Department of Neurosurgery[1], Third Hospital ; Department of General Surgery[2], Third Hospital; Department of Neurosurgery[3], First Hospital, Jilin University, Changchun, Jilin, China

# Thiabendazole, a well-known antifungal drug, exhibits anti-metastatic melanoma B16F10 activity via inhibiting VEGF expression and inducing apoptosis

JINNAN ZHANG[1,3], CONGHAI ZHAO[1], YUFEI GAO[1], YANG JIANG[2], HUAXIN LIANG[1], GANG ZHAO[3]

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*Gang Zhao, MD, PhD, Department of Neurosurgery, First Hospital, Jilin University, Changchun, Jilin, 130021,China LXL21@sina.com*

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Thiabendazole, an orally available antifungal drug, has been used in clinical practice for 40 years. Previous studies indicated its potential in inhibiting angiogenesis in both animal models and in human cells. Malignant melanoma is associated with angiogenesis and it is unknown whether thiabendazole is effective for malignant melanoma or not. In our research, the effects of thiabendazole on the proliferation of the murine metastatic melanoma cell line B16F10 *in vitro* and *in vivo* and the molecular mechanism were investigated. Assay of cell viability, chick embryo chorioallantoic membrane assay, quantitative real-time PCR, Western blot, wound healing assay, annexin V/propidium iodide (AV/PI) assay and B16F10-xenograft model were applied to elucidate the mechanism of thiabendazole on B16F10 cells. Thiabendazole inhibited B16F10 proliferation *in vitro* in a dose- and time-dependent manner with an $IC_{50}$ of $532.4 \pm 32.6$, $322.9 \pm 28.9$, $238.5 \pm 19.8$ μM at 24, 48, and 72 h, respectively. Moreover, thiabendazole inhibited the angiogenesis and the migration of B16F10 cells *in vitro*. Furthermore, thiabendazole restrained transcription and translation of the *VEGF* gene in B16F10 *in vitro*, and the apoptotic percentage of B16F10 cells was increased after exposure to thiabendazole. Finally, in the B16F10-bearing mice model, thiabendazole significantly suppressed tumor growth with inhibitory rates of 16.5%, 35.4% and 48.7% at the treatment of thiabendazole 20, 40 and 80 mg/kg, respectively. These results further indicated that thiabendazole may be a potential candidate for the treatment of malignant melanoma.

## 1. Introduction

Angiogenesis is the growth of new blood vessels from pre-existing blood supply (Emmett et al. 2011) and is essential for tumor growth and metastasis (Hanahan and Weinberg 2000). It has been proven that most tumors cannot grow beyond 1–2 mm without blood supply (Corrieet al. 2010). In 1971, Judah Folkman proposed that the inhibition of angiogenesis might be an effective therapeutic approach against cancer (Folkman 1971). Vascular endothelial growth factor (VEGF), a heparin-binding family of glycol-proteins (including VEGF-A, VEGF-B, VEGF-C, and VEGF-D), is a key player in angiogenesis for promoting endothelial cell proliferation, migration, survival, vasodilatation, and vasculogenesis via recruiting bone marrow-derived haematopoetic progenitor cells (Ferrara and Henzel 1989). Hence, anticancer drugs that could prevent VEGF expression attracted the scientists' attention.

Melanoma, occupied 65% of all skin cancer-related deaths (Cockerell 2012), is an aggressive, fatal skin cancer, and its incidence has risen rapidly during the past few years. Melanoma originates from the uncontrolled proliferation of specialized melanocytes, which could produce pigments in the epithelial layers and migrate to many locations within the body, such as eyes, bone, and heart (Mansh 2011). In clinical practice, surgery was used to cure local melanoma in the early stages, but this treatment is not effective for metastatic melanoma (Pawlik and Sondak 2003; Eggermont and Kirkwood 2004). Recent research has shown that angiogenesis is relevant to melanoma progression (Streit and Detmar 2003). The role of neovascularisation in human cutaneous melanomas was demonstrated to indicate the activity of angiogenesis and the role of VEGF in melanoma angiogenesis was firstly demonstrated with successfully transplanted human melanoma fragments into a hamster cheek pouch (Warren and Shubik 1966). It was also shown that aggressive melanoma cell lines express higher levels of VEGF (Pötgens et al. 1995), and melanoma patients with evaluated serum VEGF levels have been observed with advanced disease and a higher rate of relapse (Osella-Abate 2002). Moreover, inhibition of tumor growth has been achieved in different melanoma xenograft models by various anti-VEGF strategies (Li et al. 2002; Wedge et al. 2002).

