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Inhibition of VEGF expression in cancer cells and endothelial cell differentiation by synthetic stilbene derivatives

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ABSTRACT

We here report the synthesis of several stilbene derivatives. They show a measurable inhibitory effect on angiogenesis, some of them to a higher degree than resveratrol. Test methods included cell proliferation and tube formation assays using bovine aorta endothelial cells. In addition, it has been confirmed through the reverse transcriptase/polymerase chain reaction experiment that these stilbene derivatives down-regulate the expression of the gene related to the production of the angiogenesis factor VEGF in cancer cells.

1. Introduction

It is widely known that cancer, one leading cause of death in developed countries,[1] may be caused by both external and internal factors, including genetic mutations.[2,3] One main therapeutic line is the use of cytotoxic drugs, which exert their effect in many cases by means of inducing cell apoptosis (programmed cell death).[4] Another useful strategy is based on the use of compounds with an inhibitory ability on the formation of blood vessels (angiogenesis).[5,6] Tumor angiogenesis is a very complex process and involves the tight interplay of many factors.[7] One of these is a protein called vascular endothelial growth factor (VEGF), a key regulator of angiogenesis which drives endothelial cell survival, proliferation and migration while increasing vascular permeability.[8] In fact, overexpression in the production of VEGF has been reported to occur in many types of tumors.[9] It is therefore not surprising that VEGF has become one further target molecule in cancer therapy.[10,11]

Many phytochemicals, often present in food, have been shown to have an influence in tumor angiogenesis.[12,13] Among the promising dietary phytochemicals known to exhibit antiangiogenic activity,[14] some pertain to the stilbene class of natural polyphenols.[15] Resveratrol (Fig. 1), one naturally occurring member of this class, has been found to exhibit a remarkable both chemopreventive and chemotherapeutic potential.[16] It has been identified in various food sources including red grapes, peanuts and mulberries. Even though its molecular target is still unknown,[17] it is well documented that resveratrol has broad-spectrum health beneficial effects, such as anti-infective, antioxidant, and cardioprotective functions.[18] It has also been found to alter tumor cell growth and survival by means of a downregulation of the expression of genes involved in cancer.[19] For that reason, resveratrol and many of its synthetic analogues[18,20] have been intensively investigated in relation to their possible therapeutic use.

2. Research purpose

As part of our research project related to design, synthesis and biological evaluation of anticancer agents, we focused on the search of small molecules that could inhibit both endothelial cell differentiation and expression of certain oncogenes and proteins in tumor cells. Furthermore, and in order to prevent damaging of healthy cells, we are looking for molecules that exert these inhibitory properties at non toxic concentrations.

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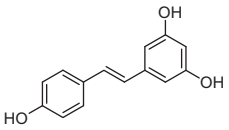


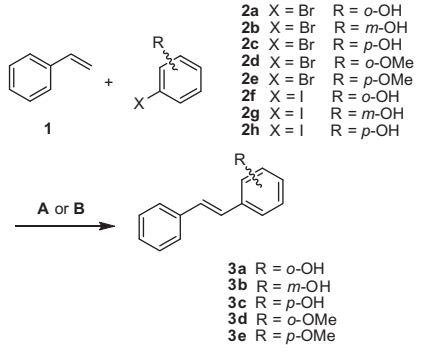
Figure 1. Structure of resveratrol.

We initially focused our attention on resveratrol because of its well-known antiangiogenic properties.[19] Unfortunately, its bioavailability is low because it exhibits a high metabolization rate due to the fact that it binds to plasma proteins forming sulphates and glucuronide conjugates.[20] With these precedents in mind, we decided to evaluate the antitumoral properties of structurally simple resveratrol analogues that exhibit both strong antitumoral properties and a higher bioavailability than the parent compound. Our first generation of analogues includes simple stilbene derivatives with less free hydroxyl groups than resveratrol itself (compounds **3a**–**3e** and **4a**–**4b**, Scheme 1). Furthermore, we have investigated the effect on the transcription of genes related to the production of the angiogenesis-associated factor VEGF. In all biological assays, resveratrol was used as the reference compound for comparison.

