

# Therapeutic potential of thalidomide for gemcitabine-resistant bladder cancer

YEN TA HUANG<sup>1,2</sup>, CHUAN CHU CHENG<sup>3</sup>, TED H. CHIU<sup>5</sup> and PEI CHUN LAI<sup>1,4</sup>

<sup>1</sup>Department of Medicine, Tzu Chi University; <sup>2</sup>Surgical Intensive Care Unit,

Departments of <sup>3</sup>Medical Research and <sup>4</sup>Pediatrics, Buddhist Tzu Chi General Hospital;

<sup>5</sup>Department of Pharmacology, College of Medicine, Tzu Chi University, Hualien, Taiwan, R.O.C.

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**Abstract.** Controversial effects of thalidomide for solid malignancies have been reported. In the present study, we evaluate the effects of thalidomide for transitional cell carcinoma (TCC), the most common type of bladder cancer. Thalidomide precipitates were observed when its DMSO solution was added to the culture medium. No precipitation was found when thalidomide was dissolved in 45%  $\gamma$ -cyclodextrin, and this concentration of  $\gamma$ -cyclodextrin elicited slight cytotoxicity on TCC BFTC905 and primary human urothelial cells. Thalidomide- $\gamma$ -cyclodextrin complex exerted a concentration-dependent cytotoxicity in TCC cells, but was relatively less cytotoxic (with  $IC_{50}$  of 200  $\mu$ M) in BFTC905 cells than the other 3 TCC cell lines, possibly due to upregulation of Bcl-xL and HIF-1 $\alpha$  mediated carbonic anhydrase IX, and promotion of quiescence. Gemcitabine-resistant BFTC905 cells were chosen for additional experiments. Thalidomide induced apoptosis through downregulation of survivin and securin. The secretion of VEGF and TNF- $\alpha$  was ameliorated by thalidomide, but they did not affect cell proliferation. Immune-modulating lenalidomide and pomalidomide did not elicit cytotoxicity. In addition, cereblon did not play a role in the thalidomide effect. Oxidative DNA damage was triggered by thalidomide, and anti-oxidants reversed the effect. Thalidomide also inhibited TNF- $\alpha$  induced invasion through inhibition of NF- $\kappa$ B, and downregulation of effectors, ICAM-1 and MMP-9. Thalidomide inhibited the growth of BFTC905 xenograft tumors in SCID mice via induction of DNA damage and suppression of angiogenesis. Higher average body weight, indicating less cachexia, was observed in thalidomide treated group. Sedative effect was observed within one-week of treatment. These pre-clinical results suggest therapeutic potential of thalidomide for gemcitabine-resistant bladder cancer.

## Introduction

Bladder cancer is still one of the most common malignancies in the world (1). Pathologically, >90% of bladder cancer is transitional cell carcinoma (TCC) (2). Surgery, radiation, and chemotherapy are evidence-based treatments depending on clinical staging (3,4). Chemotherapy with standard regimen MVAC (methotrexate, vinblastine, adriamycin, and cisplatin) or more tolerable regimen GC (gemcitabine and cisplatin) for advanced or metastatic bladder cancer has shown poor response (5). However, once diagnosed as a muscle-invasive type, the 5-year survival and freedom from relapse rate under conservative multimodality therapies were 54 and 42%, respectively (6). Therefore, we need to investigate new strategies for bladder cancer treatment.

Thalidomide possesses anti-angiogenic and immunomodulatory effects (7). It has been widely used for the standard therapies of multiple myeloma (MM) (8), based not only on its anti-angiogenic and immunomodulatory mechanisms but also on inducing apoptosis (9). Its new analogues, so called IMiDs (immunomodulatory drugs), lenalidomide and pomalidomide, have also been approved by US FDA for MM therapy with satisfactory and tolerable responses (10-12). Thalidomide has also been used clinically for the treatments of some solid tumors, such as hepatocellular carcinoma (13,14) and glioblastoma multiforme (15,16), but controversial effects were observed in these studies. Beyond its well-known anti-angiogenic property, only few studies of cytotoxic effect (17) and migratory inhibition (18) on solid tumor cells have been reported. In the present study, we demonstrated the therapeutic effects of thalidomide via induction of intracellular reactive oxygen species (ROS) to elicit apoptosis, inhibition of angiogenesis, and suppression of invasion in gemcitabine-resistant TCC BFTC905 cells *in vitro* and *in vivo*.

## Materials and methods

**Cell lines and cell culture.** Four TCC cell lines were used: BFTC905 (19), BFTC909 (19), T24, and TSGH8301. Cells were maintained as described previously (20,21). BFTC905 cells were gemcitabine-resistant (22). We also used primary human urothelial cells (HUCs) (ScienCell Research Laboratories, Carlsbad, CA, USA) (22).

**Correspondence to:** Dr Pei Chun Lai, Department of Pediatrics, Buddhist Tzu Chi General Hospital, 707, Section 3, Chung Yang Road, Hualien 970, Taiwan, R.O.C.  
E-mail: chunta.lab@gmail.com

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**MTT cytotoxicity assay.** Procedures as previously described were followed (21,23). Thalidomide (Sigma-Aldrich, St. Louis, MO, USA), gemcitabine (Sigma-Aldrich), pomalidomide (kindly provided by Professor Chinpiao Chen, Department of Chemistry, National Don Hwa University, Hualien, Taiwan), lenalidomide (LC Laboratories, Woburn, MA, USA), CRBN siRNA/scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), N-acetylcysteine (NAC, Sigma-Aldrich), and dl-dithiothreitol (DTT, Sigma-Aldrich) were used in individual experiments. Gemcitabine, NAC, and DTT were dissolved in distilled water. Dimethyl sulfoxide (DMSO, J.T. Baker, Phillipsburg, NJ, USA), (2-hydroxypropyl)- $\beta$ -cyclodextrin (Sigma-Aldrich),  $\gamma$ -cyclodextrin (Sigma-Aldrich), carboxymethyl cellulose (CMC, Sigma-Aldrich), and Cremophor-EL (Sigma-Aldrich) were chosen as the solvent to dissolve thalidomide, and the viability changes after administration of these solvents were tested. The protocol for siRNA transfection provided by Santa Cruz Biotechnology was followed. The cell viability was calculated according to the following formula: cell viability = (absorbance of the experimental group)/(absorbance of reference group)  $\times$  100%. Reference group was administered with equal volume of PBS (phosphate-buffered saline) as control.

**Cell proliferation by trypan blue exclusion test.** For cell proliferation assay, thalidomide at 0–200  $\mu$ M was administered (day 1) after overnight seeding of  $1 \times 10^4$  BFTC905 cells in 10-cm dish with complete medium. Subsequently, the cells were re-cultured with fresh medium and administration of each concentration of thalidomide on day 5. Administration of equal volume of vehicle  $\gamma$ -cyclodextrin was used as control. Viable cells do not take up impermeable trypan blue (24), so counts of cells with negative stainings of trypan blue (Sigma-Aldrich) were calculated by a hemocytometer on days 5 and 9. In another experiment, thalidomide at 200  $\mu$ M plus 100 nM recombinant human vascular endothelial growth factor (VEGF, PreproTech, Rocky Hill, NJ, USA), 100 nM human recombinant basic fibroblast growth factor (bFGF, Merck Millipore, Darmstadt, Germany), or 100 ng/ml human recombinant tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , Merck Millipore) were administered after overnight seeding of  $1 \times 10^4$  BFTC905 cells in 10-cm dish (day 1). Administration of equal volume of PBS was used as control. Trypan blue-negative cells were calculated on day 3. To determine the appropriate concentration of TNF- $\alpha$  for experiment of invasion without the proliferative effects, a 48-h administration of TNF- $\alpha$  at 0–100 ng/ml on BFTC905 cells was cultured in the medium with 1% FBS. Then trypan blue-negative cells were counted.

**Western blotting.** Conventional protocols as described previously were followed (20). Primary antibodies were purchased from the following vendors: Cell Signaling Technology (Danvers, MA, USA), anti-survivin (no. 2808), anti-LC3B (no. 2775), cell cycle regulation (no. 9932), and cell cycle/checkpoint antibody sampler kits (no. 9917); Abcam (Cambridge, MA, USA), anti-securin (ab3305) and anti-cleaved PARP [poly (ADP-ribose) polymerase, ab4830]; R&D Systems (Minneapolis, MN, USA), anti-CAIX (carbonic anhydrase 9, AF 2188); GeneTex (Irvine, CA, USA), anti-

HIF-1 $\alpha$  (hypoxia-inducible factor 1 alpha, GTX 127309), anti-caspase 3 (GTX110543), anti-Bcl2 (B-cell lymphoma 2, GTX127958), anti-Bax (Bcl-2-associated X protein, GTX109683), anti-cIAP1 (cellular inhibitor of apoptosis 1, GTX110087), anti-cIAP2 (GTX113128), anti-Bcl-xL (B-cell lymphoma-extra-large, GTX105661), anti-TCTP (translationally controlled tumor protein, GTX63597), anti-cyclin A1 (GTX103042), anti-cyclin B1 (GTX100911), anti-cyclin D1 (GTX112874), anti-cyclin E1 (GTX103045), and anti-cereblon (GTX 45011); and Santa Cruz Biotechnology (Dallas, TX, USA), anti-Ki-67 (sc-15402), anti-MMP-9 (matrix metalloproteinase 9, sc-6840), anti-ICAM-1 (intercellular adhesion molecule 1, sc-7891), anti-CD34 (sc-9095),  $\alpha$ -tubulin (sc-8305), and actin (sc-1616). Expression of  $\alpha$ -tubulin or actin was used as the internal standard.

