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# $\alpha_v\beta_3$ Integrin-dependent antiangiogenic activity of resveratrol stereoisomers

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## Abstract

**Angiogenesis is target for antineoplastic and chemopreventive therapies. The natural phytoalexin resveratrol is found in grapes and red wine as *cis* and *trans* stereoisomers. *trans*-Resveratrol shows antiangiogenic activity, but its mechanism of action is not fully elucidated. Recently, *trans*-resveratrol has been shown to interact with the $\beta_3$ integrin subunit, raising the possibility that inhibition of endothelial $\alpha_v\beta_3$ integrin function may concur to its angiosuppressive activity. To get novel insights about the antiangiogenic activity of resveratrol, we compared *cis*- and *trans*-resveratrol stereoisomers for their effect on the angiogenesis process and endothelial $\alpha_v\beta_3$ integrin function. *trans*-Resveratrol inhibits endothelial cell proliferation and the repair of mechanically wounded endothelial cell monolayers. Also, it prevents endothelial cell sprouting in fibrin gel, collagen gel invasion, and morphogenesis on Matrigel. *In vivo, trans*-resveratrol inhibits vascularization of the chick embryo area vasculosa and murine melanoma B16 tumor growth and neovascularization. In all the assays, *cis*-resveratrol exerts a limited, if any, effect. In keeping with these observations, *trans*-resveratrol, but not *cis*-resveratrol, inhibits $\alpha_v\beta_3$ integrin-dependent endothelial cell adhesion and the recruitment of enhanced green fluorescent protein-tagged $\beta_3$ integrin in focal adhesion contacts. In conclusion, stereoisomery affects the antiangiogenic activity of resveratrol, the *trans* isomer being significantly more potent than the *cis* isoform. The different antiangiogenic potential of resveratrol stereoisomers is related, at least in part, to their different capacity to affect $\alpha_v\beta_3$ integrin function. This may have profound implications for the design of synthetic antiangiogenic/angiopreventive phytoalexin derivatives. [Mol Cancer Ther 2008;7(12):3761–70]**

## Introduction

Angiogenesis 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 interendothelial tight junctions and deposition of new basement membrane (1). Uncontrolled neovascularization is observed in tumors, angioproliferative diseases, and angiogenesis-dependent diseases (2, 3). Thus, angiosuppressive molecules able to affect different steps of the angiogenesis process may have broad applicability for the therapy of a wide spectrum of diseases, including cancer (4). Also, antiangiogenic compounds may act as cancer chemopreventive drugs, the so-called ''angiopreventive'' agents (5, 6). To this respect, dietary phytochemicals derived from edible plants may represent a promising class of compounds (7).

The polyphenolic phytoalexin *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), abundant in grapevines and red wine, exists as *cis* and *trans* stereoisomers with *trans* to *cis* isomerization facilitated by UV exposure (8). *trans*-Resveratrol has received wide attention because of its possible role in the prevention of human pathologic processes, including cardiovascular diseases and cancer (9–11). Much less is known about the biological activity of *cis*-resveratrol that appears to exert antioxidant (12) and anti-inflammatory (13) activities similar to those exerted by the *trans* form.

Relevant to its putative antineoplastic and chemopreventive activity (5, 7), *trans*-resveratrol inhibits fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor-triggered neovascularization and tumor angiogenesis (14, 15). Although the molecular bases for its antiangiogenic activity are not fully elucidated (16), recent observations have shown that *trans*-resveratrol binds purified $\beta_3$ integrin chain *in vitro* (17). Given the role of endothelial $\alpha_v\beta_3$ integrin receptors in angiogenesis (18)

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and the antiangiogenic potential of $\alpha_v\beta_3$ integrin antagonists (19–22), this observation raises the hypothesis that inhibition of $\alpha_v\beta_3$ integrin function may concur to the angiosuppressive activity of resveratrol.

On this basis, to get novel insights about the mechanisms responsible for the antiangiogenic activity of resveratrol, we have compared *cis*- and *trans*-resveratrol stereoisomers for their capacity to affect angiogenesis and endothelial $\alpha_v\beta_3$ integrin activity. The results show that *trans*-resveratrol is significantly more potent than the *cis* isomer in inhibiting different steps of the angiogenesis process *in vitro* and tumor growth and neovascularization *in vivo*. This is due, at least in part, to the previously unrecognized capacity of the two resveratrol stereoisomers to differently affect $\alpha_v\beta_3$ integrin function.

## Materials and Methods

### Chemicals

*trans*-Resveratrol and bibenzyl were from Sigma. *cis*-Resveratrol was synthesized as described (12). All the compounds were 95% pure or better. For *in vitro* and chick embryo studies, stock solutions of these compounds (whose chemical structure is shown in Fig. 1A) were prepared in DMSO and diluted directly in cell culture medium. Final concentration of DMSO (vehicle) was ≤0.5% (v/v) and control cells were treated with the same concentrations of vehicle that did not exert any affect in all the assays. Vascular endothelial growth factor was from Calbiochem. FGF2 was expressed and purified from *Escherichia coli* cell extract as described (23).

### Cell Cultures and Enhanced Green Fluorescent Protein-Tagged $\beta_3$ Integrin Chain Transfectants

Immortalized BALB/c murine aortic endothelial cells (MAEC) were obtained from R. Auerbach (University of Wisconsin) and grown in DMEM added with 10% FCS (Life Technologies, Invitrogen). FGF2-T-MAECs, a highly tumorigenic subclone of FGF2-transfected MAECs (24), were grown in DMEM supplemented with 4.0 mmol/L glutamine (Life Technologies) and 10% FCS. Bovine aortic endothelial cells (BAEC; provided by A. Vecchi, Istituto Clinico Humanitas) were cultured in DMEM supplemented with 10% heat-inactivated donor calf serum. Cultures were used between the 6th and the 10th cell passages. Human umbilical vein endothelial cells (HUVEC) were from Clonetics (Biowhittaker) and cultured in growth factor-enriched EGM-2 medium (Clonetics). Transformed fetal GM7373 BAEC cells (25) were transfected with the

