0026-895X/05/6705-1451–1459$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 5
9043/1198162
Mol Pharmacol 67:1451–1459, 2005
Printed in U.S.A.

Antiangiogenic and Vascular-Targeting Activity of the Microtubule-Destabilizing *trans*-Resveratrol Derivative 3,5,4′-Trimethoxystilbene[S]

Mirella Belleri, Domenico Ribatti, Stefania Nicoli, Franco Cotelli, Luca Forti, Vanio Vannini, Lucia Anna Stivala, and Marco Presta

Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy (M.B., S.N., M.P.); Department of Human Anatomy and Histology, University of Bari, Bari, Italy (D.R.); Department of Biology, University of Milan, Milan, Italy (F.C.); Department of Chemistry, University of Modena and Reggio Emilia, Modena, Italy (L.F.); and Unit of General Pathology, Department of Experimental Medicine, University of Pavia, Pavia, Italy (V.V., L.A.S.)

Received November 10, 2004; accepted February 9, 2005

ABSTRACT

Neovascularization plays an important role in neoplasia and angioproliferative diseases. Two major modalities have been developed so far to affect neovascularization: its prevention by antiangiogenic compounds, and immature vessel disruption by vascular-targeting agents. *trans*-Resveratrol, found in grapes and wine, exerts antioxidant, antineoplastic, and antiangiogenic activities. Here, among various synthetic *trans*-resveratrol derivatives tested, 3,5,4′-trimethoxystilbene was an antiangiogenic agent 30 to 100 times more potent than parent compound in inhibiting endothelial cell proliferation, sprouting, collagen gel invasion, and morphogenesis (ID_{50} = 0.3–3.0 μM). In addition, 3,5,4′-trimethoxystilbene acts as a vascular-targeting agent by causing microtubule disassembling and tubulin depolymerization and by impairing the repositioning of the microtubule organization center and the formation of membrane ruffles in migrating endothelial cells. In keeping with a vascular-targeting ability, 3,5,4′-trimethoxystilbene induced apoptosis only in subconfluent endothelial cells and apoptotic regression of immature vessels in the ex vivo rat aorta ring assay. In vivo, 3,5,4′-trimethoxystilbene caused the rapid stasis of blood flow and regression of intersegmental vessels in the trunk of zebrafish embryos. In addition, it inhibited blood vessel growth and caused the disappearance of pre-existing blood vessels in the area vasculosa of the chick embryo. In conclusion, 3,5,4′-trimethoxystilbene associates an antiangiogenic profile to a significant vascular-targeting activity.

In the adult, the proliferation rate of endothelial cells is very low compared with many other cell types in the body. Uncontrolled endothelial cell proliferation is observed in tumor neovascularization, angioproliferative diseases, and angiogenesis-dependent diseases (Folkman, 1995; Carmeliet, 2003). Thus, chemical agents able to affect neovascularization may have broad applicability for the therapy for a wide spectrum of diseases, including cancer. Two major modalities have been developed so far to affect neovascularization: the prevention of new blood vessel formation by antiangiogenic compounds, and the disruption of the neovasculature by vascular-targeting agents.

New blood vessel formation is a multistep process that begins with the degradation of the basement membrane by activated endothelial cells that will migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed, and capillary tubes develop with formation of tight junctions and deposition of new basement membrane (Carmeliet, 2000). Each step of this process represents a potential target for the inhibitory action of angiosuppressive molecules (Keshet and Ben-Sasson, 1999). Indeed, various angiogenesis inhibitors have been developed so far; their efficacy has been evaluated in different in vitro and in vivo assays, and their clinical

This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Centro di Eccellenza "IDET", Firb 2001, Cofin 2004), Associazione Italiana Ricerca sul Cancro, Fondazione Berlucchi, Istituto Superiore di Sanità (Onco-technological Program), and Fondo Sociale Europeo-Regione Lombardia (SPAFARM project).

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.009043.

ABBREVIATIONS: VEGF, vascular endothelial growth factor; BAE cells, bovine aortic endothelial cells; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified minimal essential medium; FCS, fetal calf serum; FGF2, fibroblast growth factor-2; FGF2-*T*-MAE, fibroblast growth factor-2–transfected murine aortic endothelial; HUVE, human umbilical vein endothelial; MAE, murine aortic endothelial cells; MTOC, microtubule organization center; hpf, hours postfertilization.

evaluation is in progress (Kerbel and Folkman, 2002). Recently, the anti-VEGF antibody bevacizumab has been shown to exert antiangiogenic effects in patients with cancer, leading to U.S. Food and Drug Administration approval for colorectal cancer treatment (Fernando and Hurwitz, 2003).

The properties of neovasculature differ from those of quiescent endothelium. Small-molecule vascular-targeting agents exploit differences in cell proliferation, permeability, maturation, and reliance on tubulin cytoskeleton to induce selective blood vessel occlusion and destruction (Thorpe, 2004). In particular, microtubule-destabilizing agents, including combretastatin-derived prodrugs and analogs, disrupt rapidly proliferating and immature tumor endothelium, leading to reduced tumor blood flow and hypoxia (Tozer et al., 1999, 2001, 2002). Combining vascular-targeting agents with angiogenesis inhibitors may result in additive or synergistic effects on tumor growth and vascularization (Siemann et al., 2002; Siim et al., 2003). Microtubule-destabilizing agents (e.g., combretastatin A-4 and vinblastine) may also show a distinct antiangiogenic activity (Vacca et al., 1999; Ahmed et al., 2003).

The polyphenolic phytoalexin *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is an antioxidant constituent of many plant species, abundant in grapevines and present at high concentrations (up to 30 μM) in red wine (Frémont, 2000; Cao et al., 2002). *trans*-Resveratrol has received wide attention because of its possible role in the prevention of human pathological processes, including cardiovascular diseases and cancer (Jang et al., 1997; Frémont, 2000). In a study aimed at identifying the structural determinants responsible for the antioxidant and antiproliferative activity of *trans*-resveratrol, *trans*-derivatives were synthesized, differing for the lack of hydroxylic functions, the reduction of the stilbenic bond, or the substitution of hydroxylic groups with methoxy groups. These derivatives differently affect the proliferative capacity of human fibroblasts and tumor cells independently of their antioxidant activity (Stivala et al., 2001).

Recently, *trans*-resveratrol has been shown to exert antiangiogenic activity (Brakenhielm et al., 2001; Tseng et al., 2004). Here, we evaluated the capacity of *trans*-resveratrol derivatives to affect endothelial cell behavior in vitro and in vivo. The results identify 3,5,4′-trimethoxystilbene as a microtubule-destabilizing agent endowed with antiangiogenic and vascular-targeting activity.

Materials and Methods

Reagents. *trans*-Resveratrol, bibenzyl, and *trans*-stilbene were from Sigma-Aldrich (St. Louis, MO). *trans*-Resveratrol derivatives (Fig. 1) were synthesized as described previously (Stivala et al., 2001). Stock solutions were prepared in dimethyl sulfoxide and diluted directly in cell-culture medium. VEGF was from Calbiochem (San Diego, CA). Recombinant FGF2 was expressed and purified from *Escherichia coli* cell extract (Bastaki et al., 1997).

Cell Cultures. Immortalized BALB/cMAE cells were obtained from R. Auerbach (University of Wisconsin, Madison, WI) and grown in DMEM (Invitrogen, Carlsbad, CA) added to 10% FCS (Integro, Zaandam, The Netherlands). FGF2-*T*-MAE cells, a highly tumorigenic subclone of FGF2-overexpressing MAE cells (Gualandris et al., 1996), were grown in DMEM supplemented with 4 mM glutamine (Invitrogen) and 10% FCS. BAE cells (provided by A. Vecchi, Istituto Mario Negri, Milan, Italy) were cultured in DMEM supplemented with 10% heat-inactivated donor calf serum. HUVE cells were from Cambrex Bio Science Walkersville (Walkersville, MD) and were cultured in endothelial growth medium-2 (Cambrex).

