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Antiproliferation in human EA.hy926 endothelial cells and inhibition of VEGF expression in PC-3 cells by topotecan

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Protracted administration of topotecan (TPT), a topoisomerase I inhibitor, exhibited high anticancer efficacy both in animal models and human cancers. This phenomenon is related to the TPT-induced inhibition of angiogenesis in tumor, but the potential mechanism remains largely unknown. In the present study, we reported that TPT (1–10 μM) could inhibit angiogenesis in a dose-dependent manner in Chick embryo chorioallantoic membrane (CAM) assay. TPT showed strong inhibitory activity against proliferation on human EA.hy926 endothelial cells with an IC_{50} value of 0.13 μM (MTT assay), lower than that of most sensitive cancer cell lines (IC_{50} range, 0.17 μM to 5.1 μM). TPT could induce EA.hy926 cells undergoing apoptosis, and the percentage of apoptotic cells induced by TPT (0.05 μM–5.0 μM) were 17.9%–52.3%. The similar results were observed with AO/EB staining. Flow cytometry assay also revealed that various concentrations of TPT induced cell cycle disturbance in EA.hy926 cells. Western blotting results showed that TPT caused an obvious increase of p53 expression and a decline of ERK expression in EA.hy926 cells. In addition, the VEGF expression of PC-3 cells is inhibited by TPT in hypoxia. Altogether, inhibiting proliferation of endothelial cells and down-regulating VEGF expression in cancer cells may involve in the antiangiogenesis mechanism of TPT.

1. Introduction

There is substantial preclinical and clinical evidence that angiogenesis plays an important role in the development of tumors and the progression of malignancies. Every increment of tumor growth requires an increment of vascular growth. Tumors lacking angiogenesis remain dormant indefinitely, and rapid logarithmic growth follows the acquisition of blood supply. Thus, inhibition of tumor blood supply has been realized as a unique approach to suppress tumor growth and metastasis. An antiangiogenic effect could be accomplished by known chemotherapeutic agents such as paclitaxel, flavone acetic acid, bleomycin, methotrexate and mitoxantrone, suggesting that a hidden "antivascular" effect may be operating in a number of conventional anticancer therapies (Fayette et al. 2005; Sridhar 2003; Clements et al. 1999).

Topotecan (TPT), a semi-synthetic analogue of camptothecin, has a considerable anti-cancer activity alone in a wide range of experimental tumor models and human cancers (Stuart 2003; Akbas 2004). TPT belongs to the group of topoisomerase I (topo I) inhibitors. In the presence of TPT, topo I cleaves the DNA but is unable to religate the single-strand break. This leads to stabilization of topo I-DNA-bound complexes and the accumulation of DNA strand breaks that may interfere with DNA replication (Chrencik 2004). Interestingly, it is found that protracted administration of TPT appears to be more efficacious both in animal models and human cancers. Although the mechanism of this phenomenon remains largely unknown, antiangiogenesis activity of TPT is thought to be involved in this process (Clements et al. 1999; O'Leary et al. 1999; McCrudden et al. 2002). We reported here that TPT induces apoptosis in human EA.hy926 endothelial cells and attenuated VEGF expression in PC-3 prostate cancer cells.

2. Investigations and results

2.1. Endothelial cells and cancer cells proliferation assay

To evaluate the possible selective inhibitory activity against endothelial cells, the IC_{50} values of TPT were measured in human EA.hy926 endothelial cells and various cancer cell lines from a diverse set of target organs, including leukemia and solid tumors (prostate, glioma, lung, sarcoma and breast cancer cell lines). Treatment of TPT for 48 h exhibited high inhibitory activity against human EA.hy926 endothelial cell proliferation with an IC_{50} of 0.13 μM, which was lower than IC_{50} values for eight cancer cell lines, ranging from 0.17 μM to 5.1 μM (Table).