**Cell cycle analysis.** After treatment, the supernatant and the dead BFTC905 cells were removed. Only the cell cycle changes in the viable BFTC905 cells were analyzed. Procedures using flow cytometry (Bedford, MA, USA), as described, were followed (22).

**DNA damage assay.** The Cell Death Detection ELISA<sup>plus</sup> (Roche, Mannheim, Germany) assay kit was used to differentiate apoptotic or necrotic condition of BFTC905 cells after thalidomide treatment. Procedures as previously described were followed (25). To determine the oxidative DNA damage, OxiSelect<sup>TM</sup> Oxidative DNA Damage ELISA kit (Cell Biolabs, San Diego, CA, USA) was used for the detection and quantitation of 8-hydroxy-2'-deoxyguanosine (8-OHdG).

**Immunofluorescent stainings of DCF (2',7' dichlorodihydrofluorescein).** The procedures using OxiSelect<sup>TM</sup> Intracellular ROS (reactive oxygen species) Assay kit (Cell Biolabs) were followed. The intensity of greenish fluorescence of DCF is proportional to the levels of ROS production in the cytoplasm of BFTC905 cells. The cells were photographed (x100) 48 h after exposure to IMiDs or vehicle.

**Total oxidant status (TOS) assay.** TOS levels were measured by commercial assay kit (Rel Assay Diagnostics, Turkey) (26). The oxidants in BFTC905 cells oxidized the ferrous ion-chelator complex to ferric ion. The oxidation reaction was prolonged by the enhanced molecule, glycerol, which was abundant in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, measured as OD (optic density) at 530 nm by a spectrophotometer, was recorded at 6, 12, 24, 48 h after BFTC905 cells were treated with 200  $\mu$ M thalidomide or vehicle. Due to significant differences of cell counts between vehicle and thalidomide treatment, the changes of TOS at each time-point were calculated according to the following formula: TOS ratio per cell = [(OD value of thalidomide treatment)/(cell counts of thalidomide treatment)]  $\div$  [(OD value of vehicle treatment)/(cell counts of vehicle treatment)]. Cell counts were calculated conventionally by a hemocytometer.

**Matrigel invasion assay.** The BioCoat<sup>TM</sup> growth factor reduced Matrigel<sup>TM</sup> Invasion Chamber (BD Biosciences) was used for *in vitro* invasion study. TNF- $\alpha$  at 10 ng/ml and/or

50  $\mu$ M of thalidomide were added to the chamber with medium containing 1% FBS. Subsequently, the chambers were put into 24-well plate containing medium plus 10% FBS for 24 h. The protocol as described previously was followed (23). BFTC905 cells attached on the bottom layer of chambers were counted from 10 randomized fields (x200).

**Immunocytochemistry.** The protocol as described previously was followed (20). Primary antibody of anti-NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) p65 (SC-109, Santa Cruz Biotechnology) was used. After 48-h treatment with thalidomide or vehicle with/without TNF- $\alpha$  stimulation, BFTC905 cells fixed on the slides with positive immunostaining for nuclear NF- $\kappa$ B p65 were counted from 5 randomized fields (x200).

**Enzyme-linked immunosorbent assay (ELISA).** Human BDNF (brain-derived neurotrophic factor) Quantikine™ ELISA kit purchased from R&D Systems and human VEGF (vascular endothelial growth factor), as well as bFGF (basic fibroblast growth factor) and TNF- $\alpha$  ELISA kit purchased from Millipore were used to detect the above molecules in culture medium. The procedures were recommended by the manufacturer and used in our previous study (20). MMP-9 activity in culture medium was also measured by ELISA method (Biotrak activity assay system, Amersham Pharmacia Biotech, Little Chalfont, UK). After 48-h treatment with thalidomide or vehicle with/without TNF- $\alpha$  stimulation, MMP-9 activity in the medium was assayed according to the manufacturer's instructions.

**Mouse xenograft model.** BFTC905 xenograft model and protocol in NOD.CB17-Prkdc<sup>scid</sup>/Tcu (SCID) male mice were established in our previous study (20). BFTC905 cells ( $1 \times 10^6$ ) were implanted s.c. into the right inguinal area of SCID mice, concomitantly 250 mg/kg of thalidomide or vehicle was administered s.c. into the loading site of cancer cells when the tumor was palpable or directly into the tumor 3 times per week (W1, 3, 5) since the day of implantation. In another experiment to evaluate the therapeutic effects, the same dosage of thalidomide with the same frequency was administered for 2 weeks after implantation of BFTC905 cells for 3 weeks. Body weight was measured after sacrifice, and necropsy of xenografts was performed immediately. Tumor volumes were calculated using the formula:  $[1/2] \times a \times b^2$ , where a and b represent the largest and smallest tumor diameters, respectively. Expression of cleaved PARP and CD34 was determined by western blotting, and expression of human VEGF was measured by ELISA.

**Locomotor activity.** Locomotor activity as described were followed (27). Briefly, a 30-min habituation period was used prior to the first episode of administration of thalidomide or vehicle. Images of traveled distance, the so-called locomotor activity, were captured by a video camera and the recorded images were analyzed by TrackMot software (Diagnostic & Research Instruments Co., Taoyuan, Taiwan). The activity was summated consecutively for two 10-min intervals following the drug injection. All animals without xenograft implantation were used only once with 3 drug injections (W1, 3, 5).

**Statistical analysis.** Data were analyzed by Student's t-test, Mann-Whitney U test, or one-way/two-way ANOVA based on individual data, and presented as mean  $\pm$  SEM (standard error of mean). In all cases,  $p < 0.05$  was considered statistically significant, and indicated in the figures as: \* $0.01 \leq p < 0.05$ , \*\* $0.005 \leq p < 0.01$  and \*\*\* $p < 0.005$ , respectively.

## Results

**Only the  $\gamma$ -cyclodextrin did not elicit cytotoxicity in BFTC905 cells.** Based on the literature, initially we used DMSO as the solvent for thalidomide (Fig. 1A). However, precipitation of thalidomide was observed immediately following addition of thalidomide-DMSO solution into culture medium (Fig. 1B). Therefore, several solvents or substances were tested for their ability to carry thalidomide into culture medium without precipitation. We observed that cremophor-EL, (2-hydroxypropyl)- $\beta$ -cyclodextrin and  $\gamma$ -cyclodextrin can dissolve thalidomide, while carboxymethyl cellulose (CMC) can keep thalidomide in a suspension state. To test the cytotoxicity of these solvents, BFTC905 cells were exposed to different v/v ratios of solvent/culture medium (0-1%) for 48 h. At 1% of v/v, only 45%  $\gamma$ -cyclodextrin did not exert cytotoxicity in BFTC905 cells ( $p = 0.198$ ,  $n = 3$ ; Fig. 2A). Whereas,  $\gamma$ -cyclodextrin did not elicit significant cytotoxicity in primary HUC cells, except at the concentration of 1% v/v ( $p = 0.002$ ,  $n = 3$ ; Fig. 2B). No precipitation was observed when thalidomide/45%  $\gamma$ -cyclodextrin solution was added into the culture medium (Fig. 2C). Thalidomide dissolved in 45%  $\gamma$ -cyclodextrin was used for the subsequent experiments.

**Cytotoxicity of thalidomide on TCC and primary human urothelial cells.** After 48-h treatment, thalidomide (dissolved in 45%  $\gamma$ -cyclodextrin) caused a concentration-dependent cytotoxicity on TCC cells ( $p < 0.05$  in all TCC cell lines by one-way ANOVA,  $n = 3$ ; Fig. 3A). Thalidomide exerted significant less cytotoxic effect in BFTC905 cells compared to other TCC cell lines (all  $p < 0.05$  by two-way ANOVA). The viability of BFTC905 cells after administration of thalidomide dissolved in  $\gamma$ -cyclodextrin appeared to plateau at 200  $\mu$ M ( $51.0 \pm 7.0\%$  vs.  $47.1 \pm 7.1\%$  for 200 and 500  $\mu$ M, respectively;  $p = 0.714$ ). The v/v of thalidomide and 45%  $\gamma$ -cyclodextrin mixture at 200 and 500  $\mu$ M was 0.4 and 0.8%, respectively. The viability of HUCs was not altered after administration of 200  $\mu$ M thalidomide dissolved in 45%  $\gamma$ -cyclodextrin ( $p = 0.2$ ,  $n = 3$ ), but 500  $\mu$ M thalidomide elicited significant cytotoxicity in HUC cells ( $p = 0.006$ ). Therefore, 200  $\mu$ M thalidomide dissolved in 45%  $\gamma$ -cyclodextrin was used for the subsequent experiments.

