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# Resveratrol Derivative, *trans*-3,5,4′-Trimethoxystilbene, Exerts Antiangiogenic and Vascular-Disrupting Effects in Zebrafish Through the Downregulation of *VEGFR2* and Cell-Cycle Modulation

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## ABSTRACT

Angiogenesis plays an important role in the development of neoplastic diseases such as cancer. Resveratrol and its derivatives exert antiangiogenic effects, but the mechanisms of their actions remain unclear. The aim of this study was to evaluate the antiangiogenic activity of resveratrol and its derivative *trans*-3,5,4′-trimethoxystilbene in vitro using human umbilical vein endothelial cells (HUVECs) and in vivo using transgenic zebrafish, and to clarify their mechanisms of action in zebrafish by gene expression analysis of the vascular endothelial growth factor (VEGF) receptor (VEGFR2/KDR) and cell-cycle analysis. *trans*-3,5,4′-Trimethoxystilbene showed significantly more potent antiangiogenic activity than that of resveratrol in both assays. In zebrafish, *trans*-3,5,4′-trimethoxystilbene caused intersegmental vessel regression and downregulated *VEGFR2* mRNA expression. *Trans*-3,5,4′-trimethoxystilbene also induced G2/M cell-cycle arrest, most specifically in endothelial cells of zebrafish embryos. We propose that the antiangiogenic and vascular-targeting activities of *trans*-3,5,4′-trimethoxystilbene result from the downregulation of *VEGFR2* expression and cell-cycle arrest at G2/M phase. J. Cell. Biochem. 109: 339–346, 2010. © 2009 Wiley-Liss, Inc.

**KEY WORDS:** 3,5,4′-TRIMETHOXYSTILBENE; ANGIOGENESIS; HUVEC; ZEBRAFISH; VEGFR2; CELL CYCLE

Ever since Folkman [1971] reported the relationship between angiogenesis and tumor growth, efforts have been made to explore the possibility of treating cancer by targeting angiogenesis. In recent years, the study of pharmacological means to disrupt new blood-vessel formation from existing vessels (antiangiogenic strategy) and/or the disruption of newly formed blood vessels (vascular-targeting strategy) has gathered pace, with many new compounds undergoing clinical trials [Siemann et al., 2004; Kanthou and Tozer, 2007]. The inhibition of vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) activity has been in the forefront of antiangiogenic therapies [Ferrara et al., 2003], with several small molecular drugs now undergoing clinical trials [Veeravagu et al., 2007]. Another area of intense research is the potential of vascular-disrupting agents to target tumor vasculature, disrupting the existing vasculature and causing tumor cell necrosis [Cai, 2007; Kanthou and Tozer, 2007]. However, recent evidence suggests that a more aggressive management of tumors by combining antiangiogenic, vascular-disrupting, and conventional chemotherapies and/or radiotherapy might be the way forward [O'Reilly, 2007; Citrin and Camphausen, 2008].

Resveratrol (Res, *trans*-3,5,4′-trihydroxystilbene), a constituent of red wine, vegetables, and Chinese medicines, such as *Rhizoma polygoni cuspidati*, has been reported to possess antioxidant, antiplatelet-aggregation, anticancer, and antiangiogenic activities

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[Gescher, 2008]. Recently, we showed that resveratrol modulates the differentiation of cardiomyoblasts, indicating the novel mechanism underlying its cardioprotective effect [Leong et al., 2007]. Attempts to design resveratrol analogues with more potent biological activities have yielded many new stilbenes with different aryl substituents, of which *trans*-3,5,4′-trimethoxystilbene (TMS) is a primary candidate. TMS is more potent than resveratrol as an anticancer agent in various cell systems [Simoni et al., 2006; Pan et al., 2008]. It possesses potent antiangiogenic and vascular-targeting activities both in vitro and in vivo [Belleri et al., 2005]. In this study, we evaluated the cytotoxicity of resveratrol and its derivative TMS in human cancer cell lines and their antiangiogenic effects on human endothelial cells and on zebrafish blood-vessel formation in vivo. To understand the mechanisms underlying the effects of TMS on vascular changes in zebrafish, we studied the cell cycle and gene expression in zebrafish.

Zebrafish (*Danio rerio*) is fast becoming a powerful model for drug discovery [Langheinrich, 2003; Crawford et al., 2008]. Many cardiovascular, antiangiogenic, and anticancer drugs elicit similar responses in zebrafish embryos as in mammalian systems [Langheinrich, 2003]. Its rapid development and rather simple, short assay time make zebrafish a useful tool in large-scale drug screening [Norrby, 2006]. In particular, it is a perfect model for studying angiogenesis because transgenic zebrafish that express green fluorescent protein (GFP) in the vasculature allow rapid drug response analysis in live embryos [Lawson and Wienstein, 2002; Norrby, 2006]. In our recent study, we reported the proangiogenesis effects of *Angelica sinensis* extract in a zebrafish model [Lam et al., 2008].