2.2. Chick embryo chorioallantoic membrane (CAM) assay

The antiangiogenic activity of TPT was tested with the CAM assay, a popular model for studying various aspects of

Table: IC_{50} values of TPT for tumor cell lines and human EA.hy926 endothelial cell. Cells were treated with TPT for 72 h

Original tumor types	Cell Lines	IC_{50} (μM)	95% confidence interval (μM)
Breast cancer	MCF-7	2.2	2.0–2.4
Mouse leukaemia tumor	P388	0.17	0.025–1.1
Erythromyeloid tumor	K562	2.6	1.4–4.9
Non small cell lung tumor	A549	0.19	0.02–1.7
Androgen-independent Prostate tumor	PC-3	2.3	1.3–4.3
Mouse sarcoma	s180	1.7	0.73–4.0
Lewis lung cancer	LLC	5.1	0.58–44
Glioma	U251	1.2	0.2–7.6
Human endothelial cell	EA.hy926	0.12	0.077–0.19

blood vessel development including angiogenesis. Fig. 1 shows the dose-dependent inhibition action of angiogenesis mediated by 48 h treatment of TPT. TPT at a concentration of 10 μM substantially inhibited new blood vessel growth of chick embryos with a clearly avascular zone in CAM. In contrast, no avascular zones were observed in the control embryos treated with 0.9% sodium chloride injection or DMSO alone.

2.3. Acridine orange/ethidium brimide (AO/EB) staining

To test the promotive effect of TPT on endothelial cells apoptosis, EA.hy926 cells pretreated with various concentrations of TPT were stained by AO/EB and morphologies were immediately assessed in florescence microscopy. As

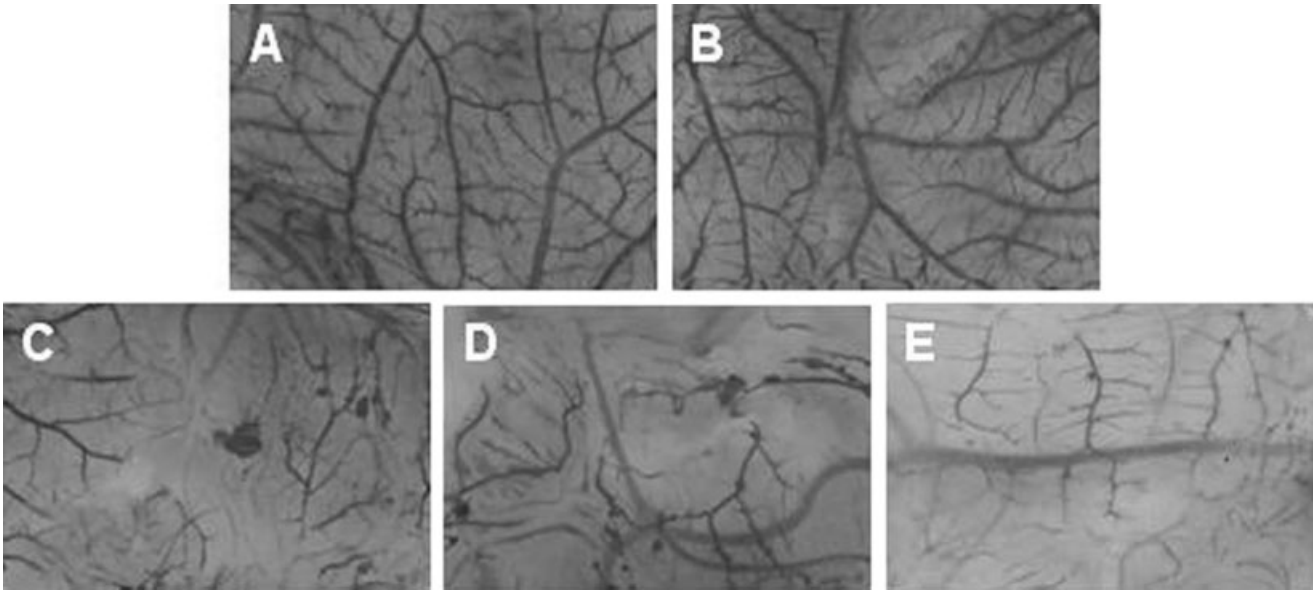

Fig. 1: Inhibitory effect of TPT on angiogenesis *in vivo*. CAMs were treated with various concentrations of TPT for 48 h. A: control, B: DMSO, C: TPT 1.0 μM, D: TPT 5.0 μM, E: TPT 10.0 μM

