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Anti-tumor Properties of *cis*-Resveratrol Methylated Analogues in Metastatic Mouse Melanoma Cells

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Abstract

Resveratrol (*E*-3,5,4'-trihydroxystilbene) is a polyphenol found in red wine that has been shown to have multiple anti-cancer properties. Although *cis* (*Z*) and *trans* (*E*) isomers of resveratrol occur in nature, the *cis* form is not biologically active. However, methylation at key positions of the *cis* form results in more potent anti-cancer properties. This study determined that synthetic *cis*-polymethoxystilbenes (methylated analogues of *cis*-resveratrol) inhibited cancer-related phenotypes of metastatic B16 F10 and non-metastatic B16 F1 mouse melanoma cells. In contrast with *cis or trans*-resveratrol and *trans*-polymethoxystilbene which were ineffective at 10 μM, *cis*-polymethoxystilbenes inhibited motility and proliferation of melanoma cells with low micromolar specificity (IC50 <10 μM). Inhibitory effects by *cis*-polymethoxystilbenes were significantly stronger with B16 F10 cells and were accompanied by decreased expression of β-tubulin and pleckstrin homology domain-interacting protein, a marker of metastatic B16 cells. Thus, *cis*-polymethoxystilbenes have potential as chemotherapeutic agents for metastatic melanoma.

Keywords

polymethoxystilbenes; cell motility; proliferation; β-tubulin; PHIP; anti-cancer

Introduction

Melanoma is a deadly form of skin cancer that is often related to excessive exposure to ultraviolet light. In view of the rising incidence of melanoma over the past decade, research has focused on approaches using drugs that would prevent metastasis of the primary lesion (1). In recent years, activating mutations of B-raf and N-ras were implicated in mechanisms that drive proliferation and metastasis of 50% of human melanomas (2). These enzymes are now being used successfully in the clinic as targets for small molecule inhibitors and immunotherapies. However, for melanomas that express wildtype B-raf and N-ras and therefore arise from other, undefined mechanisms, there is a need for alternate strategies. In particular, *trans*-resveratrol (*E*-3,5,4'-trihydroxystilbene, **1**; Chart 1), a polyphenol found in

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red wine, and its related analogues are being investigated as potential drugs for melanoma and other cancers (3-6).

Although **1** was once thought to act exclusively as a free radical scavenger and thus as an anti-oxidant, its pro-oxidant effects (resulting in oxidative breakage of DNA) are apparent at low micromolar concentrations. In its pro-oxidant role, resveratrol (4-8 μM) treatment of human leukemia cells leads to increased superoxide levels (7). Resveratrol can cause pro-oxidant effects in cultured tumor cells (8), and a protective effect in neuronal cells by inducing pro-oxidant heme oxygenase (9). It has been argued that its pro-oxidant effects may be part of a general anti-cancer and anti-aging mechanism of plant polyphenolic compounds. The dual nature of resveratrol as anti-oxidant or pro-oxidant is thought to be dependent on hydroxylation pattern, concentration and cell type, as previously discussed (10).

As an anti-cancer agent, resveratrol (**1**) has been studied extensively for its anti-inflammatory and anti-proliferative effects (11-13). Multiple cellular targets include the estrogen and androgen receptors (14), Akt/PKB (4), protein kinase C (15,16), ribonucleotide reductase (12,17), mTOR (13,18), and tubulin (19). In addition, **1** was found to be a radiation sensitizer for melanoma (20). In view of its multifocal effects, resveratrol and its synthetic analogues have potential therapeutic value for the treatment of certain cancers (16).

Resveratrol was shown to be selectively inhibitory of drug-resistant human melanoma cells DM738 and DM443 (at 50 μM) in comparison with normal dermal cells (5), and to inhibit highly invasive B16 F10 and B16 BL6 mouse melanoma cells (at 100 μM) (4). With regard to other cancer cells, resveratrol (at 50 μM) was demonstrated to have anti-estrogenic and anti-migration effects with metastatic human breast cells (14), and (at 1 μM) to promote apoptosis of human prostate cells via the MAPK pathway (21). Efforts to use resveratrol (**1**) in a mouse xenograft model were compromised by the difficulty in maintaining a high serum level of this drug over time due to its rapid clearance and metabolism (5). Consequently, analogues of resveratrol that have enhanced bioavailability are needed (22,23).