The remarkable antiangiogenic effect of TMS demonstrated in this study provides a better understanding of the biological activities of TMS and new evidence for the further development of this compound as an antitumor and antiangiogenesis treatment.

## MATERIALS AND METHODS

### CELL LINES AND CHEMICALS

Human umbilical vein endothelial cells (HUVECs), the human hepatoma cell line HepG2, and the human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA). Resveratrol and TMS were from Sigma (St. Louis, MO). HUVECs were cultured in Kaighn's modification of Ham's F12 medium (F-12K) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 μg/ml heparin, 30 μg/ml endothelial cell growth supplement (Sigma), 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), and 100 U/ml penicillin–streptomycin (Gibco). Tissue culture flasks were pre-coated with 0.1% gelatin. All assays were conducted with low-passage cells (3–5 passages). All cancer cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin (Gibco). The cells were incubated under standard cell culture conditions at 37°C and 5% $CO_2$ in a humid environment.

### MAINTENANCE OF ZEBRAFISH AND EMBRYOS

Transgenic zebrafish- Tg(*fli1*-EGFP), that expresses green fluorescent protein(GFP) under the control of *fli1* promoter in endothelial cells, was kindly provided by ZFIN (OR) and wild-type zebrafish was purchased from local pet shop, for use as in vivo model. Both were maintained as described in the Zebrafish Handbook [Westerfield, 1993]. The embryos were cultured at 28.5°C in distilled water.

### GROWTH INHIBITORY ASSAY OF CELL LINES

HepG2, MCF-7, and MDA-MB-231 cells were seeded in 96-well microplates at $1 \times 10^4$ cells/well in 100 μl of medium. Drugs were added to the cells at serially diluted concentrations, from a 100 mM stock solution in DMSO, and incubated for 24 h. The controls were treated with 1% DMSO. 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltrtrazolium bromide (MTT, USB Corporation, USA) solution (30 μl; 5 mg/ml in PBS) was added, and after incubation for 4 h, the blue formazan crystals were dissolved with 100 μl of DMSO. Optical density (OD) was measured with a Multilabel Counter (Perkin Elmer, Singapore) at 570 nm. MTT solution with DMSO was used as the blank. Cell viability (percentage of the control) was calculated relative to the untreated control. The inhibition of cell proliferation was calculated using the following formula: growth inhibition $(\%) = ([OD_{control} - OD_{treated}]/OD_{control}) \times 100\%$.

### CELL PROLIFERATION ASSAY

HUVECs were seeded into 96-well gelatin-coated plates at a density of $10^4$ cells/well. To achieve a quiescent state, the complete medium was replaced after incubation for 24 h with low-serum (0.5% FBS) medium and incubated for a further 24 h. The medium was replaced with various doses of drugs together with 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen, USA). The plates were incubated for an additional 48 h and cell proliferation was assessed with the Cell Proliferation Kit II (XTT, Roche Diagnostics GmbH, Germany), in accordance with the manufacturer's protocol. The spectrophotometric absorbance was measured with a Multilabel Counter (Perkin Elmer) at 490 nm, with the reference wavelength at 690 nm.

### MORPHOLOGICAL OBSERVATIONS OF ZEBRAFISH

Transgenic Tg(*fli1*:EGFP) zebrafish embryos at 48 h post-fertilization (hpf) were treated with different concentrations of TMS, vehicle-control and SU5416 as positive control. After 20 h of drug treatment, the embryos were anesthetized with 0.02% tricaine (Sigma), and observed for viability and gross morphological changes under a fluorescence microscope (Olympus MVX10, Japan) equipped with a digital camera (ColorView II, Soft Imaging System, Olympus). The images were analyzed with Adobe Photoshop 7.0 and ACDSee 7.0.

### TOTAL RNA EXTRACTION, REVERSE TRANSCRIPTION, AND REAL-TIME PCR

Zebrafish embryos at 48 hpf were treated with different concentrations of TMS for 20 h. Total RNA was extracted from 40 zebrafish embryos of each treatment group using the RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. RNA was reverse transcribed to single-strand cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™, USA),

followed by real-time PCR using the TaqMan® Universal PCR Master Mix and 250 nM custom TaqMan primer for zebrafish *VEGFR2* (Applied Biosystems, USA) in the ABI 7500 Real-Time PCR System (Applied Biosystems). The expression of *VEGFR2* mRNA was normalized to the amount of 18S rRNA using the relative quantification method described by the manufacturer.

The zebrafish *VEGFR2* primers were 5′-AGCAGTGGATGGA-GTTTGACAATAA-3′ (F) and 5′-GACTTTGACCCCATCATATGT-GAGA-3′ (R) (Applied Biosystems).

### WHOLE-MOUNT IN SITU HYBRIDIZATION

Digoxigenin-labeled antisense riboprobe to *flk1* was made from zebrafish flk1 cDNA clone (a generous gift from Prof. AH Leung of University of Hongkong) using a DIG RNA labeling kit (Roche Applied Science, Mannheim, Germany). Whole-mount in situ hybridization was carried out according to standard protocols [Thisse et al., 1993]. Digoxigenin-labeled probe was detected with alkaline phosphatase conjugated with antidigoxigenin antibody. BCIP/NBT was used as substrate to develop purple color (all from Roche Applied Science).