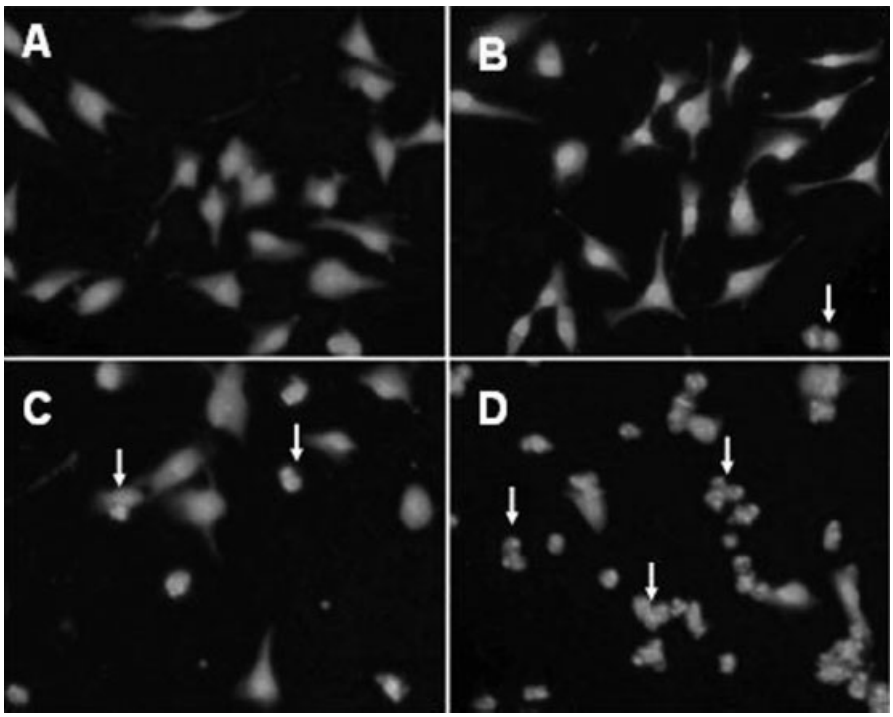

Fig. 2:
Effects of TPT on the morphology of EA.hy926 cell stained by AO/EB. EA.hy926. Cells were treated with DMSO (A), TPT 0.05 μM (B), TPT 0.5 μM (C) and TPT 5.0 μM (D) for 24 h. Apoptotic cell percentage increased in a dose-dependent manner