Although both *cis* (*Z*) and *trans* (*E*) isomers of resveratrol occur in nature, the *cis* form (*Z*-3,5,4’-trihydroxystilbene; **2**) exhibits little biological activity. However, recent reports identified several methylated derivatives of both **1** and **2** that display greatly improved bio-activity with cultured cancer cells. The trimethylated cognate of *trans*-resveratrol (*E*-3,5,4’-trimethoxy stilbene; **3**) proved to be a potent inhibitor of cell motility and invasion of hepatocarcinoma cells ($IC_{50} \leq 1$ μM) (24). In other studies, the trimethylated cognate of *cis*-resveratrol (*Z*-3,5,4’-trimethoxystilbene; **4**) was tested for anti-tumor activity. With IC_{50} = 300 nM, **4** was nearly 100-fold more effective than **3** in inhibiting proliferation of colon carcinoma cells (25, 26). Other modifications of the aromatic rings of resveratrol have been developed, notably addition of more hydroxy groups or acyloxy groups as substituents were found to be effective. In this regard, *E*-3,4,5,4’-tetrahydroxystilbene (at 500 nM) activated apoptosis in transformed fibroblasts with 100-fold higher potency than in non-transformed

fibroblasts (27), while *trans*-resveratrol analogues bearing various acyl residues at C-4' were effective in the 10-50 μM range with human melanoma and pancreatic cancer cells (28).

Since resveratrol was shown to interact directly with tubulin protein monomers and microtubules, the structure of tubulin has been used as a template to correlate with the cellular potency of an analogue with its docking characteristics at the colchicine binding site in β-tubulin (26). This site was previously established to be a target for mitotic inhibitors of microtubule polymerization and depolymerization. Unlike the resveratrol-related *trans*-polymethoxystilbenes, most of the *cis*-polymethoxystilbenes, such as **4**, produced potent anti-proliferative effects with human colorectal SW480 cells. Interestingly, in docking studies, **4** and the other *cis*-analogues largely overlapped the docked configuration of combretastatin A-4 at the colchicine binding site, implicating this region in β-tubulin as a potential target.

The development of analogues of resveratrol with improved biological activity continues to be a focus of current research (29). The present study explores the effects of several resveratrol-related *cis*- and *trans*-polymethoxystilbenes on metastatic mouse melanoma B16 F10 cells, non-metastatic B16 F1 cells, and immortalized melanocytes all which were isolated from C57BL/6 mice (30, 31). Because both B16 cell lines express wildtype B-raf and wildtype N-ras (32, 33), they offer a model system for studying alternative mechanisms that give rise to malignant melanoma. For the studies presented here, *cis (Z)* and *trans (E)* isomers of resveratrol are compared with their polymethylated analogues for differential effects on motile behavior and proliferation of melanoma cells.

Materials and Methods

Materials

Cell culture media, fetal bovine serum, antibiotics, trypsin, and Alamar Blue were purchased from Life Technologies (Carlsbad, CA). Rabbit polyclonal antibodies for β-tubulin and β-actin were obtained from Cell Signaling Technology (Beverly, MA) and rabbit polyclonal anti-PHIP was purchased from Bethyl Laboratories (Montgomery, TX). Secondary antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein dye reagent and protein markers were from Bio-Rad (Hercules, California). Chemiluminescence reagents (West Pico Super Signal) were purchased from Pierce (Rockford, Il). *Trans*-resveratrol (**1**), protease inhibitors, phosphatase inhibitors, phenylthiourea, and tetradecanoyl phorbol acetate were purchased from Sigma-Aldrich (St. Louis, MO). *Trans*-resveratrol (**1**), *cis*-resveratrol (**2**), *trans*-3,5,4'-trimethoxystilbene (**3**) and *cis*-3,5,4'-trimethoxystilbene (**4**) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Immortalized mouse melanocytes (melan-a cells) were obtained from the Wellcome Trust Functional Genomics Cell Bank and the laboratory of Dr. Dorothy Bennett (St. George's, University of London, UK).