### CELL-CYCLE ANALYSIS OF ZEBRAFISH ENDOTHELIAL CELLS

At 48 hpf, 10 Tg(*fli1*:EGFP) zebrafish embryos were exposed to 2 ml of medium containing drug in 12-well plates. After treatment for 20 h, the embryos were washed in PBS and digested in 500 μl of trypsin lysis solution (0.5 g/L trypsin [1:250] in a solution of 0.14 M $CaCl_2$, 0.05 M KCl, 0.005 M glucose, 0.007 M $NaHCO_3$, 0.7 mM EDTA), incubated for 30 min at 28°C, and triturated through a narrow-bore Pasteur pipette until the cells were completely dissociated. Trypsin digestion was terminated by the addition of one volume of stop solution (2 mM $CaCl_2$, 20% FBS). The cell suspensions were centrifuged at 1,000*g* for 5 min at 4°C. The pellets were resuspended in 1 ml of cold PBS containing 1% FBS and then centrifuged again at 1,000*g* for 5 min at 4°C. The cell pellets were resuspended in 500 μl of PBS. DRAQ5™ (Biostatus Ltd., Leicestershire, UK), a deep red/infrared fluorescent cell-permeant DNA probe, was added to the cells at a concentration of 20 μM and incubated at 37°C for 30 min. Cell clumps and debris were removed by passing the cell suspension through a nylon strainer with a pore size of 40 μm (BD Falcon, USA). The stained cell suspension was analyzed immediately using a flow cytometer (BD FACS Canto™, USA). Measurements were recorded using a fluorescein isothiocyanate (FITC) filter (to detect the GFP-positive cells from the transgenic zebrafish) and the PerCP-Cy5 channel to detect the DNA content of the cells. Cell-cycle analysis was performed with the ModFitLT 3.0 software (Verity Software House, USA).

### STATISTICAL ANALYSIS

Each experiment was performed at least three times, and all values are presented as the means ± SEM. Student's *t*-test was used to analyze the statistical significance of the results. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

### TMS IS MORE POTENT THAN RESVERATROL IN INHIBITING bFGF-STIMULATED HUVEC PROLIFERATION

Anti-cancer effect of a drug can be achieved by either direct cytotoxicity to cancer cells or by antiangiogenesis thereby cutting off blood supply to the tumor. To study the anti-cancer effect of *trans*-resveratrol and its derivative TMS, we performed a comparative cytotoxic and cell proliferation study of various cancer cell lines and endothelial cells using MTT and XTT assays. As shown in Figure 1A, resveratrol showed higher cytotoxic efficacy against the three cancer cells lines tested compared with TMS, although at very high $IC_{50}$s (more than 150 μM for each of the cell lines tested). At lower concentrations (40–100 μM), TMS exerted partial cytotoxic effect (30–50%) of all the cancer cell lines. The reduced cytotoxicity of resveratrol and TMS on these cancer cell lines indicates that their reported anticancer effects may act via an indirect mechanism such as antiangiogenesis. To further investigate this hypothesis, the effects of resveratrol and TMS on the proliferation of bFGF-stimulated HUVECs were evaluated by XTT assay. After 24 h starvation, HUVECs were treated with 1 ng/ml bFGF and different concentrations of resveratrol or TMS for 48 h. Figure 1B showed that both resveratrol and TMS inhibited bFGF-stimulated HUVEC proliferation in a dose-dependent manner ($IC_{50}$ values of 44 and 3.75 μM, respectively). HUVECs treated with 40 nM SU5416 were used as the positive control as it is an established VEGF receptor kinase inhibitor that specifically inhibits endothelial cell proliferation, migration, and vessel formation in tumors [Shaheen et al., 1999]. The results indicated that TMS is more effective than resveratrol in inhibiting bFGF-stimulated HUVEC proliferation. The inhibitory potency of TMS against bFGF-stimulated HUVEC proliferation was approximately 11 times higher than that of resveratrol.

### ANTI-ANGIOGENIC EFFECT OF TMS IN ZEBRAFISH EMBRYOS

To confirm the antiangiogenic activity of resveratrol and TMS, we used Tg(*fli1*:EGFP) transgenic zebrafish embryos that express EGFP under the control of *fli-1* and in which the endothelial cells can be directly observed under a fluorescence microscope. Figure 2 shows the effects of TMS on the blood-vessel formation of intersegmental vessels (ISV; shown with arrows) in Tg(*fli1*:EGFP) zebrafish embryos. The exposure of 48 hpf zebrafish embryos to TMS for 20 h clearly caused blood-vessel regression in the ISVs (B–C) compared with the control (A) and was similar to the effects of SU5416 treatment (D). Same experiment was repeated using resveratrol. However, even the highest tested concentration (100 μM) of resveratrol had no observable effect on the ISV phenotype of the zebrafish embryos. Close observation of the pattern of ISV regression indicated a loss of GFP cells within the region in which new blood vessels were formed, at the edges of the ISVs [Leslie et al., 2007].