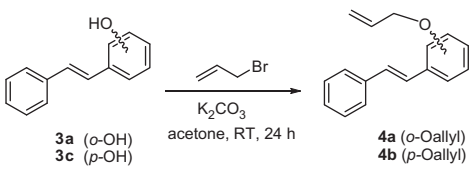
3. Chemical results

A range of synthetic strategies has been developed for the preparation of stilbene derivatives. Some of them rely on the creation of the C=C bond through Wittig-like olefinations.[21,22] Other methods center on the formation of the C_{sp2}–Ar bond by means of palladium-catalyzed cross couplings.[23] In the present case, we have employed Heck couplings of styrene **1** with halogenated derivatives of either phenol (**2a**–**c** and **2f**–**2h**) or methyl phenyl ether (**2d**–**e**) under two different reaction conditions A[23] and B[24] (for more experimental details and yields, see Experimental section). Couplings under conditions A are carried out with very low catalyst loadings and involve the use of microwaves (MW), which have often been found to accelerate coupling reactions.[25]

In order to see the effect of the *O*-alkyl group size on the aforementioned biological properties, we have subjected stilbenes **3a** and **3c** to *O*-allylation as depicted in Scheme 2. Details about yields and reaction conditions are given in the Experimental section.



Scheme 1. Synthesis of stilbenes **3a**–**3e** via Heck coupling.



Scheme 2. Synthesis of *O*-allyl derivatives **4a** and **4b**.

4. Biological results

4.1. Cytotoxicity and inhibition of tube formation by stilbene derivatives in bovine aortic endothelial cells

Since angiogenesis involves the local proliferation of endothelial cells, we investigated the ability of stilbenes **3a**–**3e** and **4a**–**4b**, as well as resveratrol as the reference compound, to inhibit the growth of bovine aortic endothelial (BAE) cells. IC_{50} values of this antiproliferative effect are shown in Table 1. We found an IC_{50} = 48 μmol/L for resveratrol, which is in the range of concentrations described by others for the endothelial cell growth inhibition by this compound.[26] Our data indicate that the IC_{50} values for stilbene derivatives range from 33.6 to >400 μmol/L, indicating that some of these compounds exhibit a lower toxicity than resveratrol. The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes, as shown in Figure 2a for a control experiment using dimethylsulfoxide (DMSO). In vitro, endothelial cells plated on Matrigel align themselves forming cords, already evident a few hours after plating. Figure 2b shows that 54.8 μmol/L resveratrol was able to completely inhibit BAE cell alignment and cord formation, what is in good agreement with previously reported data.[26] A complete inhibition of endothelial morphogenesis on Matrigel was obtained with a concentration of 32 μmol/L for compounds **3a**, **3b** and **3c** (Fig. 2c–e), about 50–60 μmol/L for compounds **3d** and **4a** (Fig. 2f and h), 106 μmol/L for compound **4b** (Fig. 2i) or with 475 μmol/L for compound **3e** (Fig. 2g).

4.2. Effect of resveratrol and stilbenes 3a, 3d and 4a on the transcription of the VEGF gene in human colon adenocarcinoma cell line (HT-29)

In relation to the fact that angiogenesis begins with the release of VEGF from cancer cells, we decided to examine whether our resveratrol analogues were able to inhibit or at least decrease the activation of VEGF genes in HT-29 tumoral cells. First, we have investigated the ability of stilbenes **3a**–**3e**, **4a**–**4b** and resveratrol to inhibit the growth of HT-29 cells (see IC_{50} values in Table 1).

Table 1
Cytotoxicity and inhibition of angiogenesis in vitro by resveratrol and stilbene derivatives **3a**–**3e** and **4a**–**4b**

Compound	IC_{50}[a,b]	MIC tube formation[c]	IC_{50}[d,b]
3a	107 ± 15	32	112 ± 15
3b	91.7 ± 10	32	127 ± 5
3c	33.6 ± 2.5	32	34.6 ± 2
3d	152 ± 19	59.5	42.8 ± 5
3e	418 ± 90	475[e]	26 ± 2.5
4a	313 ± 31	53	55 ± 4
4b	>400	106[e]	23 ± 3
Resveratrol	48 ± 4	54.8	110 ± 13

[a] IC_{50} values related to BAE cells are expressed as the compound concentration (in μmol/L) that causes 50% inhibition of cell growth.
[b] Mean values (±sd) of three different experimental values, as described in the Materials and methods section.
[c] Minimal inhibitory concentration (in μmol/L) of BAE cell differentiation for the different tested compounds. The differentiation assay was carried out in the presence of different concentrations of resveratrol and related stilbenes, as described in the Materials and methods section.
[d] IC_{50} values related to human colon adenocarcinoma cancer cells (HT-29) are expressed as the compound concentration (in μmol/L) that causes 50% inhibition of cell growth.
[e] Compound partially insoluble at this concentration.