Thiabendazole (TBZ; 4-(1*H*-1,3-benzodiazol-2-yl)-1,3-thiazole), as an anti-fungal and anti-helminthic drug, had already been approved by the U.S. Food and Drug Administration (FDA) since 1967, so its safety has been well established. Moreover, scientists recently have found that thiabendazole had the potential in inhibiting angiogenesis (Cha et al. 2012). However whether thiabendazole had effects on the melanoma cells and its relative mechanisms has not yet been investigated. In this study, the inhibitive effect of thiabendazoleand on murine metastatic melanoma cell line B16F10 proliferation *in vitro* and *in vivo* and its relative mechanisms were firstly investigated. The

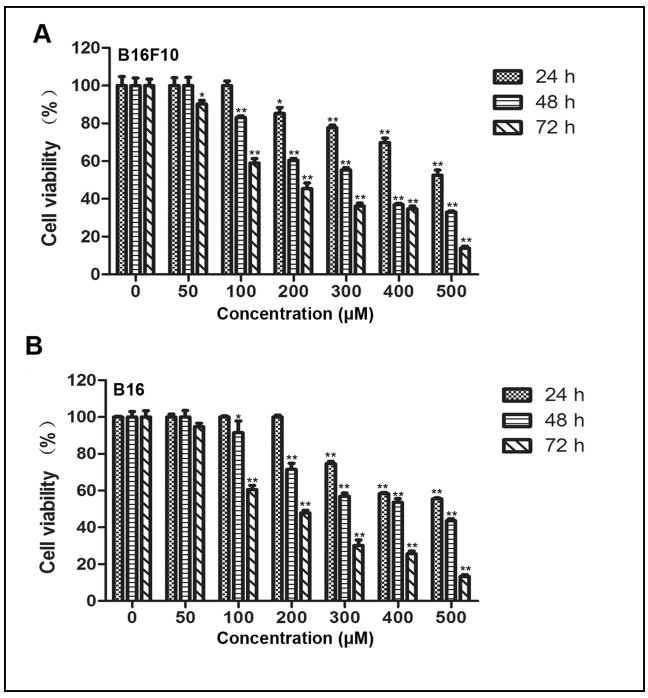


Fig. 1: Drug sensitivity assays were performed by MTT assay using thiabendazole (range from 0–500 μM) at 24 h, 48 h and 72 h post-treatment. Inhibition of proliferation of B16F10 cells and B16 cells by thiabendazole was in a dose- and time-dependent manner. Values are presented as mean ± SD from three separate determinations with six replicates each. (*$p < 0.05$, **$p < 0.01$, compared with the untreated control at each time point). (A) B16F10 (B) B16.

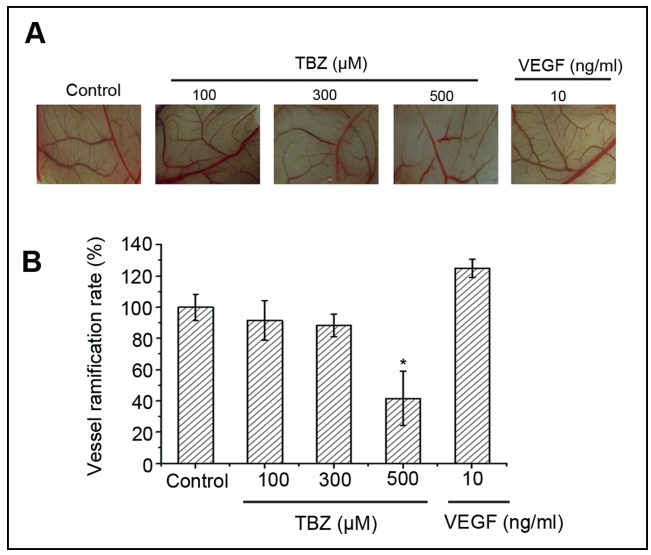


Fig. 2: Effect of thiabendazole on the angiogenesis of CAM. (A) Various concentrations of thiabendazole (0, 100, 300, 500 μM per egg) were loaded on the CAMs with or without VEGF (10ng/ml). After 72 h incubation, methanol and acetone (1:1) was added onto the surface of CAMs to fix the blood. The disc and surrounding CAMs were harvested and photographed. Thiabendazole effectively inhibited angiogenesis *in vivo*. (B) Quantitative analysis of neovascularizationin the capillary bed area of CAM. Thiabendazole treated groups had fewer blood vessels than the untreated negative control groups and the VEGF treated positive control grous. The blood vessels in the untreated negative control group were treated as 100% and the blood vessels in thiabendazole treated groups were compared to the negative control group. Data are presented as the mean ± SD (n = 6. *$P < 0.05$, compared with negative control group).

results obtained revealed thiabendazole suppressed metastatic melanoma cell proliferation *via* inducing apoptosis in B16F10 cells and preventing transcription and translation of the *VEGF* gene in B16F10 cells *in vitro*. These interferences then further inhibited angiogenesis, B16F10 migration and tumor growth of B16F10 in mice. These results highlight the therapeutic potential of thiabendazole in metastatic melanoma.

## 2. Investigations and results

### *2.1. Thiabendazole inhibited cell proliferation of murine melanoma cell lines B16 and B16F10*

To clarify whether thiabendazole could inhibit the growth of murine melanoma cells, we treated B16 and B16F10 cells with thiabendazole at concentrations of 0, 50, 100, 200, 300, 400 and 500 μM for 24, 48, and 72 h. Cell viability was examined by a MTT cell viability assay. Thiabendazole time- and dose-dependently inhibited cell proliferation (Fig. 1). The 50% inhibitory concentrations ($IC_{50}$) of B16 cells were 540.8 ± 40.8, 410.7 ± 30.6, 280.4 ± 22.3 μM at 24, 48, and 72 h, respectively (Table). And the $IC_{50}$ value of B16F10 cells were 532.4 ± 32.6, 322.9 ± 28.9, 238.5 ± 19.8 μM at 24, 48, and 72 h, respectively (Table). These data suggested that thiabendazole exhibited inhibitory effects to murine melanoma cell lines *in vitro*.