### DOWNREGULATION OF *VEGFR-2* mRNA IN ZEBRAFISH EMBRYOS BY TMS

The VEGF receptors, VEGFR1 and VEGFR2, are the most important receptors involved in neovascularization. We used real-time

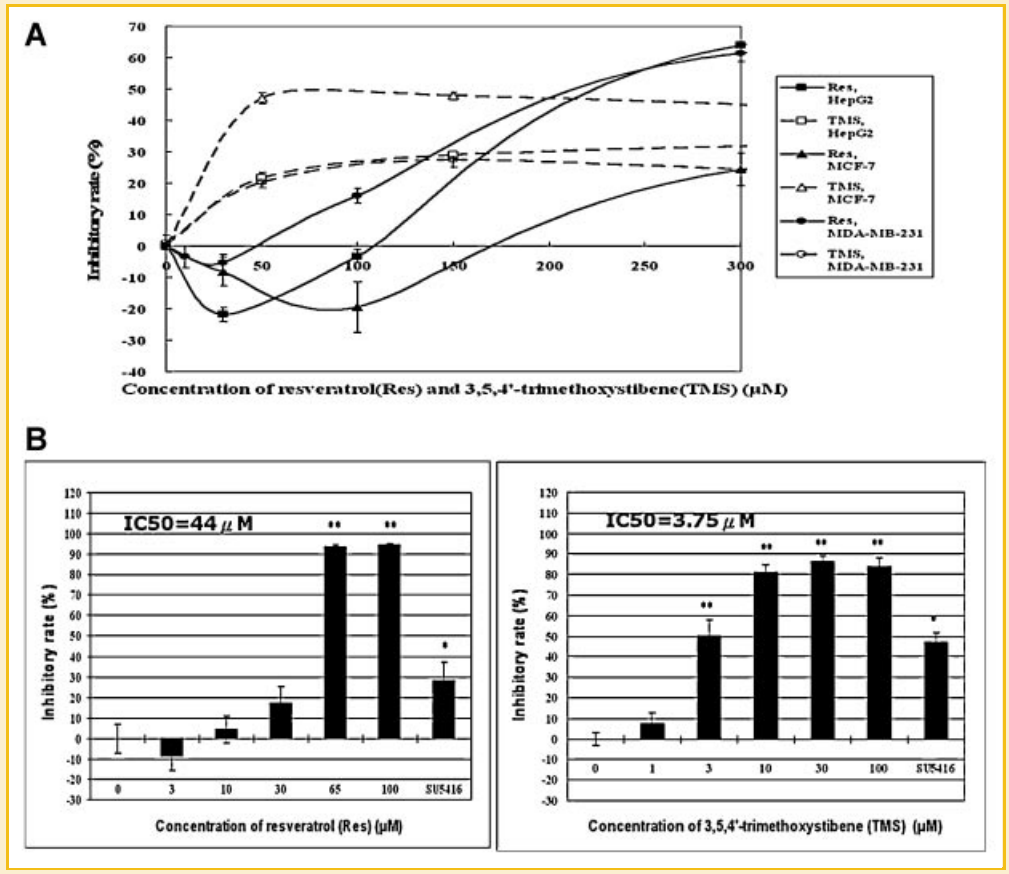


Fig. 1. Cytotoxicity of resveratrol and TMS to cancer cells MDA-MB-231, MCF-7, and HepG2 (A). Three cancer cell lines (two human breast adenocarcinoma cell lines, MDA-MB-231 and MCF-7, and the human hepatoma HepG2 cell line) were treated with drug-free medium or medium containing different concentrations of resveratrol or TMS for 24 h. Cell growth was determined by the MTT assay. Data are presented as means ± SEM of three individual experiments. Resveratrol and 3,5,4′-trimethoxystilbene (TMS) inhibited bFGF-induced HUVEC proliferation (B). HUVECs were seeded at $10^4$ cells/well in a 96-well plate and incubated with bFGF and resveratrol or TMS. After 48 h, cell proliferation was assessed with the XTT assay. SU5416 (40 nM) was used as the positive control. Error bars represent the means ± SD of duplicate experiments. $^{*}P < 0.05$ versus the bFGF group. $^{**}P < 0.01$ versus the bFGF group.

quantitative PCR to measure the relative mRNA levels expressed from these genes. Figure 3A shows that TMS downregulated the expression of *VEGFR-2(flk-1)* mRNA in zebrafish, with a four-fold reduction in mRNA expression at a TMS concentration of 100 μM. Milder downregulation were observed at lower concentrations (1 and 10 μM) but the mRNA expression of *VEGFR1* remained unaffected (data not shown). To confirm the role of TMS in inhibiting *VEGFR-2(flk-1)* in endothelium, we conducted in situ hybridization using *VEGFR-2(flk-1)* probe. Figure 3C demonstrated that 3 μM and 10 μM TMS treatment significantly downregulated the gene expression in endothelium (ISVs are represented by arrows). Moreover, this inhibition pattern closely resembled the pattern observed in TMS treated Tg(*fli1*:EGFP) zebrafish embryos.