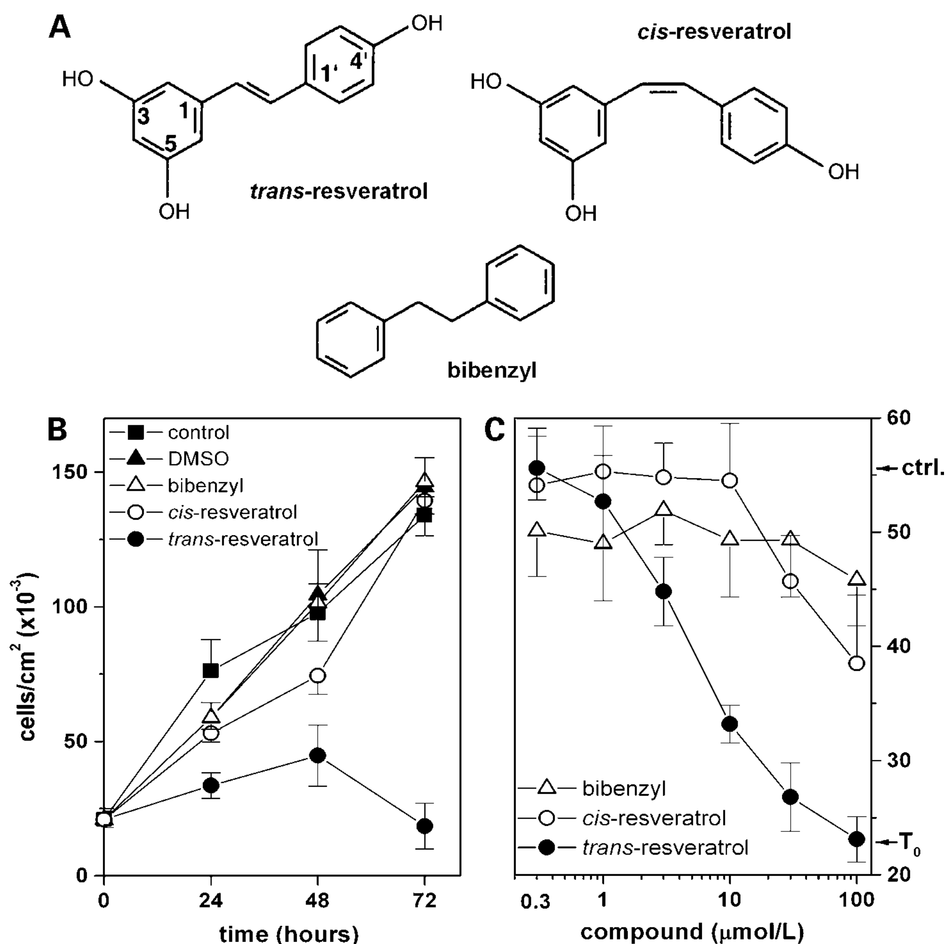


**Figure 1.** Effect of resveratrol stereoisomers on endothelial cell proliferation. **A,** chemical structure of resveratrol stereoisomers and control bibenzyl. **B,** endothelial GM7373 cells were seeded at 20,000 cells/cm$^2$. After overnight incubation, cells were incubated in fresh medium in absence (■) or presence of 30 μmol/L *trans*-resveratrol (●), *cis*-resveratrol (○), bibenzyl (△), or the corresponding amount of vehicle (▲). At different time points, cells were trypsinized and counted in a Burker chamber. **C,** GM7373 cells seeded at 20,000 cells/cm$^2$ ($T_0$) were left untreated (*ctrl.*) or treated with increasing concentrations of *trans*-resveratrol (●), *cis*-resveratrol (○), or bibenzyl (△) and counted 24 h thereafter. Mean ± SD of three determinations. All the experiments were repeated three times with similar results.

pcDNA3/enhanced green fluorescent protein (EGFP) vector harboring the full-length mouse $\beta_3$ integrin cDNA (kindly provided by B. Wehrle-Haller, Centre Medical Universitaire; ref. 26). After G418 selection, cell clones ($\beta_3$-EGFP-GM7373 cells) were screened for stable expression and correct subcellular localization of the $\beta_3$-EGFP fusion protein by Western blotting and fluorescence microscopy of cells adherent to different substrata (see below). GM7373 cells transfected with EGFP devoid of the $\beta_3$ integrin chain moiety (EGFP-GM7373 cells) were used as controls.

#### Cell Proliferation Assay

GM7373 cells were seeded at 20,000 per cm$^2$. After overnight incubation, cells were incubated in fresh medium in the presence of the compound under test. At the indicated time points, cells were trypsinized and counted in a Burker chamber. MAECs, FGF2-T-MAECs, BAECs, and HUVECs were seeded at 25,000, 75,000, 13,000, and 6,250 cells/cm$^2$, respectively, treated as above, and counted after 48 h.

#### Wounding of Endothelial Cell Monolayers

Confluent GM7373 and MAEC monolayers were wounded with a 1.0-mm-wide rubber policeman. After washing, monolayers were incubated in fresh medium added with 10% FCS and the compound under test (23). After 16 h, wounds were photographed at ×40 magnification using an inverted phase-contrast photomicroscope and endothelial cells invading the wound were quantified by computerized analysis of the digitalized images (27).

#### Three-Dimensional Gel Invasion Assays

Fibrin-embedded FGF2-T-MAEC aggregates were prepared as described (24). Then, culture medium containing 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 48 h, photographed at ×40 magnification, and quantified by computerized analysis of the digitalized images (23). BAECs were seeded on the top of rat tail tendon type I collagen (100,000 cells per well; ref. 28) and allowed to reach confluence. Cell cultures were then treated with growing medium containing recombinant FGF2 plus vascular endothelial growth factor (both at 30 ng/mL) in the absence or presence of the compound under test. After 24 h, cells were photographed at ×100 magnification using an inverted phase-contrast photomicroscope, 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 (23). HUVECs were seeded onto Matrigel-coated dishes (Becton Dickinson) at 40,000 cells/cm$^2$ in the absence or presence of the compound under test. Cells were photographed after 16 h at ×100 magnification using an inverted phase-contrast photomicroscope and 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.

#### Cell Adhesion Assay

The assay was done as described (25). Briefly, parental and transfected GM7373 cells (50,000 cells in 200 μL culture medium containing 1% FCS) were allowed to adhere at 37°C for 3 h to wells coated with 20 μg/mL of different substrata in the absence or presence of the compound under test. Then, adherent cells were fixed and stained with methylene blue/Azur II (1:1, v/v). Plates were read with a microplate reader at 595 nm. Focal adhesion contacts were visualized in $\beta_3$-EGFP-GM7373 cells seeded in medium containing 10% FCS in the absence or presence of resveratrol stereoisomers on coverslips coated with different substrata. After 3 h of incubation, adherent cells were fixed in 3% paraformaldehyde/2% sucrose in PBS, permeabilized with 0.5% Triton X-100, and analyzed for $\beta_3$-EGFP localization and paxillin immunostaining by fluorescence microscopy.

#### Chick Embryo 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) were cut to a size of 1 mm$^3$, adsorbed with the compound under test (3 μL/implant of a 10 or 100 μmol/L solution, corresponding to 30 and 300 pmol/embryo, respectively), and placed on top of the area vasculosa. After 48 h, membranes were fixed *in ovo* for 2 h in Bouin's fluid, removed from the eggs, placed on a light box, and photographed under a stereomicroscope at ×8 magnification. Blood vessel area surrounding the gelatin implant was quantified *in ovo* by an image analyzer system connected to the microscope (23).