Cell Proliferation Assays. Routinely, MAE and FGF2-*T*-MAE cells were seeded at 25,000 cells/cm^2. HUVE cells were seeded at 6250/cm^2 on gelatin-coated dishes. After overnight incubation, cells were incubated in fresh medium in the presence of the compound under test. Cells were trypsinized and counted in a Burker chamber after 24 (MAE and FGF2-*T*-MAE cells) or 72 h (HUVE cells). In one set of experiments, MAE cells were incubated for 3 h with the compound under test. Then, cells were washed and allowed to grow for the next 21 h in fresh medium with no addition.

BAE cell proliferation assays were performed on both confluent and subconfluent cell cultures. To this purpose, cells were seeded at 40,000/cm^2 or 20,000/cm^2 and maintained for 4 days or 24 h, respectively. Then, all cell cultures were incubated with the compound under test in fresh medium with 10% FCS. After 24 h, cells were trypsinized and counted. Parallel cell cultures were fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 0.5 μg/ml DAPI to visualize apoptotic nuclei or immunostained with anti-tubulin antibody (see below).

Preparation of Three-Dimensional Gels. FGF2-*T*-MAE cell aggregates were prepared on agarose-coated plates and embedded in fibrin gel as described previously (Gualandris et al., 1996). Then, culture medium containing increasing concentrations of the compound under test was added on the top of the gel in the presence of 10 μg/ml aprotinin to prevent the dissolution of the substrate. The formation of radially growing cell sprouts was observed during the next 24 h. Sprouts were photographed at 40× magnification (Olympus IX51 inverted microscope with Camedia C-4040 digital camera; Olympus Biosystem, Munich, Germany) and quantified by computerized analysis of the digitalized images.

Three-dimensional gels of reconstituted rat tail tendon type I collagen fibrils (Roche Diagnostics, Milan, Italy) were prepared as described previously (Bastaki et al., 1997). Then, BAE cells were seeded on the top of collagen gel (80,000 cells/well) and allowed to reach confluence. Cell cultures were then treated with fresh medium

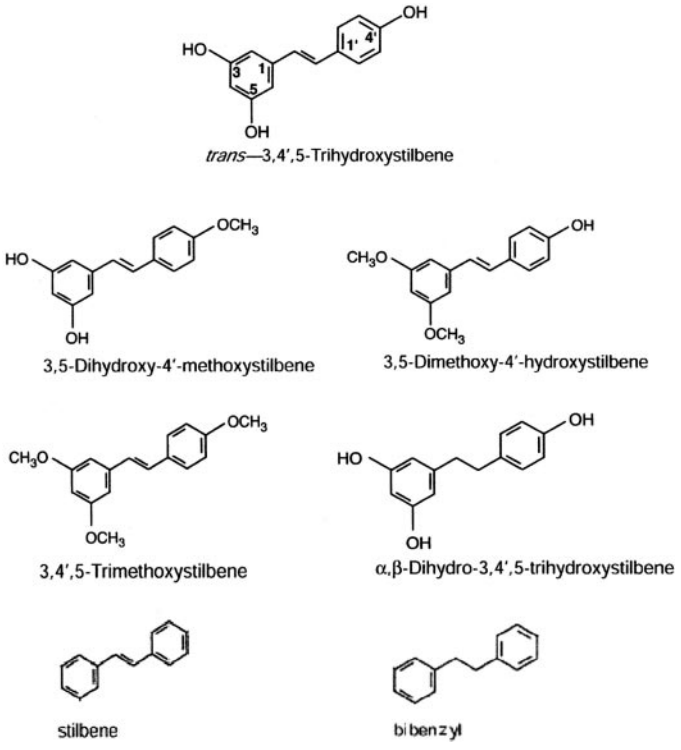

Fig. 1. Chemical structure of *trans*-resveratrol and its derivatives.

containing FGF2 plus VEGF (both at 30 ng/ml) and added to 10% FCS in the absence or in the presence of the compound under test. After 24 h, cells were photographed at 100× magnification (Olympus IX51 inverted microscope), and endothelial cells invading the gel, in a plane of focus beneath the cell monolayer surface, were quantified by computerized analysis of the digitalized images.

Matrigel (10 mg/ml; BD Biosciences, Milan, Italy) was used to coat 48-well plates at 4°C (150 μl/well) (Bastaki et al., 1997). After gelification at 37°C, HUVE cells were seeded onto Matrigel-coated dishes at 40,000 cells/cm^2 in the absence or in the presence of the compound under test. Newly formed endothelial cell "cords" and "tubes" were photographed after 16 h at 40× magnification (Olympus IX51 inverted microscope), scored by two investigators without knowledge of the samples tested, and graded on an arbitrary scale of 0 to 4+, with 0 representing no morphogenic response and 4+ representing the strongest response.

Immunocytochemistry. Cells were seeded on gelatin-coated glass coverslips in DMEM added to 10% FCS. After overnight incubation, cells were treated with the compound under test for 3 h, washed, fixed in 3% paraformaldehyde/2% sucrose in phosphate-buffered saline, permeabilized with 0.5% Triton X-100, and saturated with goat serum in phosphate-buffered saline. Then, cells were incubated with rodaminate-phalloidin, a monoclonal anti-paxillin antibody (BD Transduction Laboratories, Lexington, KY) followed by Alexa Fluor 594 anti-mouse IgG (Molecular Probes, Eugene, OR), a monoclonal anti-α-tubulin antibody or a monoclonal anti-vimentin antibody, followed by fluorescinated anti-mouse IgG (all from Sigma-Aldrich). Cells were photographed at 63× magnification [Axiovert S100 epifluorescence microscope (Carl Zeiss GmbH, Jena, Germany) with Camedia C-4040 digital camera].

Tubulin Polymerization Assay. MAE cells were incubated with the compound under test for 3 h. After cell lysis with hypotonic buffer (1 mM $MgCl_2$, 2 mM EGTA, 20 mM Tris-HCl, pH 6.8, and 0.5% Nonidet P-40) containing protease inhibitors (Complete; Roche Diagnostics), insoluble pellets containing cytoskeletal polymerized tubulin were analyzed by Western blotting using a monoclonal anti-α-tubulin antibody.

Wounding of Endothelial Cell Monolayer. Wounds were created in MAE cell monolayers with a 1.0-mm wide rubber policeman. Then, cells were incubated in fresh medium added to 10% FCS and the compound under test. At different time points, the percentage of cells at the edge of the wound showing cell-membrane ruffles were counted under a inverted microscope at 400× magnification (Olympus IX51 microscope with Camedia C-4040 digital camera). After 16 h, wounds were photographed, and endothelial cells invading the wound were quantified by computerized analysis of the digitalized images. In some experiments, cells were fixed 3 h after the wound, and repositioning of the MTOC was evaluated by immunostaining with an anti-pericentrin antibody (BabCO, Richmond, CA). To determine MTOC position, 70 to 80 cells in the first row of cells adjacent to the wound and in the inner monolayer were then selected per experimental point. Each cell was visually divided by drawing two perpendicular lines across the cell nucleus, thus defining a front quadrant covering 25% of the cell surface and containing the leading edge (Fig. 4B). The percentage of migrating and quiescent cells with MTOC located inside the front quadrant was then calculated. Statistically, a random distribution of the MTOC around the nucleus, as observed in a quiescent monolayer, will result in 25% of cells with MTOC located in the front quadrant.

Rat Aorta Ring Assay. This assay was performed as described previously (Nicosia and Ottinetti, 1990), with minor modifications. In brief, aortic rings were obtained by cross-sectioning the thoracic aorta of 5- to 10-week-old Fisher 344 male rats at 1-mm intervals. Rings were placed individually on the bottom of 24-well plates, with the luminal axis lying parallel to the bottom of the plate. Next, 30 μl of polymerizing fibrin solution (prepared as described above) were applied onto each ring. After 5 min, wells were added to 600 μl of serum-free endothelial cell basal medium (Cambrex Bio Science Walkersville) plus 10 μg/ml aprotinin in the absence or in the presence of the compound under test. The medium was changed three times a week starting from day 3. The angiogenic response was measured by counting the number of neovessels sprouting out of the rings over time.

For neovessel-regression experiments, rings were cultured with no addition. At day 7, rings were treated with the compound under test, and neovessels were photographed at 100× magnification (Olympus IX51 inverted microscope) during the following hours. At the end of treatment, rings were fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 0.5 μg/ml DAPI to visualize apoptotic nuclei.