**Table: $IC_{50}$ values (μM) of B16F10 and B16 cells at different times**

| | 24 h | 48 h | 72 h |
|---|---|---|---|
| B16F10 | 532.4 ± 32.6 | 322.9 ± 28.9 | 238.5 ± 19.8 |
| B16 | 540.8 ± 40.8 | 410.7 ± 30.6 | 280.4 ± 22.3 |

### *2.2. Thiabendazole inhibited angiogenesis in the CAM assay*

Angiogenesis is critical during tumor progression (Hanahan and Weinberg 2000) and is associated with melanoma progression (Streit and Detmar 2003). To investigate the effect of thiabendazole on angiogenesis *in vivo*, we performed a chick embryo chorioallantoic membrane (CAM) assay, a well established angiogenesis model. As indicated in Fig. 2A, the blood vessels formed dense branching vascular networks in the untreated negative control group and the positive VEGF group. In contrast, thiabendazole dose-dependently suppressed CAM neovascularization, presenting as reducing blood vessel numbers and branching patterns. In addition, these results were supported by statistically data that new microvessels formation rate was 91.6%, 88.3% and 41.6%, at the concentrations of 100, 300, 500 μM compared to the untreated negative control group (treated as 100%), respectively (Fig. 3B).

### *2.3. Thiabendazole suppressed the VEGF gene expression in B16F10 cells*

Previous reports have indicated that VEGF, which is produced by tumor cells under hypoxic conditions, is considered to be one of the most crucial and specific regulators in angiogenic signaling cascades (Ferrara 2002). Additionally, increased levels of VEGF are associated with aggressive and metastatic phenotypes in melanoma cells (Dewing et al. 2012). Therefore, the effect of thiabendazole on VEGF expression in B16F10 cells was determined. Quantitative real-time PCR (qRT-PCR) was performed to determine the RNA levels of VEGF when B16F10 after exposure to thiabendazole for 48 h. As presented in Fig. 3A, the decrease in VEGF RNA levels occurred in a dose-dependent manner and showed significant differences ($P < 0.05$) at the 300 μM and 500 μM of thiabendazole when compared to the controls.

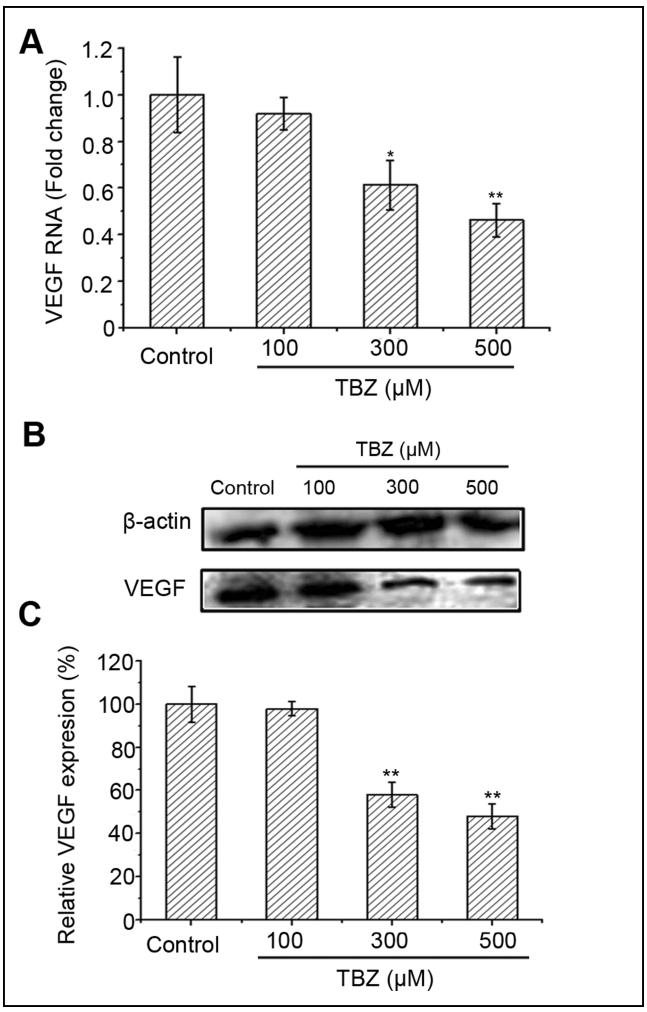


Fig. 3: Inhibition of VEGF in thiabendazole-treated B16F10 cells. (A) RNA extracted from B16F10 cells treated with or without thiabendazole (100, 300 and 500 μM) for 48 h, and then were measured by real-time PCR assay as indicated in the methods. The VEGF RNA levels were dose-dependently suppressed by thiabendazole. (*$P < 0.05$, **$P < 0.01$, compared with control group). Experiments repeated three times with similar results. (B) Protein extracted from the B16F10 cells that treated with or without thiabendazole (100, 300 and 500 μM) for 48 h were analyzed by Western blot as described in the methods. The western blot bands were photographed, scanned and analyzed by Quality One 1-D analysis software. Thiabendazole inhibited VEGF protein expression in a dose-dependent pattern. (C) Densitometric analysis of VEGF protein levels in Western blot (**$P < 0.01$, compared with control group). Shown were representative blots from three independent experiments with similar results.