Furthermore, we examined the cytotoxicity of gemcitabine, the main current therapy for bladder cancer plus thalidomide on TCC cells (Fig. 3B). After 48-h treatment, the viability of BFTC905, BFTC909, and TSGH8301 cells was similar between thalidomide alone (200  $\mu$ M) and thalidomide plus gemcitabine (100 nM) group. Significant additive cytotoxicity for the combination of gemcitabine and thalidomide was only observed in T24 cells ( $37.6 \pm 3.4$  vs.  $18.7 \pm 5.0\%$  for thalidomide alone vs. thalidomide plus gemcitabine treatment, respectively;  $p = 0.03$ ,  $n = 3$ ). Therefore, gemcitabine-resistant TCC cell line BFTC905 (22) was

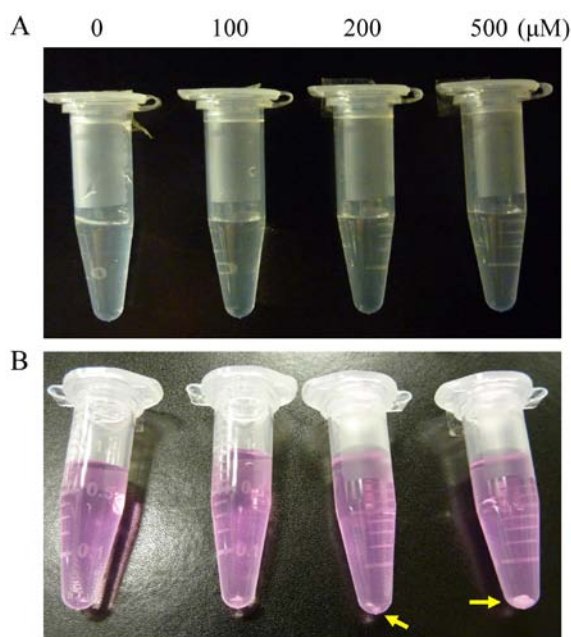


Figure 1. The precipitation of thalidomide. (A) Clear solutions of thalidomide  $\leq 500 \mu\text{M}$  dissolved in DMSO. (B) Precipitates (yellow arrow) of thalidomide dissolved in DMSO settled to the bottom of Eppendorf tube when mixed with culture medium.

chosen to investigate the therapeutic effects of thalidomide in 45%  $\gamma$ -cyclodextrin in the following experiments.

Autophagy mediated survival of cancer cells by overcoming hypoxia and a shortage of nutrients (28). To delineate why thalidomide elicited less efficacy on BFTC905 cells, the expression of LC3B-II, HIF-1 $\alpha$ , and CAIX was determined after thalidomide treatment. Expression of LC3B-II, a marker of autophagy and converted from LC3B-I, was not changed after thalidomide treatment (Fig. 3C). CAIX, a marker of hypoxia induced by HIF-1 $\alpha$ , may be a factor contributing to drug resistance in cancer cells (29). Upregulation of HIF-1 $\alpha$  started 4 h after thalidomide treatment and returned to basal level at 48 h (Fig. 3D). Increase in CAIX expression was observed 48 h after thalidomide treatment (Fig. 3D).

*Thalidomide inhibition of BFTC905 cell growth is not via VEGF, bFGF, or TNF- $\alpha$ .* Treatment with 100 and 200  $\mu\text{M}$  thalidomide for 8 days significantly inhibited the number of viable BFTC905 cells ( $p=0.02$  and  $<0.001$ , respectively, in two-way ANOVA;  $n=3$ ; Fig. 4A). The difference between 100 and 200  $\mu\text{M}$  thalidomide treatment was statistically significant ( $p=0.003$ ). Thalidomide at 200  $\mu\text{M}$  maintained the static growth of BFTC905 cells. VEGF (30) and bFGF (31) were secreted from TCC cells as growth factors, and thalidomide has been reported to decrease the expression of both (32). Exogenous administration of TNF- $\alpha$  promoted proliferation of TCC cells (33), and thalidomide enhanced the degradation of TNF- $\alpha$  mRNA (34). Besides, BDNF was also a survival factor for TCC in our previous study (20). Therefore, the secretion of these molecules after thalidomide treatment for 48 h was determined. Thalidomide at 200 and 500  $\mu\text{M}$  significantly inhibited the secretion of TNF- $\alpha$  and VEGF ( $n=3$ , Fig. 4B). Furthermore, exogenous VEGF (100 nM), bFGF (100 nM),

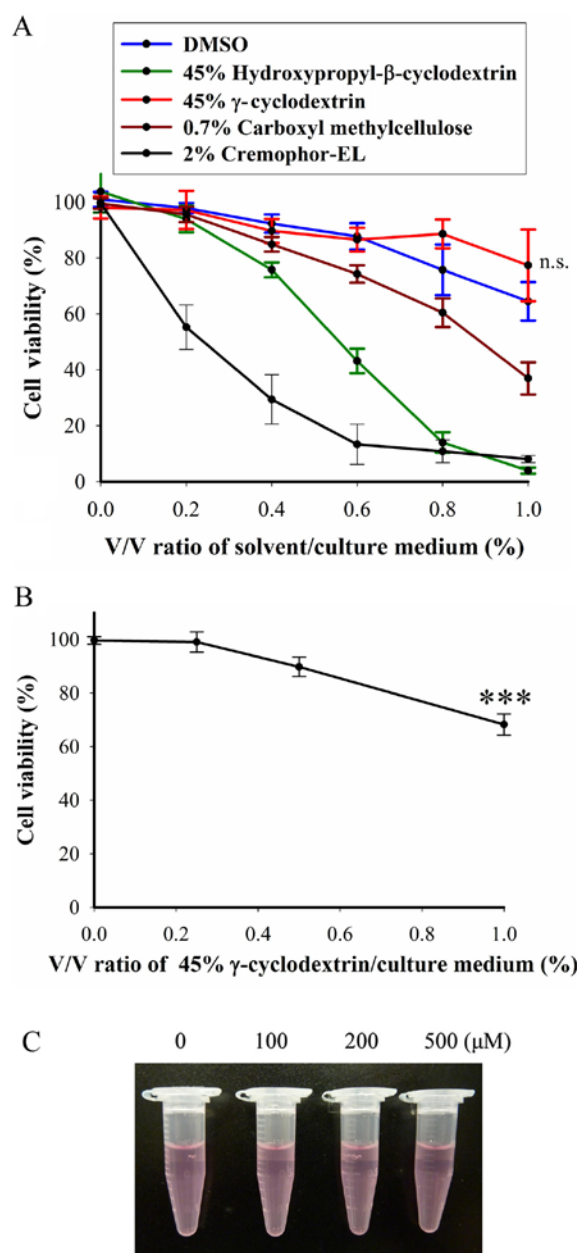


Figure 2. MTT assay was used to determine the viability of (A) BFTC905 cells after exposure to various solvents, and (B) primary human urothelial cells (HUC) after exposure to 45%  $\gamma$ -cyclodextrin for 48 h. (C) When thalidomide dissolved in 45%  $\gamma$ -cyclodextrin was added to the culture medium, no sediment was observed.  $^{**}0.005 \leq p < 0.01$ .

or TNF- $\alpha$  (100 ng/ml) did not reverse thalidomide induced growth inhibition (Fig. 4C).

*Thalidomide elicits quiescence in BFTC905 cells.* After 48-h treatment, a significant accumulation of G0/G1 phase [ $58.2 \pm 0.1$  vs.  $65.1 \pm 0.6\%$  after vehicle ( $\gamma$ -cyclodextrin) vs. 200  $\mu\text{M}$  thalidomide treatment,  $n=3$ ,  $p=0.002$ , Fig. 5A and B] was observed. Cell cycle analysis by flow cytometry only reveals the proportional changes in each phase, so we further examined the specific cell cycle related proteins by western blotting (Fig. 5C and D). The dynamic expression of cyclins, cyclin dependent kinases, and related molecules in cell cycles is well established (35,36). Downregulation of cyclin B and phospho-CDC2, the