#### Tumor Growth Assay

Animal experiments were approved by the Animal Ethical Committee of the School of Medicine of the University of Brescia and conducted in conformity with the Italian and European guidelines for the protection of animals used for scientific experiments. Seven-week-old female C57BL/6N mice (Charles River Laboratories) were given *trans*- or *cis*-resveratrol in the drinking water (30 μg/mL in 0.15% ethanol) with a change every day throughout the whole experimental period starting 4 days before tumor cell transplantation. Control animals received the same concentration of ethanol. Murine B16 melanoma cells ($1.5 \times 10^5$ cells suspended in 100 μL PBS) were injected s.c. in both flanks of the animals. The growth of the tumors was monitored every other day by measuring tumor diameters (mm) in three dimensions with a metric caliper and tumor volume (mm$^3$) was calculated according to the formula: $a \times b \times c \times 0.5$. Both stereoisomers had no effect on body weight of tumor-bearing mice when compared with controls.

At sacrifice, frozen tumor sections were immunostained with the rat monoclonal anti-mouse CD31 antibody MEC 13.3 (provided by A. Vecchi, Istituto Clinico Humanitas) for the evaluation of microvessel density (29). For each tumor section, the number of CD31$^+$ vessels was counted in the five most vascularized areas at ×200 magnification.

Also, five tumors for each experimental group were snap-frozen in liquid nitrogen for the evaluation of steady-state *CD31* mRNA levels by two-step quantitative real-time PCR analysis. Briefly, cDNA was generated from 2.0 μg total RNA using the MMLV-RT kit (Invitrogen). Real-time PCR was carried out on a iCycler Real-time PCR Detection System (Bio-Rad) using 25 μL reactions containing iQ SYBR Green Supermix, 150 to 300 nmol/L forward and reverse primers, and 5 μL cDNA diluted template. The PCR cycling profile was as follows: 3 min at 95°C and 40 cycles for 15 s at 95°C, 60°C for 1 min. After PCR amplification, melting curve analysis was done for each reaction. Each PCR was done in triplicate on one plate and fluorescence data were recorded using iCycler software (Bio-Rad). Relative expression ratios were calculated by use of Pfaffl equation and Relative Expression Software Tool.[5] The *CD31* mRNA expression levels were normalized to the levels of murine *β-actin* gene. The primers were *murine CD31* (5′-CGGTTATGATGATGTTTCTGGA-3′ and 5′-AAGGGAGGACACTTCCACTTCT-3′) and *murine β-actin* (5′-CGTAAAGACCTCTATGCCAACA-3′ and 5′-CCACCGATCCACACAGAGTA-3′).

### Statistics

The significance of differences between means was assessed by Student's *t* test.

## Results

### *In vitro* Effects of Resveratrol Stereoisomers on Different Steps of the Angiogenesis Process

Because of endothelial cell heterogeneity (30, 31), the antiangiogenic potential of *trans*- and *cis*-resveratrol was assessed using endothelial cells of bovine, murine, and human origin. Bibenzyl, a resveratrol analogue here used as negative control (23), did not exert any effect in all the assays.

As shown in Fig. 1B, *trans*-resveratrol is significantly more potent than the *cis* isoform in inhibiting the proliferation of bovine endothelial GM7373 cells. Accordingly, dose-response experiments showed that *trans*- and *cis*-resveratrol inhibit the proliferation of GM7373 after 24 h of treatment with an $ID_{50}$ equal to 3.0 and 100 μmol/L, respectively (Fig. 1C). Similar results were obtained in MAECs and BAECs stimulated by serum, in FGF2-T-MAECs stimulated by endogenous overexpressed FGF2 (24), and in HUVECs stimulated by angiogenic growth factor-enriched medium (data not shown). The inhibitory effect exerted by the two stereoisomers was reversible as shown by the ability of both GM7373 cells and FGF2-T-MAECs to recover their proliferative capacity after 24 h of incubation with the compound followed by an extensive washing and incubation with fresh medium (data not shown). Also, in keeping with previous observations on different cell types (12), *trans*-resveratrol caused the reversible arrest of FGF2-T-MAECs in the S phase of the cell cycle (data not shown). Thus, *trans*-resveratrol is more potent than the *cis* isoform in exerting a cytostatic effect on endothelial cells of different origin activated by either exogenous or endogenous stimuli.

The capacity of resveratrol isomers to affect different steps of the angiogenesis process was investigated further in various *in vitro* assays. *trans*-Resveratrol significantly inhibits the ability of GM7373 cells (Fig. 2A) and MAECs (data not shown) to repair a wounded monolayer, a phenomenon that depends on the migratory and proliferative activity of the cells at the edge of the wound (32). Also, as shown in Fig. 2B, addition of *trans*-resveratrol to the cell culture medium prevents the capacity of endogenously activated FGF2-T-MAECs to invade a fibrin gel and to form radially growing sprouts (24). Similarly, *trans*-resveratrol inhibits the capacity of BAECs to invade a three-dimensional type I collagen gel and to organize capillary-like structures when stimulated by exogenously added FGF2 plus vascular endothelial growth factor (Fig. 2C). Finally, *trans*-resveratrol prevents vascular tube formation by HUVECs seeded on Matrigel (Fig. 2D), a morphogenic phenomenon known as ''spontaneous angiogenesis'' (33). No activity was instead exerted by *cis*-resveratrol in all these assays.

### Effect of Resveratrol Stereoisomers on Endothelial $\alpha_v\beta_3$ Integrin Function

Endothelial GM7373 cells express $\alpha_v\beta_3$ integrin that specifically mediates their adhesion to the prototypic $\alpha_v\beta_3$ integrin-binding protein vitronectin (25). Recent observations have shown the capacity of *trans*-resveratrol to bind the $\beta_3$ integrin chain *in vitro* (17), raising the possibility that inhibition of $\beta_3$ integrin function may be responsible, at least in part, for the antiangiogenic potential of resveratrol. To assess this hypothesis, resveratrol stereoisomers were evaluated for their capacity to affect GM7373 cell adhesion to the $\alpha_v\beta_3$ integrin substratum vitronectin as well as to the $\beta_1$ integrin substrata laminin and fibronectin (8). As shown in Fig. 3, *trans*-resveratrol inhibits the adhesion of GM7373 cells to immobilized vitronectin, without affecting their adhesion to fibronectin or laminin. No effect was instead exerted by *cis*-resveratrol or bibenzyl on any substratum.

Next, to follow the fate of $\beta_3$ integrin in resveratrol-treated cells, we generated stable GM7373 cell transfectants ($\beta_3$-EGFP-GM7373 cells) coexpressing fluorescent EGFP tagged to the COOH terminus of the cytoplasmic domain of the $\beta_3$ integrin subunit (26). Cells transfected with EGFP devoid of the $\beta_3$ chain moiety were used as controls (EGFP-GM7373 cells). As shown in Fig. 4A, $\beta_3$-EGFP-GM7373 cells express significant levels of the $\beta_3$-EGFP protein chimera and levels of the endogenous $\beta_3$ chain were similar to those expressed by parental and EGFP-transfected cells. Also, $\beta_3$-EGFP-GM7373 cells adhere to vitronectin, laminin, and fibronectin in a manner undistinguishable from parental cells (data not shown). Moreover, $\beta_3$-EGFP chimera properly localizes in focal adhesion contacts when $\beta_3$-EGFP-GM7373 cells are seeded on vitronectin but not when seeded on laminin (Fig. 4B). Finally, as observed for parental GM7373 cells, *trans*-resveratrol significantly