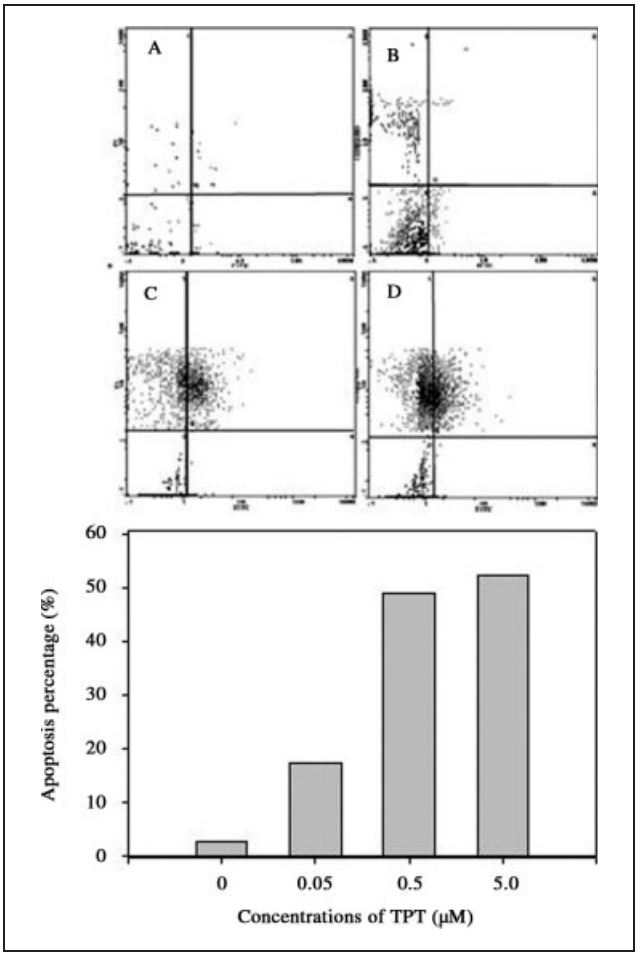

Fig. 3: Effects of TPT on the cell cycle of EA.hy926 cell. EA.hy926 cells were treated without (A) or with TPT 0.05 μM (B), 0.5 μM (C) and 5.0 μM (D) for 48 h

shown in Fig. 2, the normal morphologies of EA.hy926 cells were with green nuclei and intact structure, whereas a few EA.hy926 cells, treated with 0.05 μM TPT for 48 h, exhibited cell shrinkage, membrane blebbing, chromatin condensation, and formation of apoptotic bodies. The percentages of apoptotic cells exhibited a dose-dependant manner.

2.4. Flow cytometry

The cell cycle distribution patterns of EA.hy926 cells treated with TPT (0.05 μM, 0.5 μM or 5.0 μM) for 48 h were analyzed by flow cytometry. Fig. 3 shows that various

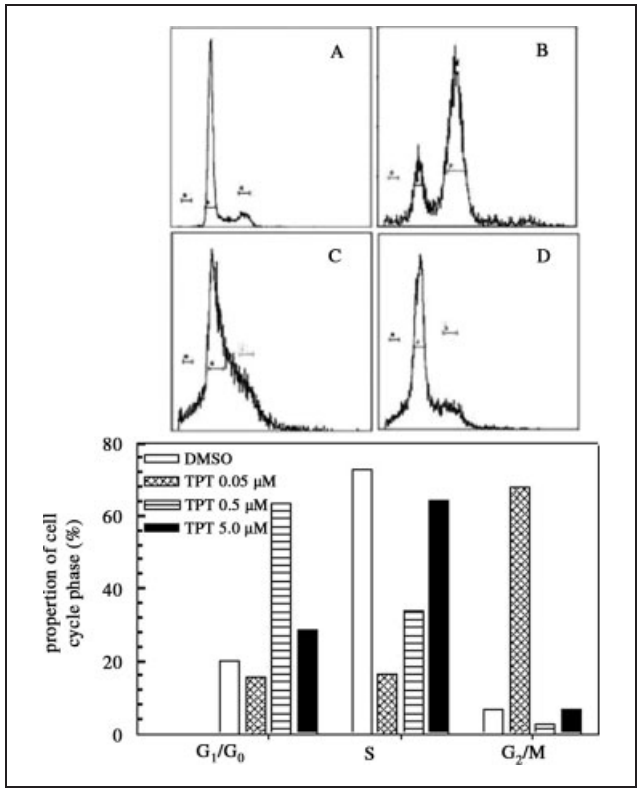

Fig. 4: TPT induced EA.hy926 cell apoptosis. Cells were treated without (A) or with TPT 0.05 μM (B), 0.5 μM(C) and 5.0 μM (D) for 48 h

concentrations of TPT induced cell cycle disturbance in EA.hy926 cells. TPT caused G2/M phase accumulation at a dose of 0.05 μM, S phase accumulation at a dose of 0.5 μM and G1/G0 phase accumulation at a dose of 5.0 μM.
Fig. 4 shows that the TPT-induced apoptosis in EA.hy926 cells was exhibited in a dose-dependent manner. The percentages of apoptotic cells induced by TPT were 17.3% (0.05 μM), 48.9% (0.5 μM), and 52.3% (5.0 μM) of the total number of cells, respectively.