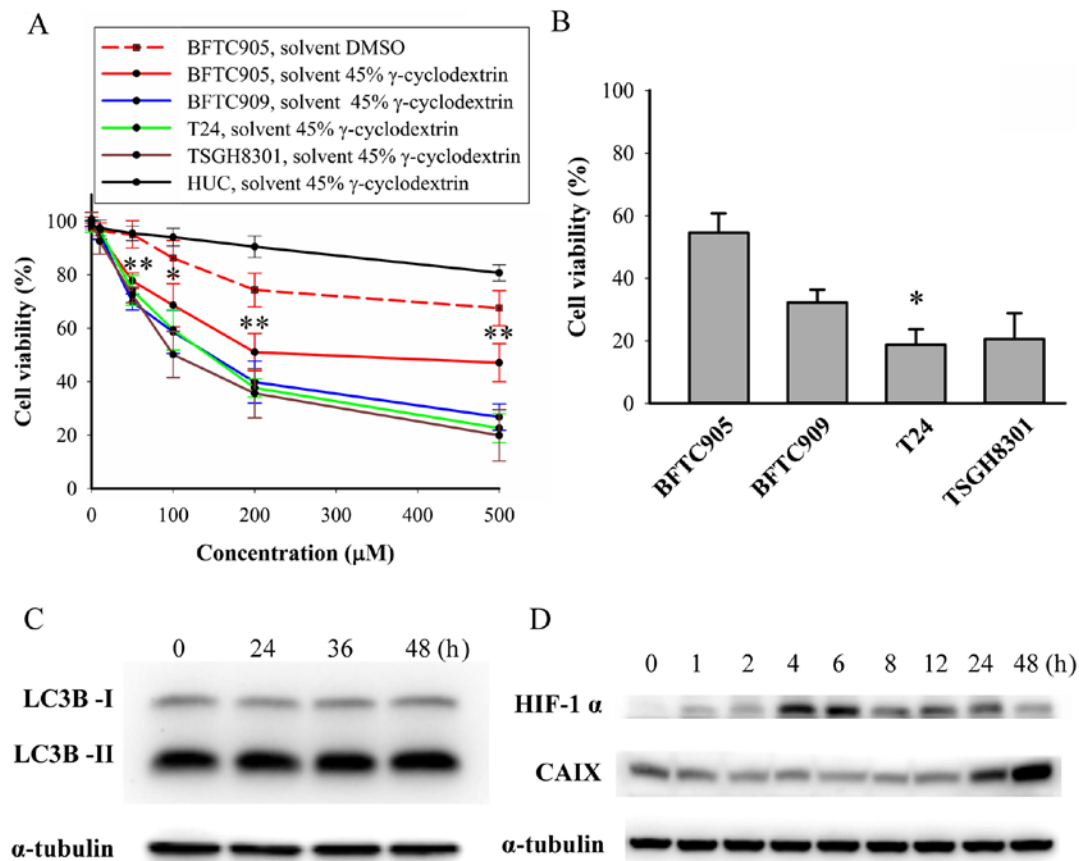


Figure 3. Cytotoxicity of thalidomide on TCC cells measured with MTT assay. (A) The viability of BFTC905, T24, TSGH8301, and BFTC909 cells was measured 48 h after treatment with thalidomide dissolved in 45%  $\gamma$ -cyclodextrin. The viability of BFTC905 cells treated with thalidomide dissolved in DMSO was also examined. The symbols '\*' and '\*\*' indicate statistical significance of viability between thalidomide treatment and vehicle 45%  $\gamma$ -cyclodextrin alone. (B) The viability of BFTC905, T24, TSGH8301, and BFTC909 cells was measured 48 h after co-treatment with thalidomide (200  $\mu$ M, dissolved in 45%  $\gamma$ -cyclodextrin) and gemcitabine (100 nM). (C and D) Representative western blots of (C) LC3B and (D) HIF-1 $\alpha$  and CAIX expression were examined within 48 h after treatment with 200  $\mu$ M thalidomide. \*0.01 $\leq$ p<0.05, \*\*0.005 $\leq$ p<0.01.

markers of G2/M phase marker, indicated no G/M arrest. Downregulation of cyclin A, which is expressed in S phase, indicated no S phase arrest. Decrease in phospho-CDK2 expression, which is expressed in late G1 phase or S phase, indicated no cell cycle stasis in late G1 and S phase. Decrease in expression of cyclin D1 and CDK4/6 (expressed in G1 phase before R point) and cyclin E (expressed in late G1 phase after R point) indicated that thalidomide did not induce G1 phase arrest. Due to the increased ratio of G0/G1 phase without G1 arrest, we substantiated that thalidomide induced G0 phase stasis, the so-called quiescence. The increased expression of p27<sup>kip1</sup>, a potent inhibitor of cyclin-CDK complex formation, supported this idea (Fig. 5E). Ki-67 protein is present in the nucleus during all active phases of the cell cycle (G1, S, G2, and mitotic phase) but absent from quiescent cells (G0), so Ki-67 is the most referenced marker for cellular proliferation (37,38). As shown in Fig. 5E, expression of Ki-67 after 48-h thalidomide treatment was lower than control. Thus, we found that thalidomide promoted quiescence in BFTC905 cells.

**Thalidomide induces apoptosis in BFTC905 cells.** Cytoplasmic DNA fragments in BFTC905 cells, detected by binding of anti-histone plus anti-DNA antibodies, was

significantly increased 48-h after 200  $\mu$ M thalidomide treatment compared to vehicle (n=3, p=0.03; Fig. 6A). Low basal levels and no significant changes of DNA fragments were detected in the culture medium of BFTC905 cells 48 h after thalidomide treatment (p=0.73; Fig. 6A). Expression of cleaved caspase 3, activated both by extrinsic and intrinsic pathways in apoptotic cells, was also increased after thalidomide treatment (Fig. 6B). These results indicated that thalidomide induced apoptosis of BFTC905 cells. Furthermore, we examined the changes of several anti-apoptosis or apoptosis related proteins. Expression of Bcl-2, BAX, TCTP, and cIAP1 were not changed (Fig. 6C). In contrast, expression of survivin and securin were downregulated. Expression of cIAP2 increased 24 and 36 h after thalidomide treatment, and returned to baseline at 48 h (Fig. 6D). Upregulation of Bcl-xL was observed after thalidomide treatment (Fig. 6D).

**Lenalidomide and pomalidomide do not elicit cytotoxicity in BFTC905 cells.** We further evaluated cytotoxicity of other IMiDs, lenalidomide and pomalidomide, on BFTC905 cells. Both drugs can be dissolved in DMSO and  $\gamma$ -cyclodextrin. After 48-h treatment, 200  $\mu$ M of both drugs dissolved in both solvents did not elicit significant cytotoxicity, but both at 500  $\mu$ M elicited significant cytotoxicity (~15%, Fig. 7A).



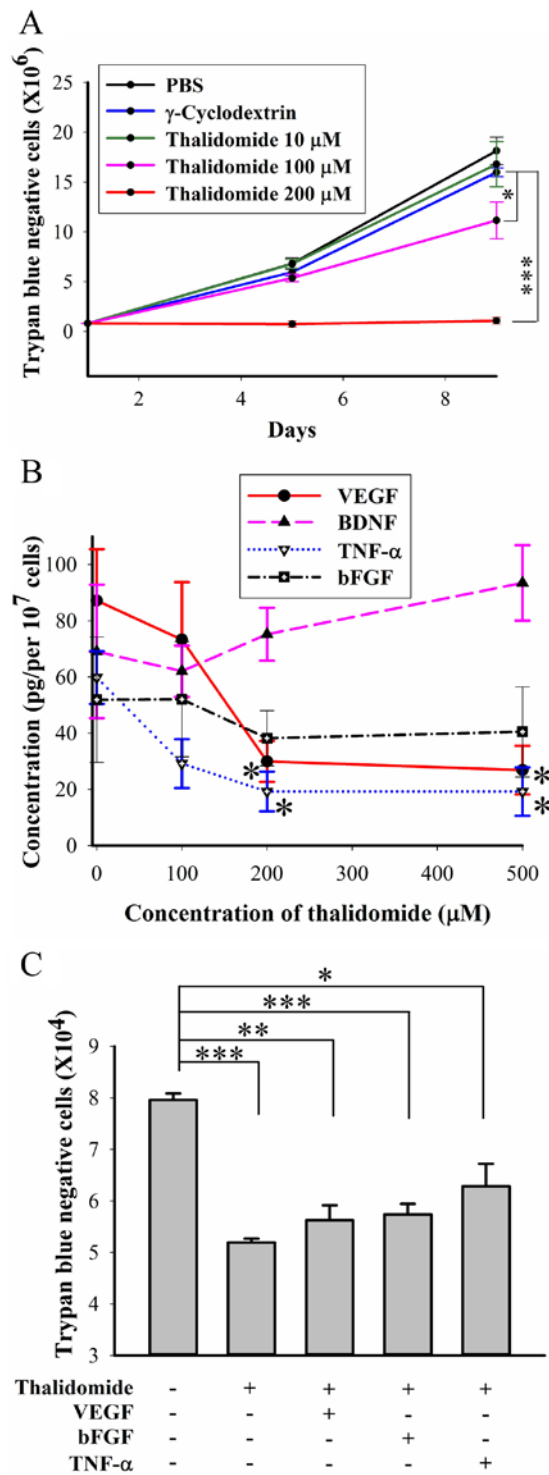


Figure 4. Roles of growth factors in BFTC905 cells treated with thalidomide (200  $\mu$ M, dissolved in 45%  $\gamma$ -cyclodextrin). (A) After administration of thalidomide on days 1 and 5, trypan blue-negative (viable) cell counts of BFTC905 cells were calculated on days 5 and 9. (B) Secretion of BDNF, VEGF, bFGF, and TNF- $\alpha$  from BFTC905 cells treated with thalidomide for 48 h was measured by ELISA. (C) After co-administration of thalidomide and 100 nM VEGF, 100 nM BDNF, 100 nM bFGF, or 100 ng/ml TNF- $\alpha$  in culture medium with 1% FBS for 48 h, trypan blue-negative cell counts of BFTC905 cells were calculated. \*0.01 $\leq$ p<0.05, \*\*0.005 $\leq$ p<0.01, \*\*\*p<0.005.

Thalidomide and other IMiDs were shown to bind to cereblon and inhibited the associated E3 ubiquitin ligase activity (39), so we determined the roles of cereblon in the mechanism of

the thalidomide effects. After 48-h treatment with IMiDs, no change of cereblon expression was observed (Fig. 7B). Furthermore, knockdown of cereblon by RNAi (Fig. 7C) did not change the cytotoxicity elicited by thalidomide, lenalidomide, or pomalidomide alone at 200  $\mu$ M (Fig. 7C). Therefore, cereblon plays no role in thalidomide for bladder cancer therapy.

*Reactive oxidative species is produced after thalidomide treatment.* Free radical-mediated oxidative damage was involved in the teratogenicity of thalidomide (40), so we evaluated the role of reactive oxidative species (ROS) in thalidomide-induced cytotoxicity. After 48-h treatment, only thalidomide, neither lenalidomide nor pomalidomide, triggered the production of ROS in BFTC905 cells (Fig. 8A). A progressive significant increase in total oxidant status (TOS) was found following 48-h treatment with thalidomide (p=0.02 by one-way ANOVA, n=3; Fig. 8B). Administration of anti-oxidant catalase (10,000 U/ml), NAC (500  $\mu$ M), and DTT (500 nM) for 48 h all ameliorated thalidomide-induced cytotoxicity (p=0.04, 0.03, and 0.02 in catalase, NAC, DTT treatment group, respectively; n=3; Fig. 8C). Thalidomide significantly elicited oxidative DNA damage detected by the formation of 8-OHdG (p=0.00004, n=3; Fig. 8D), and NAC significantly reversed the effect (p=0.0005; Fig. 8D).