Zebrafish Assay. A zebrafish (*Danio rerio*) breeding colony (wild-type AB strain) and the *VEGFR2:G-RCFP* transgenic line (Cross et al., 2003), kindly provided by A. Rubinstein (Zygogen, Atlanta, GA), were maintained according to routine procedures (Westerfield, 2000). Maintenance of adult fishes, collection of fertilized eggs, and compound exposure were all performed in system water adjusted to a conductivity of approximately 1500 AS/cm, pH 7.5 to 8.0, with Instant Ocean Salts (Aquarium System, Mentor, OH). Exposures, initiated at 72 h postfertilization (hpf), were carried out in 96-well plates with 1 embryo per well in 100 μl of 0.1 μM 3,5,4′-trimethoxystilbene or 0.1 μM bibenzyl at 28°C in system water. For each compound, 10 embryos in two independent experiments were tested. To avoid pigment formation, phenylthiourea (200 mM) was included in the embryo medium. After 5 to 30 min, embryos were anesthetized with tricaine (Sigma-Aldrich) in system water, and videos were acquired at 10× magnification using an epifluorescence Leica MZ FLIII stereomicroscope equipped with a Leica DFC480 camera and IM50 software (Leica Microsystems, Inc., Deerfield, IL). At 6 h, embryos were anesthetized again and photographed at 8× magnification.

Chick Area Vasculosa Assay. Fertilized White Leghorn chicken eggs were incubated under conditions of constant humidity at a temperature of 37°C. At day 3, gelatin sponges (Gelfoam; Upjohn Company, Kalamazoo, MI) were cut to a size of 1 mm^3, adsorbed with the compound under test (300 pmol/implant), and placed on top of the area vasculosa. After 48 h, membranes were photographed in ovo under a Zeiss SR stereomicroscope (Carl Zeiss GmbH) equipped with the MC 63 Camera System at 8× magnification.

In some experiments, the percentage of blood vessels of the area vasculosa surrounding the gelatin implant showing an arrest of the blood flow was quantified in ovo 10 min after treatment under the stereomicroscope at 20× magnification.

Results

Antiangiogenic Activity of *trans*-Resveratrol Derivatives. To assess their anti-angiogenic potential, *trans*-resveratrol and a series of synthetic derivatives were evaluated for the capacity to affect different steps of the angiogenesis process, including endothelial cell proliferation, sprouting activity within fibrin gel, type-I collagen gel invasion, and morphogenesis on Matrigel. Because of endothelial cell heterogeneity (Bastaki et al., 1997; Chi et al., 2003), the assays were performed using endothelial cells of murine, bovine, and human origin. The compounds were tested at concentrations ranging between 0.1 and 100 μM, and the dose (ID_{50}) causing 50% inhibition of the response compared with vehicle-treated cell cultures was then calculated (Table 1).

trans-Resveratrol exerts an inhibitory activity in all of the assays with a similar potency (ID_{50} = 30–100 μM). The 3,5,4′-trimethoxystilbene derivative seems to be at least 30 times more potent than *trans*-resveratrol in all of the assays (ID_{50} = 0.3–3.0 μM), whereas 3,5-dimethoxy-4′-hydroxystilbene showed an intermediate efficacy. All the other deriva-

tives did not elicit a significant response in any assay, with the only exception for 3,5-dihydroxy-4′-methoxystilbene that showed some inhibitory effect on HUVE cell proliferation and morphogenesis on Matrigel. Representative experiments demonstrating the inhibitory activity of 3,5,4′-trimethoxystilbene are shown in Fig. 2. The high potency of 3,5,4′-trimethoxystilbene prompted us to investigate in more detail the biological activity and mechanism of action of this derivative.

3,5,4′-Trimethoxystilbene As an Endothelial Cell Microtubule-Destabilizing Agent. The chemical structure of 3,5,4′-trimethoxystilbene resembles that of the *trans*-isomer of the microtubule-depolymerazing agent combretastatin A-4 (Cushman et al., 1991). In addition, the *cis*-isomer of 3,5,4′-trimethoxystilbene affects tubulin polymerization in cancer cells (Schneider et al., 2003). From this, we evaluated the effect of 3,5,4′-trimethoxystilbene in its *trans* configuration on microtubule organization in endothelial cells.

In a first set of experiments, we evaluated the effect of 3,5,4′-trimethoxystilbene on the different components of MAE cell cytoskeleton. A 3-h treatment with 1.0 μM 3,5,4′-trimethoxystilbene causes a dramatic depolymerization of microtubules (Fig. 3, a and b) with minor, if any, effects on actin organization (Fig. 3, c and d) and intermediate filament integrity (Fig. 3, e and f). Accordingly, 3,5,4′-trimethoxystilbene did not hamper the capacity of adherent cells to form focal adhesion contacts as shown by anti-paxillin immunostaining (Fig. 3, g and h). In keeping with previous observations (Pettit et al., 2002), no significant effect on microtubule assembly was exerted by *trans*-resveratrol (data not shown). Time-course experiments showed that microtubule disassembly occurred within 30 min from 3,5,4′-trimethoxystilbene administration.

To confirm the antitubulin activity of 3,5,4′-trimethoxystilbene, MAE cells were treated for 3 h with the compound, and polymerized tubulin was assessed by Western blotting of the insoluble fraction of the cell lysate. As anticipated, 1.0 μM 3,5,4′-trimethoxystilbene causes a significant decrease in the amount of polymerized tubulin (Fig. 3, a and b, insets).

Migration of endothelial cells after the mechanical wounding of the cell monolayer is characterized by the repositioning of cell MTOC from a random distribution to a biased localization in front of the nucleus, toward the direction of cell migration (Ueda et al., 1997). Consistently, 3 h after wounding, the MTOC was located at the leading edge in the front of the nucleus in 55% of MAE cells adjacent to the wound, compared with 24% of cells of the inner quiescent monolayer (Fig. 4A). 3,5,4′-Trimethoxystilbene (1–3 μM) impaired the repositioning of the MTOC that was localized in front of the nucleus in only 30% of cells at the edge of the wound, thus maintaining an essentially random distribution (Fig. 4A). This was paralleled by the inhibition of the formation of cell-membrane ruffles and suppression of cell migration, with a consequential significant delay in the healing of the

TABLE 1

In vitro effects of *trans*-resveratrol derivatives on endothelial cells

trans-Resveratrol and its derivatives were tested on the indicated endothelial cell types for the capacity to inhibit cell proliferation, sprouting in 3D-fibrin gel, type-I collagen gel invasion, and morphogenesis on Matrigel. Data are the means of two to three experiments performed in triplicate and are expressed as the dose (ID_{50}) of the compound exerting 50% inhibition of the response compared with vehicle-treated cells.

Compound	Cell proliferation			FGF2-*T*-MAE Sprouting in Fibrin Gel	BAE Collagen Gel Invasion	HUVE Morphogenesis on Matrigel
	MAE	FGF2-*T*-MAE	HUVE			
				μM		
trans-Resveratrol	30	30	30	30	30–100	30–100
3,5-Dihydroxy-4′-methoxystilbene	>100	>100	30–100	>100	N.D.	30–100
3,5-Dimethoxy-4′-hydroxystilbene	30	10	10	3–10	10	10
3,5,4′-Trimethoxystilbene	1	1	1	0.3	1–3	1
α,β-Dihydro-3,4′,5-trihydroxystilbene	>100	>100	100	>100	>100	>100
trans-Stilbene	>100	>100	>100	>100	>100	>100
Bibenzyl	>100	>100	>100	>100	>100	>100

N.D., not determined.

