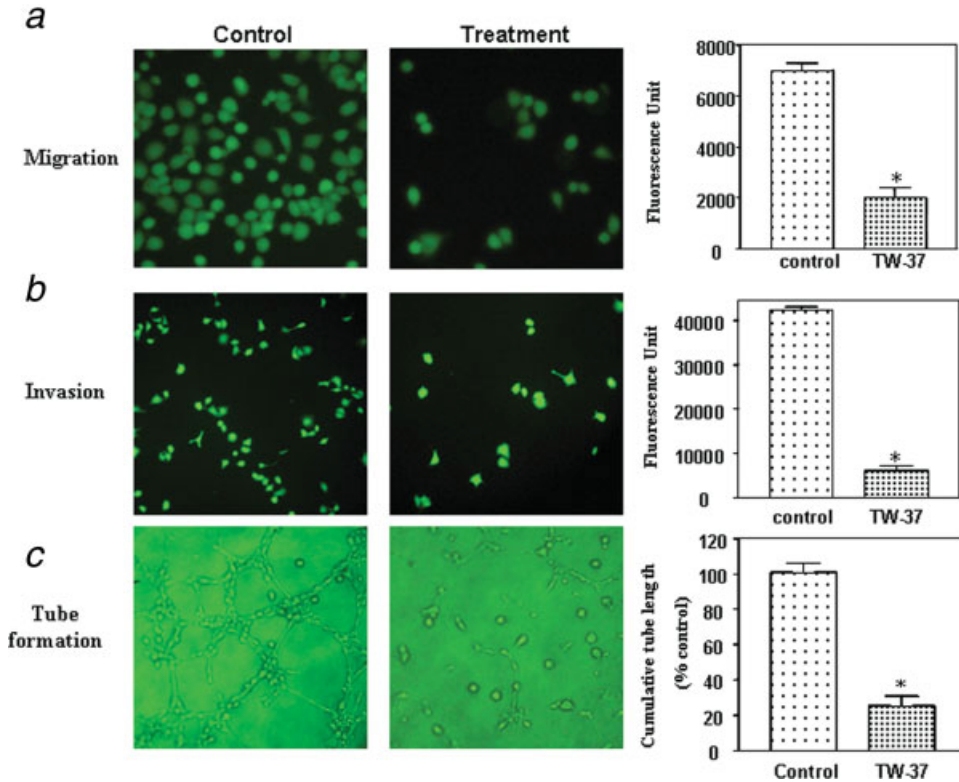
FIGURE 5 – TW-37 decreased pancreatic cancer cell migration, invasion and reduced the HUVECs tube formation. (*a*) Left panel: Migration assay showing that 250 nM TW-37 decreased Colo-357 pancreatic cancer cell migration at 24 hr. Right panel: value of fluorescence from the migrated cells as presented in the left panel. *$p < 0.05$, compared to the control. (*b*) Left panel: invasion assay showing that 250 nM TW-37-treated cells resulted in low penetration through the Matrigel-coated membrane at 24 hr, compared to control cells. Right panel: value of fluorescence from the invaded cells. The value indicated the comparative amount of invaded cells as shown in the left panel. *$p < 0.05$, compared to the control. (*c*) Left panel: conditioned media from 250 nM TW-37-treated Colo-357 cells were able to significantly reduce the tube formation of HUVECs in 6 hr incubation compared to the medium from control cells. Right panel: Image analysis of tubule/capillary length was carried out using software image analysis program Scion Image. Quantification of cumulative tube length of endothelial cells is also shown. *$p < 0.05$, compared to the control.

It is well-known that NF-κB is composed of a heterodimer of p65 and p50 subunits in most cell types and is sequestered in the cytoplasm by its inhibitory proteins, the IκBs.[26,27] During the phosphorylation and degradation of IκBs, NF-κB p65 is activated and rapidly transported from the cytoplasm to the nucleus in cancer cells.[26,27] Therefore, NF-κB p65 has been described as an important therapeutic target in cancer. In this study, we found that TW-37 inhibited the p65 expression. To further investigate whether the enhanced cell growth inhibition and apoptosis by TW-37 was mediated through the NF-κB pathway, we conducted NF-κB cDNA and NF-κB p65 siRNA transfection studies. We found that p65 cDNA transfection induced the activity of NF-κB. However, NF-κB p65 siRNA was functioning similarly as TW-37, which inhibited NF-κB DNA-binding activity. Moreover, TW-37 treatment combined with p65 siRNA transfection exerted greater inhibitory effect on the activation of NF-κB and caused greater degree of apoptotic cell death, suggesting that TW-37-induced cell growth inhibition and apoptosis is partly mediated through the NF-κB pathway. Importantly, we also found that TW-37 could abrogate the activation of NF-κB induced by the p65 cDNA transfection. Therefore, our results clearly show that TW-37 inhibits cell proliferation and induces of apoptotic cell death, which could be due to both the inhibition of NF-κB p65 expression and NF-κB DNA binding activity.

NF-κB activation has been reported to regulate several genes such as VEGF, COX-2, Survivin and MMP-9 that are directly associated with metastatic processes.[28–32] Indeed, in this study, we showed that TW-37 reduced NF-κB DNA binding activity

and concomitantly inhibited the expression of VEGF, MMP-9, Survivin and COX-2. We also found that TW-37 reduced the secretion of VEGF in the culture medium and inhibited the activity of MMP-9 in PC cells. Since we observed that TW-37 inhibited the expression and activities of MMP-9 and VEGF, we tested the effects of TW-37 on the migration and invasion of PC cells and tube formation (angiogenesis) of HUVECs. We found that TW-37 inhibited migration and invasion of pancreatic cancer cells through matrigel and reduced tube formation of HUVECs. These results were consistent with inactivation of MMP-9 and VEGF, documenting that TW-37 could inhibit cancer cell migration and invasion which is likely due in part through the downregulation of MMP-9 and VEGF, mediated by inactivation of NF-κB.