*Thalidomide suppresses the invasion of BFTC905 cells induced by TNF- $\alpha$ .* For Matrigel invasion assay, chamber containing BFTC905 cells with medium deficient in nutrient is placed above the well containing full nutrient medium to create a nutrient gradient for chemotaxis of invasion by BFTC905 cells. Thalidomide exerted less cytotoxicity on BFTC905 cells in the medium containing 1% FBS compared to normal medium containing 10% FBS (Fig. 9A vs. Fig. 3A). We found that 50  $\mu$ M of thalidomide did not alter the viability of BFTC905 cells (Fig. 9A; p=0.142, n=3), and this concentration was chosen for the invasion experiment. However, no changes of invasion ability and related signalings were noted. Therefore, TNF- $\alpha$  induced invasion of TCC cells was followed (33). In agreement with a previous report (33), we found that exogenous administration of 100 ng/ml TNF- $\alpha$  for 48 h, promoted proliferation of BFTC905 cells with medium containing 1% FBS (p=0.002, n=3; Fig. 9B). However, in order to avoid the effects of proliferation in invasion experiment, TNF- $\alpha$  at 10 ng/ml, which did not increase the number of BFTC905 cells in 1% FBS, was used (p=0.4, n=3; Fig. 9B). TNF- $\alpha$  promoted the invasive ability of BFTC905 cells in Matrigel assay (p=0.004, n=10), and thalidomide significantly suppressed it (p=0.03, Fig. 9C). Transcription of NF- $\kappa$ B is involved in TNF- $\alpha$  triggered signaling pathways, the nuclear translocation of P65 after treatment with TNF- $\alpha$  and/or thalidomide was examined. Nuclear P65-positive cells were increased after TNF- $\alpha$  stimulation (p=0.01, n=5), which were reversed to baseline by thalidomide treatment (p=0.03, Fig. 9D). The levels of MMP-9 and ICAM-1 have been found to be higher in high grade and more invasive type of bladder cancer (41,42). Thus, we examined the total expression of MMP-9 and ICAM-1 and MMP-9 activity in the culture medium. TNF- $\alpha$  upregulated the expression of MMP-9 and ICAM-1, and thalidomide ameliorated their expression

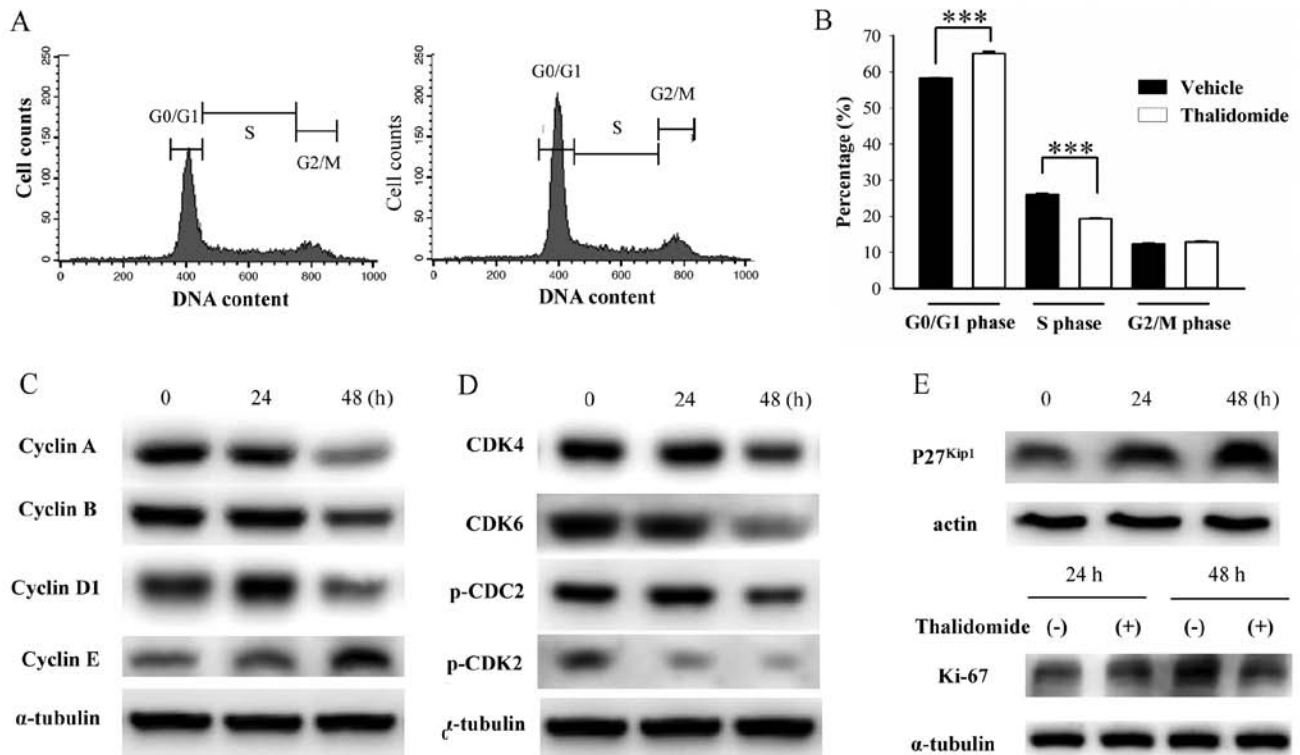


Figure 5. Changes of cell cycle and related proteins in BFTC905 cells after thalidomide (200  $\mu$ M, dissolved in 45%  $\gamma$ -cyclodextrin) treatment. (A and B) Distribution and ratios of cell cycle changes were analyzed by flow cytometry 48 h after thalidomide treatment. Representative western blots of (C and D) cell cycle related protein as well as (E) p27<sup>Kip1</sup> and Ki-67 were examined within 48 h after thalidomide treatment. Experiments were repeated 3 times with similar results. Vehicle, 45%  $\gamma$ -cyclodextrin. \*\*\*p<0.005.

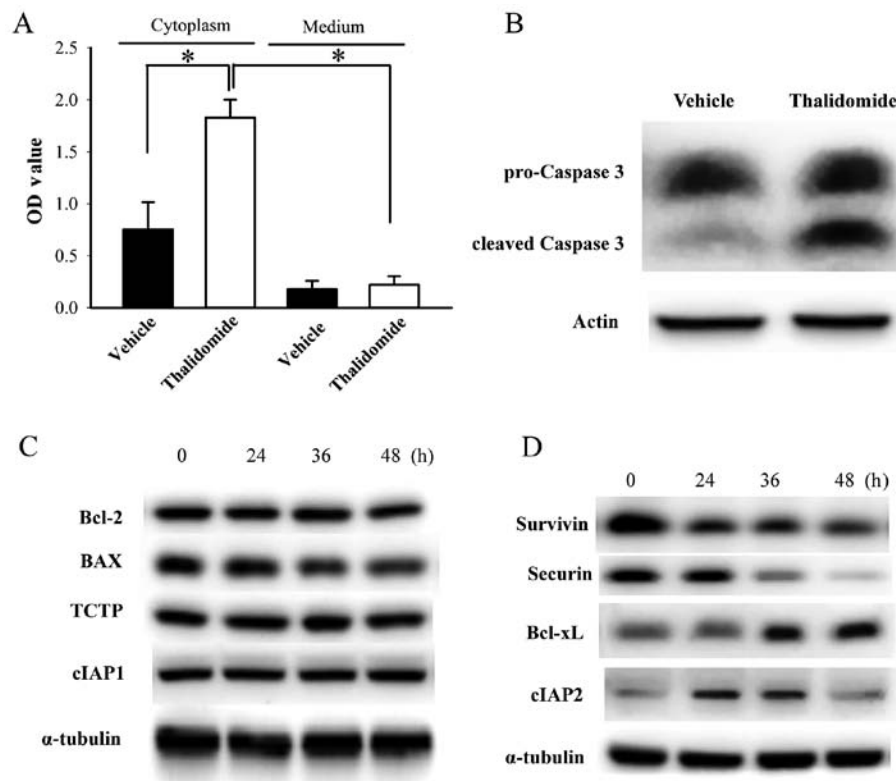


Figure 6. Apoptosis in BFTC905 cells after thalidomide (200  $\mu$ M, dissolved in 45%  $\gamma$ -cyclodextrin) treatment. After thalidomide treatment for 48 h, the supernatants of both the culture medium and the cytoplasmic fraction were collected. Increased expression of DNA fragments in the medium indicates necrosis due to membrane rupture, while increased expression in the cytoplasm alone indicates apoptosis. Representative western blots of (B) caspase 3 as well as (C and D) apoptosis and anti-apoptosis proteins were examined within 48 h after thalidomide treatment. Experiments were repeated 3 times with similar results. Vehicle, 45%  $\gamma$ -cyclodextrin. \*0.01≤p<0.05.