To explore whether thiabendazole could affect the protein expression of VEGF, western blotting was performed. As the Figs. 3B and 3C show, the effect of thiabendazole on B16F10 cells on the protein levels of VEGF was consistent with its effect on RNA levels. These data indicated that thiabendazole could inhibit the transcription and translation of the *VEGF* gene.

### *2.4. Thiabendazole inhibited B16F10 cell migration*

As tumor cell migration is an important process in tumor development and metastasis, we detected the effects of thiabendazole on B16F10 cells motility using a scratch wound assay. As shown in Figs. 4A and 4B, in the absence of thiabendazole group, B16F10 cells migrated along the edges of the wound and showed a large-scale migration, whereas a dose- and time-dependent inhibition of cell spreading and flattening could be observed in the presence of thiabendazole. Specifically, the average widths of the wound in thiabendazole-treated groups were 52.2% and 64.7% (compared to the width at 0 h) at 300 and 500 μM thiabendazole, respectively, after 48 h incubation.

### *2.5. Thiabendazole induced apoptosis of B16F10*

To test whether thiabendazole induced cell death *via* apoptosis, the percentage of apoptotic cells was determined by an AV/PI assay. Positive staining with AV-FITC correlated with the loss of membrane polarity, and the complete loss of membrane integrity lead to apoptosis or necrosis. PI entered the cells after the loss of membrane integrity. Thus, dual staining with AV and PI adopted for discriminating between unaffected and apoptotic cells. The results indicated that thiabendazole induced the apoptotic effects on B16F10 cells (Figs. 5A and 5B). Specifically, in the treatment of B16F10 cells with 300 μM thiabendazole, the induced apoptotic cell accumulation reached approximately 31.9%. Therefore, apoptosis induction could be another mechanism for thiabendazole in preventing B16F10 cells' proliferation.

### *2.6. Thiabendazole suppressed the growth of B16F10-transplanted tumor in mice*

Considering that thiabendazole significantly inhibited the proliferation of B16F10, suppressed the angiogenesis and induced apoptosis it was tested whether thiabendazole could inhibit tumor growth *in vivo*, and a B16F10 xenograft model was established. After thiabendazole treatment, the tumor weight was measured. As presented in Figs. 6A, 6B and 6C, thiabendazole dose-dependently suppressed the tumor growth of B16F10 in C57BL/6J mice. Compared to the model group, the tumor inhibitory rates of thiabendazole-treated groups were of 16.5%, 35.4% and 48.7% at treatment of 20, 40 and 80 mg/kg, respectively (Fig. 6D). These results suggested that thiabendazole administration prevented B16F10 tumor growth in mice.

## 3. Discussion

Angiogenesis was first associated with malignancy 100 years ago (Goldmann 1908), and defined as the new vessel growth from a pre-existing blood supply and was as an absolute requirement for tumor survival and progression (Carmeliet and Jain 2000). Angiogenesis is physiologically tightly regulated in the progress of wound healing, embryogenesis, and female reproduction and occurs under several pathological conditions, including malignant melanoma (Dewing et al. 2012). Thiabendazole, an orally available antifungal drug in clinical use for 40 years, potently inhibited angiogenesis in animal models and in human cells (Cha et al. 2012). However, its antitumor effects on malignant melanoma had not yet been studied in detail.

Thiabendazole remarkably inhibited the proliferation of two murine melanoma cells, B16 and B16F10, indicating that thiabendazole possessed an anti-B16F10 activity. Additionally, B16F10 cell line was a malignant melanoma cell line. And malignant melanoma, a highly metastatic tumor with poor prognosis and high resistance to treatment, has been documented as an angiogenic tumor type, demonstrating new vessel formation as a crucial progress in disease progression from atypical melanocytes, through radial growth to the aggressive vertical growth phase (Streit and Detmar 2003). Hence, B16F10 cell line, a malignant melanoma cell line, was chosen as investigated object in this study.

Thiabendazole inhibited the neovascularization as shown in the CAM assay (Fig. 2). Whether the thiabendazole could affect the angiogenesis related gene expression in B16F10 cells was further investigated.