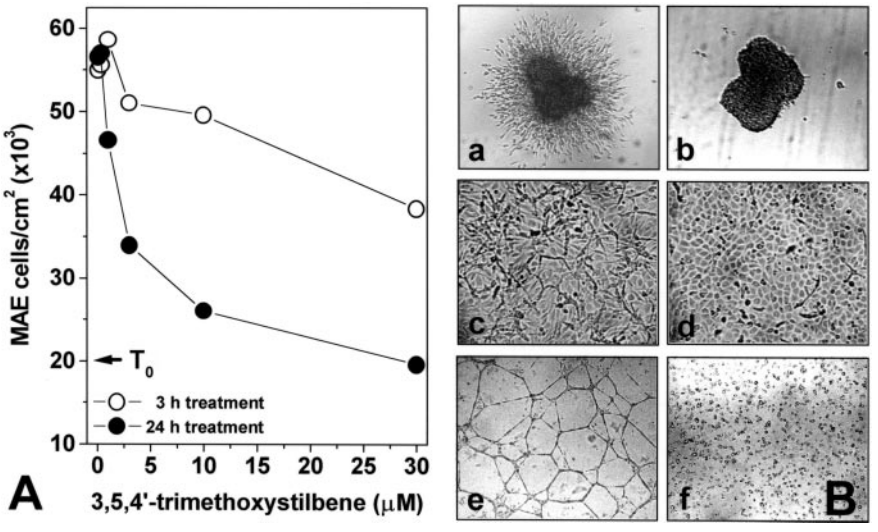

Fig. 2. Effect of 3,5,4′-trimethoxystilbene on endothelial cells. A, MAE cells were seeded at 25,000 cells/cm^2. After overnight incubation (T_0), cells were incubated in fresh medium in the presence of increasing concentrations of 3,5,4′-trimethoxystilbene for 3 h, were washed and allowed to grow for the next 21 h (○), or were incubated with the compound for 24 h throughout the whole experimental period (●). Then, cells were trypsinized and counted in a Burker chamber. Data are the means of three determinations. B, representative images of FGF2-*T*-MAE cell aggregates embedded in fibrin gel (a and b), BAE cells invading a collagen gel (c and d), and HUVE cells seeded on Matrigel (e and f) in the presence of vehicle (a, c, and e) or 3.0 μM 3,5,4′-trimethoxystilbene (b, d, and f). Original magnification, 40×.

wounded monolayer (Fig. 5). In contrast, no effect on cell proliferation was observed when MAE cells were exposed to 1 to 10 μM 3,5,4′-trimethoxystilbene for a 3-h pulse compared with the strong inhibitory effect exerted on cells exposed to the compound for 24 h (Fig. 2A). Thus, the early effects exerted by low doses of 3,5,4′-trimethoxystilbene on endothelial cell motility are not the direct consequence of its cytotoxic activity. Similar results were obtained on BAE cells (data not shown).

Tubulin-destabilizing agents have profound effects on proliferating immature endothelial cells but not on quiescent endothelium (Dark et al., 1997; Iyer et al., 1998). Accordingly, 24-h exposure to 3,5,4′-trimethoxystilbene caused microtubule degradation and a significant inhibition of the proliferation of subconfluent BAE cells, with a parallel increase in the number of apoptotic cells (Fig. 6). In contrast, despite the observed degradation of the microtubule network and cell contraction, 3,5,4′-trimethoxystilbene did not affect the number of quiescent confluent BAE cells that did not show any sign of apoptosis (Fig. 6). In addition, no apoptotic cells were observed in *trans*-resveratrol–treated endothelial cells independently on their origin and proliferative status (data not shown).

As anticipated for an antiangiogenic compound, 3,5,4′-tri-

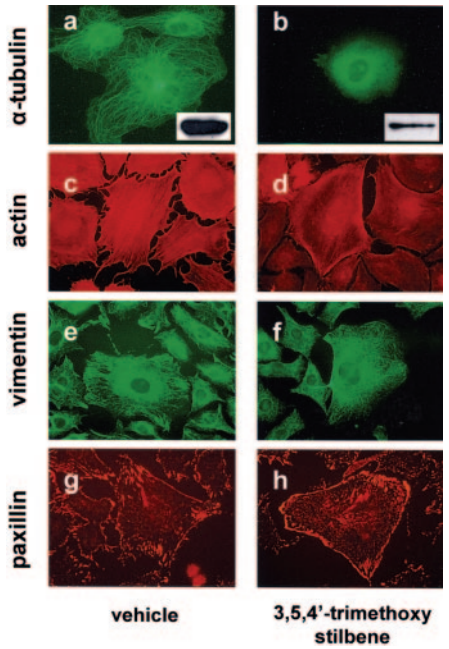

Fig. 3. Effect of 3,5,4′-trimethoxystilbene on endothelial cell cytoskeleton. Subconfluent MAE cells were treated for 3 h with vehicle (a, c, e, and g) or 1.0 μM 3,5,4′-trimethoxystilbene (b, d, f, and h). Then, cells were incubated with anti-α-tubulin antibody (a and b), rodaminate-phalloidin (c and d), anti-vimentin antibody (e and f), or anti-paxillin antibody (g and h) and photographed at 63× magnification. Insets, Western blot of the polymerized tubulin fraction in vehicle (a) or 1.0 μM 3,5,4′-trimethoxystilbene (b)-treated MAE cells.

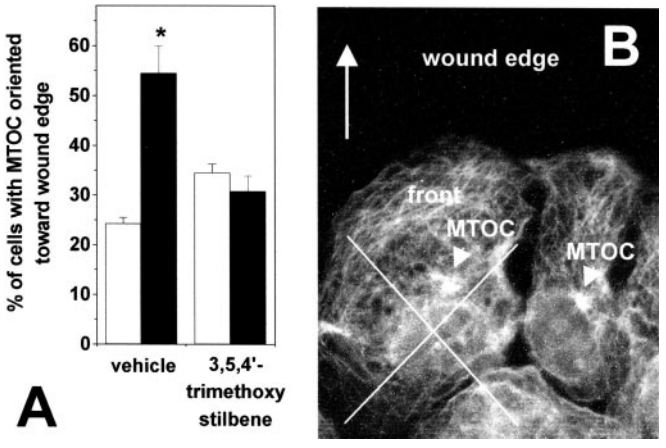

Fig. 4. Effect of 3,5,4′-trimethoxystilbene on MTOC repositioning. A, confluent MAE cells were wounded with a 1.0-mm wide rubber policeman. After 3 h of incubation with vehicle or 3.0 μM 3,5,4′-trimethoxystilbene, cells were immunostained with an anti-pericentrin antibody. The percentage of cells at the edge of the wound (■) and in the inner monolayer (□) with MTOC localized in the front quadrant was then calculated ($n = 70–80$). ⋆, $P < 0.05$ versus all the other groups, Student's t test. B, wound edge of a vehicle-treated, pericentrine/tubulin double-stained cell culture to show the front quadrant used to score MTOC position. The arrow points to the direction of migration. Original magnification, 63×.

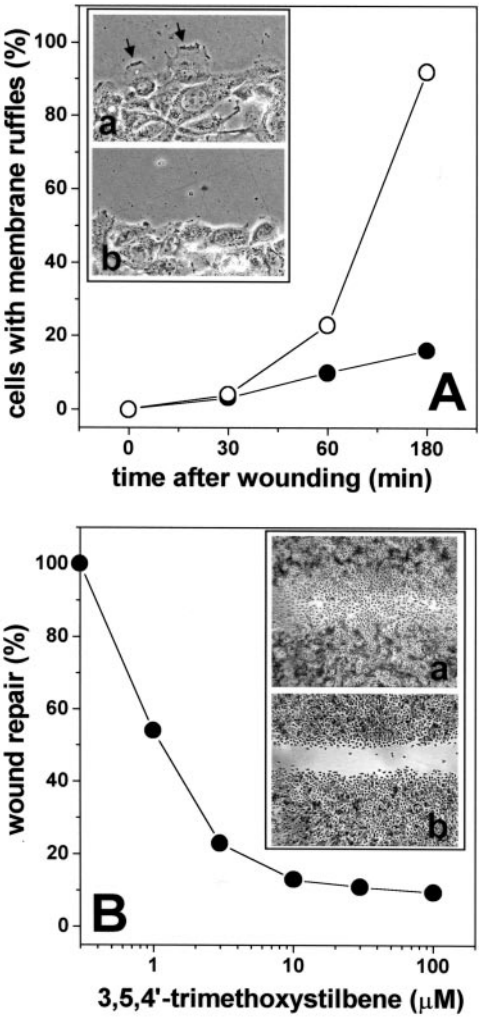

Fig. 5. Effect of 3,5,4′-trimethoxystilbene on endothelial wound repair. A, confluent MAE cells were wounded with a 1.0-mm wide rubber policeman and incubated with vehicle (○) or 10 μM 3,5,4′-trimethoxystilbene (●). At different time points, the percentage of cells showing membrane ruffles were counted. B, MAE cell wounded monolayers were incubated with increasing concentrations of 3,5,4′-trimethoxystilbene. After 16 h, wounds were photographed, and endothelial cells invading the wound were quantified by computerized analysis of the digitalized images. Insets, representative images of vehicle (a) and 3,5,4′-trimethoxystilbene (b)-treated monolayers photographed 3 h (A) and 16 h (B) after wounding. Arrows point to membrane ruffles. Original magnifications, 400× (A) and 40× (B).

