Cytotoxic, antiangiogenic and antitelomerase activity of glucosyl- and acyl-resveratrol prodrugs and resveratrol sulphate metabolites.

Eva Falomir[1], Rosa Martí-Centelles[1], Ricardo Lucas[2], Alexia Dupont[2], Miguel Carda[1]*, Juan C. Morales[2]*

[1]*Department of Inorganic and Organic Chemistry, University Jaume I, 12071 Castellón, Spain.* [2]*Department of Bioorganic Chemistry, Instituto de Investigaciones Químicas (IIQ), CSIC-Universidad de Sevilla, 41092 Sevilla, Spain.*

Running Title: Cytotoxicity of resveratrol prodrugs and resveratrol metabolites.

*To whom correspondence should be addressed: M.C. (E-mail: mcarda@qio.uji.es) or J.C.M. (E-mail: jcmorales@iiq.csic.es).

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Abbreviations: anh., anhydrous, *t*-BuOH, *tert*-butanol, C_{max}, maximum concentration; DCM, dichloromethane; DMF, dimethylformamide; MeOH, methanol; MS, molecular sieves, MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MW, molecular weight; NMR, nuclear magnetic resonance spectroscopy; r.t., room temperature; TBDMS, *tert*-butyldimethylsilyl; THF, tetraydrofuran; TLC, thin layer chromatography.

Abstract

Resveratrol (3,5,4'-trihydroxystilbene, RES) is a natural product reported to display relevant and varied biological activity. However, its low bioavailability and rapid metabolism to its glucuronate and sulfate conjugates has opened a debate on the mechanisms underlying its bioactivity. At the same time, resveratrol derivatives and prodrugs are being developed to circumvent these difficulties. We have synthesized a series of resveratrol prodrugs and resveratrol metabolites and evaluated their cytotoxicity, VEGF expression and telomerase inhibition. One healthy (human embryonic kidney, HEK-293) and two tumoral cell lines (human colon HT-29 and breast adenocarcinoma MCF-7) were used for the assays. Cytotoxic measurements revealed that resveratrol glucosylated prodrugs **2**-**4** and piceid octanoate **7** were more cytotoxic than resveratrol itself and more interestingly piceid **2**, resveratrol-3,5-diglucoside **3**, and resveratrol-3,4'disulfate **10** combine relatively high cytotoxicity and high therapeutic safety margins. VEGF production in HT-29 cells was decreased by piceid **2** and resveratrol-3,4'disulfate **10** whereas resveratrol-3,5-diglucoside **3** and piceid octanoate **7** diminished it to a higher extent than resveratrol. Finally, **3** and **10** were also able to inhibit the expression of the hTERT gen that could be correlated to a lower transcription of the c-Myc gene for **3** but not for RES disulfate metabolite **10**. In conclusion, resveratrol prodrugs such as resveratrol-3,5-diglucoside **3** could be promising candidates as anticancer drugs. In addition, RES sulphate metabolites have shown their own biological activity indicating they are not simply RES reservoirs.

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene, **1**, see Figure 1) is a phytoalexin generated in response to environmental stress or pathogenic attack in grapes, blueberries, peanuts, cocoa and plants such as the Japanese knotweed *Polygonum cuspidatum*. Resveratrol has raised a great attention due to its relevant and **varied biological activity** such as its cardiovascular protective properties (*1*), antiinflammatory activity (*2*) or its capacity to extend lifespan in a variety of species (*3*). The **cancer** chemopreventive and chemotherapeutic potential of resveratrol has been demonstrated in different models of carcinogenesis in vitro and in vivo. It inhibits the proliferation of a variety of cancer cell lines (*4*) and in animal studies, resveratrol is able to interfere with the formation of azoxymethane-(AOM) induced aberrant crypt foci in rat colon (*5*), reduce mammary tumour formation in N-methyl-N-nitrosourea-(NMU) treated rats (*6*) or suppress prostate cancer in SV-40 tag rats (*7*). In fact, extensive in vitro studies have revealed **multiple intracellular targets** of resveratrol, which affect cell growth, inflammation, apoptosis and metastasis (*8*).