Chemistry

The *cis*-resveratrol methylated analogues **5**-**8** were synthesized through an Arbuzov rearrangement followed by a Horner-Emmons-Wadsworth reaction, according to a

procedure previously employed by some of us (26). The main product of this reaction was a *trans* stilbenoid (compounds **9**-**12**) that was subsequently converted to its *cis* isomer (compounds **5**-**8**) by photo-isomerization. Namely, compound **5** was obtained by photo-isomerization of **9** in turn obtained by reaction of the diethyl (4-methoxybenzyl)-phosphonate (prepared from 4-methoxybenzylchloride) with 3,4-dimethoxybenzaldehyde. Compound **6** was prepared by photo-isomerization of **10** in turn obtained by reaction of diethyl (3,5-dimethoxybenzyl)-phosphonate (prepared from 3,5-dimethoxybenzylbromide) with 3,4-dimethoxybenzaldehyde. Compound **7** was obtained by photo-isomerization of **11** in turn obtained by reaction of the diethyl (4-methoxybenzyl)-phosphonate (prepared from 4-methoxybenzylchloride) with 3,4,5-trimethoxybenzaldehyde. Compound **8** was obtained by photo-isomerization of **12** in turn obtained by reaction of diethyl (3,5-di-methoxybenzyl)-phosphonate (prepared from 3,5-dimethoxybenzylbromide) with the 3,4,5-trimethoxybenzaldehyde. Irradiation experiments were performed using a Rayonet photochemical reactor, as previously reported (34). All photo-isomerizations were obtained with 80-82% conversion to the *Z* isomer. Each mixture was resolved by silica gel PLC column chromatography using a gradient of EtOAc-*n*-hexane to separate the *Z* product from the residual *E* isomer. Spectral data of compounds **5** - **8** are in perfect agreement with those reported in the literature (35-37).

Cell-based Assays

Cell culture—B16 cells (F1 and F10) were cultured in 60-mm or 100-mm Falcon dishes containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% penicillin/streptomycin and 0.1% fungizone. Cells were grown at 37°C in a 5% CO_2 atmosphere and sub-cultured twice per week. In the same growth medium, immortalized mouse melanocytes (melan-a cells) were cultured at 37°C with 10% CO_2 in the presence of 200 nM tetradecanoyl phorbol acetate (TPA). Prior to the experiment, cells were plated and incubated overnight. The next day, the cells were washed with PBS, and fresh medium was restored. Each compound was added to cells at the indicated concentration and incubated at 37°C and 5% CO_2 prior to the experiment.

Preparation of cell lysates—Cells were lysed in 0.25 mL lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100) containing phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/μl leupeptin) and protease inhibitors. Cells were sonicated three times for 10 s and centrifuged at 10000 × g for 10 min to remove insoluble material. The resulting lysate was analyzed for protein content using the Bio-Rad reagent and bovine serum albumin as standard.

Western blot analysis—Cell lysates were denatured by heating in sample buffer at 95 °C for 5 min. Samples were resolved by 7.5% SDS-PAGE gel and transferred to a PVDF membrane (Millipore; Billerica, MA). The membrane was incubated overnight with antibodies to β-tubulin. After washing the membrane with TBST (150 mM NaCl, 20 mM Tris pH 7.4, 0.1% Tween 20, v/v), the appropriate horseradish-peroxidase conjugated secondary antibody was added and the membrane was incubated at 4°C for 1-2 h. After

washing three times with TBST, the membrane was visualized with reagents for chemiluminescence.

Motility assay—Measurements were carried out with a 10-well glass slide and a pre-chilled cell sedimentation manifold (Creative Scientific Methods Inc., Phoenix, AZ). Cells (4×10^3/well) were pipetted into the manifold in triplicate enabling their sedimentation onto the slide as a small concentric circle. After incubation for approximately 16 h at 37 °C and 5% CO_2, the manifold was removed (t=0) allowing the cells to radiate outwardly. At this time the media in each well was replaced with media containing 10 μM of a resveratrol analogue or DMSO (0.1%, v/v). Both at t=0 and following incubation with the analogues for 18 h at 37 °C, images of the cells were recorded and analyzed using Motic Image 2.0 software. The motility of each cell sample was judged by the increase in area occupied by the cells and averaging the values for triplicate wells.

Wound healing assay—Cells were plated in a 6-well plate (Falcon) and grown for 24-48 hours until a dense monolayer was achieved. Two parallel lines were drawn with colored markers on the undersurface of the plate to identify the area to be analyzed. A “scratch wound” of uniform width was created in this region by drawing a sterile micropipette tip across the monolayer using a ruler as a guide. Triplicate wells were washed twice with serum-free media before adding 1 ml serum-free medium containing 10 μM of the resveratrol analogue. By use of a camera (Motic Image) attached to a phase contrast Nikon microscope, the area of the wound was measured in 4-8 fields/well at t=0 and t=5 h and the difference in the occupied area was measured with Motic Image 2.0 software.