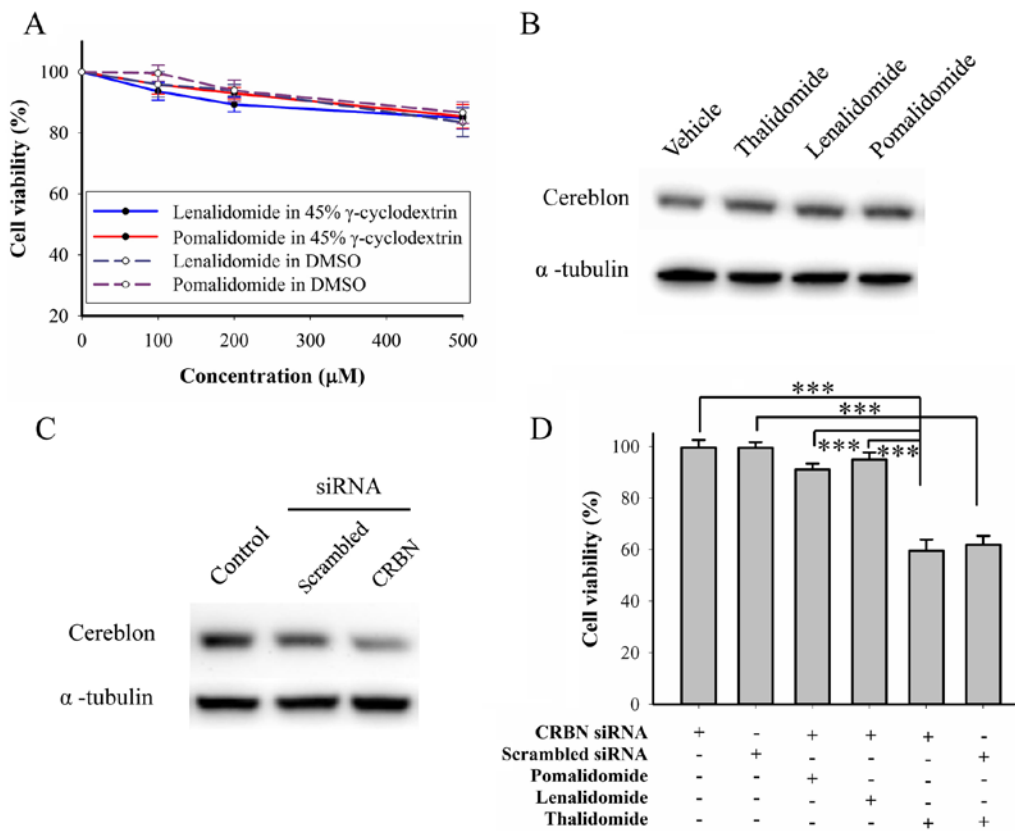


Figure 7. Role of cereblin in BFCT905 cells treated with IMiDs. (A) The viability of BFCT905 cells was measured by MTT assay 48 h after treatment with lenalidomide or pomalidomide dissolved in DMSO or 45%  $\gamma$ -cyclodextrin. Western blots of cereblin expression were examined 48 h after (B) thalidomide, lenalidomide, or pomalidomide (all at 200  $\mu\text{M}$ , dissolved in 45%  $\gamma$ -cyclodextrin) treatment, and exposure to (C) cereblin siRNA or scrambled siRNA (as negative control). (D) The viability of BFCT905 cells was measured by MTT assay 48 h after administration with IMiD and/or cereblin siRNA. \*\*\* $p < 0.005$ .

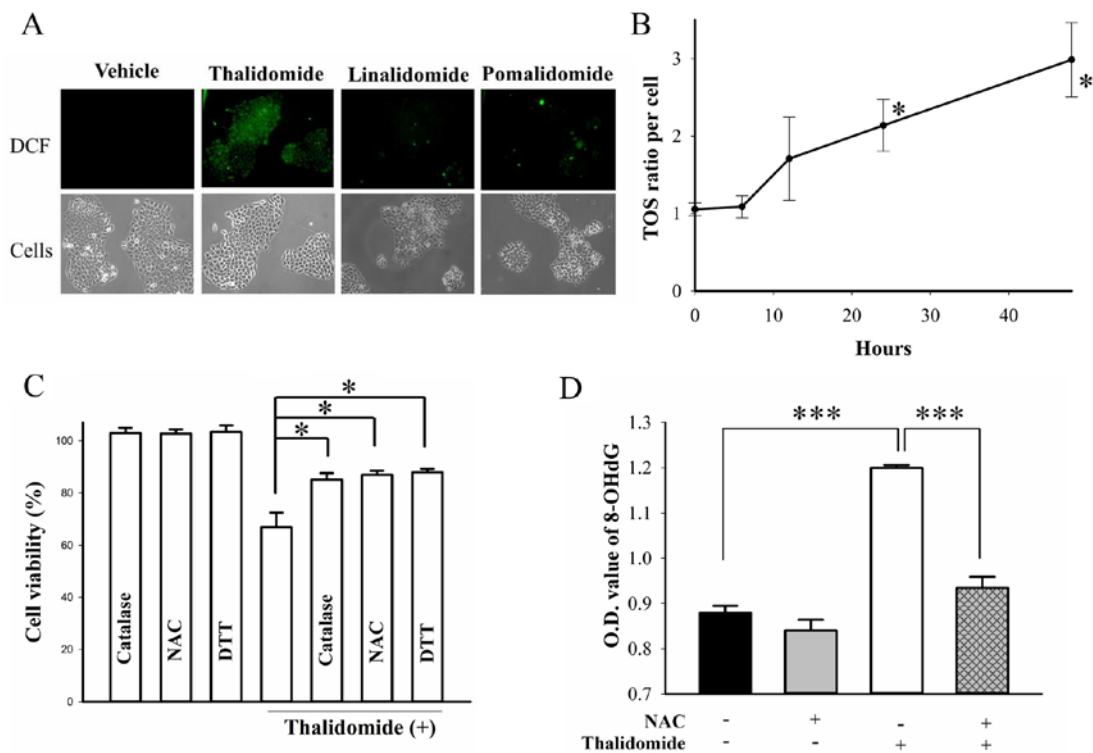


Figure 8. Role of ROS in BFCT905 cells treated with IMiDs (all at 200  $\mu\text{M}$ , dissolved in 45%  $\gamma$ -cyclodextrin). (A) Expression of DCF, proportional to the ROS level within the cell cytosol, was photographed 48 h after thalidomide, lenalidomide, or pomalidomide treatment (x100). (B) Ratios of total oxidant status (TOS) compared to vehicle exposure were measured after thalidomide treatment within 48 h. (C) The viability of BFCT905 cells was measured by MTT assay 48 h after administration with thalidomide and/or anti-oxidants, including catalase (10,000 U/ml), NAC (500  $\mu\text{M}$ ), and DTT (500 nM). (D) Formation of 8-OHdG, the marker of oxidative DNA damage after administration of thalidomide and/or NAC. Vehicle, 45%  $\gamma$ -cyclodextrin. \* $0.01 \leq p < 0.05$ , \*\*\* $p < 0.005$ .