Tumor **angiogenesis** also plays a critical role in the development of cancer. Resveratrol **1** and piceid (resveratrol-3-β-glucoside, **2**) have shown to inhibit the formation of capillary-like tube formation from human umbilical vein endothelial cells (HUVEC) (*9, 10*). At the same time, vascular endothelial growth factor (**VEGF**) is crucial for angiogenesis and tumor growth, as it is involved in blood vessel development. In breast cancer cells, a significant decrease in extracellular levels of VEGF has been associated with apoptosis following resveratrol treatment (*11*).
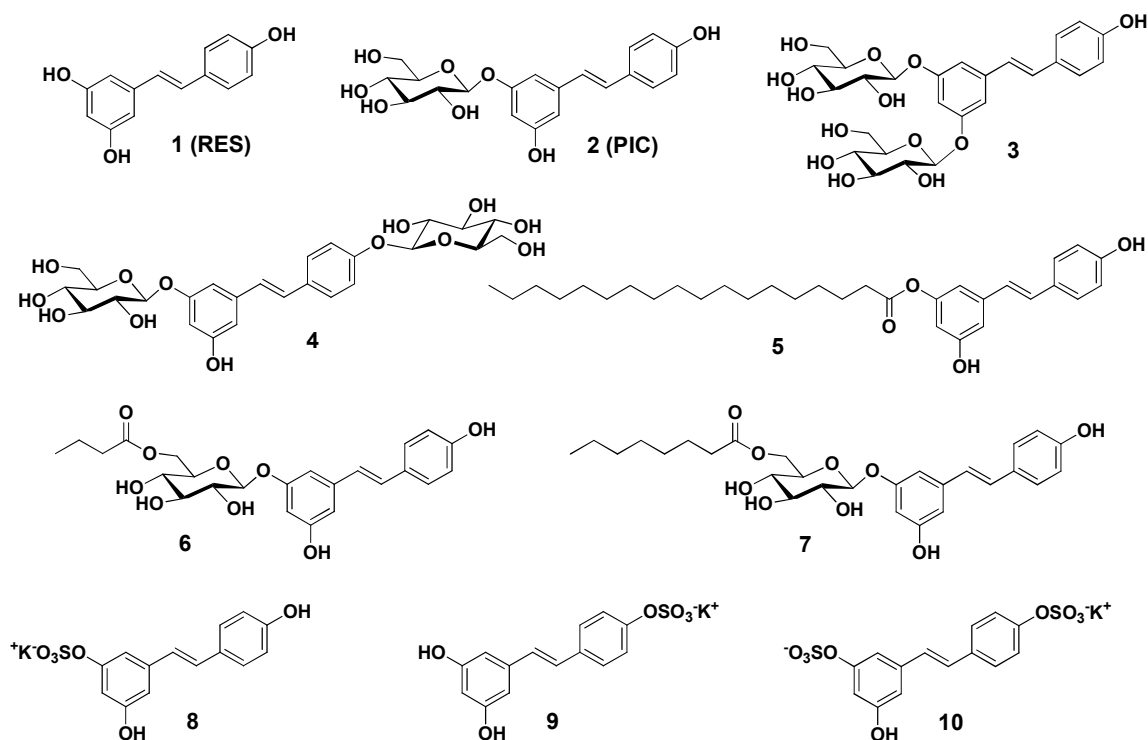
Figure 1

A relevant aspect observed in 90% of malignant tumors is the maintenance of the telomeres length within the cellular DNA through the expression of the telomerase reverse transcriptase (hTERT). This process allows cancer cells to elude the progressive shortening of the telomeres and finally avoid apoptosis. Resveratrol showed an inhibitory effect on MCF-7 tumor cell line mainly due to induce S-phase arrest and apoptosis in association with reduced expression of hTERT (*12*).

However, as is the case with many polyphenols with putative anticancer properties, the **bioavailability** of resveratrol is **very low** (*13*). Resveratrol is well absorbed but avidly glucuronidated and sulphated both in the liver and in intestinal epithelial cells (*14-17*). In order to counteract this effect, high doses of resveratrol have been used in cellular, animal and clinical studies. This could be the cause of toxicity observed in certain cases such as in a multiple myeloma study in humans where 5 grams a day of a formulation of resveratrol were administered.