[5] http://www.gene-quantification.info

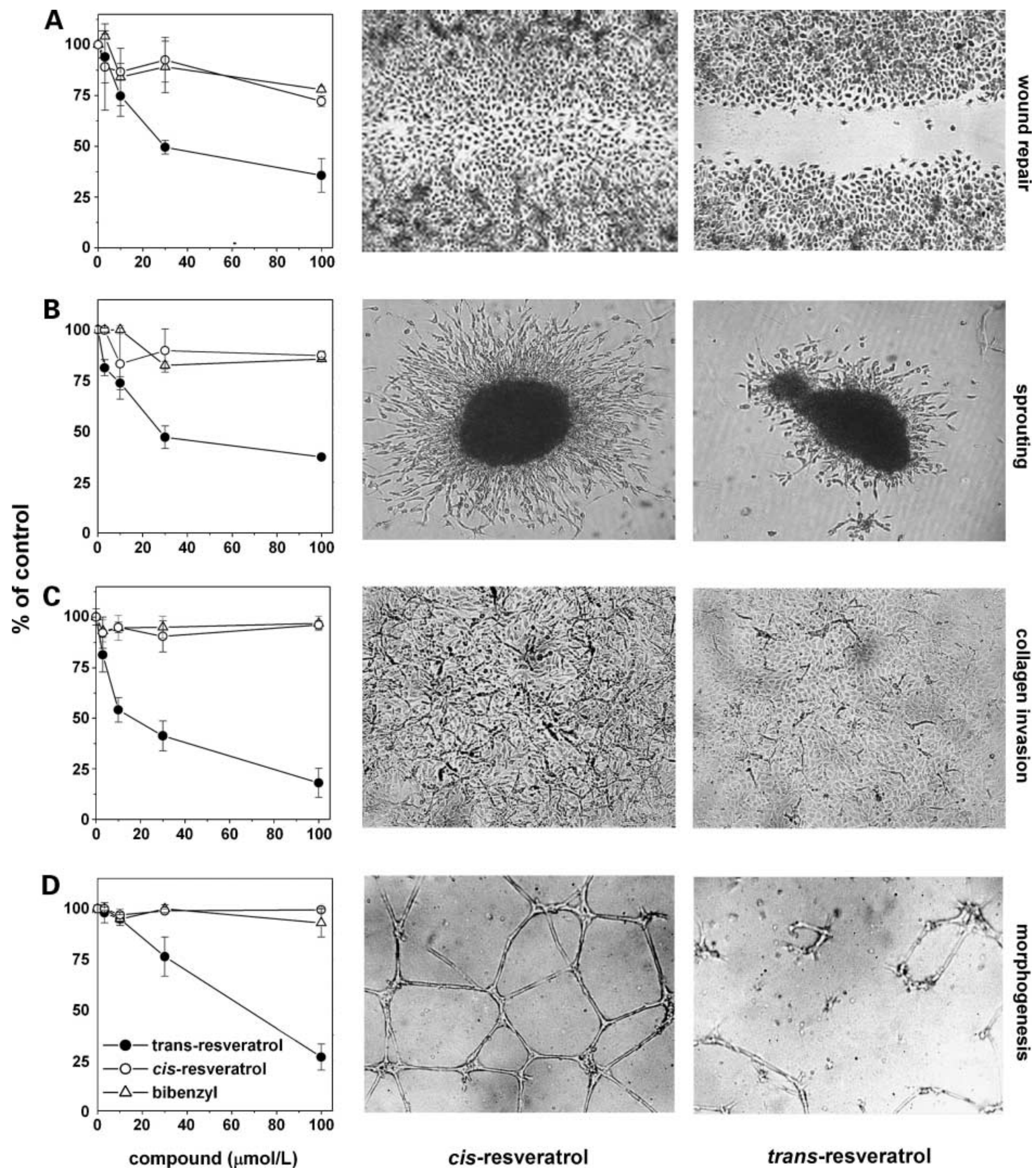


**Figure 2.** Effect of resveratrol stereoisomers on different *in vitro* steps of the angiogenesis process. **A,** confluent GM7373 cells were wounded with a 1.0-mm-wide rubber policeman and incubated with the compound under test. After 16 h, endothelial cells invading the wound were quantified by computerized analysis of the digitalized images. **B,** FGF2-T-MAEC aggregates were embedded in fibrin gel in the presence of the compound under test. After 48 h, sprouts were quantified by computerized analysis of the digitalized images. **C,** confluent monolayers of BAECs grown on type I collagen gel were incubated with FGF2 plus vascular endothelial growth factor in the presence of the compound under test. After 24 h, endothelial cells invading the gel in a plane of focus beneath the cell monolayer surface were quantified by computerized analysis of the digitalized images. **D,** HUVECs were seeded on Matrigel in the presence of the compound under test. After 16 h, newly formed endothelial cell tubular structures were scored and graded on an arbitrary scale of 0 to 4+, with 0 representing no morphogenic response and 4+ representing the strongest response. In all the experiments, data are mean ± SD of three determination in triplicate and are expressed as percent of the values measured in control cultures grown in presence of vehicle. ●, *trans*-resveratrol; ○, *cis*-resveratrol; △, bibenzyl. *Middle* and *right,* representative images of cells treated with *cis*- or *trans*-resveratrol in the various assays, respectively.