methoxystilbene caused the inhibition of neovessel formation in an ex vivo rat aorta ring assay (Fig. 7), in which activated endothelial cells of the vessel intima originate capillary-like structures in the surrounding fibrin gel (Nicosia and Ottinetti, 1990). In addition, when added to control aortic rings after 7 days in culture, 3,5,4′-trimethoxystilbene exerted its vascular-targeting activity by causing endothelial cell apoptosis in immature, newly formed vessels (Fig. 8A). Time-course experiments showed that vessel regression was already apparent 3 h after drug administration (Fig. 8B). Taken together, the data indicate that 3,5,4′-trimethoxystilbene is endowed with both antiangiogenic and tubulin-destabilizing/vascular-targeting activities.

Effect of 3,5,4′-Trimethoxystilbene on Blood Vessels in Vivo. Zebrafish embryos have been proposed for the screening of antiangiogenic drugs (Serbedzija et al., 2000). Here, transgenic *VEGFR2:G-RCFP* zebrafish embryos (Cross et al., 2003), in which green fluorescent protein expression is driven in endothelium by the VEGF receptor 2 promoter, were exposed to 0.1 μM 3,5,4′-trimethoxystilbene at 72 hpf. In keeping with its vascular-targeting activity, 3,5,4′-trimethoxystilbene caused the rapid stasis (within 30 min after exposure) of blood flow in the dorsal aorta, posterior cardinal vein, and intersegmental vessels of the trunk in the absence of significant changes in tissue integrity (as shown in the zebrafish movie in Supplemental Video S1 associated with Fig. 9). At later time points (6 h after incubation), 3,5,4′-trimethoxystilbene caused the regression of intersegmental vessels (Fig. 10). When embryos were exposed to 3,5,4′-trimethoxystilbene at earlier time points during somitogenesis (19 hpf), before intersegmental angiogenesis has occurred, the compound exerted a potent toxic effect, leading to profound morphological changes and developmental arrest of the embryo (data not shown), similar to other microtubule-destabilizing agents (Moon et al., 2002). No effect was exerted at both time points by the control compound bibenzyl.

On this basis, 3,5,4′-trimethoxystilbene was assessed for its antiangiogenic and vascular-targeting activity on the chick embryo area vasculosa, an extraembryonal adnexum copiously found in vascularization (Ribatti, 1995). To this purpose, 3,5,4′-trimethoxystilbene (300 pmol/embryo) was applied topically on the area vasculosa membrane via a gelatin sponge implant at day 3 of development (Fig. 11A). As observed in zebrafish embryos, 3,5,4′-trimethoxystilbene caused the rapid stasis (within 5 min after delivery of the molecule) of the blood flow in the vessels surrounding the implant, leading to erythrocyte stagnation and vessel dilation (Fig. 11B). A quantitative analysis (n = 10) performed at 10 min of treatment demonstrated that approximately 80% of blood vessels surrounding 3,5,4′-trimethoxystilbene implants underwent blood flow stasis, compared with approximately 30% of the vessels surrounding vehicle-treated implants. After 48 h, when area vasculosa continues to expand and becomes denser under normal conditions (Fig. 11A), we observed a significant inhibition of the vascularization in 3,5,4′-trimethoxystilbene-treated embryos, characterized by the lack of blood vessel growth and the disappearance of the majority of pre-existing blood vessels (Fig. 11C). This occurred in the absence of any effect on embryonic development and survival. In addition, no effect on area vasculosa was exerted by vehicle or by the control compound bibenzyl (data not shown).

Discussion

Pharmacological interventions aimed at affecting the neovasculature may represent the basis for novel therapeutic approaches in a variety of pathological conditions, including neoplasia. Basically, two major conceptually distinct mechanisms of interventions have been envisaged so far. The first

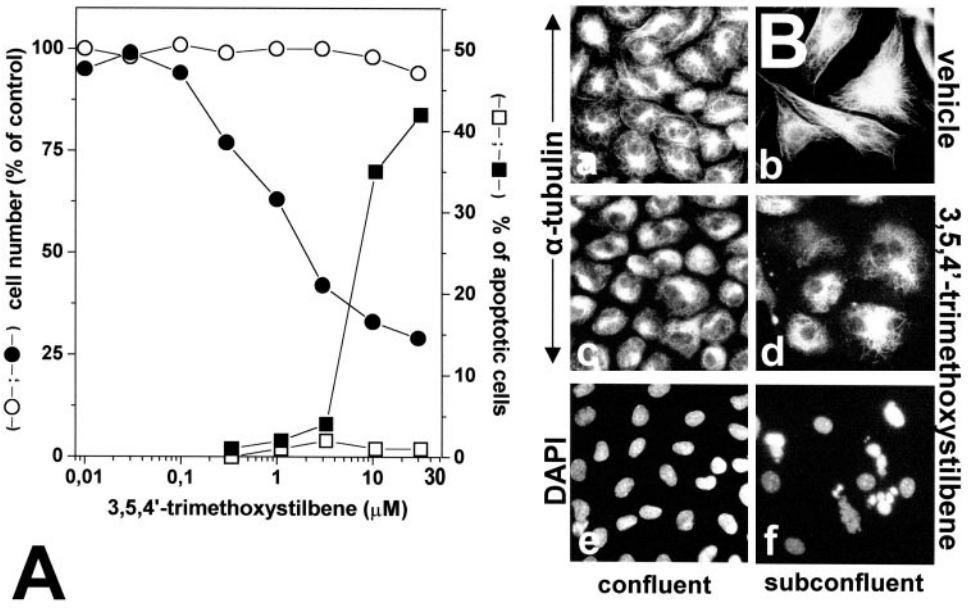

Fig. 6. Different response of confluent and subconfluent endothelial cells to 3,5,4′-trimethoxystilbene. A, BAE cells were seeded at 40,000/cm^2 and maintained for 4 days (○, □) or were seeded at 20,000/cm^2 and grown for 24 h (●, ■). Then, both confluent and subconfluent cell cultures were incubated with vehicle or 3,5,4′-trimethoxystilbene in fresh medium with 10% FCS (T_0). After 24 h, cells were stained with DAPI to calculate the percentage of apoptotic cells (□, ■) or were trypsinized and counted (○, ●). B, parallel cell cultures immunostained with anti-tubulin antibody (a–d) or stained with DAPI (e and f). In A, subconfluent vehicle-treated cell cultures underwent approximately 1.5 cell population doublings during the 24 h incubation (from 23,600 to 66,400/cm^2 at T_0 and 24 h, respectively), whereas confluent cell cultures were fully contact-inhibited (from 85,600 to 87,800/cm^2 at T_0 and 24 h, respectively). Quantification of apoptosis was performed on at least 200 cells per experimental point. Similar results were obtained in a second independent experiment.

one is based on the prevention of new blood vessel formation from existing vessels (antiangiogenic approach); the second one is based instead on the occlusion/destruction of the newly formed blood vessels (vascular-targeting approach) (Thorpe, 2004). Here, the synthetic *trans*-resveratrol derivative 3,5,4′-trimethoxystilbene has been identified as a microtubule-destabilizing agent endowed with both antiangiogenic and vascular-targeting activity.