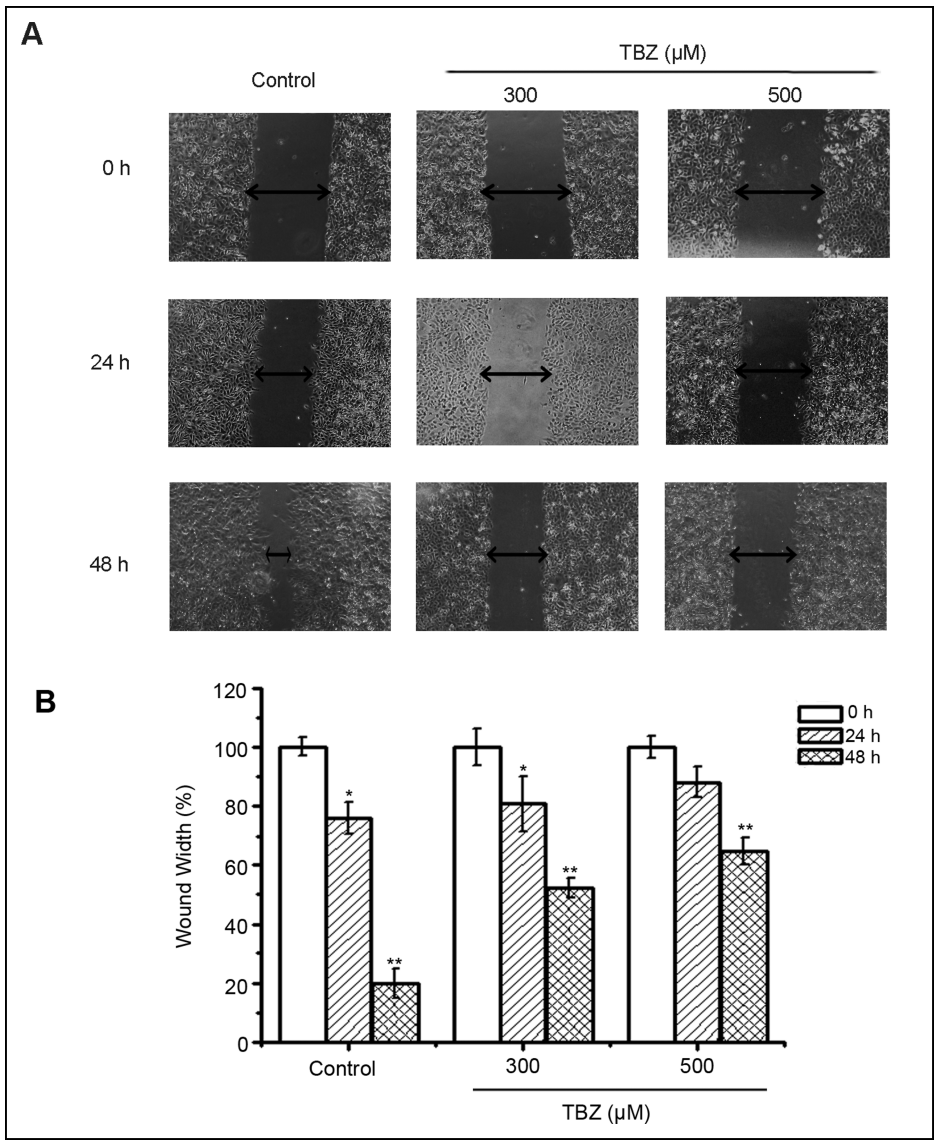


Fig. 4: Suppressive effects of thiabendazole on the migration of B16F10 cells. B16F10 cells were seeded in a 12-well plate and the confluent monolayers were wounded and then incubated in serum-free medium with 0, 300, and 500 μM thiabendazole. At 0, 24 h and 48 h after wounding, the cells were photographed and analyzed. Thiabendazole-treated B16F10cells migrated more slowly than the control group after wounding. (A) Representative micrographs are shown (×100). (B) Quantitative analysis of the wound healing assay. The width of the wound was determined after incubation by using Image Pro Plus 5 software. Data are presented as the mean ± SD, n = 3, $*P < 0.05$, $**P < 0.01$, compared with the wound width at 0 h.

As well known, angiogenesis is critical in the development of tumor growth and is regulated by several growth factors (Roskoski 2007) and VEGF is a crucial regulator in the angiogenic signaling cascades (Ferrara 2002). Researchers have reported that increased levels of VEGF correlated with the migration and angiogenesis of melanoma (Dewing et al. 2012), so VEGF was an important therapeutic target for malignant melanoma. Additionally, bevacizumab (Avastin), a combination of the monoclonal antibody, which targets VEGF, has been combined with interferon or cytotoxic chemotherapy in the treatment of metastatic melanoma in clinical (Corrie et al. 2010). Considering that thiabendazole suppressed the proliferation of B16F10 cells, malignant melanoma cells, and inhibited the neovascularization. We therefore hypothesized that thiabendazole might suppress *VEGF* gene expression in B16F10 cells. Further results illustrating that thiabendazole significantly inhibited the transcription and translation of the *VEGF* gene in B16F10 cells in a dose-dependent manner (Figs. 3A, 3B and 3C).

Since malignant melanoma is an aggressive cancer, the inhibitory effect of thiabendazole on migration was determined. As the Fig. 4 shown, the migration of B16F10 cells was dose-dependently inhibited by thiabendazole. The average widths of the wound in 300 and 500 μM thiabendazole-treated groups were 52.2% and 64.7%, respectively, after 48 h incubation, while the wound in the control group was nearly healed.

Thiabendazole dose-dependently induced B16F10 cell apoptosis *in vitro*, and the apoptosis rate was 31.9% at the concentration of 300 μM. Hence, induced apoptosis may be another mechanism for thiabendazole inhibiting the proliferation of B16F10 cells. Moreover, studies have found that VEGF expression was correlated with apoptosis (Benjamin et al. 1999; Alon et al. 1995; Benjamin and Keshet 1997) and VEGF suppressed cell death through autocrine mechanism (Ferrer et al. 1999; Langer et al. 2000; Wu et al. 2003). It is possible that thiabendazole inhibited the VEGF expression, which might lead to induce the apoptosis of B16F10 cells.