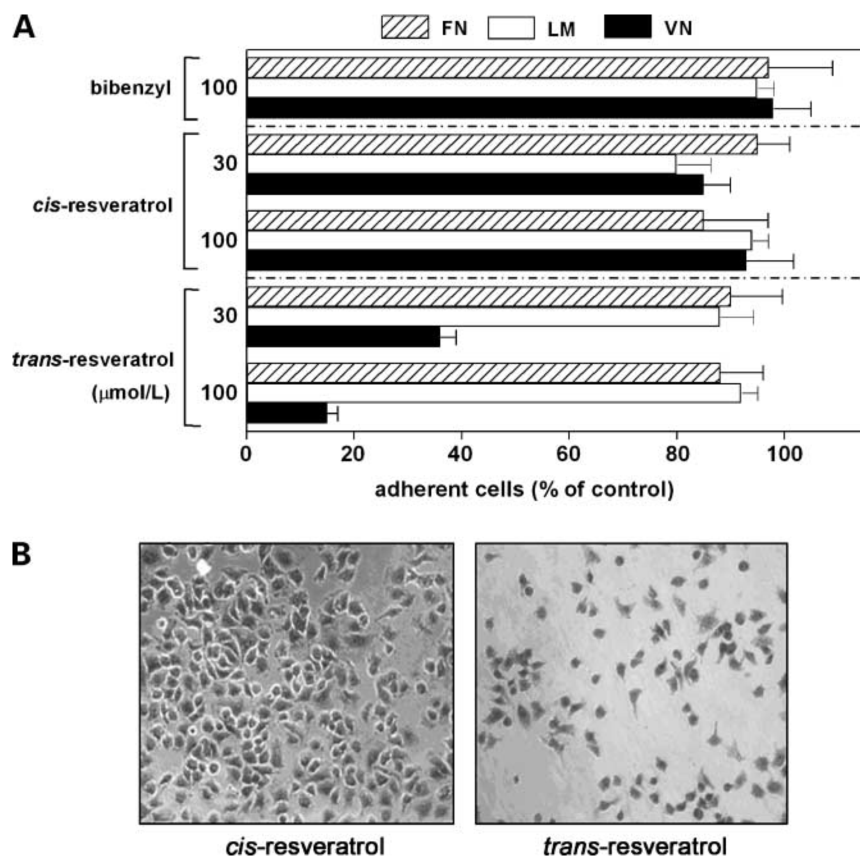


**Figure 3.** Effect of resveratrol stereoisomers on endothelial cell adhesion. **A,** GM7373 cells were seeded on 24-well plates coated with vitronectin (*black columns*), fibronectin (*dashed columns*), or laminin (*open columns*) in medium containing 1% FCS and the molecule under test. After 3 h at 37°C, the number of adherent cells was evaluated. Mean ± SD of five determinations, expressed as percent of the values measured in control cultures seeded on the corresponding substratum in presence of vehicle. **B,** representative images of vitronectin-seeded GM7373 cells treated with *cis*- or *trans*-resveratrol. Untreated cells and cells treated with bibenzyl or vehicle alone were undistinguishable from *cis*-resveratrol-treated cells (data not shown).

inhibits $\beta_3$-EGFP-GM7373 cell adhesion to vitronectin, but not to fibronectin or laminin, whereas *cis*-resveratrol was ineffective (Fig. 4C). Thus, $\beta_3$-EGFP transduction does not affect the adhesive behavior of GM7373 cells and their response to resveratrol isomers. On this basis, *cis*- and *trans*-resveratrol were assessed for their capacity to affect $\beta_3$-EGFP recruitment in focal adhesion contacts of vitronectin-adherent $\beta_3$-EGFP-GM7373 cells. As shown in Fig. 4D, *trans*-resveratrol, but not the *cis* isoform, prevented the formation of paxillin-positive focal adhesion contacts in $\beta_3$-EGFP-GM7373 cells adherent to vitronectin and the recruitment of the $\beta_3$-EGFP chimera in these contacts.

### Effect of Resveratrol Stereoisomers on *In vivo* Angiogenesis and Tumor Growth

*trans*- and *cis*-Resveratrol were assessed for their antiangiogenic activity on the chick embryo area vasculosa, a copiously vascularized extraembryonal adnexum (34). To this purpose, 30 or 300 pmol/embryo of the polyphenol under test were applied topically on the area vasculosa membrane via a gelatin sponge implant at day 3 of development. Control embryos were treated with the same doses of bibenzyl or vehicle. At day 5 of incubation, no effect on area vasculosa was observed in embryos treated with vehicle, with both doses of bibenzyl, or with low-dose *cis*- or *trans*-resveratrol (data not shown). In contrast, area vasculosa treated with 300 pmol *trans*-resveratrol were characterized by the lack of blood vessel growth and the disappearance of the majority of preexisting blood vessels (Fig. 5) in the absence of any effect on embryonic development and survival. This resulted in 50% reduction of the vascular density when compared with vehicle- and bibenzyl-treated embryos (Fig. 5C). The same dose of *cis*-resveratrol had only a limited effect on area vasculosa, resulting in ~25% inhibition of vascular density (Fig. 5C).

Next, resveratrol isomers were assessed for their capacity to affect tumor growth in mice. Oral administration of *trans*-resveratrol at the concentration of 30 μg/mL in drinking water inhibits the growth of s.c. grafts of murine B16 melanoma cells in C57BL/6N mice. Inhibition of tumor growth was statistically significant from day 8 after grafting and onward when compared with animals given vehicle alone ($P < 0.01$, Student's $t$ test). No significant inhibition was instead exerted by the *cis* isomer at all the time points investigated (Fig. 6A). Accordingly, analysis of tumor neovascularization done at day 18 after grafting (Fig. 6B; Supplementary Fig. S1)[6] showed that *trans*-resveratrol administration causes an evident decrease of tumor blood vessel density (−39%), as assessed by CD31 immunostaining of tumor sections, and of CD31 mRNA expression (−38%), as assessed by quantitative real-time

[6] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

PCR analysis done on total tumor RNA. Again, administration of the *cis* isomer caused instead only a limited effect (−14% and −20% inhibition of the number of $CD31^+$ vessels and of CD31 mRNA levels, respectively).

## Discussion

In the present article, we compared the antiangiogenic activity of *trans*- and *cis*-resveratrol on endothelial cells of different origin activated by endogenous or exogenous stimuli. The results indicate that *trans*-resveratrol affects endothelial cell proliferation and various cell proliferation-independent aspects of the angiogenesis process, including endothelial cell sprout formation, collagen gel invasion, and morphogenesis. Thus, *trans*-resveratrol acts as an angiostatic molecule on multiple targets of the angiogenic process. In all the assays, the *cis* stereoisomer exerted only a limited effect, if any. In keeping with the *in vitro* observations, *trans*-resveratrol was more effective than the *cis* isomer in inhibiting angiogenesis and tumor growth *in vivo*.

The molecular bases of the antiangiogenic activity of resveratrol are poorly understood (16). *trans*-Resveratrol is endowed with a significant antioxidant and anti-inflammatory activity (8, 9). However, no direct relationship exists between the antiangiogenic and the antioxidant