The low amounts of resveratrol observed systemically have brought the debate on the possible **contribution of the resveratrol metabolites** to the in vivo biological activities attributed to resveratrol (*13, 18*). It has been proposed that resveratrol metabolites could contribute as a resveratrol reservoir through their deconjugation or that resveratrol metabolites could enter the cell using ABC transporters and interact directly with different intracellular targets (*19*).

Moreover, **resveratrol prodrugs** are being investigated as an alternative trying to increase resveratrol bioavailability (*20-24*). We had previously prepared a family of resveratrol prodrugs and pro-prodrugs attaching glucosyl- or acyl- groups to resveratrol **1** or piceid **2** and examined their anti-inflammatory effect on an inflammatory bowel disease (IBD) mice model (*25*). We observed that oral administration of *trans*-resveratrol-3-*O*-(6'-*O*-butanoyl)-β-D-glucopyranoside (**6**) and *trans*-resveratrol-3-*O*-(6'-*O*-octanoyl)-β-D-glucopyranoside (**7**) produced an increase delivery of resveratrol to the colon, prevented the rapid metabolism of resveratrol and reduced inflammation in a murine dextran sodium sulphate (DSS) model.
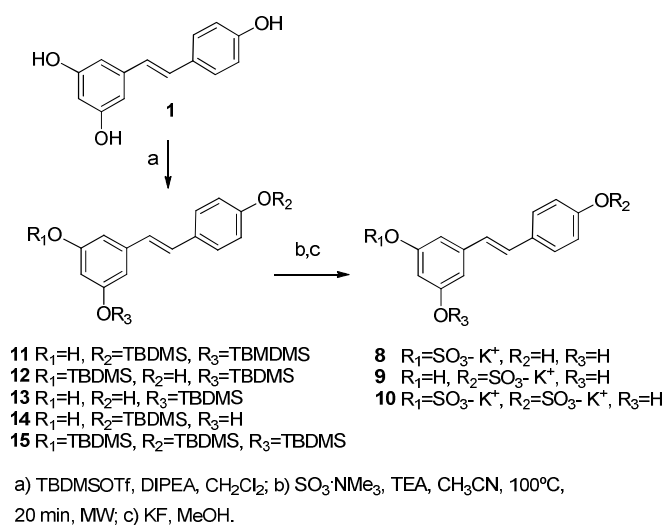
In the present study, we have measured cytotoxicity studies, VEGF expression and telomerase inhibition of five resveratrol prodrugs **3**-**7** modified with glucosyl- or acyl-groups. The idea was to find out their potential as cancer chemopreventive or chemotherapeutic agents either if they act as such or as a resveratrol reservoir. In addition, we have synthesized three resveratrol sulphate metabolites **8**-**10** using an improved synthetic route. We have also tested their biological activity and compared it with resveratrol itself trying to shed some light on the controversy of resveratrol metabolites biological activity.

CHEMISTRY

Diglucosylated resveratrol derivatives, *trans*-resveratrol-3,5-di-*O*-β-D-glucopyranoside (**3**) and *trans*-resveratrol-3,5-di-*O*-β-D-glucopyranoside (**4**) were synthesized from resveratrol (RES, **1**) as described previously (*25*). Briefly, mono-TBDMS protected resveratrol compounds obtained by random silylation of RES were glycosylated using 2,3,4,6-tetra-*O*-benzoyl-D-glucopyranosyl trichloroacetimidate as the glycosyl donor. Final one step deprotection under aqueous strong basic conditions yielded the desired products **3** and **4**. Piceid acyl derivatives, *trans*-resveratrol-3-*O*-(6'-*O*-butanoyl)-β-D-glucopyranoside (**6**) and *trans*-resveratrol-3-*O*-(6'-*O*-octanoyl)-β-D-glucopyranoside (**7**), were synthesized by enzymatic acylation using *Thermomyces lanuginosus* lipase immobilized on granulated silica (Lipozyme TL IM) as reported earlier (*25*).