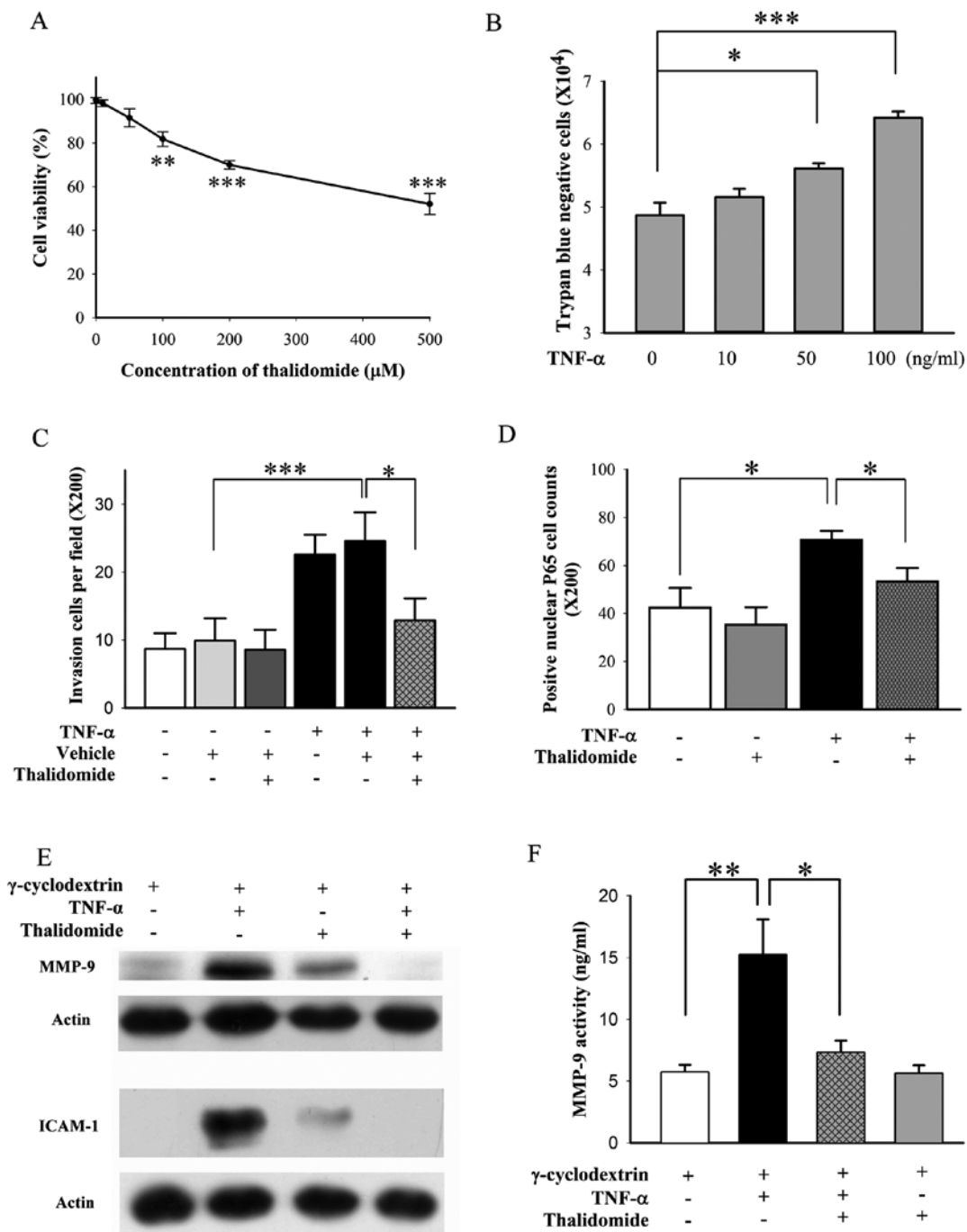


Figure 9. Thalidomide suppressed invasion of BFTC905 cells. Matrigel assay with deficient nutrient status (1% FBS) in the chamber and full nutrient environment (10% FBS) in the well to create a gradient for chemotaxis of invasion was used. (A) The viability of BFTC905 cells was measured by MTT assay 48 h after administration of thalidomide with deficient nutrient status. (B) After administration of TNF- $\alpha$  for 48 h with deficient nutrient status, trypan blue-negative cell counts were calculated. (C) Cancer cell invasion was detected by Matrigel assay after 10 ng/ml of TNF- $\alpha$  and/or 50  $\mu\text{M}$  of thalidomide. After 48-h incubation, invading cells on the bottom surface of the membrane were counted under a microscope ( $\times 200$ ). (D) Immunostaining of positive P65 expression in nuclei was counted under a microscope ( $\times 200$ ) 48 h after TNF- $\alpha$  and/or thalidomide treatment. (E) Representative western blots of MMP-9 and ICAM-1 expression were examined 48 h after treatment with 200  $\mu\text{M}$  thalidomide. (F) Extracellular MMP-9 activity of BFTC905 cells was detected by ELISA 48 h following TNF- $\alpha$  and/or thalidomide treatment. Vehicle, 45%  $\gamma$ -cyclodextrin. \* $0.01 \leq p < 0.05$ , \*\* $0.005 \leq p < 0.01$ , \*\*\* $p < 0.005$ .

after TNF- $\alpha$  stimulation (Fig. 9E). MMP-9 activity was also significantly increased by TNF- $\alpha$  stimulation ( $p=0.008$ ,  $n=5$ ; Fig. 9F). Thalidomide suppressed MMP-9 activity after TNF- $\alpha$  stimulation ( $p=0.03$ , Fig. 9F).

*Effects of thalidomide on BFTC905 xenografts in vivo.* When thalidomide treatment was initiated on the day of BFTC905

cell implantation, almost no xenograft tumor formation was observed (Fig. 10A). The difference of tumor growth curve between thalidomide and vehicle treatment revealed statistical significance ( $p < 0.0001$  by two-way ANOVA,  $n=6$ ). To evaluate the possible therapeutic effect, thalidomide treatment was administered after tumor formation (2 weeks after BFTC905 cell implantation) in another experiment

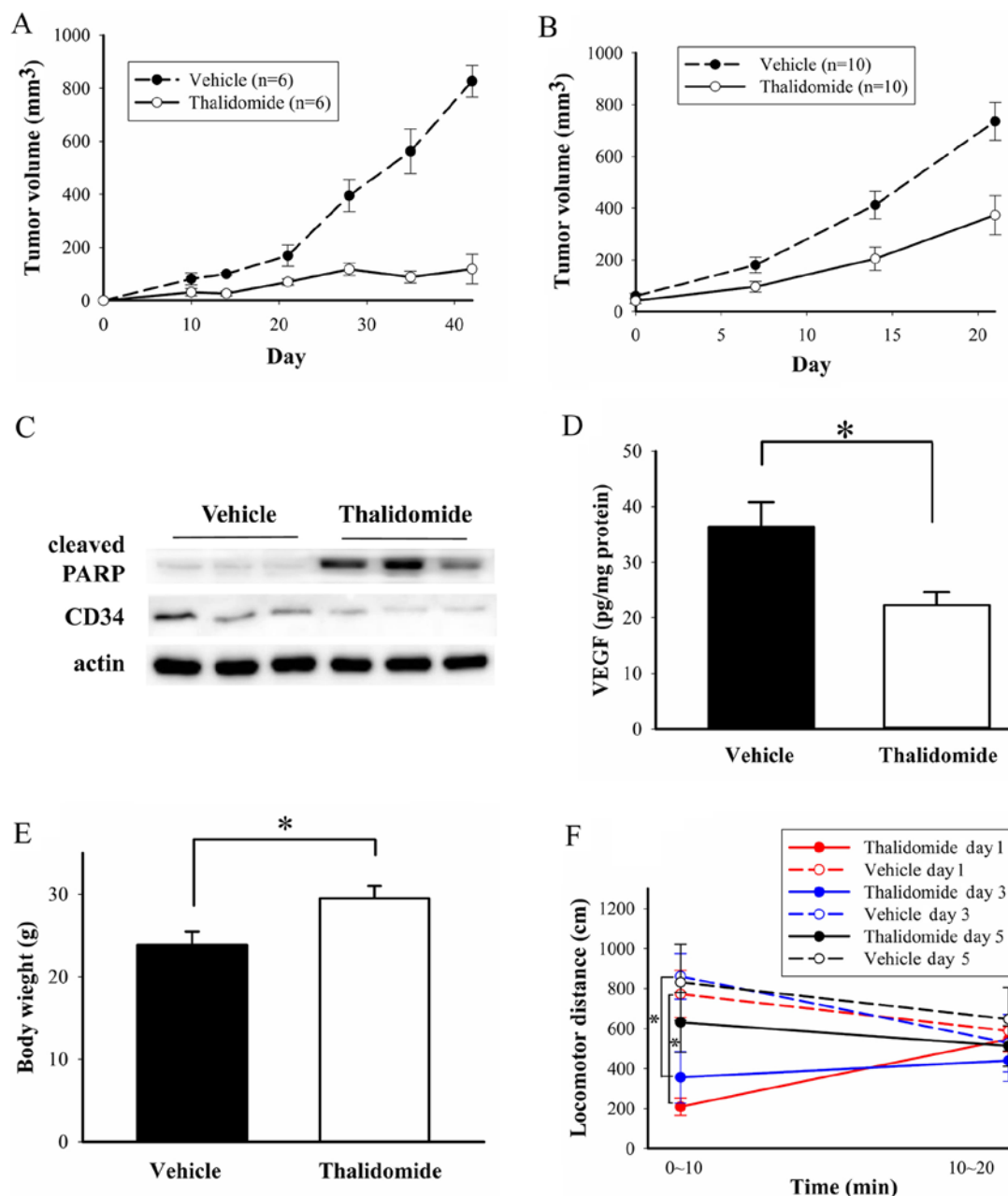


Figure 10. Effects of thalidomide in BFTC905 xenograft model of SCID mice. Treatment with 250 mg/kg of thalidomide administered s.c. into the loading site of cancer cells when the tumor was impalpable or directly into the tumor 3 times per week (W1, 3, 5) (A) on the day of implantation, and (B) 2 weeks after implantation of cancer cells. The following results were obtained after sacrifice from (B), the investigation of post-treatment strategy. (C) Representative western blots of cleaved PARP and CD34 expression in three repeated samples were examined. (D) VEGF in xenograft tumors were detected by ELISA. (E) Body weights of mice were measured. (F) Locomotor distance 0-10 min and 10-20 min after thalidomide infection in the first week was calculated. Vehicle, 45%  $\gamma$ -cyclodextrin. \* $0.01 \leq p < 0.05$ .

(post-treatment strategy). After thalidomide treatment for 3 weeks, the difference of tumor volume between thalidomide and vehicle treatment also revealed statistical significance ( $p < 0.0001$  by two-way ANOVA,  $n = 10$ ; Fig. 10B). In addition, the significant difference was observed at 7 days after treatment ( $96.4 \pm 20.7$  and  $180.3 \pm 30.1$  mm<sup>3</sup> for thalidomide and vehicle treatment, respectively;  $p = 0.03$ ). Although the tumor sizes were significantly increased after thalidomide treatment ( $p < 0.001$  in one-way ANOVA), the average tumor sizes in the thalidomide group were only half of those in the control group ( $p = 0.003$ ). After 3-week treatment, the mean tumor volume in thalidomide and vehicle group was  $372.7 \pm 75.7$  and

$735.7 \pm 73.6$  mm<sup>3</sup>, respectively. In the following results, we examined the changes of cleaved PARP, CD34 and VEGF in the xenograft tumors for the post-treatment strategy. Cleaved PARP facilitates cellular disassembly and promotes apoptosis (43), and thalidomide treatment increased the expression of cleaved PARP in three repeated samples (Fig. 10C). CD34 is a marker for angiogenesis in bladder cancer (44). Expression of CD34 was decreased in three repeated samples of thalidomide therapy (Fig. 10C). VEGF levels detected by ELISA were also significantly decreased after thalidomide treatment ( $p = 0.02$ ,  $n = 5$ ; Fig. 10D). Grossly, no overt activity changes and no distal organ metastases were observed.