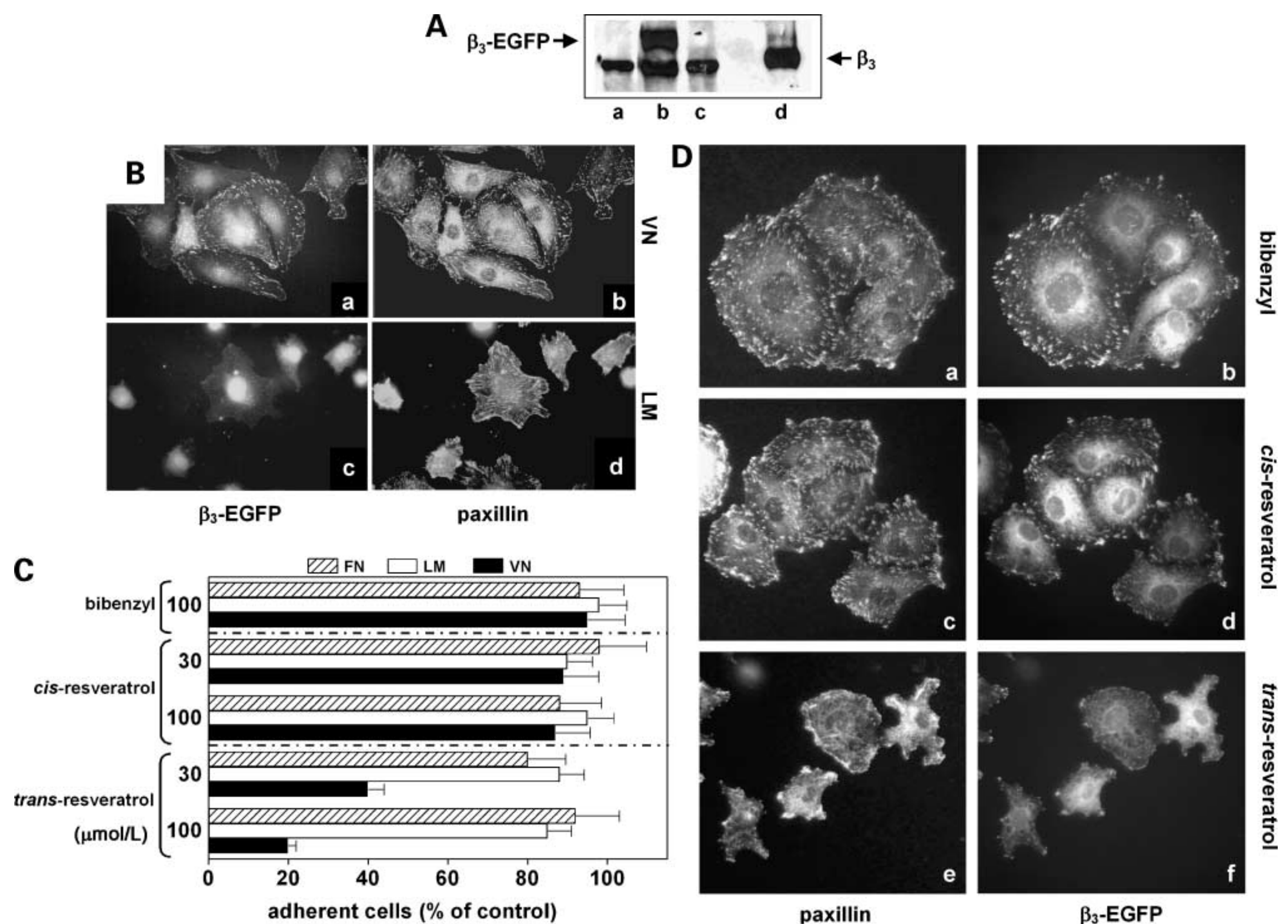


**Figure 4.** Effect of resveratrol stereoisomers on EGFP-tagged $\beta_3$ integrin localization in endothelial cells. **A,** lysates (50 μg/lane) of parental (*a*), $\beta_3$-EGFP-transfected (*b*), and EGFP-transfected (*c*) GM7373 cells were probed in a Western blot with an anti-$\beta_3$ antibody. Purified $\beta_3$ integrin was used as a positive control (*d*). **B,** $\beta_3$-EGFP-GM7373 cells were seeded on vitronectin-coated (*a* and *b*) or laminin-coated (*c* and *d*) glass coverslips. After 16 h, cells were immunostained with an anti-paxillin antibody and observed under an epifluorescence microscope for $\beta_3$-EGFP (*a* and *c*) and paxillin (*b* and *d*) localization. Note that the $\beta_3$-EGFP chimera localizes in paxillin-positive focal adhesion contacts in vitronectin-adherent but not in laminin-adherent cells. No focal contact localization of the EGFP protein was instead observed in EGFP-GM7373 cells adherent to the two substrata (data not shown). **C,** $\beta_3$-EGFP-GM7373 cells were seeded on 24-well plates coated with vitronectin (*black columns*), fibronectin (*dashed columns*), or laminin (*open columns*) in medium containing 1% FCS and the molecule under test. After 3 h at 37°C, the number of adherent cells was evaluated. Mean ± SD of five determinations, expressed as percent of the values measured in control cultures seeded on the corresponding substratum in presence of vehicle. **D,** $\beta_3$-EGFP-GM7373 cells were seeded on vitronectin-coated coverslips in medium containing 10% FCS in the presence of bibenzyl or resveratrol stereoisomers (100 μmol/L). After 3 h, cells were analyzed by paxillin immunostaining (*a, c,* and *e*) and for $\beta_3$-EGFP integrin chimera localization (*b, d,* and *f*). Paxillin and $\beta_3$-EGFP integrin chimera colocalize in cells treated with bibenzyl or *cis*-resveratrol, whereas no $paxillin^+/\beta_3\text{-}EGFP^+$ focal adhesion contacts are observed after *trans*-resveratrol incubation. Untreated cells and cells treated with vehicle alone were undistinguishable from bibenzyl-treated cells (data not shown).

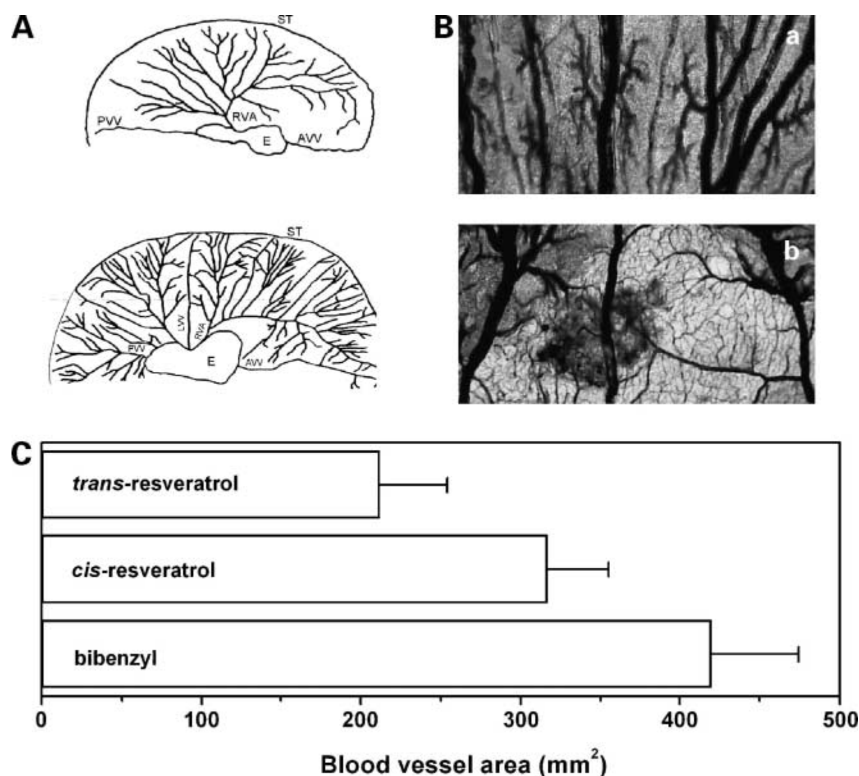


**Figure 5.** Effect of resveratrol stereoisomers on vascularization of the chick embryo area vasculosa. **A,** computer printouts of the vascular tree of area vasculosa at the right side of a control chick embryo (*E*) at day 3 (*top*) and day 5 (*bottom*) of incubation. *RVA,* right vitelline artery; *AVV,* anterior vitelline vein; *LVV,* lateral vitelline vein; *PVV,* posterior vitelline vein; *ST,* sinus terminalis. **B,** gelatin sponges adsorbed with 300 pmol of the compound under test were placed on top of the chick embryo area vasculosa at day 3 of development and photographed at day 5 (original magnification, ×8). Note the lack of blood vessel growth and the disappearance of the majority of preexisting blood vessels following *trans*-resveratrol treatment (*b*) when compared with bibenzyl-treated embryos (*a*). **C,** the blood vessel area of the area vasculosa surrounding the gelatin implant was quantified by an image analyzer system. Mean ± SD of 10 embryos per group. *, $P < 0.001$. Vehicle and bibenzyl treatment did not exert any effect on the vascularization of area vasculosa when compared with untreated embryos (data not shown).

activities of various resveratrol derivatives (23). Accordingly, despite its antioxidant potential (12, 13), *cis*-resveratrol is characterized by a limited angiostatic activity when compared with the *trans* isomer.