Resveratrol sulphate metabolites **8**-**10** were synthesized following the strategy reported by Hoshino *et al.(26)*. First, TBDMS-protected resveratrol derivatives **11**-**15** were obtained from RES and each compound separated by flash column chromatography. However, posterior sulphation of each RES derivative using $SO_3 \cdot NMe_3$ as sulphating reagent under acetonitrile reflux followed by silyl deprotection resulted in very low yields (10-26%). Difficulties may come from no completion of the sulphation reaction and the difficult purification of these highly polar products.

We have used instead microwave-assisted synthesis as reported by the group of Desai (*27, 28*) for highly sulphated organic scaffolds. TBDMS-protected resveratrol derivatives **11**-**13** were treated with $SO_3 \cdot NMe_3$ and NEt_3 in acetonitrile and microwaves applied at 100 ºC for 20-40 minutes (Scheme 1). The crude was purified using LH-20 chromatography and desilylation was carried out using KF in MeOH during 18 h. Final crude purification was carried out using reversed-phase chromatography to obtain **8**-**10** in good yields (73-89%).

11 R_1=H, R_2=TBDMS, R_3=TBMDMS
12 R_1=TBDMS, R_2=H, R_3=TBDMS
13 R_1=H, R_2=H, R_3=TBDMS
14 R_1=H, R_2=TBDMS, R_3=H
15 R_1=TBDMS, R_2=TBDMS, R_3=TBDMS

8 R_1=SO_3- K^+, R_2=H, R_3=H
9 R_1=H, R_2=SO_3- K^+, R_3=H
10 R_1=SO_3- K^+, R_2=SO_3- K^+, R_3=H

a) TBDMSOTf, DIPEA, CH_2Cl_2; b) $SO_3 \cdot NMe_3$, TEA, CH_3CN, 100ºC, 20 min, MW; c) KF, MeOH.

Scheme 1

BIOLOGICAL RESULTS AND DISCUSSION

In vitro cytotoxicity. The cytotoxicity of resveratrol derivatives **2**-**7** and resveratrol sulphate metabolites **8**-**10** was evaluated in vitro against two cancer cell lines, the human colon adenocarcinoma HT-29 and the breast adenocarcinoma MCF-7 cell lines. In addition, one normal cell line, the human embryonic kidney cell line (HEK-293) was employed in the assays for comparison (*29*). The standard MTT assay was used as described in the Experimental Section. Resveratrol **1** was used as the positive control. Cytotoxicity values, expressed as the compound concentration (µM) that causes 50% inhibition of cell growth (IC_{50}), are shown in Table 1. The observed values are in most cases in the low to medium

micromolar range. Among the resveratrol derivatives, glucosylated compounds (piceid **2**, **3** and **4)** and piceid octanoate **7** showed the highest cytotoxicities for the HT-29 cell line, with IC_{50} values lower than those of resveratrol. A similar trend is observed for the MCF-7 cell line where the lowest IC_{50} values were observed for piceid **2** and piceid octanoate **7**.

Cytotoxic values have been reported for piceid **2** in several cancer cell lines with a wide range of IC_{50} values, being for example on MCF-7 cells from 1.5 to 300 µM (*30-32*). When piceid is compared with resveratrol, IC_{50} values were in the same range or higher (*31, 33*). In our case, piceid IC_{50} values measured were in between those reported previously and in both cell lines (HT-29 and MCF-7) were lower than those obtained for resveratrol. The differences found in cytoxicity values for piceid may come from the different methodologies used to measure cell viability or from the different purity of piceid used in these studies.

It is important to remark that glucosyl resveratrol derivatives **2**-**4** tested are more toxic for tumoral cells than for normal ones, an obviously desirable feature. This can be better appreciated with the α and β coefficients, obtained by dividing the IC_{50} values of the normal cell line (HEK-293) by those values of one or the other tumoral cell line (see footnote in Table 1). The higher value of the α or the β coefficient, the higher is the therapeutic safety margin of the compound in the corresponding cell line. Thus, resveratrol is relatively cytotoxic for the normal cell line compared with cytotoxicity exhibited in both cancer cell lines. In contrast, piceid **2** and 3,5-diglucosyl-resveratrol **3** show a good selectivity in the case of the HT-29 line (α> 5.6 and 4.2 and respectively). Zhang et al. (*30*) have also described that piceid **2** is more potent eliminating cancer cells than non-cancer cells for a variety of cell lines.