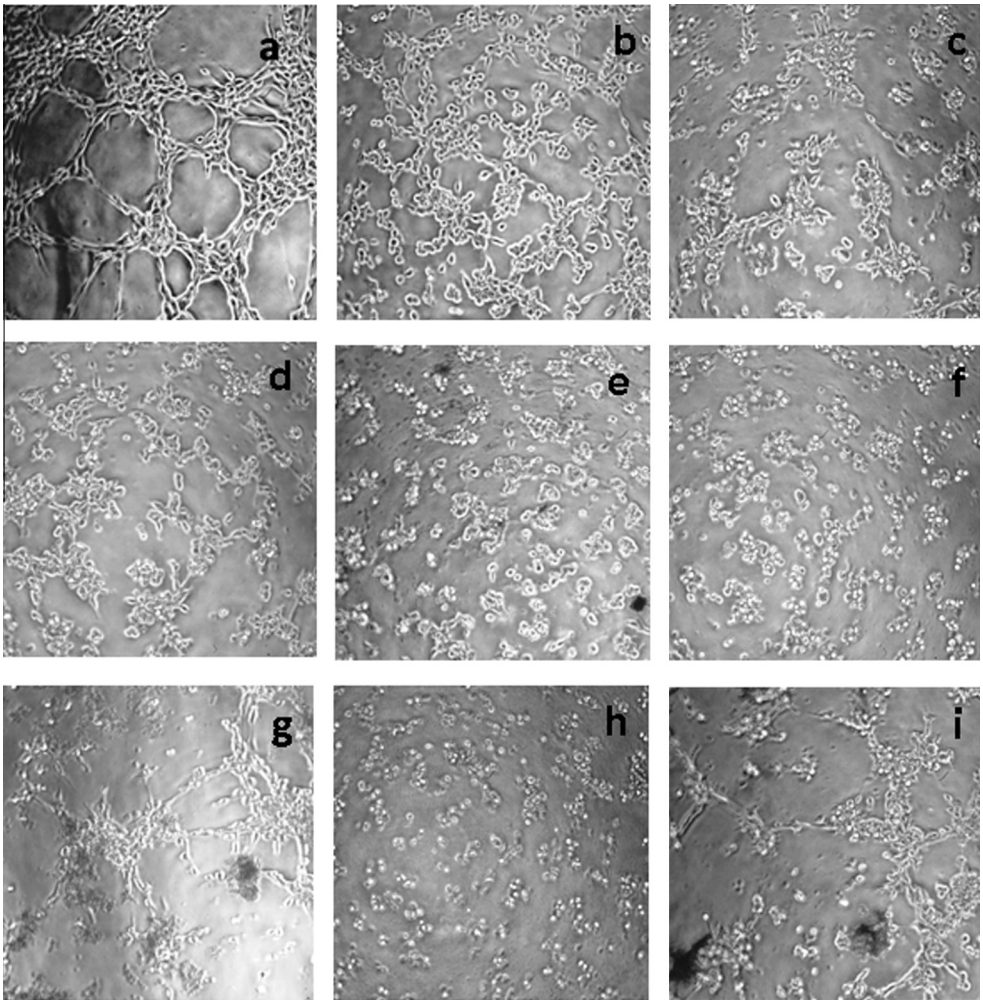


Figure 2. Effect of resveratrol and stilbene derivatives on endothelial cell tubulogenesis in vitro: (a) control (DMSO); (b) resveratrol, 54.8 μmol/L; (c) **3a**, 32 μmol/L; (d) **3b**, 32 μmol/L; (e) **3c**, 32 μmol/L; (f) **3d**, 59.5 μmol/L; (g) **3e**, 475 μmol/L; (h) **4a**, 53 μmol/L; and (i) **4b**, 106 μmol/L.