Thiabendazole exhibited a remarkable inhibitory effect on B16F10 cells *in vitro*, suppressed *VEGF* gene expression, and inhibited angiogenesis and migration. Whether thiabendazole had the inhibitory effect *in vivo* was further explored by establishing B16F10-xenograft tumor models in C57BL/6J mice. Compared to the model control, thiabendazole dose-

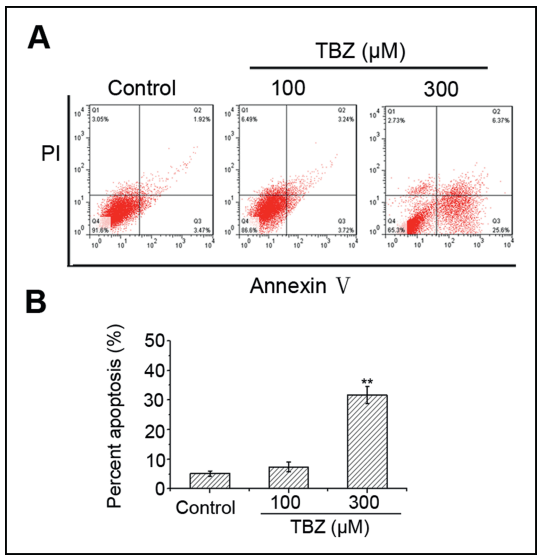


Fig. 5: Induction of apoptosis by thiabendazole in B16F10 cells. (A) Apoptosis analysis in B16F10 cells was assessed by Annexin V/PI double staining. After cells were exposed to the three designated concentrations of thiabendazole (100, 300 μM, dissolved in 1%DMSO) for 48 h, the attached and detached cells were collected. Then staining with Annexin V and PI, cells were subjected to flow cytometry analysis. Bottom right quadrant, cells mainly stained by Annexin V were early apoptotic cells; top right quadrant, cells were stained by both PI and Annexin V were late apoptotic/necrotic secondary necrosis; top left quadrant, cells mainly stained by PI viable cells; bottom left quadrant, cells negative for both Annexin V and PI. (B) Percentage of apoptosis. The data presented represents the mean ± SD from three independent experiments (**$P < 0.01$, compared with the control group).

dependently suppressed the proliferation of B16F10 cells in tumor-bearing mice with 48.7% inhibition at 80 mg/kg. Hence, thiabendazole could inhibit the B16F10 cells proliferation not only *in vitro* but also *in vivo*.

In summary, our results indicated that at least two mechanisms contributed to the outstanding anti-B16F10 action of thiabendazole. Thiabendazole inhibited the proliferation of B16F10 cells *in vitro and in vivo*, through inducing apoptosis and inhibiting the expression of VEGF which further prevented angiogensis and the migration of B16F10 cells. These results further suggested that thiabendazole might be a potential candidate for the treatment of malignant melanoma.

## 4. Experimental

### 4.1. Cell culture

The B16 cell line and B16F10 cell line were obtained from ATCC (Manassas, Virginia, USA) and grown in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/ml of penicillin and 100 U/ml of streptomycin, respectively. Cells were cultured at 37°C in a humidified incubator containing 5% $CO_2$.

### 4.2. Experimental animals

Female C57BL/6J mice (6–8 weeks of age) were purchased from the Academy of Military Medical Science and acclimatized for 1 week before use. Animals were housed in a rodent facility at 22 ± 1 °C with a 12 h light–dark cycle and provided with continuous standard rodent chow and water for acclimatization. All procedures involving animals and their care in this study were in strict accordance with protocols approved by the Ethics Committee of Jilin University.

### 4.3. Analysis of cell viability

The effect of thiabendazole on the proliferation of B16 and B16F10 cells was detected by MTT assay as described (Zhang et al. 2012). B16 and B16F10 cells in the logarithmic phase of growth were inoculated on 96-well plates at a concentration of $5 \times 10^3$ cells/well with RPIM-1640 medium that contained 10% fetal calf serum. After 12 h, different concentrations (range from 0 to 500 μM) of thiabendazole (dissolved in 1% DMSO) (Sigma-Aldrich) were added to the cells and further incubated for 24 h, 48 h and 72 h. After incubation, the culture medium was removed, and the cells were washed with PBS. Then the cells were further incubated with an MTT solution (5 μg/ml) for 4 h. Next, the cells were lysed and the purple formazan crystals were solubilized for detection at 570 nm with an ELISA reader. The cell viability was calculated with the following formula: cell viability (%) = $OD_A/OD_B \times 100\%$. where $OD_A$ is the absorbance at 570 nm of the experimental group and $OD_B$ is that of the control group.