Storniolo et al. has reported recently that piceid **2** inhibited Caco-2 cell growth (1-50 µM). Since the authors did not observed β-glucosidase activity they proposed that piceid presents antiproliferative effects on intestinal epithelial cells by itself and not as a resveratrol

reservoir. This could also be the case in our assays for piceid **2** and the glycosylated RES derivatives **3** and **4**. However, we observed previously (*25*) that glucosyl- or acyl-modifications in resveratrol prodrugs **2**, **3**, **4**, **6** and **7** retarded their metabolism in Caco- 2 cells but were indeed metabolized to RES and its conjugates (glucuronates, sulfates, etc.) after 6-24h incubation times. Therefore, compounds **2-4**, **6** and **7** seem to acting just as prodrugs delivering RES to the cells.

Table 1. Cytotoxicity of resveratrol and compounds **2-10** [a]

	HT-29	MCF-7	HEK-293	α[b]	β[c]
RES (1)	70 ± 3	127 ± 22	12 ± 3.5	0.2	0.1
2	18 ± 8	38 ± 18	>100	>5.6	>2.6
3	24 ±0.5	72 ± 18	>100	>4.2	>1.4
4	45 ± 9	89 ± 16	>100	>2.2	>1.1
5	99 ±24	**115 ± 12**	>100	>1.0	>0.9
6	**137 ± 20**	**110 ± 7**	>100	>0.7	>0.9
7	39 ± 2	52 ± 2	15 ± 4	0.4	0.3
8	**115 ± 17**	17 ± 1	>100	>0.9	>5.9
9	>100	**144 ± 32**	>100	>1.0	>0.7
10	56 ± 4	26 ± 4	>100	>1.8	>3.8

[a]IC_{50} values are expressed as the compound concentration (µM) that causes 50% inhibition of cell growth. The values are the average (± s.d.) of three different measurements performed as described in the Experimental section. [b]α = IC_{50} (HEK-293)/IC_{50} (HT-29). [c]β = IC_{50} (HEK-293)/IC_{50} (MCF-7). Values of α and β have been rounded off to a decimal figure.

When cytotoxicity was measured for the resveratrol sulphated metabolites **8**-**10**, we found low IC_{50} values for resveratrol-3-sulphate **8** and resveratrol-3,4'-disulphate **10** on MCF-7 cells (17 and 26 µM, respectively). In fact, these two metabolites were more toxic than resveratrol itself (127 µM). In the case of HT-29 cells, only resveratrol-3,4'-disulphate **10** was slightly more toxic than resveratrol. It is relevant to point out that sulphate resveratrol metabolites **8-10** had low toxicity on the normal cell line HEK-293, and it is especially interesting the high safety margin for **8** and **10** in the MCF-7 line ($\beta > 5.9$ and 3.8, respectively).

Several reports have found low or no cytoxicity (>100 µM) of resveratrol sulphated metabolites in a variety of cancer cells (MDA-MB-231, ZR-55-1, KB and neuroblastoma cells) (*26, 34, 35*). In contrast, more recent studies have described higher cytoxicity for resveratrol-3-sulphate **8** (11-35 µM) in different cancer cell lines (Caco-2, CCL-228, HCT-116, SW620 and SW480) (*36-38*). In the same three studies, resveratrol 3-*O*-D-glucuronide and resveratrol 4'-*O*-D-glucuronide, another common resveratrol metabolites, also resulted cytotoxic (10-35 µM), except on SW620 and SW480 cell lines. In the specific case of MCF-7 cells, Hoshino *et al.* (*26*) reported IC_{50} values >50 µM for resveratrol-3-sulphate **8.** We obtained slightly higher toxicity for compound **8**, showing similar values than the corresponding resveratrol-3,4'-disulphate **10**. Polycarpou *et al.* (*38*) suggested that the differences reported on cytoxicity for the resveratrol metabolites could be due to the use of different assays to measure cell viability.