Previous observations have shown that *trans*-resveratrol is endowed with angiostatic activity in vitro and in vivo by inhibiting FGF2 or VEGF-triggered neovascularization in the mouse cornea and angiogenesis in tumor models (Brakenhielm et al., 2001; Tseng et al., 2004). The molecular bases for its antiangiogenic activity are not fully elucidated. *trans*-Resveratrol exerts anti-inflammatory and antioxidant activities (Frémont, 2000). In addition, *trans*-resveratrol affects intracellular signaling in various cell types, including endothelium (Cao et al., 2002; Lin et al., 2003). Here, *trans*-resveratrol inhibits various cell proliferation-independent aspects of the angiogenesis process (i.e., sprout formation, collagen gel invasion, and morphogenesis). These effects are observed at high concentrations of the compound (ID_{50} = 30–100 μM), concentrations that are cytostatic but not cytotoxic for endothelial cells. Indeed, in keeping with previous observations (Stivala et al., 2001), *trans*-resveratrol treatment prolonged up to 48 h does not cause endothelial cell death and/or apoptosis, and the inhibitory effect is reversible (M. Belleri, unpublished observations). Thus, *trans*-resveratrol acts as an angiostatic molecule on multiple targets of the angiogenic process.

Methylation of the hydroxyl group in the 4′ position (3,5-dihydroxy-4′-methoxystilbene) or reduction of the double bond in the stilbenic skeleton (α,β-dihydro-3,4′,5-trihydroxystilbene) abolishes the antiangiogenic activity of these compounds. Methylation of hydroxyl groups in 3,5 positions (3,5-dimethoxy-4′-hydroxystilbene) results instead in a significant increase in the angiostatic activity of the compound. This was further increased in the presence of the 4′ methoxyl group (3,5,4′-trimethoxystilbene). Thus, subtle modifications in the chemical structure of *trans*-resveratrol deeply affect its antiangiogenic capacity. Both 3,5-dihydroxy-4′-methoxystilbene and α,β-dihydro-3,4′,5-trihydroxystilbene retain a significant, albeit reduced, antioxidant activity compared with *trans*-resveratrol, whereas 3,5,4′-trimethoxystilbene does

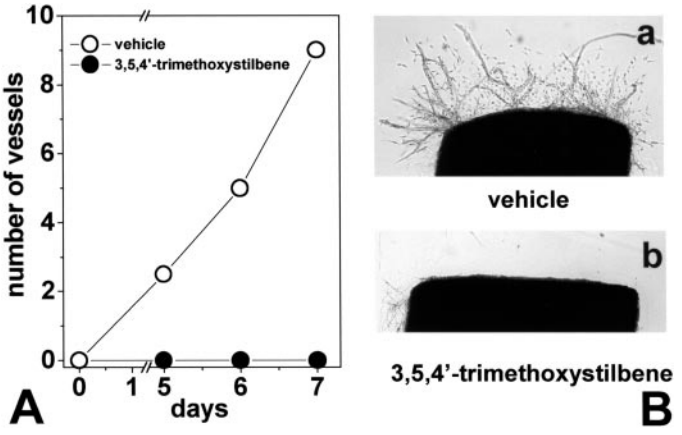

Fig. 7. Antiangiogenic activity of 3,5,4′-trimethoxystilbene in a rat aorta ring assay. A, rat aorta rings (n = 6) were embedded in fibrin gel in the presence of vehicle (○) or 1.0 μM 3,5,4′-trimethoxystilbene (●). Neovessel sprouts were counted during the next 7 days. Similar results were obtained in a second independent experiment. B, representative images of vehicle (a) and 3,5,4′-trimethoxystilbene (b)-treated aorta rings after 7 days of incubation. Original magnification, 40×.

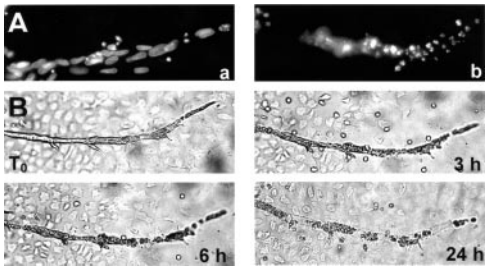

Fig. 8. Neovessel regression induced by 3,5,4′-trimethoxystilbene. A, after 7 days in culture, fibrin-embedded rat aorta rings were treated with vehicle (a) or 1.0 μM 3,5,4′-trimethoxystilbene (b) for 24 h. Then, neovessel sprouts were stained with DAPI. B, representative sequential images of a neovessel undergoing regression during 3,5,4′-trimethoxystilbene treatment. Original magnifications, 400× (A) and 100× (B).

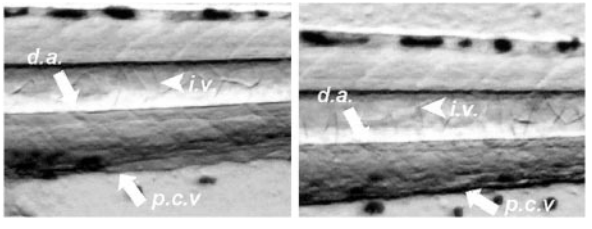

Fig. 9. 3,5,4′-Trimethoxystilbene causes blood flow stasis in zebrafish embryos (see Supplemental Video S1). Zebrafish embryos were exposed to 0.1 μM bibenzyl (left) or 0.1 μM 3,5,4′-trimethoxystilbene (right) at 72 hpf. After 30 min, embryos were anesthetized, and videos (10 frames per second) were acquired at 10× magnification under a stereomicroscope (lateral views, anterior is to the left). Stasis of blood flow in the dorsal aorta (d.a.), posterior cardinal vein (p.c.v.), and intersegmental vessels (i.v.) of the trunk occurs in 3,5,4′-trimethoxystilbene-treated embryos compared with bibenzyl-treated animals, as showed in the associated video, with no major alterations of tissue integrity.

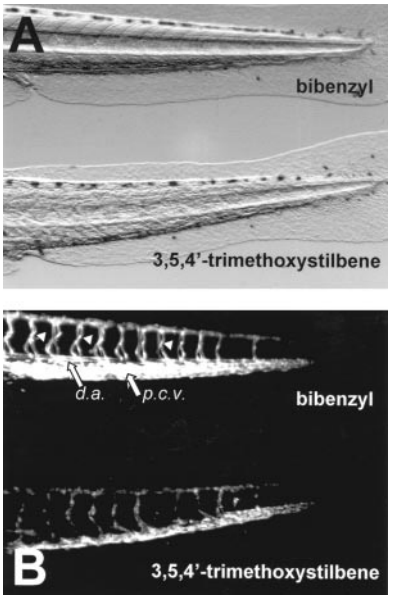

Fig. 10. 3,5,4′-Trimethoxystilbene causes blood vessel regression in zebrafish embryos. *VEGFR2:G-RCFP* transgenic zebrafish embryos were exposed to 0.1 μM bibenzyl or 0.1 μM 3,5,4′-trimethoxystilbene at 72 hpf (n = 10). At 6 h, embryos were anesthetized and photographed at 8× magnification. Phase contrast (A) and corresponding epifluorescence photographs (B) of two representative embryos (lateral views, anterior is to the left). Similar results were obtained in all the embryos. d.a., dorsal aorta; p.c.v., posterior cardinal vein; arrows, intersegmental vessels.

not exert any antioxidant effect (Stivala et al., 2001). These observations rule out the possibility of a direct relationship between the antiangiogenic and antioxidant activities of these polyphenols.

It is interesting that the three hydroxyl substitutions conferred to 3,5,4′-trimethoxystilbene a potent antitubulin vascular-targeting activity absent in *trans*-resveratrol. Various antitubulin agents are endowed with a vascular-targeting activity including, among others, combretastatin A-4 and the antitumor drug vinblastine (Vacca et al., 1999; Thorpe, 2004). The chemical structure of 3,5,4′-trimethoxystilbene resembles that of combretastatin A-4 in the *trans* configuration. However, the two compounds differ for the number of methoxyl groups in the stilbene ring and for the presence of a 3′ hydroxyl substitution in combretastatin A-4. Even though *cis*-isomers of combretastatin derivatives are usually more potent antitubulin agents than their *trans* counterparts, some exceptions exist (Cushman et al., 1991). In addition, structure-activity studies had shown that some *trans*-resveratrol derivatives exert a potent inhibitory effect on the proliferation of different tumor cell lines (Pettit et al., 2002). The comparison of the vascular-targeting activity of the two 3,5,4′-trimethoxystilbene isomers deserves further investigation.