Cell proliferation assay—Proliferation of cells treated with selected analogues was measured by using Alamar Blue (Life Technologies) in a fluorescence-based assay (38). B16 F10 cells or B16 F1 cells were added to a 96-well plate (3×10^3 cells/200 μl) and incubated for 4 h at 37°C and 5% CO_2. Melan-a mouse melanocytes (9×10^3 cells/200 μl) were added to a 96-well plate and incubated for 2 h at 37°C and 10% CO_2 in complete growth medium. The growth medium was replaced with medium containing 10 μM resveratrol or resveratrol analogue or the vehicle control (0.1%, v/v) which was added to six replicate wells. Wells containing growth medium without cells served as the blank. Unless otherwise specified, cells were incubated for 40 h at 37°C, followed by addition of Alamar Blue to a final concentration of 5% (v/v). After 3 h at 37°C, the fluorescence was measured using a plate reader (SpectraMax M5 Microplate Reader) set at λ_{ex} = 544 nm and λ_{em} = 590 nm. The number of cells measured was within the range of cells that gave a linear response to Alamar Blue, and therefore was directly proportional to the number of actively metabolizing cells.

Statistical analysis—Statistical significance was judged by applying the Welch’s unpaired two sample *t*-test.

Results

Comparison of trimethoxystilbenes 3 and 4, analogues of *cis-(Z)-* and *trans-(E)-*resveratrol, on motility of B16 F10 cells

The replacement of the hydroxy groups with methoxy groups on the aromatic rings in resveratrol structure can engender increased biological activity, as previously shown. In the initial experiment, the effect of 50 μM *trans*-resveratrol (**1**) and *cis*-resveratrol (**2**) and their corresponding trimethylated analogues (namely *E*-3,5,4'-trimethoxystilbene **3** and *Z*-3,5,4'-trimethoxystilbene **4**) (Chart 1) was tested in an assay of cell motility. As shown in Figure 1A, 50 μM of both **1** and **2** were without effect on the motility of F10 cells. However, 50 μM of **3** and **4** produced significant decreases in motility, where **4** was the more potent inhibitor. Compared to the control, the effect by **4** was statistically significant. A dose-response curve, shown in Figure 1B, indicated that **4** inhibited F10 cell motility with IC_{50} = 10 μM. This curve was biphasic in nature as it consisted of a high affinity component produced by the initially steep slope at 1 μM (the lowest concentration of **4** that was tested), and a lower affinity component as shown by the shallow slope of the curve at concentrations above 1 μM.

It was further observed that when F10 cells were treated with **4** at 10 μM, there was substantial loss of β-tubulin protein, as demonstrated by the Western blot in Figure 2. However, the parent resveratrol isomers **1** and **2**, as well as the *trans*-trimethoxystilbene **3** had no detectable effect on β-tubulin expression. It was noted that treatment of non-metastatic F1 cells with **4** also led to decreased expression of β-tubulin, whereas **1**-**3** had no effect (Figure 2). Analyzed in the same blot was pleckstrin homology interacting protein (PHIP), a recently identified marker of metastatic B16 cells (39). In F10 cells, PHIP was observed to be down-regulated slightly by **1** and **2**, but a stronger inhibitory effect was observed with trimethoxystilbenes **3** or **4**, irrespective of their geometry. However with F1 cells, PHIP was seen to be more resistant to the effects of **1**-**4**.

Testing of additional polymethoxystilbene analogues of *cis*-(*Z*)-resveratrol 5 – 8 on motility of F10 cells

In view of the impact of trimethoxystilbene **4** on F10 cell movement at low micromolar concentrations, additional polymethoxystilbene analogues of *cis*-resveratrol were synthetized, namely *cis*-3,4,4'-trimethoxystilbene (**5**), *cis*-3,5,3',4'-tetramethoxystilbene (**6**), *cis*-3,4,5,4'-tetramethoxystilbene (**7**) and *cis*-3,4,5,3',5'-pentamethoxystilbene (**8**). These compounds were evaluated for their effect on the motility of B16 F10 cells to establish the substitution pattern that improves upon the properties of **4**. As can be observed in a wound healing assay (Figure 3A), compounds **5** - **8** (at 10 μM) substantially inhibited cell motility relative to the control (Figure 3B). Trimethoxy- (**5**) and pentamethoxy- (**8**) analogues were similar to **4** in that they produced inhibition in the 50-60% range. However, tetramethoxy analogues **6** and **7** showed a somewhat higher potency of inhibition (80-90%). These findings suggest that a tetramethoxylated *cis*-stilbenoid is more effective in inhibiting motility than either a tri- or pentamethoxy *cis*-analogue.