Resveratrol sulfate metabolites have been suggested to provide an intracellular pool for resveratrol generation (*39*). Our results show that sulfate resveratrol metabolites are equally or more active as antiproliferative agents that RES itself pointing out they probably contribute to the in vivo biological activity observed for resveratrol.

Effect of resveratrol derivatives and sulfate metabolites on VEGF production. One of the key factors in the angiogenesis process is the release of VEGF from cancer cells. We decided to examine whether our resveratrol derivatives and metabolites were able to inhibit or at least decrease the activation of VEGF genes in HT-29 tumoral cells. We selected for this study glucosylated resveratrol compounds **2** and **3**, piceid octanoate **7**, and resveratrol sulphate metabolites **8**, **9** and **10**. Again, resveratrol **1** was used as the positive control. We determined VEGF protein production by ELISA in culture supernatants. Figure 1 shows the results obtained in ELISA measurements after treatment of HT-29 cells with resveratrol derivatives in DMSO. Piceid **2**, 3,5-diglucosyl-resveratrol **3**, piceid octanoate **7**, and resveratrol 3,4'-disulphate **10** decreased VEGF secretion in HT-29 cells in comparison with untreated cells. In fact, compounds **3** and **7** are more effective than resveratrol **1** itself.

Piceid **2** had been shown previously to inhibit angiogenesis on human umbilical vein endothelial cells at concentrations of 100 to 1000 µM what is in accordance to our experiments. Two permethylated stilbene derivatives (*40-42*) and a family of resveratrol dimers (*43*) have also been reported to inhibit VEGF production. In the case of the sulfate resveratrol derivatives, it is surprising that resveratrol 3,4'-disulphate **10** reduces VEGF secretion whereas the monosulfates **8** and **9** do not. In this scenario, it is difficult to imagine that resveratrol 3,4'-disulphate **10** could be a resveratrol reservoir since neither resveratrol-3-sulfate **8** or resveratrol-4'-sulfate **9** display antiangiogenic activity.
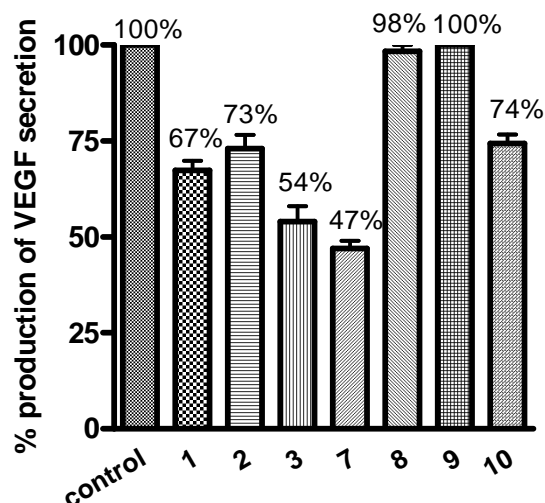
Figure 1. Percentage of production of VEGF from HT-29 cells treated with DMSO, resveratrol **1**, piceid **2**, **3**, **7**, **8**, **9** and **10** (conc. 20 μg/mL for **1** and 10 μg/mL for the rest of compounds) related to not treated cells. At least three measurements were performed in each case. Bars shown represent mean values of VEGF expression percentage related to control. Error bars indicate standard errors of the mean. Statistical significance was evaluated using one-sample t-tests (P < 0.001).

Effect of resveratrol derivatives on telomerase production. Human telomerase contains an RNA component (hTER) that serves as a template for the addition of the repeat nucleotide sequences and a protein subunit (hTERT) which catalyzes the nucleotide polymerization process. Human telomerase is regulated during development and differentiation, mainly through transcriptional control of the hTERT gene, the expression of which is restricted to cells that exhibit telomerase activity. This indicates that hTERT is the rate limiting factor of the enzyme complex (*44*). Transcriptional factors c-Myc and Sp1 are, among others, implicated in the expression of the hTERT gene. These factors upregulate mRNA encoding the hTERT protein subunit of telomerase (*45-47*). Thus, and as a preliminary study of the potential anti-telomerase activity of resveratrol derivatives, we have

investigated their ability to inhibit the expression of the hTERT and c-Myc genes. For that purpose, we selected the same group of resveratrol derivatives (**2**, **3** and **7**) and metabolites (**8**, **9** and **10**) as previously investigated for their VEGF inhibition activity. Resveratrol **1** was used again as control.