### 4.4. Chicken embryo chorioallantoic membrane (CAM) assay

The chick embryo chorioallantoic membrane (CAM) model was performed as described previously (Dai et al. 2008). A small hole (approximately to 3 cm diameter) was made in the 8-day-old chick embryos' eggshell at the air sac to create a false air sac. Next, prepared aseptic filter papers with different concentration of thiabendazole (100, 300 and 500 μM, dissolved in PBS with 1% DMSO) were placed onto the CAMs. As the untreated controls, aseptic filter papers contained with PBS with 1% DMSO were loaded on the CAMs. In the positive control group, 10 ng/ml VEGF (PeproTech) were treated in the same manner. After 3 days of treatment, CAMs were fixed with methanol and acetone 1:1 (v/v) for 15 min, then the CAM area was cut off and photographed. The percentage of vessels in the thiabendazole treated groups was calculated as: vessel numbers of CAM in the drug-treated/vessel numbers of CAM in the untreated group × 100%. Each group contained 6 separate CAMs.

### 4.5. Quantitative real-time polymerase chain reaction

Total RNA was extracted from B16F10 cells treated with or without thiabendazole using the RNeasy Plus Mini Kit (KeyGEN) following the manufacturer's instructions. cDNA was generated with the iScript Select cDNA Synthesis Kit (KeyGEN) and then analyzed by quantitative real-time polymerase chain reaction (PCR) via using SyberGreenqPCR primer assays (KeyGEN) and the iCycleriQ multicolor real time PCR detection system (KeyGEN). Relative expression levels were normalized against β-actin expression run concurrently as a reference control.

The cDNA fragment for VEGF was amplified by PCR using the forward primer 5'-CAACATCACCATGCAGATTATGC-3' and the reverse primer 5'-GCTTTCGTTTTTGCCCCTTTC-3'.

### 4.6. Western blot analysis

B16F10 cells treated with or without thiabendazole were lysed in lysis buffer (Generay, Shanghai, China) and then centrifuged at 13000 × g for 15 min at 4 °C. The concentration of protein in the supernatants was determined using the BCA kit (Tiangen, Beijing, China). Equal amounts of protein sample (50 μg) were separated via sodium sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA). Next, membranes were blocked with TBST supplemented with 5% BSA for 2 h at 37 °C and incubated with the primary anti-VEGF antibody (Santa Cruz, CA, USA) overnight at 4 °C, then followed by incubation with secondary antibody (Santa Cruz, CA, USA) for 1 h at 37 °C. After washing with PBST, the membranes were exposed using chemiluminescence (ECL) detection kit (Beyotime Biotech, China). The blots were detected on the Bio-Imaging System using Quality One 1-D analysis software (Bio-Rad, USA). All of the Blots were stripped and reprobed by using a monoclonal anti-β-actin antibody (Santa Cruz, CA, USA) to ascertain whether the proteins were loaded equally.

### 4.7. Wound healing assay (scratch assay)

The effect of thiabendazole on B16F10 cell migration was determined by a scratch assay as previously described (Kumar et al. 2012). The B16F10 cells ($3 \times 10^5$/well) in logarithmic phase were inoculated on a 12-well plate for 24 h. A confluent cell monolayer was starved using serum-free medium for 8 h and wounded by scratching with a 200-μl pipette tip. After being rinsed with PBS, the cells were further incubated in serum-free 1640 medium (contained 1% DMSO) supplemented with thiabendazole (0, 300 or 500 μM) for 48 h at 37 °C in a 5% $CO_2$ cell incubator. Photographs were taken at 0, 24, and 48 h after wounding. The width of the wound was measured using Image J Pro Plus 5 software.

### 4.8. Flow cytometric detection of apoptotic cells

The extent of apoptosis was measured followed the AV-FITC/PI apoptosis detection kit (Mbchemic, Shanghai, China). Briefly, B16F10 cells were incubated in 6-well plates with different concentrations of thiabendazole for 48 h. After incubation, the cells were harvested and washed twice with PBS. Next, the cells used for the AV/PI assay were deal with as instructions of