Subsequently, we treated these cells with each of the aforementioned compounds at a concentration below their corresponding IC_{50} values. We used untreated cells as a negative control (−) and cells treated only with DMSO a positive control (+). Finally, we have performed a reverse transcriptase/polymerase chain reaction (RT-PCR) analysis which has shown that stilbene derivatives such as those described here are able to reduce the transcription of VEGF mRNA in HT-29 cells. As shown in Figure 3, treatment of the cells with **3a**, **3d**, **4a** and resveratrol in DMSO (10 μg/mL of each compound, which corresponds to concentrations in the range between 42 and 51 μmol/L), did in fact reduce the transcription of VEGF mRNA to up half its value as compared with control cells.

We have also determined VEGF protein production by ELISA in culture supernatants. Figure 4 shows the results obtained in the ELISA measurements after treatment of HT-29 cells with **3a**, **3d**, **4a** and resveratrol in DMSO (10 μg/mL each compound, approximate concentrations in the range between 42 and 51 μmol/L). In comparison with untreated cells, compounds **3a** and **4a** are more effective in decreasing VEGF secretion in HT-29 cells than resveratrol.

5. Discussion

A few years ago, Kimura and coworkers investigated the antitumor and antimetastatic properties of a library of stilbenes, among them resveratrol and other mono and polyhydroxylated stilbene derivatives, including compounds **3a**–**3c**.[20a] Working with human umbilical vein endothelial cells, these authors found that some dihydroxylated resveratrol derivatives were able to inhibit VEGF-induced endothelial cell migration/differentiation and angiogenesis. However, they did not test the monohydroxylated derivatives **3a**–**c** as inhibitors of endothelial cell differentiation, a key step of the angiogenic process.

As a matter of fact, our results using BAE cells indicate that structurally simple stilbene derivatives are able to inhibit endothelial cell differentiation at non toxic concentrations. Furthermore, three of these compounds (**3a**–**3c**) exhibit a higher antiangiogenic activity than resveratrol itself in the in vitro assay (see Table 1). It should also be pointed out that some of our compounds (**3a**, **3b**, **3d**, **4a** and **4b**) are able to completely inhibit tube formation by endothelial cells at concentrations that are far below their IC_{50} values in the MTT assay (see Materials and methods section). This indicates that their toxicities at the concentrations needed for inhibition of angiogenesis are lower than in the case of resveratrol, therefore suggesting a lower probability of the appearance of antiproliferation-derived side effects.

As a further outcome of this study, we have established that resveratrol and several stilbene derivatives lead to a decrease in the production of VEGF by HT-29 cells. ELISA measurements of culture supernatants after treatment of HT-29 cells with each of the compounds under study have revealed that **3a** and **4a** decrease VEGF production to a higher extent than resveratrol. As a matter of fact, while the latter diminishes the VEGF production to about 65% of