The results are depicted in figures 2 and 3 and show that treatment of HT-29 cells with resveratrol and the aforementioned derivatives leads in fact to various degrees of reduction in the transcription of hTERT and c-Myc mRNA as compared with control cells. The most active compounds as regards to inhibition of hTERT expression are 3,5-diglucosylated resveratrol **3** and resveratrol-3,4'-disulphate **10** which showed higher inhibition of hTERT gene expression than resveratrol itself. Particularly appealing is resveratrol metabolite **10** which was able to reduce the expression of this gene to 39% of the control value. For the sake of comparison, resveratrol **1** caused a reduction to 61% of the control value in the case of the hTERT gene.

Regarding c-Myc inhibition our study revealed that resveratrol **1** and 3,5-diglucosyl-resveratrol **3** are the most active compounds (48% and 66% respectively, see figure 3). Compounds **2**, **8**, **9** and **10** proved inactive in the inhibition of c-Myc gene expression. Resveratrol and compound **3** show some correlation between inhibition of c-Myc and hTERT gene expression (61% and 52%, see figure 2), indicating that resveratrol and compound **3** could downregulate the expression of the hTERT gene by lowering the transcription of the c-Myc gene. In contrast, this is not the case for resveratrol metabolite **10** which showed the highest inhibition of hTERT on this series but does not downregulate c-Myc gene expression. A different mechanism seems to be used by metabolite **10** pointing out again the fact that resveratrol-3,4'-disulfate is not just a resveratrol reservoir but instead that this resveratrol metabolite displays its on biological responses.
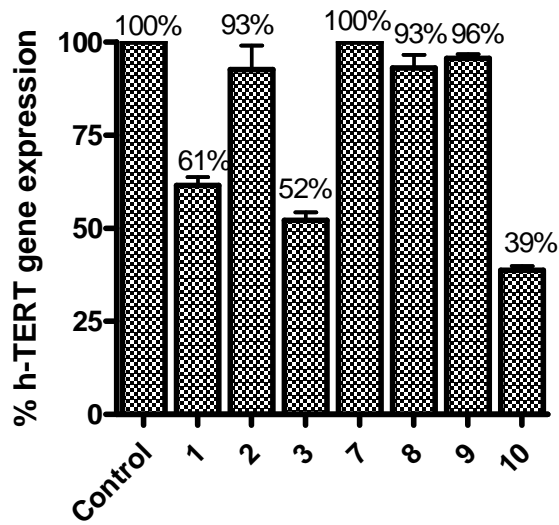
Figure 2. Gene expression of hTERT was normalized to that of the housekeeping gene β-actin. At least three measurements were performed in each case. Bars shown represent mean values of hTERT gene expression and error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample t-tests (P <0.001).
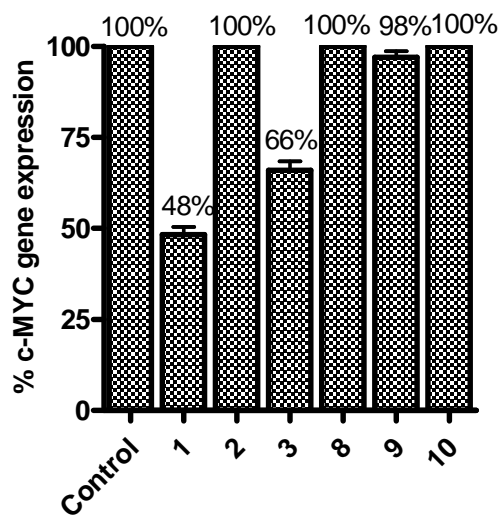

Figure 3. Gene expression of c-Myc was normalized to that of the housekeeping gene β-actin. At least three measurements were performed in each case. Bars shown represent mean values

of c-Myc gene expression and error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample t-tests (P <0.001).