Our results show that stereoisomery plays an important role in defining the antiangiogenic potential of resveratrol. This suggests that the inhibitory effects exerted by this phytoalexin on endothelial cells are mediated, at least in part, by a stereoselective receptor-dependent mechanism of action. Recent observations have shown the capacity of *trans*-resveratrol to bind $\alpha_v\beta_3$ integrin receptor *in vitro* near the RGD recognition site by interacting with the $\beta_3$ integrin chain (17). Our results extend these observations by showing that *trans*-resveratrol, but not the *cis* stereoisomer, affects $\alpha_v\beta_3$ integrin function by inhibiting the adhesion of parental and $\beta_3$-EGFP-transfected GM7373

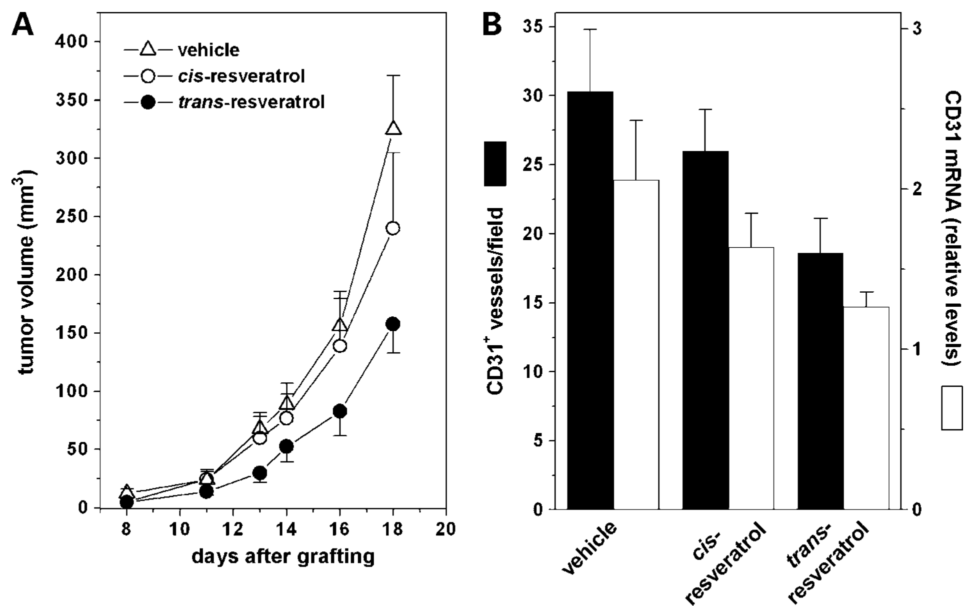


**Figure 6.** Effect of resveratrol stereoisomers on tumor growth and neovascularization. **A,** female C57BL/6N mice were transplanted s.c. in both flanks with $1.5 \times 10^5$ murine B16 melanoma cells. Animals were given *trans*-resveratrol (●) or *cis*-resveratrol (○) in the drinking water (30 μg/mL in 0.15% ethanol) with a change every day throughout the whole experimental period starting 4 d before cell transplantation. Control animals received the same concentration of ethanol (△). *Points,* mean of 16 tumors; *bars,* SE. **B,** animals were sacrificed 18 d after grafting and tumor vascularization was assessed by counting the number of $CD31^+$ blood vessels per field in the most vascularized areas of five sections per each tumor (*black columns*) and by evaluating the levels of *CD31/β-actin* mRNA ratio by quantitative real-time PCR analysis of total tumor RNA (*open columns*). *Points,* mean of 5 tumors; *bars,* SE.

cells to the prototypic $\alpha_v\beta_3$ substrate vitronectin. In contrast, *trans*-resveratrol does not affect $\beta_1$ integrin-mediated GM7373 cell adhesion to fibronectin or laminin. This indicates that *trans*-resveratrol exerts its action by affecting $\beta_3$ integrin function rather than by exerting a generic effect on cytoskeleton organization and/or integrin activity. Indeed, previous observations have shown that *trans*-resveratrol does not perturb cytoskeleton organization even when delivered to endothelial cells at doses as high as 300 μmol/L (23). In keeping with its $\alpha_v\beta_3$ antagonist function, *trans*-resveratrol selectively prevents the formation of paxillin-positive focal adhesion contacts and the recruitment of the EGFP-$\beta_3$ integrin chimera in endothelial $\beta_3$-EGFP-GM7373 cells seeded on vitronectin.

Similar to *trans*-resveratrol, RGD-derived $\alpha_v\beta_3$ integrin antagonists affect endothelial cell adhesion, proliferation, invasion, and morphogenesis when added to endothelial cell cultures in a micromolar range of concentrations (19, 21). Thus, our results support the hypothesis that interaction of *trans*-resveratrol with the $\beta_3$ integrin chain may lead to inhibition of $\alpha_v\beta_3$ integrin activity. This may be responsible, at least in part, for the antiangiogenic potential of the phytoalexin. Nevertheless, the antiangiogenic activity of *trans*-resveratrol is likely the sum of several distinct mechanisms. Indeed, besides inhibiting $\alpha_v\beta_3$ integrin function, our results indicate that *trans*-resveratrol can exert an antiangiogenic effect in different *in vitro* assays where endothelial cells are adherent to various substrata distinct from vitronectin, including Matrigel, fibrin, and type I collagen. This may represent the consequence of the capacity of *trans*-resveratrol to affect cell cycle progression, cell survival, and intracellular signaling in endothelium (16, 35). Further studies are required to further dissect the complex mechanisms of action of *trans*-resveratrol on the angiogenesis process.

Dietary phytochemicals derived from edible plants may represent a promising class of tumor chemopreventive compounds by acting directly on tumor cells (7) and/or by inhibiting the angiogenesis process, thus acting as ''angiopreventive'' agents (5, 6). As confirmed in the present work, resveratrol is endowed with antiangiogenic and antineoplastic effects *in vivo* (14, 15) and exerts a significant cancer chemopreventive activity (10). However, results from pharmacokinetic studies indicate that resveratrol is rapidly metabolized in the body via glucuronidation, sulfation, and hydroxylation reactions (11, 36), casting some doubts about the physiologic relevance of the high concentrations that are effective in the *in vitro* experiments. This has led to the hypothesis that resveratrol metabolites may retain a significant biological activity (discussed in ref. 11). For instance, the resveratrol derivatives piceatannol and resveratrol-3-*O*-β-D-glucoside are endowed with significant anticancer and antiangiogenic activities (37, 38). Relevant to this point, resveratrol stereoisomers can undergo regioselective and stereospecific metabolic conversions (39, 40), thus suggesting that their bioavailability and metabolism may differ *in vivo*.