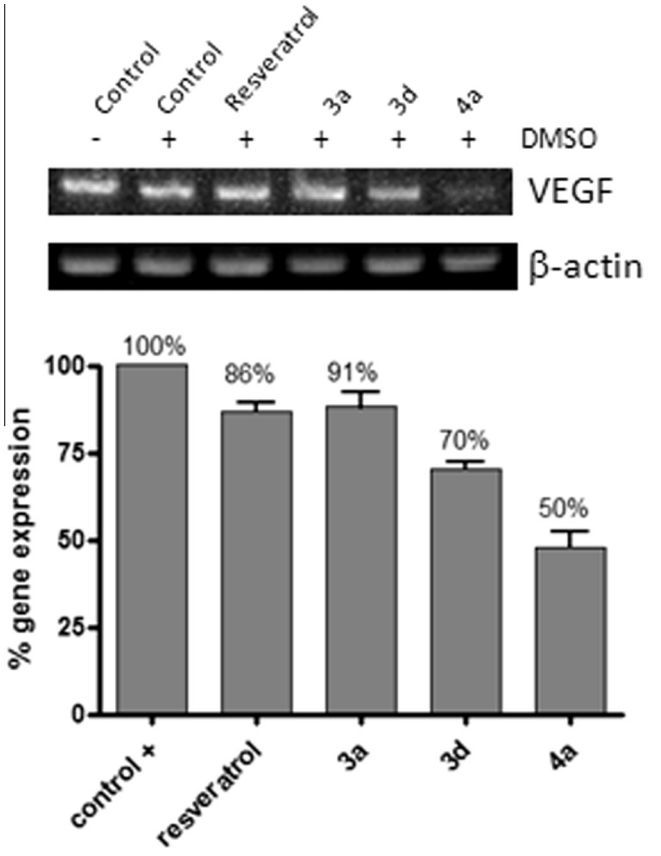


Figure 3. Agarose gel profile of products resulting from RT-PCR amplification. The total RNA of HT-29 cells previously treated with resveratrol, **3a**, **3d** and **4a** was isolated (10 μg/mL each compound), converted into cDNA, and amplified by PCR as described in the Materials and methods section (primers used for the RT-PCR are shown in Table 2). Gene expression of VEGF and β-actin was quantified using the Image J program and normalized to that of the housekeeping gene β-actin. At least three measures were performed in each case. Bars shown represent mean activations of VEGF gene expression and error bars indicate standard errors of the mean. Statistical significance was evaluated using one-sample *t*-tests (P <0.001).

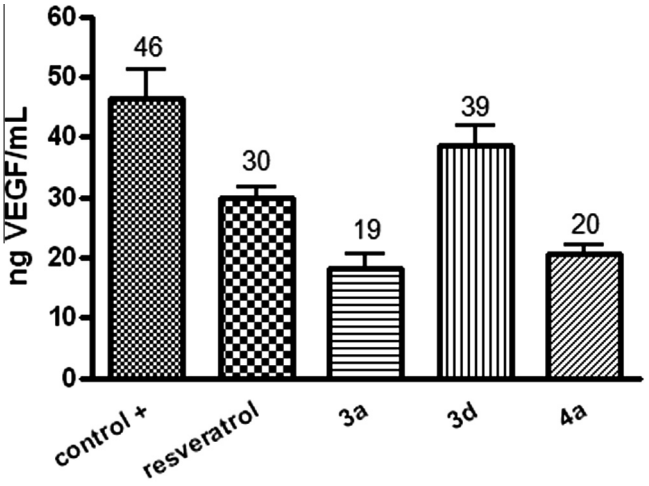


Figure 4. Expression of VEGF from HT-29 cells treated with DMSO, resveratrol, **3a**, **3d** and **4a**. At least three measurements were performed in each case. Bars shown represent mean values of VEGF expression (in ng/mL) and error bars indicate standard errors of the mean. Statistical significance was evaluated using one-sample *t*-tests (P <0.001).

the value observed with DMSO (control +), compounds **3a** and **4a** cause a decrease in the VEGF production to a higher extent (to about only 41% of the control + value, see Figure 4).

A comparison of data obtained from ELISA experiments with those obtained in relation to VEGF gene transcription indicates that, except for compound **4a**, there is no correlation between VEGF mRNA levels and VEGF secreted levels. Indeed, compound **4a** strongly suppressed the expression of VEGF gene whereas compound **3a** only caused a weak suppression (50% vs 91%, Fig. 3). However, the secreted VEGF levels in the media were very similar (Fig. 4). In addition, compound **3d** suppressed the expression of VEGF mRNA to a moderate extent compared with resveratrol (70% vs 86%, Fig. 3) but the secreted VEGF level was higher than of the latter compound. These results indicate on one hand that compound **4a** does indeed cause decrease in VEGF production by means of a downregulation in the expression of the corresponding gene. On the other hand, the results also suggest that **3a** and resveratrol lead to a decrease in VEGF production through interference at a different phase in the genesis of the growth factor.

It is worth noting here that stilbene derivative **4a** is able to decrease VEGF transcription by 50% at a concentration of 10 μg/mL (compared with 86% for resveratrol at the same concentration), clearly below its IC_{50} value with BAE cells (Fig. 3 and Table 1). This indicates that **4a** retards tumor angiogenesis at non toxic concentrations by means of reduction in VEGF production in tumoral cells and, consequently, by complete inhibition of capillary-like tube formation. These features suggest that **4a** may be a potentially valuable anticancer agent with fewer side effects than other anticancer agents. Moreover, the fact that compound **4a** presents no free hydroxyl groups could be an advantage because it may exhibit a lower metabolization rate as it is not able to form glucuronide conjugates.

6. Materials and methods

6.1. Chemistry: general procedures

General features. NMR spectra were measured at 25 °C. The signals of the deuterated solvent ($CDCl_3$) were taken as the reference. Multiplicity assignments of ^{13}C signals were made by means of the DEPT pulse sequence. IR spectra were measured as KBr pellets. Commercially available reagents were used as received.