2.5. Western blot

The abundance of VEGF protein in PC-3 cells and p53, ERK proteins in EA.hy926 cells were determined by Western blot analysis. Fig. 5 shows the expression of p53 in EA.hy926 cells was obviously elevated with the increasing concentration of TPT (0.05–5.0 μM), while the protein expression of ERK was obviously reduced. In hypoxia, the reduced expression of VEGF in PC-3 cells treated with TPT (0.05–5.0 μM) for 48 h was observed (Fig. 5).

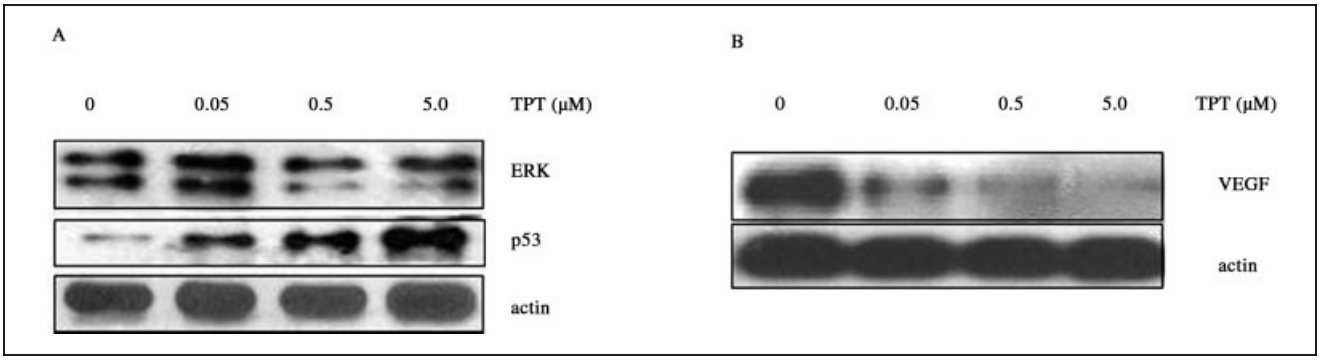

Fig. 5: P53 and ERK protein expression in EA.hy926 cells (A) and VEGF expression in PC-3 cells (B). 40 μg protein/lane was loaded. The expression of p53 was up-regulated and the expression of VEGF, and ERK were down-regulated by TPT

3. Discussion

Tumor angiogenesis is essential for cancer growth and metastasis (Harris 2003). Whereas angiogenesis is a common feature during embryogenesis, the blood vessels in healthy adults are predominantly quiescent, with the exception of the cyclic angiogenesis occurring in the uterus and the ovaries during the female reproductive cycle. Other exceptions are hair growth, wound healing, psoriasis and cancer (Detmar 2000.). There is substantial preclinical and clinical evidence that angiogenesis plays an important role in the development of tumors and the progression of malignancies. Inhibiting angiogenesis has been considered as an important anticancer strategy to suppress tumor growth and metastasis.

TPT, a topoisomerase I inhibitor, had been demonstrated as a considerable anti-cancer agent alone in a wide range of experimental tumor models and human cancers (Stuart 2003; Akbas 2004). In the present study, TPT exhibited high inhibitory activity on the proliferation of human EA.hy926 endothelial cells and the formation of new blood vessel in CAM. The IC_{50} value of TPT on EA.hy926 cells was 0.13 μM, lower than TPT on most of sensitive cancer cell lines, indicating that TPT could exert antiangiogenesis action in low concentration. These results were consistent with previous reports which showed that antiangiogenesis ability of TPT contributed to its anticancer activity, but the mechanism was still unclear (Clements et al. 1999; O'Leary et al. 1999; McCrudden et al. 2002).