Tubulin protein content was evaluated following treatment with 10 μM concentration of the *cis*-polymethoxystilbenes **4**–**7**. Compared to the control, dramatic decreases in intracellular β-tubulin protein were observed with all tested *cis*-polymethoxystilbenes and in the case of **6**, the level of expression fell below detectable limits (Figure 3C).

Effects of resveratrol compounds on proliferation of B16 melanoma cells and melanocytes

Compound **4** had previously been described as anti-proliferative towards colon carcinoma cells and colorectal cells (25, 26, 35, 36). Resveratrol compounds (**1**,**2**) and their trimethoxystilbenes (**3**,**4**) were tested at 10 μM for 40 h to determine their effects on proliferation of F10 and F1 melanoma cells, and melanocytes. In a comparison of the four compounds (Figure 4), **4** was the most effective in decreasing proliferation of F10 cells (hatched bars), F1 cells (dark grey bars), and melanocytes (light grey bars). Relative to control-treated cells, **4** produced 75% inhibition of proliferation with F10 cells, whereas it produced 50-60% inhibition with F1 cells, and only 20-25% inhibition of melanocytes. These results suggest that these cells exhibit differential sensitivity to the anti-proliferative effects of **4**.

Differential effects of *cis*- and *trans*-polymethoxystilbene analogues on proliferation of F10 melanoma cells

In a proliferation assay, *cis*/*trans* pairs of compounds (Chart 1) were compared at 10 μM. The *cis*-polymethoxy analogues (hatched bars) were generally found to decrease cell proliferation by 55-70% relative to control-treated cells (Figure 5), of which **4** was the strongest inhibitor of cell proliferation (71% inhibition) followed by **7** (66% inhibition), whereas analogues **5**, **6**, and **8** were significantly less potent (45% inhibition). It is notable that neither *trans*-resveratrol (**1**) nor any of the counterpart *trans*-polymethoxystilbenes (**3, 9**-**12**) (grey bars) had a significant effect on the proliferation of F10 cells.

Discussion

The findings of this investigation identified a group of synthetic polymethoxystilbenes, analogues of *cis*-resveratrol (**2**), as inhibitory to both motility (an aspect of metastasis) and proliferation of metastatic melanoma cells with effective potencies in the low micromolar range (IC_{50} = 1 - 10 μM). Our findings indicate that these methylated analogues of *cis*-resveratrol are 5-fold more effective at inhibiting motility of B16 F10 cells than previously measured with *trans*-resveratrol (**1**) (4). The impact on melanoma cells by the *cis*-analogues contrasted sharply with the lower or absent inhibitory activity observed with *trans*-resveratrol or the *trans*-trimethoxy analogue **3** (*E*-3,5,4’-trimethoxystilbene) at 10 μM. When compared against the trimethylated cognate of *cis*-resveratrol (*Z*-3,5,4’-trimethoxystilbene, **4**) as a reference compound, the present work established the potency of **4** for inhibiting motility of metastatic melanoma cells. Furthermore, we identified additional *cis*-resveratrol analogues (Chart 1) obtained by synthesis, namely *Z*-3,4,4’-trimethoxystilbene (**5**), *Z*-3,5,3’,4’-tetramethoxystilbene (**6**), *Z*-3,4,5,4’-tetramethoxystilbene (**7**) and *Z*-3,4,5,3’,5’-pentamethoxystilbene (**8**), as having similar potency in the low micromolar range. Notably, at 10 μM, inhibition of motility by (**6**) and (**7**) was reproducibly stronger than that observed for (**4**), (**5**), and (**8**). These results confirm the observation by

others (26, 40, 41) that subtle modification of the substitution pattern in resveratrol-related structure may have significant effects on their anti-tumor properties.