6.2. Reaction conditions

6.2.1. Heck couplings

Conditions ***A***.[23] The synthesis of 4-methoxystilbene **3e** is described as a representative example: In a 10-mL glass tube was placed 4-bromoanisole **2e** (125 μL, 1 mmol), styrene (230 μL, 2 mmol), K_2CO_3 (511 mg, 3.7 mmol), tetra-*n*-butylammonium bromide (322 mg, 1 mmol), palladium stock solution (0.4 mL of a 1000 ppm solution in water), and water (1.6 mL) to give a total volume of water of 2 mL and a total palladium concentration of 200 ppm. The vessel was sealed with a septum, shaken, and placed into the microwave cavity. Initial microwave irradiation of 70 W was used, the temperature being increased from room temperature to the final value of 170 °C. Once this was reached, the reaction mixture was held at this temperature for 10 min. After allowing the mixture to cool to room temperature, the reaction vessel was opened and the contents poured into a separating funnel. Water (30 mL) and ethyl acetate (30 mL) were added and the organic material was extracted and removed. After further extraction of the aqueous layer with ethyl acetate, combination of the organic layers and dessication over anhydrous $MgSO_4$, the ethyl acetate was removed in vacuo leaving **3e** in 88% yield. The product was characterized by comparison of its physical and spectral data with those in the literature.[22,23]

The same conditions were used for the synthesis of stilbenes **3a**–**d** from the corresponding bromoarenes **2a**–**d**. Yields were: **3a** (63%), **3b** (68%), **3c** (26%), and **3d** (81%). Products were character-

ized by comparison of their physical and spectral data with those in the literature.[22,23]

Conditions ***B***.[24] The synthesis of 2-hydroxystilbene **3a** from 2-iodophenol **2f** is described as a representative example: 2-iodophenol (115 μL, ca. 1 mmol), styrene (172 μL, 1.5 mmol), tri-*n*-butylamine (480 μL, 2 mmol), tetra-*n*-butylammonium bromide (322 mg, 1 mmol) were suspended in water (4 mL). The mixture was the stirred to homogeneity and then treated with $Pd(NH_3)_2Cl_2$ (3 mg, 0.015 mmol). The mixture was heated at reflux (bath temperature, 140 °C) for 24 h, then cooled and extracted three times with EtOAc. The organic layers were dried on anhydrous $MgSO_4$ and filtered. Removal of volatiles under reduced pressure gave a solid (119 mg, 86%), which was shown to be **3a** by comparison of their physical and spectral data with those in the literature.

The same conditions were used for the synthesis of stilbenes **3b** and **3c** from the corresponding iodoarenes **2g** and **2h**. Yields were: **3b** (65%) and **3c** (97%). For stilbenes **3d** and **3e**, bromoarenes **2d** and **2e** were used instead of the corresponding iodoarenes. Yields were: **3d** (65%) and **3e** (78%).

6.2.2. *O*-Allylation of phenols

A solution of **3a** (196 mg, 1 mmol) in acetone (15 mL) was treated with allyl bromide (260 μL, 3 mmol) and K_2CO_3 (415 mg, 3 mmol). The mixture was stirred at reflux under N_2 for 24 h. Subsequently, the mixture was filtered through silica gel, with additional washing of the silica gel pad with EtOAc. Removal of all volatiles under reduced pressure gave **4a** (187 mg, 79%). The product was characterized by means of comparison of its physical and spectral data with those in the literature.[27]

Under the same conditions, stilbene **3c** was converted into its *O*-allyl derivative **4b** in 90% yield: white solid, mp 120–121 °C; ^{1}H NMR (500 MHz) δ 7.48 (2H, br d, $J \sim 7.4$ Hz), 7.43 (2H, br d, $J \sim 8.8$ Hz), 7.33 (2H, t, $J \sim 7.6$ Hz), 7.22 (1H, tt, $J \sim 7.6$, 1.5 Hz), 7.05 (1H, d, $J = 16.5$ Hz), 6.96 (1H, d, $J = 16.5$ Hz), 6.90 (2H, br d, $J \sim 8.8$ Hz), 6.05 (1H, ddt. $J = 17.5$, 10.4, 5.5 Hz), 5.42 (1H, dq, $J = 17.5$, 1.5 Hz), 5.29 (1H, dd, $J = 10.4$, 1.5 Hz), 4.54 (2H, dt, $J = 5.5$, 1.5 Hz); ^{13}C NMR (125 MHz) δ 158.3, 137.6, 130.3 (C), 133.2, 128.6 (2×), 128.2, 127.7 (2×), 127.2, 126.7, 126.3 (2×), 115.0 (2×) (CH), 117.7, 68.9 (CH_2); HR EIMS *m/z* 236.1206 (M^+). Calcd for $C_{17}H_{16}O$, 236.1201.