### TMS INDUCES CELL-CYCLE ARREST IN ENDOTHELIAL CELLS OF ZEBRAFISH EMBRYOS

Resveratrol and TMS have previously been shown to exert their antitumor and antiproliferative activities by modulating cell-cycle progression in different cell types [Schneider et al., 2003; Lee et al., 2004; Notas et al., 2006]. To further evaluate their mechanisms of action, we undertook cell-cycle analysis of zebrafish embryo cells using flow cytometry (Fig. 4). We used transgenic zebrafish- Tg(*fli1*-EGFP), that express GFP under *fli1* promotor which is specifically expressed in endothelial cells. The endothelial GFP-expressing cells (measured on the FITC channel) and the DNA content of the cells (measured by PerCP-Cy5) were plotted to differentiate between the GFP-positive and GFP-negative cells (gate P5 and gate P4, respectively, as shown in Fig. 4A). For reference, the dot plot of wild-type zebrafish embryos is shown in Figure 4Aa. Figure 4B shows the cell-cycle distribution in the endothelial (GFP-positive) and nonendothelial cells (GFP-negative) from five independent experiments after treatment with TMS. TMS induced the accumulation of cells in G2/M phase, and significantly more so in endothelial cells (in about 20–30% of GFP-positive cells but in only 5–10% of GFP-negative cells). However, there was still an overall increase in G2/M phase cells in the whole cell population, indicating that TMS also caused cell-cycle arrest in some other cell types.

## DISCUSSION

The present study showed that TMS is a more potent antiangiogenic and vascular-disrupting agent when compared with resveratrol.

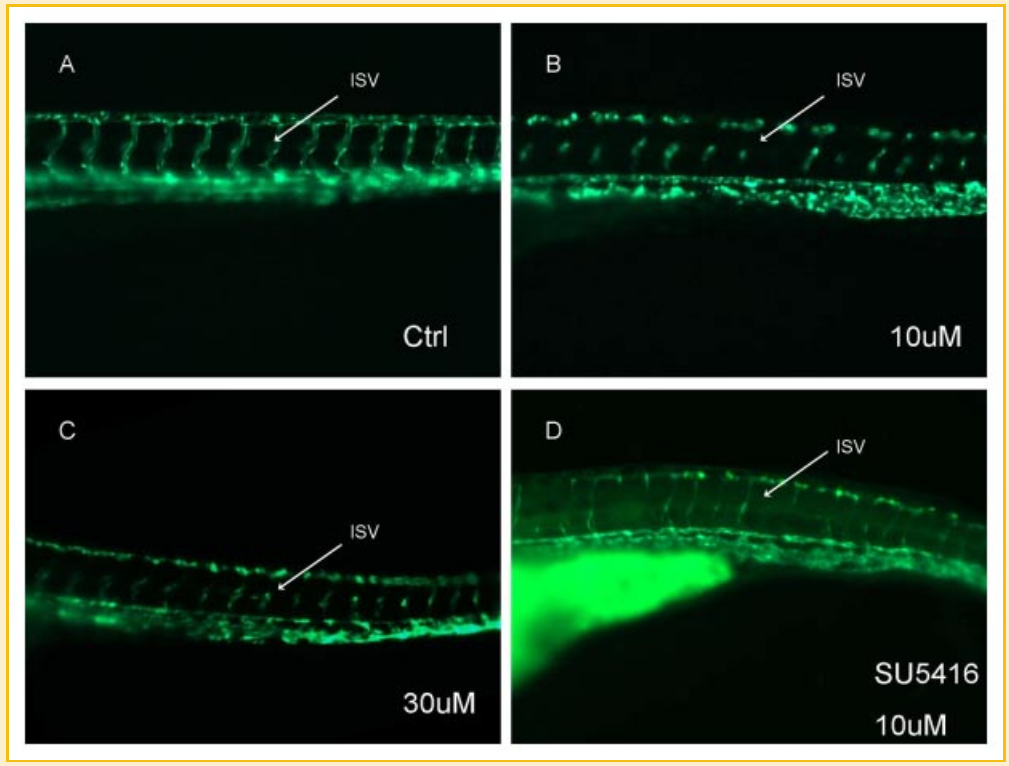


Fig. 2. The effects of 3,5,4′-trimethoxystilbene on blood vessel formation in ISVs (intersegmental vessels) of Tg(*fli*:EGFP) zebrafish. Zebrafish embryos at 48 hpf were treated with varying concentrations of TMS for 20 h and observed under an epifluorescence microscope at 6.3× magnification. Representative images of transgenic zebrafish embryos showing ISV regression (white arrows) when treated with 10 μM TMS (B), 30 μM TMS (C), or 10 μM SU5416 (D) are compared with that of the control (A) (lateral views, anterior is to the left). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Furthermore, we demonstrated that its antiangiogenic and vascular-disrupting activities might act via the downregulation of *VEGFR2* expression and cell-cycle arrest at G2/M phase. To the best of our knowledge, this is the first study using a live transgenic zebrafish model to show the cell-cycle arrest of specific cells (here, endothelial cells). This study provides novel insight into the mechanisms of action of TMS in a living organism.