Bcl-2 inhibitor has been used *in vitro* and, as such, cell-based assays has been done to show that Bcl-2 inhibitor is defined as a molecularly targeted agent. However, the major challenge in developing a molecularly targeted therapeutic agent is that it needs to demonstrate efficacy against human pancreatic cancer. Here, we tested TW-37 against Colo-357 in a SCID xenograft model. Our results show that TW-37 was effective in decreasing tumor weight significantly ($p = 0.015$) compared to untreated animals (Fig. 6*b*). Most importantly, we have done the combination of TW-37 with gemcitabine on cell growth and apoptosis assay in BxPC-3 and Colo-357 cell lines. We found that TW-37 sensitizes these 2 cell lines to gemcitabine-induced growth inhibition and apoptosis (unpublished data). Therefore, Bcl-2 inhibitor could be a novel agent for designing innovative approaches for demonstrat-
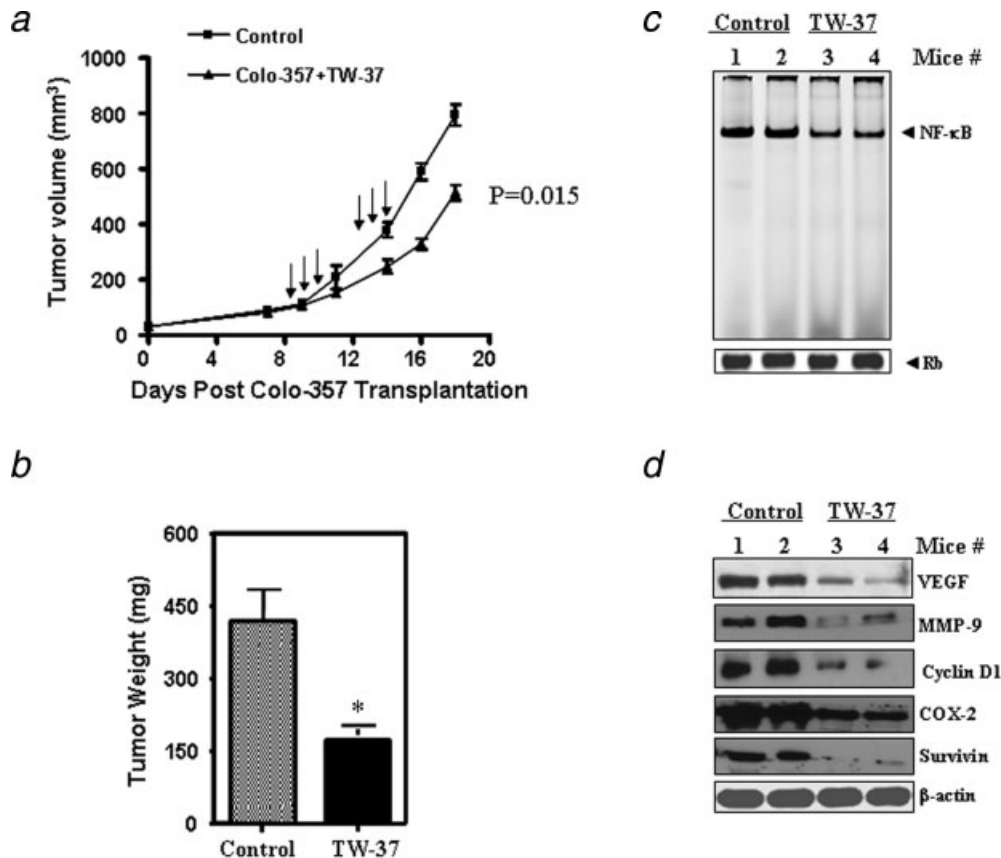
FIGURE 6 – TW-37 inhibits tumor growth and the expression of NF-κB target genes *in vivo*. Colo-357 xenografts were generated by inoculating cells subcutaneously (s.c.) in SCID mice. Once transplanted, fragments developed into palpable tumors (about 80 mg), and groups of 9 animals were removed randomly and assigned to different treatment groups. Mice were injected with TW-37 at 20 mg/kg iv x3 days, for 2 cycles. The control group received vehicle only. (*a,b*) TW-37 retards the growth of Colo-357 tumor xenografts in nude mice. Tumor volumes in SCID mice were plotted against time (*a*) and total tumor weight at time of sacrifice. (*b*) *p* < 0.05, compared to the control. (*c*) TW-37 inhibits NF-κB DNA-binding activity *in vivo*. Tumor xenografts were removed, and nuclear protein extracts were prepared. Binding of NF-κB consensus element with nuclear extracts was detected by EMSA. Retinoblastoma (*Rb*) protein level was used as a nuclear protein loading control. (*d*) The expression of NF-κB target genes was detected by Western blotting of tumor tissue extracts. TW-37 inhibited the expression of NF-κB target genes, including VEGF, MMP-9, COX-2, Cyclin D1 and Survivin in tumor tissue of control compared to TW-37 treated animals.

ing their antitumor activity against PC and, as such, could also be useful in enhancing the antitumor activity of conventional therapeutic agents for the treatment of PC patients.

In summary, we presented experimental evidence, which strongly supports the antitumor effects of TW-37 in PC *in vitro* and *in vivo*. Thus, we believe that TW-37 could potentially be an effective therapeutic agent for the inactivation of Bcl-2, NF-κB and its downstream target genes such as MMP-9 and VEGF, resulting in the inhibition of cell growth, invasion and metastasis of PC. Our study suggests that SMI TW-37 represent a promising novel agent that should be developed for the treatment of pancreatic cancer.

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