6.3. Biological procedures

6.3.1. Reagents and cell culture

Cell culture media were purchased from Gibco (Grand Island, NY, USA) and Biowhittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, U.K.). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark). Stilbenes **3a**–**e** and **4a**–**b** (samples purified by crystallization) as well as resveratrol were dissolved in DMSO at a concentration of 10 mg/mL and stored at −20 °C until use.

BAE cells were obtained as reported[28] by collagenase digestion. Human colon adenocarcinoma (HT-29) cells were obtained from American Type Culture Collection. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 μg/mL) and amphoterycin (1.25 μg/mL), supplemented with 10% FBS.

6.3.2. Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates was used, as previously described.[29] Some 3×10^3 BAE cells or 5×10^3 in case of HT-29 cells in a total volume of 100 μL of their respective growth media were incubated with serial dilutions of the tested compounds. After 3 days of incubation (37 °C, 5% CO_2 in a humid atmosphere) 10 μl of MTT (5 mg/ml in PBS) were added to each well and the plate was incubated for further 4 h (37 °C). The resulting formazan was dissolved in 150 μL of 0.04 N HCl/2-propanol and read at 550 nm. All determinations were carried out in triplicate. IC_{50} values mean the concentration of compound yielding a 50% of cell survival.

Table 2
Primers used and sizes of the PCR products

VEGF	Sense: 5′-CCTGATGAGATCGAGTACATCTT-3′ Antisense: 5′-ACCGCCTCGGCTTGTCAC-3′	379
β-Actin	Sense: 5′-TCATGAAGTGTGACGTTGACATC CGT-3′ Antisense: 5′-CGTAGAAGCATTTGCGGTGCAC GATG-3′	287

6.3.3. Endothelial cell differentiation assay: tube formation on Matrigel

Matrigel (50 μL of about 10.5 mg/mL) at 4 °C was used to coat each well of a 96-well plate and allowed to polymerize at 37 °C for a minimum of 30 min as previously described.[30] Some 5×10^4 BAE cells were added with 200 μL of DMEM. Finally, different amounts of the tested compounds were added and incubated at 37 °C in a humidified chamber with 5% CO_2. After incubation for 7 h, cultures were observed (200× magnifications) and photographed with a NIKON inverted microscope DIAPHOT-TMD (NIKON Corp., Tokyo, Japan). Two different observers evaluated the results of tube formation inhibition.

6.3.4. RT-PCR analysis

HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated with 20 μg/mL resveratrol in DMSO and with 10 μg/mL stilbene **3b** in DMSO for 48 h. Cells were collected and the total cellular RNA from HT-29 cells was isolated using Ambion RNA extraction Kit according to the manufacturer's instructions. The cDNA was synthesized by MMLV-RT with 1–21 μg of extracted RNA and oligo(dT)15 according to the manufacturer's instructions. Gene-specific PCR primers (see Table 2) were then added for amplification. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining under UV transillumination. The sequences of primers used in the RT-PCR are listed in Table 2. The PCR conditions were as follows: VEGF at 94 °C for 30 s, at 58 °C for 1 min, and at 72 °C for 1 min 50 s; and β-actin at 94 °C for 30 s, at 58 °C for 50 s, and at 72 °C for 50 s. Analysis of β-actin was used to monitor RNA integrity and accuracy of loading.[31]

6.3.5. ELISA analysis

HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated with 20 μg/mL resveratrol in DMSO and with 10 μg/mL of the corresponding stilbene in DMSO for 72 h. Culture supernatants were collected and VEGF secreted by HT-29 cells was determined using Invitrogen Human Vascular Endothelial Growth Factor ELISA Kit according to the manufacturer's instructions.

Acknowledgments

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