The in vitro study of resveratrol and TMS showed rather weak cytotoxic effects on three cancer cell lines (HepG2, MCF-7, and MDA-MB-231), which contradicted a previous study reporting that resveratrol inhibited MCF-7 cells with an $IC_{50}$ of about 10 μM [Lu and Serrero, 1999]. This discrepancy might be explained by the fact that the measurements were made 24 h after drug treatment, whereas the measurements of the previous study were taken 6 days after. The fact that the cytotoxic effect of TMS was lower than that of resveratrol is surprising, because in many studies, TMS is the most active analogue of resveratrol [Simoni et al., 2006; Cardile et al., 2007; Pan et al., 2008], although resveratrol shows much stronger antioxidant effects than that of TMS [Stivala et al., 2001]. It is unlikely that the mild cytotoxic activity of resveratrol or TMS can sufficiently lead to the tumor chemotherapeutic effects reviewed by Athar et al. [2007], at least in the cancer models tested in this study. Therefore, we further explored their antiangiogenic effects and found that TMS showed significantly stronger antiangiogenic activity than resveratrol in both the in vitro and in vivo assay. Zebrafish embryos offer great advantage over their adults as well as other in vivo models because of the external development and optical transparency during their first few days, making them invaluable in the inspection of developmental processes. These unique advantages can even be made more useful when specific cell types are labeled with fluorescent probes [reviewed by Norrby, 2006]. Results of both assays were similar to a previous study in which TMS showed antiangiogenic and vascular-targeting activities in various in vitro and in vivo models [Belleri et al., 2005]. It is interesting to note that the blood vessel regression observed in zebrafish embryos was towards the outer edges of the ISVs, indicating that perhaps newly formed blood vessels are the targets of TMS. Taken together, our in-depth study comparing TMS and resveratrol in different cancer cell lines in vitro, HUVECs in vitro, and zebrafish embryo in vivo, suggests that TMS has both more potent antiangiogenic activity and more importantly, stronger specific cytotoxic effects on endothelial cells than does resveratrol.

Controversial results have been reported regarding whether resveratrol and related compounds cause cell-cycle arrest through G1 or G2/M phase in cell culture in vitro [Ragione et al., 1998; Notas et al., 2006]. This phenomenon has been explained by their probable differential effects on different cell types, but without solid evidence. Our study in zebrafish embryos, which showed the induction of G2/M cell-cycle arrest in GFP-positive endothelial cells by TMS in a whole live organism, provides insight into a more physiologically relevant effect of the compound. Moreover, we