Cachexia, regardless of disease category or treatment modality, is a problem for cancer patients (45). However, in our study, average body weight in thalidomide group was significantly higher than vehicle group ( $29.5 \pm 1.5$  vs.  $23.9 \pm 1.6$  g for thalidomide and vehicle group, respectively;  $n=10$ ,  $p=0.01$ , Fig. 10E). Sedative effects of thalidomide have been reported in human (46), so we also examined this adverse effect in mice. A significantly sedative effect with lower locomotor distance ( $209.3 \pm 43.7$  and  $772.9 \pm 117.7$  cm for thalidomide and vehicle treatment, respectively;  $n=3$ ,  $p=0.01$ ) was observed within the first 10 min after injecting the first dose of thalidomide to mice (Fig. 10F). The locomotor distance in the first 10 min after the second administration of thalidomide also significantly less ( $356.6 \pm 129.6$  and  $861.7 \pm 114.0$  cm for thalidomide and vehicle treatment, respectively;  $n=3$ ,  $p=0.04$ ). The locomotor distance in the first 10 min after the third administration showed no difference in either group ( $p=0.21$ ). The locomotor distance in the 10-20 min after the first, second, and third administration was similar for both groups ( $p=0.66$ ,  $0.65$  and  $0.52$ , respectively).

## Discussion

In previous studies, thalidomide was usually dissolved in DMSO (18,47-49) or CMC (50-52). However, there are only a few *in vitro* thalidomide studies, and few researchers pay attention to the solubility of thalidomide. Some *in vitro* studies reported that the inhibitory effect of thalidomide may be partially attributed to the solvent DMSO alone (53,54), and our results were compatible with these studies. Cyclodextrins, cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity to form inclusion complexes, can improve solubility and permeability (55). Some studies reported that thalidomide dissolved in ether-7  $\beta$ -cyclodextrin or hydroxypropyl- $\beta$ -cyclodextrin improved the aqueous solubility (56-58). Kratz *et al* tested the solubility of thalidomide with  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin, and  $\gamma$ -cyclodextrin (56). Thalidomide dissolved in  $\beta$ -cyclodextrin showed the best solubility, however, cytotoxicity was not determined.

There are few *in vitro* studies of thalidomide on solid malignancy cells. The concentration of thalidomide used in this study might be relative high for BFTC905 cells, so we tried to find the reasons. Release rate of drugs using cyclodextrin as a carrier might be rapid, so insufficient intracellular concentration due to thalidomide  $\gamma$ -cyclodextrin complex could be excluded (59). Compensatory increase in the expression of anti-apoptotic protein Bcl-xL was found in our experiment, but overexpression of Bcl-xL was not correlated with the recurrence and survival of bladder cancer patients (60). In our previous study, upregulation of securin and Bcl-2 in BFTC905 cells may be a compensatory mechanism for gemcitabine resistance (22). Downregulated expression of securin, but no changes of Bcl-2 expression were observed after thalidomide treatment. This may explain the lack of additively therapeutic effects of gemcitabine plus thalidomide combination in this study, and the much less potency of thalidomide than YM155, the survivin inhibitor which decreased both securin and Bcl-2 expression in our previous study (22). However, thalidomide has some advantages for the potential therapy of bladder cancer clinically, for examples, available in the market and possible oral administration. Second, hypoxia-inducible CAIX

generates protons that contribute to acidification of external pH and provides bicarbonate to neutralize intracellular pH through the inward transporter. Such pH maintenance plays a role in the regulation of cell proliferation, cell adhesion, and tumor progression to mediate drug resistance (61). Any treatment aimed to inhibit HIF-1 $\alpha$ -CAIX axis signaling pathways as a therapy potential for bladder cancer has been investigated (62). HIF-1 $\alpha$  induced CAIX upregulation was observed after thalidomide treatment in this study. This may also contribute to the decreased potency of thalidomide on BFTC905 cells. Third, non-cycling quiescent cells can take the time to repair the damage after cancer therapy (63,64). The promoting effects of thalidomide to increase quiescent TCC cells, although only 7% in magnitude, may also partially explain the relatively high concentration of thalidomide used in this study.

In the present study, the growth inhibition of thalidomide on TCC cells resulted mainly from apoptotic effects. The cytotoxic effects of 500  $\mu$ M thalidomide on primary HUC cells may result from  $\gamma$ -cyclodextrin *per se*. Our result demonstrated the safety to normal urothelial cells with administration of 200  $\mu$ M thalidomide dissolved in 45%  $\gamma$ -cyclodextrin. Some studies also reported the apoptotic activity of thalidomide (17,65). In agreement with a previous study (66), we found no changes of cell cycle after thalidomide treatment. Downregulation of survivin and securin by thalidomide is the novel findings in our study. Survivin, a member of the inhibitor of apoptosis protein family, has been shown to be a prognostic parameter of bladder cancer (67). Previously we reported that securin was overexpressed in human TCC specimens (68). Therefore, decreased expression of survivin and securin is a good indicator of thalidomide therapy for bladder cancer. Jian *et al* demonstrated that inhibition of low-grade TCC cells by lenalidomide was attributable to not only its direct tumor apoptotic but also anti-angiogenic activity (69), so we compared cytotoxicity of three IMiDs on gemcitabine-resistant TCC cells. Previous research demonstrated cereblon-dependent anti-neoplastic activity of IMiDs in cancer cells (70,71). Depletion of cereblon endowed cancer cells with therapeutic resistance to IMiDs. However, cereblon plays no role in thalidomide-induced cytotoxicity in BFTC905 cells. Lack of correlation with cereblon and IMiDs in human myeloma cell lines has also been reported (72). ROS is a double-edged sword in cancer biology. Puskás *et al* demonstrated that lipid droplet binding thalidomide analogs induced oxidative stress in cancer cells (73). Teratogenic mechanism of thalidomide may be caused by enzymes catalyzing thalidomide to a free radical intermediate, and further generating ROS to modify DNA in many ways, including the formation of 8-OHdG (40). Our results were consistent with the pathophysiology of thalidomide-induced damage in embryos.

BFTC905 cells possess greater invasive ability (23), so any treatment which suppresses their invasion may be a good strategy for bladder cancer therapy. The limitation of cell culture experiments is that sometimes the *in vitro* environment may not mimic the *in vivo* one. Due to lack of stimulated or stress condition in cell culture system, we followed the model of TNF- $\alpha$  induced invasion in TCC cells (33). We used only 10% of TNF- $\alpha$  stimulation compared to Lee *et al* (33) to avoid the proliferative effects on invasion. Thalidomide exerted its effect on downregulation of NF- $\kappa$ B-induced ICAM-1 through

inhibition of degradation of I $\kappa$ B (18,74). Thalidomide decreased the mRNA, protein synthesis, and secretion of MMP-9 (75). This evidence was also found in our thalidomide-treated BFTC905 cells.

Although thalidomide significantly induced apoptosis and inhibited angiogenesis in our animal experiments, thalidomide only suppressed the growth rates of BFTC905 xenografts. Nonetheless, it is consistent with the *in vitro* findings that some compensatory or resistant mechanisms were found in thalidomide-treated BFTC905 cells. Comparing to other studies (200 mg/kg/d = 1,400 mg/kg/w), less weekly administration of thalidomide (250 mg/kg three times per week = 750 mg/kg/w) in our study might be another explanation (76,77). Therefore, combination of thalidomide with other drug(s) might be the next strategy. Unfortunately, gemcitabine plus thalidomide did not show additive cytotoxicity on BFTC905 cells *in vitro*. Better drug combinations should be tested in the future. Beyond the detection of VEGF in tumors, immunostaining of CD34 to analyze microvascular density, a specific marker of endothelial cells, is a common method for the evaluation of angiogenesis (78). We have also examined the localization of CD34 in BFTC905 xenograft tumors by immunohistochemistry, but few and scattered distribution of positive CD34 endothelial cells were found (data not shown). So western blotting was used instead of immunohistochemistry for CD34 in xenograft tumors. Low expression of CD34 measured by western blotting was correlated with the findings by immunohistochemistry. Thalidomide was effective in attenuating weight loss in cancer patients with cachexia (79), and our study also showed similar results. No animal studies report the sedative effect after thalidomide treatment. Our observation that thalidomide exerted significant somnolence in mice may be due to the higher dose of injections. Patients developed tolerance to the side effect of sedation (80), and the present study is compatible with clinical usage of thalidomide.

There are limitations in this study. We cannot characterize the thalidomide  $\gamma$ -cyclodextrin complex, for example, using X-ray powder diffractometry. In addition, we cannot detect the concentration of thalidomide in cells. Lack of co-culture of natural killer cells and TCC cells to examine the effects of IMiDs on immune cells (81) is another limitation. However, in the present study, we demonstrated the potential effects of thalidomide therapy, including induction of apoptosis through oxidative stress, inhibition of angiogenesis through decreases in VEGF production and secretion, and suppression of invasive ability via downregulation of MMP-9 and ICAM-1, for gemcitabine-resistant bladder cancer cells *in vitro* and *in vivo*.

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