TPT-induced EA.hy926 cells apoptosis was observed both in AO/EB staining and flow cytometry assay. Chromatin fragmentation and apoptotic bodies were caused by 0.05 μM TPT for 48 h, and the percentage of apoptotic cells was 17.3%, indicating that low concentration of TPT could inhibit the formation of new blood vessel by inducing endothelial cells apoptosis.

It was demonstrated that an accumulation of wild-type p53 protein occurs in cancer cells in response to induce apoptotic cell death (Biros et al. 2002; Hickman et al. 2002). In agreement with these studies, our data shows that TPT caused a dose-dependent increase of p53 protein expression in EA.hy926 cells. It is suggested that ERK activation contributed to mediate the growth-promoting and anti-apoptotic signals (Woessmann and Mivechi 2001). Here, we reported that TPT reduced the expression of ERK. Thus, both p53 and ERK proteins pathways were involved in TPT-caused apoptosis in endothelial cells.

It is reported that cell cycle effects vary with TPT concentration in cancer cells (Ohneseit et al. 2005), resulting in G1 arrest at high concentration, or G2/M arrest at low concentration. In our experiment, the TPT-mediated disturbance of cell cycle exhibited the same pattern in EA.hy926 cells. TPT caused G1/G0 arrest at a dose of 5 μM, S arrest at a dose of 0.5 μM, and G2/M arrest at a dose of 0.05 μM. It is suggested that the cell cycle regulation pathway is also involved in antiproliferation activity of TPT in endothelial cells.

Vascular endothelial growth factor (VEGF) is known to induce angiogenesis by increasing endothelial cell proliferation, migration and microvascular hyperpermeability. Besides normal angiogenesis (e.g., during wound healing), VEGF also stimulates pathological angiogenesis including cancer growth. VEGF is considered to be secreted from tumor cells and to act on endothelial cells in a paracrine fashion to induce blood vessel growth (Kondo 2002; Haspel 2002). Significant evidence indicates that hypoxia acts as a morphogen during vascularization-inducing and shaping the recruitment and formation of new vascular beds through critical transcriptional control pathways (Giordano and Johnson 2001). As reported, hypoxia increased the VEGF levels dramatically in cancer cells (Miyagishi et al. 2003). The present data shows that, in hypoxia, TPT obviously inhibited the expression of VEGF in PC-3 cells in a dose-dependent manner, suggesting that downregulating VEGF by TPT in cancer cells might be involved in the inhibition of new blood vessel formation.

In conclusion, TPT exhibited high antiangiogenesis activity in endothelial cells by inducing apoptosis and disturbing cell cycle of endothelial cells, and downregulating the expression of VEGF in cancer cells.

4. Experimental

4.1. Test solution

Topotecan (TPT) was dissolved at 10 mM in dimethyl sulfoxide (DMSO), stored at −20 °C and further diluted with the appropriate assay medium immediately before use.

4.2. Cell culture

EA.hy926 cells, a hybridoma of human aortic endothelium and an adenocarcinoma, were kindly provided by Professor Edgell, Department of Pathology, University of North Carolina. Cells were routinely grown in HG-DMEM supplemented with 20% FBS and antibiotics in an atmosphere of 5% CO_2 at 37 °C in a humidified incubator. Subcultures were made from confluent stock cultures by trypsinization in PBS containing 0.5 mM EDTA and 0.25% trypsin (Jones et al. 1998).