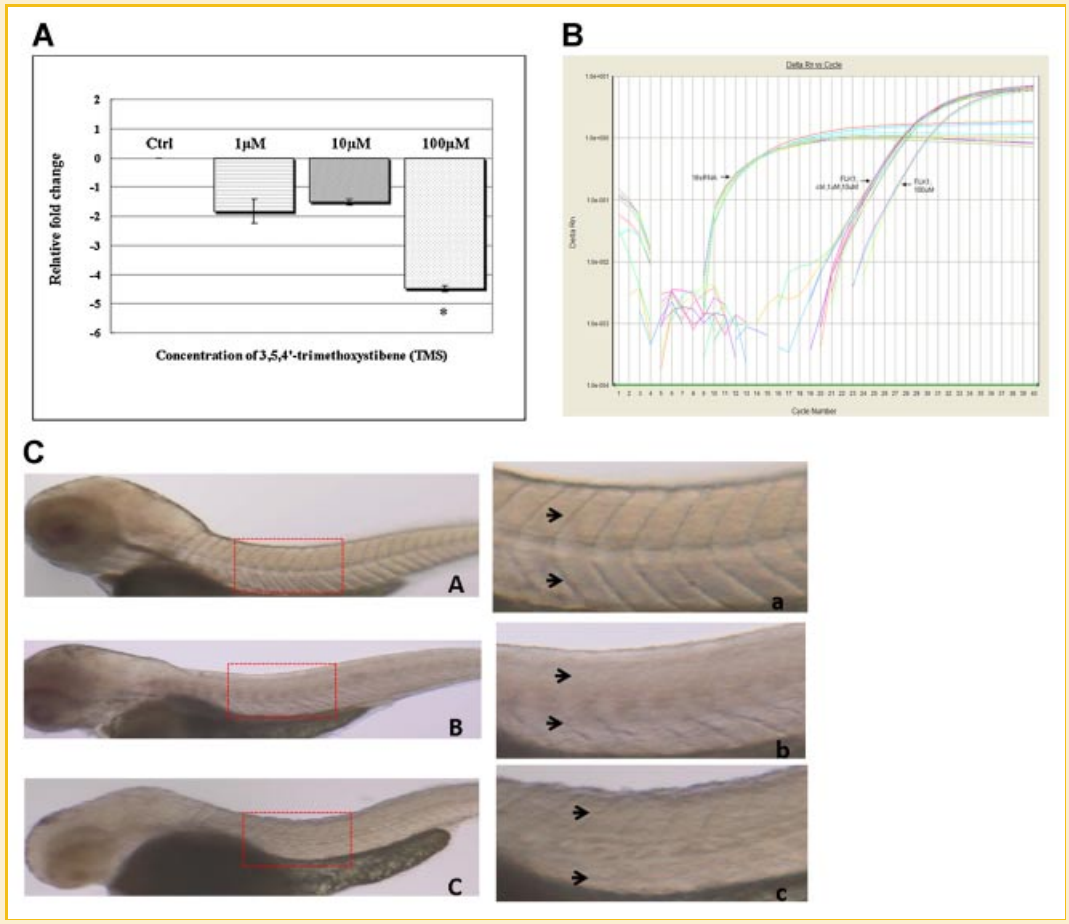


Fig. 3. TMS inhibited *VEGFR2 (flk-1)* gene expression. A: Quantitative real-time PCR of *VEGFR2(flk-1)* expression in response to 3,5,4′-trimethoxystilbene (TMS) treatment is illustrated as fold changes calculated with the relative $C_T$ method using *18S rRNA* as the internal reference. Data plotted are the means ± SEM of three independent experiments $^*P < 0.05$. B: Amplification kinetic curves for a representative experiment, done in triplicates, for *18s rRNA* and *flk-1*. C: In situ hybridization of 2 dpf wildtype fish incubated with or without 3, 10 μM of TMS for 20 h with *flk-1* probe. A,a: control, B,b; 3 μM TMS, C,c; 10 μM TMS. The magnification view of red frame area of A–C is shown in a–c. Arrows represent the ISVs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

showed that TMS induced cell-cycle arrest more effectively in endothelial cells of zebrafish embryos, confirming that TMS exerts a more specific cytotoxic effect on endothelial cells than on other cell types, both in vitro and in vivo. A previous study demonstrated that TMS caused microtubule depolymerization with a subsequent increase in the apoptosis of newly formed vessel sprouts without exerting a significant proapoptotic effect on quiescent endothelial cells in a rat aorta ring assay, proposing this as the reason for its antiangiogenic properties [Belleri et al., 2005]. One of the explanations given was that combretastatin, a potent vascular-disrupting agent currently undergoing clinical trials, causes the apoptosis of proliferating endothelial cells with the induction of apoptosis after a prolonged G2/M arrest [Kanthou et al., 2004; Belleri et al., 2005]. Our results showed that the endothelial cells in the zebrafish embryo underwent G2/M cell-cycle arrest. We also observed that TMS induced a concomitant increase in cells during the sub-G0/G1 phase in a concentration-dependent manner, suggesting the cells underwent apoptosis (data not shown). However, it is not clear whether the same endothelial cells that underwent G2/M arrest later entered apoptosis. Further work is required to address the question of whether TMS induces the apoptosis of endothelial cells after prolonged G2/M arrest, thereby disrupting newly formed blood vessels. Several previous studies have shown that methoxystilbene derivatives of resveratrol induce cell-cycle arrest at G2/M phase, although most of them are *cis* isomers [Schneider et al., 2003; Lee et al., 2004; Durrant et al., 2008], with the exception of 3,4,5,4′-tetramethoxystilbene [Ma et al., 2008]. All these results point to a common mechanism by which these tri/tetramethoxystilbenes induce the arrest of cells at G2/M phase, similar to other colchicine-site inhibitors of tubulin like combretastatin. It should be noted that combretastatin and these methoxystilbenes (in *cis* conformation) share very high structural similarity. It is possible that the *trans* isomer of trimethoxystilbene, TMS, also interacts with tubulin, as inferred from observations of cell-cycle arrest in the present study, and as reported in a previous study [Belleri et al., 2005]. In this regard, it would be interesting to conduct a comparative study of the antiangiogenic properties of the *cis* and *trans* isomers of resveratrol.