A surprising observation was the dramatic decrease in detectable β-tubulin protein levels in cells treated with the *cis*-polymethoxystilbenes (Figures 2 and 3B). Importantly, the loss of β-tubulin correlated with the effect on motile behavior by a given compound (Figures 1 and 3), as well as with extensive truncation of microtubule structure (unpublished observations). These findings suggest a mechanistic model for the substantial decreases observed in cell motility and proliferation. Microtubules are central to cell locomotion, and their contribution to B16 F10 cell movement was previously documented (42). The impact on microtubules by resveratrol analogues can be understood by their known binding activity with the colchicine binding site in β-tubulin. This interaction was shown to be strengthened with *cis*-polymethoxystilbenes (26), consequently preventing formation of polymers (or destabilizing existing polymers) of α/β-tubulin heterodimers. In this regard, **4** inhibited tubulin polymerization in colon carcinoma cells (CaCo-2 and SW480) with IC_{50} = 4 μM (19, 25), and **7** inhibited tubulin polymerization comparable to that of the known cytotoxic agent combretastatin A4 (36). The unexpected loss of β-tubulin expression in melanoma cells treated with polymethoxy *cis*-analogues **4** – **7** suggests that these analogues may also activate the proteasome, thereby leading to tubulin degradation. This outcome was previously attributed to agents that destabilize microtubules, particularly in neural cells (43). Since melanocytes derive from neural crest cells during development, the intrinsic nature of melanoma cells may render tubulin protein particularly susceptible to degradation by polymethoxy *cis*-resveratrol analogues. Alternatively, transcriptional down-regulation of tubulin mRNA may occur as proposed by others who tested trimethoxy- and tetramethoxystilbene analogues of *trans*-resveratrol in hepatoma cells (24, 44). These possible modes of action provide avenues for further investigation with polymethoxy *cis*-resveratrol compounds.

To explore additional targets of the *cis*-methoxystilbenes, we compared **1**-**4** for their effects on a molecular marker of metastatic melanoma. In this regard, 10 μM **3** or **4** led to substantial decreases in the expression of PHIP (Figure 2). Down-regulation of this protein by *cis*- trimethoxystilbene **4** correlated with the observed inhibition of motility, an aspect of metastatic behavior. However, the observation that both *cis* (**4**) and *trans* (**3**) trimethoxystilbenes were similarly effective with F10 cells implied that PHIP down-regulation was independent of the geometry of the aromatic rings in contrast with β-tubulin down-regulation which was produced exclusively by the *cis*-polymethoxystilbenes.

Additional analysis showed that ROS production was not altered in F10 or F1 cells that had been treated with 10 μM of **1**-**4** (unpublished observations). Thus, at low micromolar concentrations, neither pro-oxidant nor anti-oxidant activities of resveratrol compounds were evident in melanoma cells (unpublished observations).

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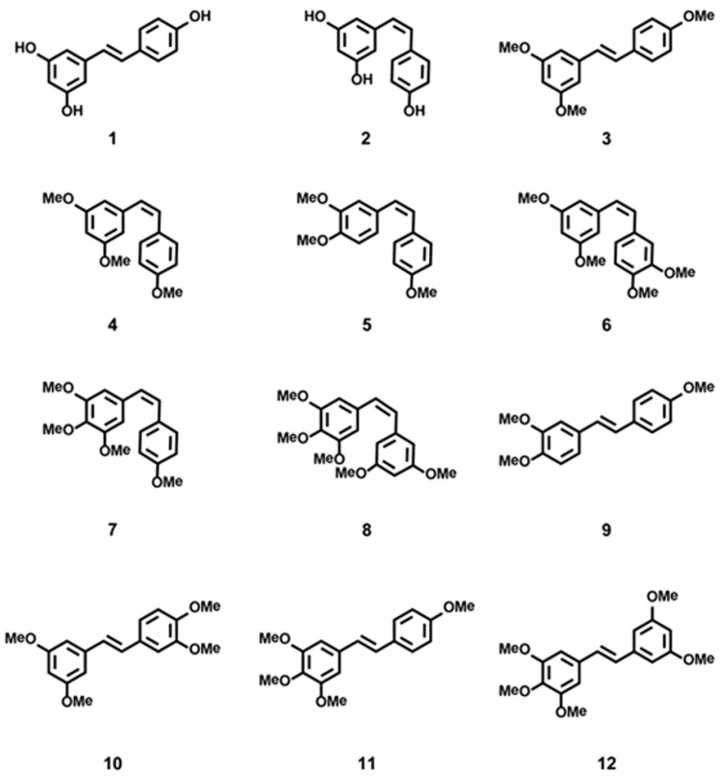

Chart 1.

Chemical structures of *cis*- and *trans*-resveratrol (**1**-**2**) and their *cis*- (**4** - **9**) and *trans*- (**3**, **10**-**13**) polymethoxystilbene analogues.