The human prostate cancer PC-3 cells, human breast cancer MCF-7 cells, human glioma U251 cells, human non small cell lung cancer A549 cells, human erythromyeloid cancer K562 cells, murine sarcoma s180 cells, murine Lewi's lung cancer LLC cells and murine lymphoma P388 cells were purchased from the Institute of Cell Biology (Shanghai, China) and maintained in RPMI 1640 supplemented with 10% FCS plus 2 mM glutamine 50 unit/ml penicillin at 37 °C in a 5% CO_2 atmosphere.

4.3. Endothelial and cancer cell proliferation assay

Growth studies *in vitro* were assessed using the MTT colorimetric assay (Sigma Chemical Co.). EA.hy926 cells and 8 cancer cell lines (4000 cell/well) were placed in medium which varying concentrations of TPT or a DMSO vehicle control were added to. After 48 h in culture, MTT reagent was added for a 4-h incubation and then medium was taken away, after which 100 ml DMSO were added. Optical density was determined at 570 nm (Moody et al. 2003).

4.4. Chick embryo chorioallantoic membrane (CAM) assay

Inhibition of angiogenesis was determined using a modified CAM assay. Fertilized chick eggs were stayed in an egg incubator regulated at 37 °C and with 50% humidity for optimal growth conditions for 6 days continuously. Then the eggshell was cracked, and gently opened into the plate to avoid any unnecessary physical stress. It was made sure that the yolk sac membrane remained intact and that the embryo was viable. Good embryos with a beating heart were chosen. The sterile filter paper square saturated with TPT (1 μM, 5 μM, 10 μM) or physiological saline solution placed in areas between vessels but never onto any large vessels. After 48 h, the CAMs were carefully isolated and fixed in methanol/acetone. Results of the samples were photographed (Koutrafouri et al. 2003).

4.5. Acridine orange/ethidium brimide (AO/EB) staining

50 μl of AO/EB (1 : 1, 100 μg/ml) cocktail mixed with 1 ml DMEM was added in the culture plates. Fields of stained cells were selected and focused using fluorescence microscopy (Nikon 4050). Viable cells stained only by AO were bright green with intact structure; early apoptotic cells stained by AO had a bright green area in the nucleus. Late apoptotic cells stained by AO and EB were red-orange with condensation of chromatin as dense orange areas and reduced cells size seen in this study (Wang and Huang 2005).

4.6. Flow cytometry

One million EA.hy926 cells treated with various concentrations of TPT or DMSO vehicle control for 48 h were collected,and resuspended with PBS. Ice-cold 75% ethanol (1 ml) was added and the cell suspension was incubated overnight at 4 °C. Fixed cells were harvested and resuspended in 1 ml of 0.1% sodium citrate containing propidium iodide (PI) 0.05 mg and

50 μg RNase for 30 min at room temperature in the dark. DNA content was measured with Coulter Epicas Elite flow cytometer (Cordova-Alarcon et al. 2005).

4.7. Western blot

EA hy926 cells and PC-3 cells were treated with TPT (0.05 μM, 0.5 μM and 5 μM) or DMSO for 48 h in hypoxia (3% O_2). Proteins were extracted in radioimmunoprecipitation assay buffer (50 mM NaCl, 50 nM Tris, 1% Triton X-100, 1% sodiumdeoxycholate, and 0.1% SDS) supplemented with a mixture of phosphatase and protease inhibitors (1 mM sodium orthovanadate and 2 mM sodium fluoride, 1 mM PMSF, aprotinin 100 μg/mL, leupeptin 100 μg/mL), and 40 μg of total protein was loaded per lane. Proteins were fractionated on 12% Tris-glycine gels, transferred to nitrocellulose membrane (Pierce Biotechnology, Inc., USA), and probed with primary antibodies (VEGF, p53, ERK and β-actin) and then HRP-labeled secondary antibodies (Santa Cruz Biotechnology, Inc.,). Antibody-positive band were visualized using ECL Western Blotting detection reagents (Pierce Biotechnology, Inc.,) (Tolis et al. 1999).

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