Here, 3,5,4′-trimethoxystilbene causes a rapid microtubule depolymerization in endothelial cells. This resulted in the incapacity of endothelial cells at the edge of a wounded monolayer to orient their MTOC at the leading front and to form cell-membrane ruffles. Centrosome repositioning stabilizes the direction of migration (Ueda et al., 1997). Accordingly, the incapacity of 3,5,4′-trimethoxystilbene–treated cells to reorient their MTOC and to stabilize pseudopod extensions results in impaired cell migration and incapacity to heal the wounded monolayer. In addition, 1 to 3 μM 3,5,4′-trimethoxystilbene abolished the chemotactic response elicited in MAE cells by tumor cell-conditioned media (M. Belleri, unpublished observations). It must be pointed out that the early effects exerted by 3,5,4′-trimethoxystilbene on endothelial cell motility are not the direct consequence of its cytotoxic activity. Indeed, no inhibitory effect on cell proliferation was observed when endothelial cells were transiently exposed to the compound. At variance, a potent inhibition of cell proliferation was observed when cells were exposed for longer periods, leading to endothelial cell apoptosis. Accordingly, when added to rat aorta rings that had been maintained in a three-dimensional fibrin gel to allow the sprouting of newly formed vessels, 3,5,4′-trimethoxystilbene caused the rapid disruption of the endothelial sprouts that underwent apoptosis at later time points. In this model, 3,5,4′-trimethoxystilbene was also able to prevent vessel sprouting when aorta rings were exposed to the compound immediately after embedding in the fibrin gel, thus confirming its angiostatic capacity.

In keeping with the ability of microtubule-destabilizing agents to affect proliferating immature endothelial cells but not the quiescent mature endothelium (Dark et al., 1997; Iyer et al., 1998), 3,5,4′-trimethoxystilbene caused microtubule depolymerization but did not exert a significant proapoptotic effect on contact-inhibited confluent endothelial cells. The molecular basis for the different response of proliferating versus quiescent endothelial cells to antitubulin drugs remains to be fully elucidated, even though recent observations indicate that induction of apoptosis in endothelial cells by combretastatin A-4 is associated with the induction of a prolonged mitotic arrest (Kanthou et al., 2004).

It is interesting that 3,5,4′-trimethoxystilbene caused the contraction of confluent endothelial cells, as observed for combretastatin A-4–treated endothelial cells (Kanthou and Tozer, 2002). Changes in endothelial cell morphology are believed to be responsible, at least in part, for the collapse of the vascular tree that occurs rapidly after drug exposure in vivo (Galbraith et al., 2001; Tozer et al., 2001). Accordingly, 3,5,4′-trimethoxystilbene caused the rapid stasis of blood flow in the vessels of the trunk of zebrafish embryos and in the area vasculosa of the chick embryo. This was followed by the collapse of the vasculature, leading to blood vessel regression in both animal models.

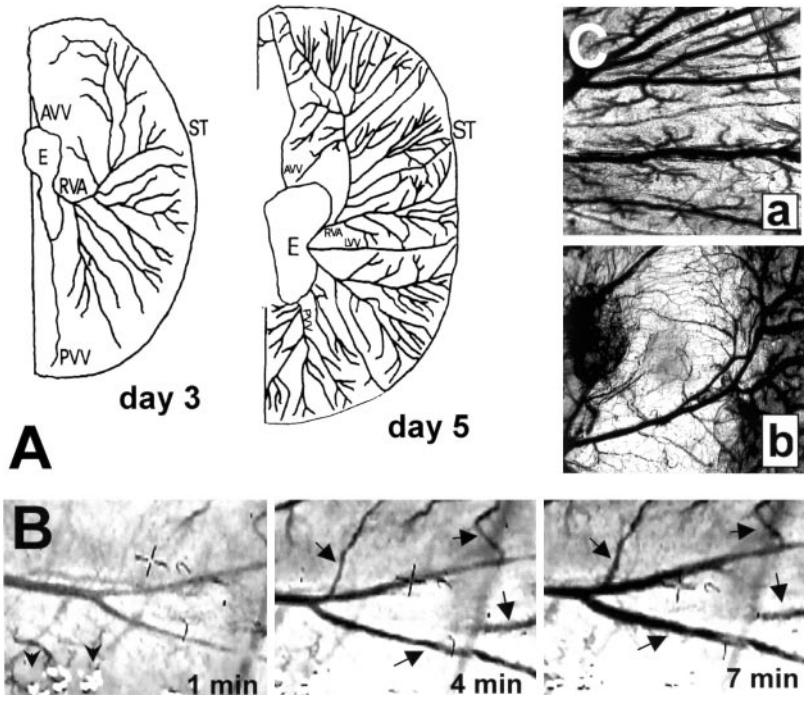

Fig. 11. Effect of 3,5,4′-trimethoxystilbene on chick embryo area vasculosa. A, computer printouts of the vascular tree at the right side of a control chick embryo (E) area vasculosa at days 3 and 5 of incubation. RVA, right vitelline artery; AVV, anterior vitelline vein; LVV, lateral vitelline vein; PVV, posterior vitelline vein; ST, sinus terminalis. B, sequential images (original magnification, 20×) of a day-3 area vasculosa adjacent to a gelatin sponge implant (arrowheads) containing 300 pmol/embryo of 3,5,4′-trimethoxystilbene. Images were taken at the indicated time points after implantation. Note the rapid erythrocyte stagnation and vessel dilation (arrows). Similar results were obtained in eight independent observations. No modifications were observed in vehicle or bibenzyl-treated embryos (data not shown). C, area vasculosa membranes (n = 10) were implanted at day 3 with gelatin sponges and photographed at day 5 after removal of the implant. Note the lack of blood vessel growth and the disappearance of the majority of pre-existing blood vessels after 3,5,4′-trimethoxystilbene treatment (b) compared with bibenzyl treatment (a) (original magnification in a and b, 8×).

Zebrafish embryos have been proposed for the screening of antiangiogenic drugs (Serbedzija et al., 2000). Accordingly, *VEGFR2:G-RCFP* zebrafish embryos have been used to study the antiangiogenic activity of VEGF receptor tyrosine kinase inhibitors at early stages of development, when intersegmental vessel angiogenesis occurs (Cross et al., 2003). Our results indicate that zebrafish may represent a model suitable for also studying the activity of vascular-targeting compounds. A limitation of the model is represented by the high susceptibility of zebrafish embryos to the cytotoxic effect of antitubulin agents (Moon et al., 2002) when exposed to the drug at early stages of development (19 hpf or earlier), hampering the possibility to assess the impact of these compounds on intersegmental angiogenesis. Nevertheless, exposure of the embryos at 72 hpf allowed us to evaluate the effect of 3,5,4′-trimethoxystilbene on the newly formed vasculature.

Vascular-targeting, antitubulin agents produce a widespread central necrosis in experimental tumors, caused by the destruction of the tumor vasculature and consequent tumor cell death (Dark et al., 1997; Tozer et al., 2002; Thorpe, 2004). However, a rim of viable parenchyma survives at the periphery of the tumor, where angiogenesis is more robust. Combining vascular-targeting agents with antiproliferative antitumor and/or antiangiogenic therapies may result in an increased efficacy with additive or synergistic effects (Siemann et al., 2002; Siim et al., 2003; Wildiers et al., 2004). Here, we have shown that 3,5,4′-trimethoxystilbene is endowed with both antiangiogenic and vascular-targeting activity. Moreover, 3,5,4′-trimethoxystilbene inhibits tumor-cell proliferation in vitro (Schneider et al., 2003). Other microtubule-destabilizing agents, like combretastatin A-4 and vinblastine, may show distinct antitumor, vascular-targeting, and antiangiogenic activities (Vacca et al., 1999; Ahmed et al., 2003). Compounds that incorporate cytostatic, antiangiogenic, and vascular-targeting features may represent a potent tool for the design of more efficacious therapeutic approaches and regimen schedules for angiogenesis-dependent diseases, including cancer.