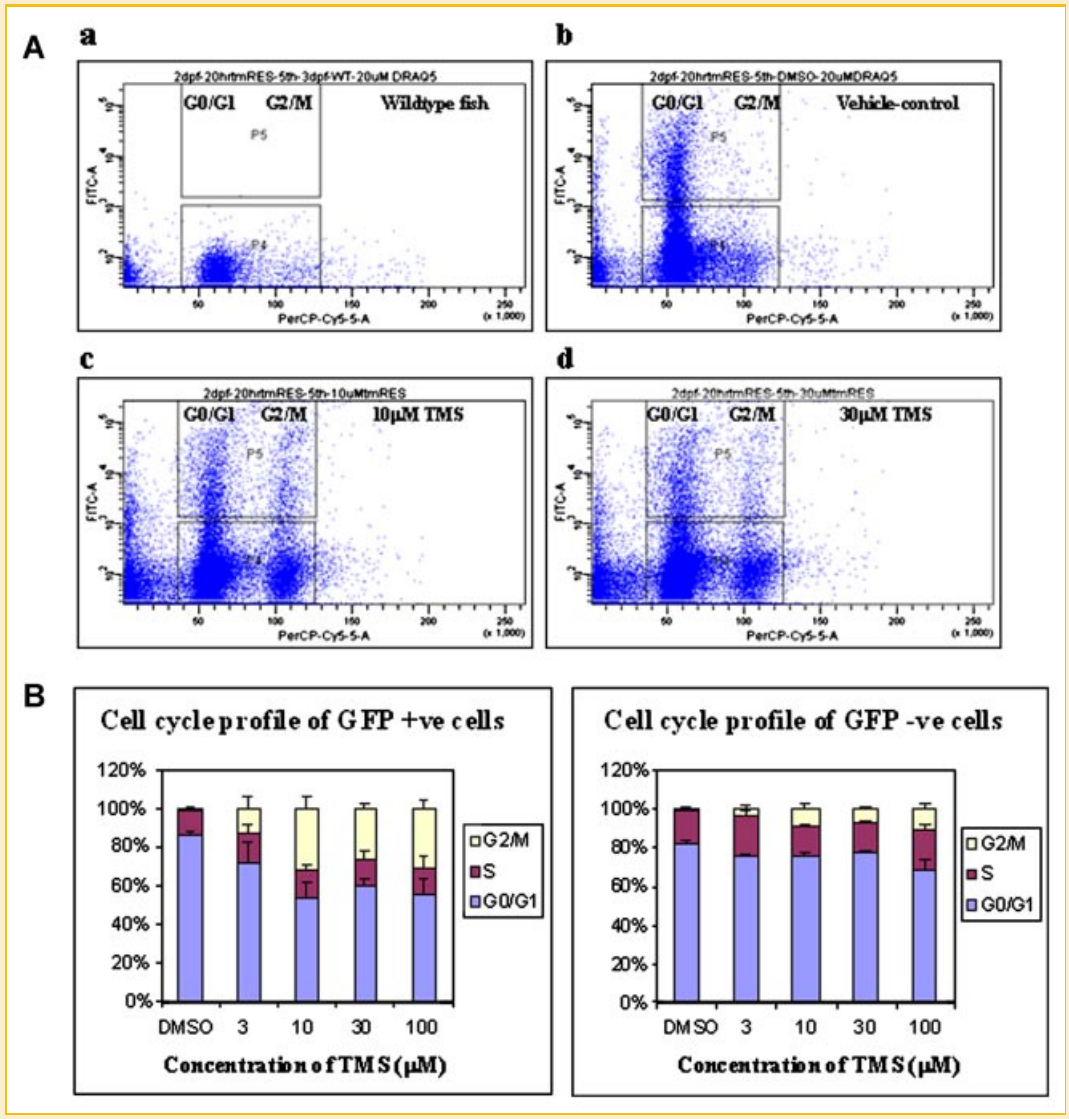


Fig. 4. Cell-cycle analysis of zebrafish embryos after treatment with different concentration of 3,5,4′-trimethoxystilbene (TMS) for 20 h. The cells were labeled with DRAQ5™ after trypsinization and analyzed by flow cytometry. A: The dot plot of green fluorescent protein (GFP) signal (FITC on the *Y*-axis) versus the DNA content (PerCP-Cy5 on the *X*-axis). GFP-positive (P5-gated) cells and GFP-negative (P4-gated) cells. The results represent the means of five independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The target genes of the antiangiogenic actions of TMS and resveratrol have not been identified or reported previously. Our gene expression studies indicated that TMS caused significant down-regulation of *VEGFR2* mRNA, albeit at a higher concentration of the drug than is needed to induce vessel regression in the ISVs of zebrafish. This requirement for a higher concentration could be because the mRNA was extracted from whole zebrafish in which the downregulation of *VEGFR2* responsible for neovascularization might have been underestimated. Our results from in situ hybridization of wildtype zebrafish embryos confirmed that indeed TMS was able to downregulate *VEGFR2* expression specifically in endothelial cells at relatively lower concentrations. Since VEGFR2 is specifically involved in neovascularization, downregulating VEGFR2 activity is one of the foremost approaches to inhibiting tumor angiogenesis [Ferrara et al., 2003; Veeravagu et al., 2007]. The antiangiogenic property of TMS could be explained, at least in part, by its downregulation of *VEGFR2* gene expression in endothelial cells.

The zebrafish is a powerful in vivo model for developmental and pharmacological studies, and has received immense attention in recent years. Our studies once again point to the merits of this system, indicating that TMS exerts its antiangiogenic effect by the G2/M arrest of endothelial cells, similar to its reported activity in mammalian cell lines.

In summary, *trans*-3,5,4′-trimethoxystilbene exerts its antiangiogenic effects both in vitro and in vivo by downregulating the VEGF/VEGFR pathway and by causing G2/M cell-cycle arrest, most specifically in the endothelial cells, indicating its potential therapeutic use as a vascular-targeting and antiangiogenic agent.

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