Previous observations from our laboratory have shown that subtle modifications in the chemical structure of *trans*-resveratrol deeply affect its antiangiogenic potency (23). Here, we have shown that stereoisomery is of importance in conferring to this phytoalexin a significant antiangiogenic and antitumor activity, possibly related to its $\alpha_v\beta_3$ antagonist activity. These data may provide the basis for the design of antiangiogenic/angiopreventive resveratrol derivatives endowed with a more potent efficacy *in vitro* and *in vivo*.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### References


1. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–95.
2. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27–31.
3. Carmeliet P. Angiogenesis in health and disease. Nat Med 2003;9:653–60.
4. Keshet E, Ben-Sasson SA. Anticancer drug targets: approaching angiogenesis. J Clin Invest 1999;104:1497–501.
5. Tosetti F, Ferrari N, De Flora S, Albini A. Angioprevention: angiogenesis is a common and key target for cancer chemopreventive agents. FASEB J 2002;16:2–14.
6. Albini A, Sporn MB. The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer 2007;7:139–47.
7. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
8. Pervaiz S. Resveratrol: from grapevines to mammalian biology. FASEB J 2003;17:1975–85.
9. Frémont L. Minireview. Biological effects of resveratrol. Life Sci 2000;66:663–73.
10. Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.
11. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the *in vivo* evidence. Nat Rev Drug Discov 2006;5:493–506.
12. Stivala LA, Savio M, Carafoli F, et al. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. J Biol Chem 2001;276:22586–94.
13. Orallo F. Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. Curr Med Chem 2006;13:87–98.
14. Brakenhielm E, Cao R, Cao Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. FASEB J 2001;15:1798–800.
15. Tseng SH, Lin SM, Chen JC, et al. Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. Clin Cancer Res 2004;10:2190–202.
16. Cao Y, Cao R, Brakenhielm E. Antiangiogenic mechanisms of diet-derived polyphenols. J Nutr Biochem 2002;13:380–90.
17. Lin HY, Lansing L, Merillon JM, et al. Integrin $\alpha_V\beta_3$ contains a receptor site for resveratrol. FASEB J 2006;20:1742–4.
18. Ruegg C, Mariotti A. Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. Cell Mol Life Sci 2003;60:1135–57.
19. Belvisi L, Riccioni T, Marcellini M, et al. Biological and molecular properties of a new $\alpha(v)\beta_3/\alpha(v)\beta_5$ integrin antagonist. Mol Cancer Ther 2005;4:1670–80.
20. Kumar CC. Integrin $\alpha_v\beta_3$ as a therapeutic target for blocking tumor-induced angiogenesis. Curr Drug Targets 2003;4:123–31.
21. Kumar CC, Malkowski M, Yin Z, et al. Inhibition of angiogenesis and tumor growth by SCH221153, a dual $\alpha(v)\beta_3$ and $\alpha(v)\beta_5$ integrin receptor antagonist. Cancer Res 2001;61:2232–8.

**22.** Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. Science 1994;264:569 – 71.

**23.** Belleri M, Ribatti D, Nicoli S, et al. Antiangiogenic and vascular-targeting activity of the microtubule-destabilizing *trans*-resveratrol derivative 3,5,4′-trimethoxystilbene. Mol Pharmacol 2005;67:1451 – 9.

**24.** Gualandris A, Rusnati M, Belleri M, et al. Basic fibroblast growth factor overexpression in endothelial cells: an autocrine mechanism for angiogenesis and angioproliferative diseases. Cell Growth Differ 1996;7:147 – 60.

**25.** Rusnati M, Tanghetti E, Dell'Era P, Gualandris A, Presta M. $\alpha_v\beta_3$ Integrin mediates the cell-adhesive capacity and biological activity of basic fibroblast growth factor (FGF-2) in cultured endothelial cells. Mol Biol Cell 1997;8:2449 – 61.

**26.** Ballestrem C, Hinz B, Imhof BA, Wehrle-Haller B. Marching at the front and dragging behind: differential $\alpha_V\beta_3$-integrin turnover regulates focal adhesion behavior. J Cell Biol 2001;155:1319 – 32.

**27.** Leali D, Moroni E, Bussolino F, Presta M. Osteopontin overexpression inhibits *in vitro* re-endothelialization via integrin engagement. J Biol Chem 2007;282:19676 – 84.

**28.** Bastaki M, Nelli EE, Dell'Era P, et al. Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. Arterioscler Thromb Vasc Biol 1997;17:454 – 64.

**29.** Giavazzi R, Sennino B, Coltrini D, et al. Distinct role of fibroblast growth factor-2 and vascular endothelial growth factor on tumor growth and angiogenesis. Am J Pathol 2003;162:1913 – 26.

**30.** Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res 2000;60:1388 – 93.

**31.** Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci U S A 2003;100:10623 – 8.

**32.** Lee TY, Gotlieb AI. Early stages of endothelial wound repair: conversion of quiescent to migrating endothelial cells involves tyrosine phosphorylation and actin microfilament reorganization. Cell Tissue Res 1999;297:435 – 50.

**33.** Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. Clin Chem 2003;49:32 – 40.

**34.** Ribatti D. A morphometric study of the expansion of the chick area vasculosa in shell-less culture. J Anat 1995;186:639 – 44.

**35.** Lin MT, Yen ML, Lin CY, Kuo ML. Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. Mol Pharmacol 2003;64:1029 – 36.

**36.** Yu C, Shin YG, Chow A, et al. Human, rat, and mouse metabolism of resveratrol. Pharm Res 2002;19:1907 – 14.

**37.** Kimura Y, Okuda H. Effects of naturally occurring stilbene glucosides from medicinal plants and wine, on tumour growth and lung metastasis in Lewis lung carcinoma-bearing mice. J Pharm Pharmacol 2000;52:1287 – 95.

**38.** Kimura Y, Baba K, Okuda H. Inhibitory effects of active substances isolated from *Cassia garrettiana* heartwood on tumor growth and lung metastasis in Lewis lung carcinoma-bearing mice (part 2). Anticancer Res 2000;20:2923 – 30.

**39.** Aumont V, Krisa S, Battaglia E, et al. Regioselective and stereospecific glucuronidation of *trans*- and *cis*-resveratrol in human. Arch Biochem Biophys 2001;393:281 – 9.

**40.** Sabolovic N, Heurtaux T, Humbert AC, Krisa S, Magdalou J. *cis*- and *trans*-Resveratrol are glucuronidated in rat brain, olfactory mucosa and cultured astrocytes. Pharmacology 2007;80:185 – 92.