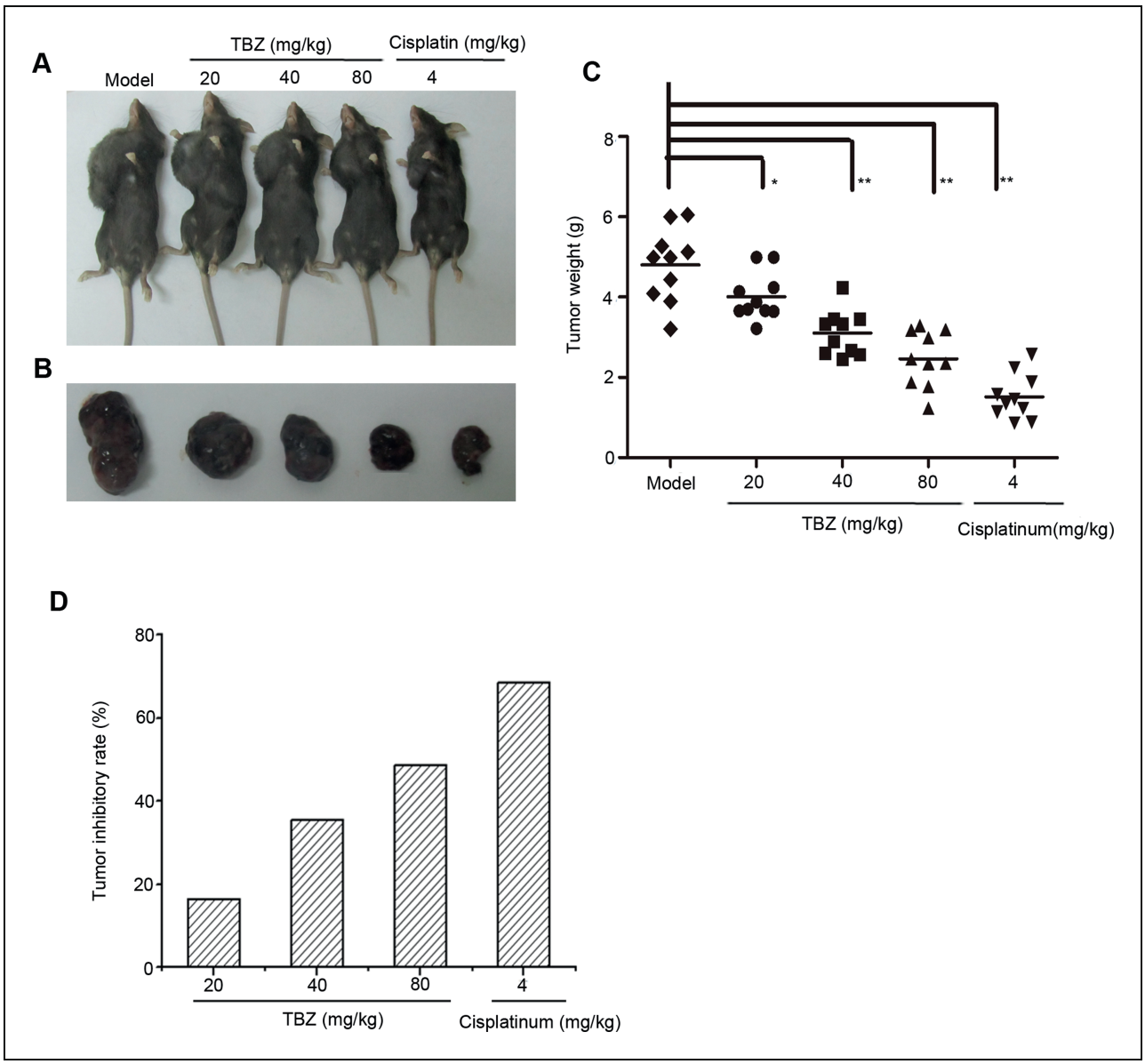


Fig. 6: Effective suppression of melanoma tumor growth in B16F10-bearing C57BL/6J mice was induced by thiabendazole. After the volume of the tumors had reached 100–300 $mm^3$, C57BL/6J mice were randomly subdivided into 5 groups: a model group, three thiabendazole groups administered intraperitoneal injection of thiabendazole (20, 40, 80 mg/kg) and a cisplatinum positive group (4 mg/kg). Mice were administrated every other day for two weeks. Then mice were sacrificed and the tumor tissues were removed. (A) Photograph of B16F10-bearing C57BL/6J mice with or without thiabendazole treatment. Tumor growth was significantly suppressed in mice treated with thiabendazole compared to those of the model group. (B) Photograph of tumors in each group. Tumors were significantly reduced in size in thiabendazole-treated animals. (C) The tumor weights in each group after thiabendazole administration. The tumor weights of thiabendazole-treated groups decreased as dose increased. (n = 10,*$P < 0.05$, **$P < 0.01$, compared with model group). (D) Inhibition rate in each group. The anti-melanoma activity of thiabendazo.

the manufacturer told. After that, cells were analyzed by a FACScan flow cytometer. The fraction of the cell population in different quadrants was analyzed by using quadrant statistics, that is cells in the lower right (LR) quadrant represented early apoptotic cells, and cells in the upper right (UR) quadrant represented late apoptotic cells. Percent apoptosis (%) = [(number of apoptotic cells)/ (number of total cells observed)] × 100% (Li et al. 2012).

### *4.9. In vivo tumor xenograft model and administration*

The B16F10 cells ($5 \times 10^5$ cells per mouse) were transplanted subcutaneously into the right axilla of each C57BL/6J mouse. After the tumor volume grew up to 100–300 $mm^3$, mice were randomly divided into five groups: a model group, three thiabendazole administrated groups (20, 40, 80 mg/kg) and a positive cisplatinum (4 mg/kg) control group. Each group contained 10 mice. In the thiabendazole treated groups mice were given intraperitoneal injections of thiabendazole (suspended in 20 μl DMSO) every other day for two weeks. As a model control, an equal volume of DMSO was injected in the same manner for the model group. In the positive control group, mice were administrated with cisplatinum in the same manner. Twenty-four hours after the last drug administration, all animals were sacrificed via cervical dislocation.

### *4.10. Antitumor activity assay in vivo*

The antitumor activity was expressed as inhibitory rate (%) and calculated as [(A-B)/A] × 100%, where A and B were the average tumor weight of the model and thiabendazole groups, respectively.

### *4.11. Statistical analysis*

All experiments elaborated above were performed in triplicate. The results were presented as the mean ± SD. Statistical analysiswas performed by one-way ANOVA, followed by Dunnett's multiple comparison or a Student's T-test. $P < 0.05$ were considered to be statistically significant.

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