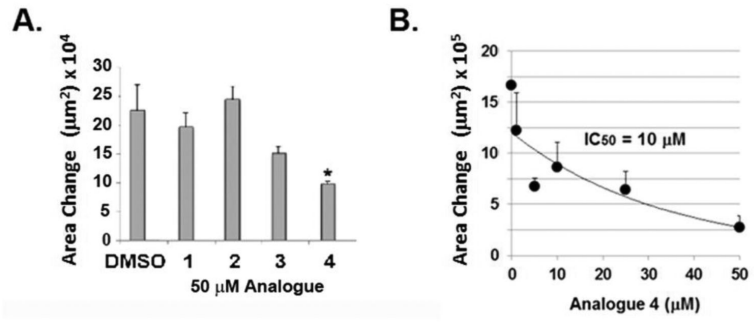

Figure 1.

Impact on motility of B16 F10 melanoma cells by *trans*- and *cis*-resveratrol and their methylated analogues. A. Cell motility was measured by the cell sedimentation assay. Triplicate samples of B16 F10 cells were treated with 50 μM *trans*-resveratrol (**1**), *cis*-resveratrol (**2**), or their corresponding methylated analogues **3** and **4**. The vehicle control was DMSO (0.1%, v/v). This experiment was performed three times with similar results. (*, $P < 0.05$) B. Dose dependence of **4** was tested in triplicate at increasing concentrations (0 - 50 μM) and the effect on cell motility was measured.

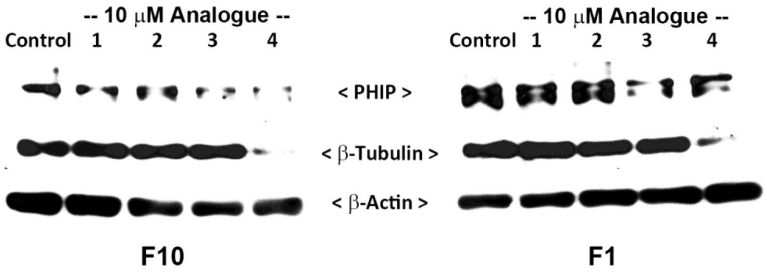

Figure 2.

Effects of resveratrol and *cis (Z)*- and *trans (E)*-trimethoxystilbene analogues (**1-4**) on β-tubulin and PHIP expression. Following treatment of cells with the indicated compound at 10 μM or ethanol (0.1%, v/v) for 18 h, cell lysates were prepared and an aliquot of each sample (10 μg per lane) was resolved by 8% SDS-PAGE, transferred to a PDVF membrane, and probed with anti-β-tubulin (1:2000), anti-PHIP (1:2000), or anti-β-actin (1:5000) (loading control), followed by HRP-conjugated anti-rabbit secondary antibody and chemiluminescence detection. The results are representative of three independent experiments.

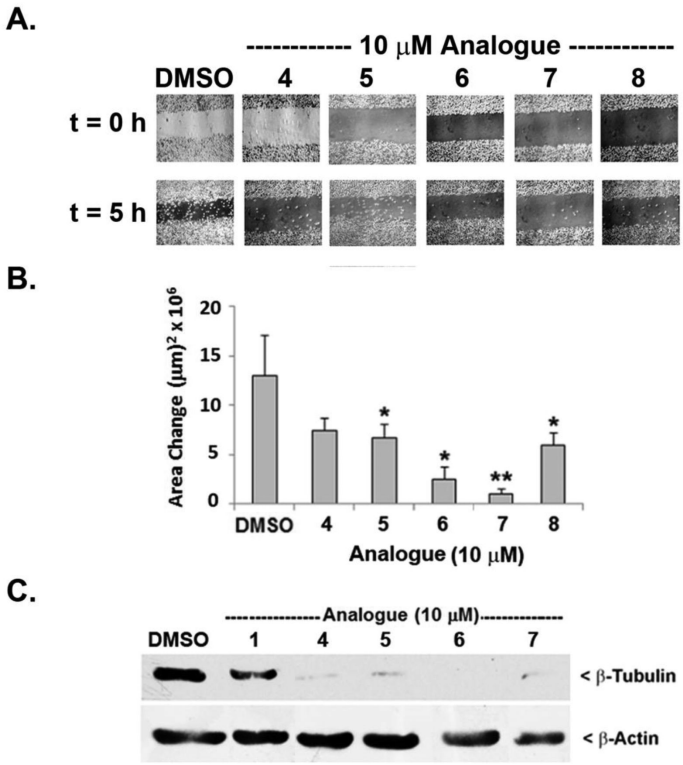

Figure 3.