References

Ahmed B, Van Eijk LI, Bouma-Ter Steege JC, Van Der Schaft DW, Van Esch AM, Joosten-Achjanie SR, Lambin P, Landuyt W, and Griffioen AW (2003) Vascular targeting effect of combretastatin A-4 phosphate dominates the inherent angiogenesis inhibitory activity. *Int J Cancer* **105:**20–25.

Bastaki M, Nelli EE, Dell'Era P, Rusnati M, Molinari-Tosatti MP, Parolini S, Auerbach R, Ruco LP, Possati L, and Presta M (1997) Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. *Arterioscler Thromb Vasc Biol* **17:**454–464.

Brakenhielm E, Cao R, and Cao Y (2001) Suppression of angiogenesis, tumor growth and wound healing by resveratrol, a natural compound in red wine and grapes. *FASEB J* **15:**1798–1800.

Cao Y, Cao R, and Brakenhielm E (2002) Antiangiogenic mechanisms of diet-derived polyphenols. *J Nutr Biochem* **13:**380–390.

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. *Nat Med* **6:**389–395.

Carmeliet P (2003) Angiogenesis in health and disease. *Nat Med* **9:**653–660.

Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, et al. (2003) Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA* **100:**10623–10628.

Cross LM, Cook MA, Lin S, Chen JN, and Rubinstein AL (2003) Rapid analysis of angiogenesis drugs in a live fluorescent zebrafish assay. *Arterioscler Thromb Vasc Biol* **23:**911–912.

Cushman M, Nagarathnam D, Gopal D, Chakraborti AK, Lin CM, and Hamel E (1991) Synthesis and evaluation of stilbene and dihydrostilbene derivatives as potential anticancer agents that inhibit tubulin polymerization. *J Med Chem* **34:**2579–2588.

Dark GG, Hill SA, Prise VE, Tozer GM, Pettit GR, and Chaplin DJ (1997) Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. *Cancer Res* **57:**1829–1834.

Fernando NH and Hurwitz HI (2003) Inhibition of vascular endothelial growth factor in the treatment of colorectal cancer. *Semin Oncol* **30:**39–50.

Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1:**27–31.

Frémont L (2000) Minireview: biological effects of resveratrol. *Life Sci* **66:**663–673.

Galbraith SM, Chaplin DJ, Lee F, Stratford MR, Locke RJ, Vojnovic B, and Tozer GM (2001) Effects of combretastatin A4 phosphate on endothelial cell morphology in vitro and relationship to tumour vascular targeting activity in vivo. *Anticancer Res* **21:**93–102.

Gualandris A, Rusnati M, Belleri M, Nelli EE, Bastaki M, Molinari-Tosatti MP, Bonardi F, Parolini S, Albini A, Morbidelli L, et al. (1996) Basic fibroblast growth factor overexpression in endothelial cells: an autocrine mechanism for angiogenesis and angioproliferative diseases. *Cell Growth Differ* **7:**147–160.

Iyer S, Chaplin DJ, Rosenthal DS, Boulares AH, Li LY, and Smulson ME (1998) Induction of apoptosis in proliferating human endothelial cells by the tumor-specific antiangiogenesis agent combretastatin A-4. *Cancer Res* **58:**4510–4514.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, et al. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science (Wash DC)* **275:**218–220.

Kanthou C, Greco O, Stratford A, Cook I, Knight R, Benzakour O, and Tozer G (2004) The tubulin-binding agent combretastatin A-4-phosphate arrests endothelial cells in mitosis and induces mitotic cell death. *Am J Pathol* **165:**1401–1411.

Kanthou C and Tozer GM (2002) The tumor vascular targeting agent combretastatin A-4-phosphate induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells. *Blood* **99:**2060–2069.

Kerbel R and Folkman J (2002) Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* **2:**727–739.

Keshet E and Ben-Sasson SA (1999) Anticancer drug targets: approaching angiogenesis. *J Clin Investig* **104:**1497–1501.

Lin MT, Yen ML, Lin CY, and Kuo ML (2003) Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. *Mol Pharmacol* **64:**1029–1036.

Moon HS, Jacobson EM, Khersonsky SM, Luzung MR, Walsh DP, Xiong W, Lee JW, Parikh PB, Lam JC, Kang TW, et al. (2002) A novel microtubule destabilizing entity from orthogonal synthesis of triazine library and zebrafish embryo screening. *J Am Chem Soc* **124:**11608–11609.

Nicosia RF and Ottinetti A (1990) Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis in vitro. *Lab Investig* **63:**115–122.

Pettit GR, Grealish MP, Jung MK, Hamel E, Pettit RK, Chapuis JC, and Schmidt JM (2002) Antineoplastic agents. 465. Structural modification of resveratrol: sodium resverastatin phosphate. *J Med Chem* **45:**2534–2542.

Ribatti D (1995) A morphometric study of the expansion of the chick area vasculosa in shell-less culture. *J Anat* **186:**639–644.

Schneider Y, Chabert P, Stutzmann J, Coelho D, Fougerousse A, Gosse F, Launay JF, Brouillard R, and Raul F (2003) Resveratrol analog (*Z*)-3,5,4′-trimethoxystilbene is a potent anti-mitotic drug inhibiting tubulin polymerization. *Int J Cancer* **107:**189–196.

Serbedzija GN, Flynn E, and Willett CE (2000) Zebrafish angiogenesis: A new model for drug screening. *Angiogenesis* **3:**353–359.

Siemann DW, Mercer E, Lepler S, and Rojiani AM (2002) Vascular targeting agents enhance chemotherapeutic agent activities in solid tumor therapy. *Int J Cancer* **99:**1–6.

Siim BG, Lee AE, Shalal-Zwain S, Pruijn FB, McKeage MJ, and Wilson WR (2003) Marked potentiation of the antitumour activity of chemotherapeutic drugs by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA). *Cancer Chemother Pharmacol* **51:**43–52.

Stivala LA, Savio M, Carafoli F, Perucca P, Bianchi L, Maga G, Forti L, Pagnoni UM, Albini A, Prosperi E, et al. (2001) Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. *J Biol Chem* **276:**22586–22594.

Thorpe PE (2004) Vascular targeting agents as cancer therapeutics. *Clin Cancer Res* **10:**415–427.

Tozer GM, Kanthou C, Parkins CS, and Hill SA (2002) The biology of the combretastatins as tumour vascular targeting agents. *Int J Exp Pathol* **83:**21–38.

Tozer GM, Prise VE, Wilson J, Cemazar M, Shan S, Dewhirst MW, Barber PR, Vojnovic B, and Chaplin DJ (2001) Mechanisms associated with tumor vascular shut-down induced by combretastatin A-4 phosphate: intravital microscopy and measurement of vascular permeability. *Cancer Res* **61:**6413–6422.

Tozer GM, Prise VE, Wilson J, Locke RJ, Vojnovic B, Stratford MR, Dennis MF, and Chaplin DJ (1999) Combretastatin A-4 phosphate as a tumor vascular-targeting agent: early effects in tumors and normal tissues. *Cancer Res* **59:**1626–1634.

Tseng SH, Lin SM, Chen JC, Su YH, Huang HY, Chen CK, Lin PY, and Chen Y (2004) Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. *Clin Cancer Res* **10:**2190–2202.

Ueda M, Graf R, MacWilliams HK, Schliwa M, and Euteneuer U (1997) Centrosome positioning and directionality of cell movements. *Proc Natl Acad Sci USA* **94:**9674–9678.

Vacca A, Iurlaro M, Ribatti D, Minischetti M, Nico B, Ria R, Pellegrino A, and Dammacco F (1999) Antiangiogenesis is produced by nontoxic doses of vinblastine. *Blood* **94:**4143–4155.

Westerfield M (2000) *The Zebrafish Book.* University of Oregon Press, Eugene, OR.

Wildiers H, Ahmed B, Guetens G, De Boeck G, de Bruijn EA, Landuyt W, and van Oosterom AT (2004) Combretastatin A-4 phosphate enhances CPT-11 activity independently of the administration sequence. *Eur J Cancer* **40:**284–290.

Address correspondence to: Dr. Marco Presta, General Pathology, Dept. Biomedical Sciences and Biotechnology, Viale Europa 11 25123 Brescia, Italy. E-mail: presta@med.unibs.it