Testing of additional *cis* (*Z*)-polymethoxystilbenes on cell motility and β-tubulin expression. A,B. B16 F10 cells that had been treated with 10 μM of the indicated analogue or DMSO (0.1% v/v) were tested for cell motility over a period of 18 h in a wound healing assay, images were taken at t=0 and t=5h. In comparing the two time points, motility was determined by the change in the wound area (A) for 4-8 microscope fields per well and averaged for each treatment. Statistical significance was determined by comparing analogue-treated cells with the DMSO control (*****, $P < 0.1$; ******, $P < 0.01$). C. Western blot analysis of lysates (50 μg protein per lane) that had been prepared from cells treated for 18 h with 10 μM of the indicated analogue. The blot was probed with anti-β-tubulin (1:2000) or anti-β-actin (1:5000) followed by HRP-conjugated secondary antibody and detected by chemiluminescence. The results are representative of three independent experiments.

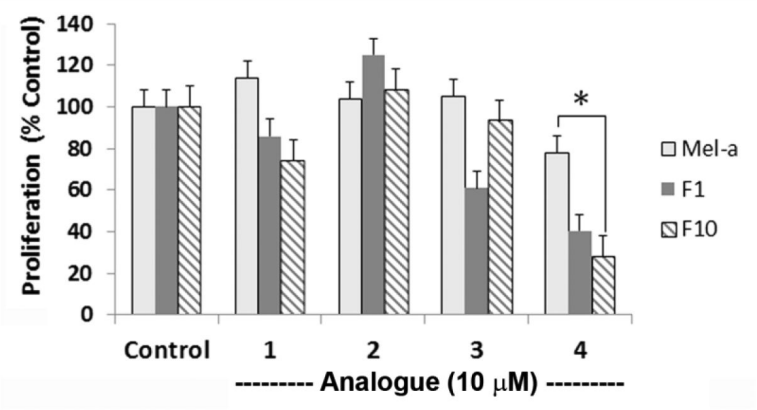

Figure 4.

The effect of resveratrol compounds (**1**-**4**) on proliferation of melanoma cells and melanocytes. Metastatic F10 cells, non-metastatic F1 cells (each plated at 3×10^3 cells per well), or melanocytes (9×10^3 cells per well) were treated for 40 h with 10 μM of the indicated analogue or an equivalent volume of ethanol (0.1%, v/v). The fluorescent signal was read after a 3-h treatment with Alamar Blue (5%, v/v). For each condition, six replicate measurements were averaged and reported as the percentage ± s.d. of the value for control-treated cells (*, $P < 0.0001$). The results are representative of two or more independent experiments.

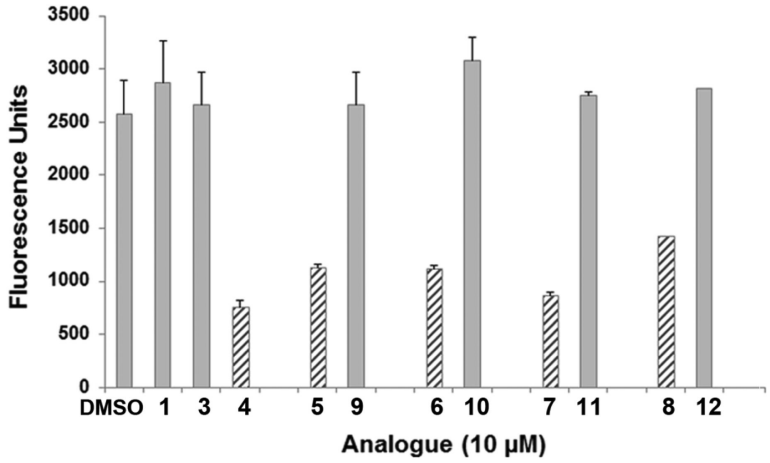

Figure 5.

Comparison of the effect of *cis (Z)*- and *trans (E)*-polymethoxystilbene isomers on the proliferation of F10 cells. Comparison of *cis* analogues (hatched bars) (**4 - 8**) and their counterpart *trans* analogues (grey bars) (**3** and **9 - 12**), where each compound was tested at 10 μM (or 0.1% DMSO, v/v) with 3×10^3 cells. After 48 h, the number of cells was analyzed by adding Alamar Blue for 3 h, followed by fluorescence detection and quantitation. Triplicate measurements were averaged and the standard deviation was calculated. The results are representative of three independent experiments.