In conclusion, resveratrol derivatives **2**, **3**, **4** and **7** displayed higher antiproliferative and antiangiogenic activity than resveratrol itself and, at the same time, these compounds showed less toxicity in HEK-293 cells. Most probably these derivatives are acting as RES prodrugs, delivering more slowly resveratrol to the cancer cells and therefore, also delaying RES metabolism. In the case of resveratrol sulfate metabolites **8**-**10**, we observed equal or higher cytotoxicity than resveratrol in HT-29 and MCF-7 cells together with lower toxicity in healthy human cells. The fact that that resveratrol disulfate **10** decreases VEGF secretion but no activity is observed for resveratrol monosulfates **8** and **9** seems to indicate an intrinsic activity of **10** more than just a resveratrol reservoir. Finally, resveratrol metabolite **10** seems to decrease hTERT expression via a different mechanism than resveratrol itself since **10** does not affect c-Myc formation. These results indicate that resveratrol metabolites contribute to the in vivo biological activity observed for resveratrol and not only act as a resveratrol reservoir.

Experimental Section

Chemistry: general procedures

All chemicals obtained from commercial sources were used without further purification, unless otherwise noted. All reactions were monitored by TLC on precoated Silica-Gel 60 plates F254, and detected by heating with Mostain (500 ml of 10% H_2SO_4, 25g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1g $Ce(SO_4)_2 \cdot 4H2O$). Products were purified by flash chromatography with Merck Silica gel 60 (200-400 mesh). Mass spectra analyses were run in the electrospray mode (ESIMS). NMR spectra were recorded on 300 or 400 MHz spectrometers, at room temperature for solutions in $CDCl_3$ or D_2O. Chemical shifts are referred to the solvent signal. Metabolites were purified by Sephadex LH-20 and RP-C18 chromatography.

Reaction Conditions

Glucosylated resveratrol derivatives

Resveratrol (**1**, RES) and piceid (*trans*-resveratrol-3-*O*-β-D-glucopyranoside, **2**, PIC) are commercially available. Diglucosylated resveratrol derivatives **3** and **4** were prepared as reported previously (*25*).

Acylated resveratrol derivatives

3-*O*-stearoyl-*trans*-resveratrol (**5**) was kindly provided by Dr. F. J. Plou. Briefly, preparation of **5** involves enzymatic acylation from RES using immobilized lipase from *Alcaligenes* sp. (lipase QLG) as reported previously (*48*). Piceid acyl derivatives (**6-7**) were also synthesized by enzymatic acylation as described earlier by our group (*25*).

Resveratrol sulphate metabolites

General procedure for the microwave-assisted O-sulfonation and desylilation: Microwave based sulfonation reactions were performed using a microwave synthesizer in sealed reaction vessels. Sulfur trioxide–trimethylamine complex was previously washed with H_2O, MeOH, and CH_2Cl_2 and dried under high vacuum. TBDMS-protected resveratrol

derivative (1.0 equiv), sulfur trioxide–trimethylamine complex (5 equiv per OH) and a magnetic stirrer bar were placed in a 2-5 mL microwave reaction vial and fitted with a septum, which was then pierced with a needle. The closed vial was then evacuated under high vacuum for 2 h. The mixture was dissolved in dry CH_3CN (2.0 mL) and NEt_3 (0.3-1.0 mL) was then added. Reaction mixture was subjected to microwave radiation for 20–40 min at 100 ºC (50-60W average power). MeOH (1 mL) and CH_2Cl_2 (1 mL) were added, and the solution was layered on the top of a Sephadex LH-20 chromatography column which was eluted with CH_2Cl_2/MeOH (1:1) to obtain the corresponding triethylammonium salt. The product and KF (2.0 equiv) were dissolved in MeOH (5 mL). The reaction mixture was stirred at room temperature for 18 h and the solvent was then removed under vacuum. The crude was purified by RP-C18 eluting with H_2O:CH_3OH from 100:0 to 70:30). Fractions containing the desired product were concentrated and freeze-dried affording the corresponding resveratrol sulphated compounds. Yields were: **8** (73%), **9** (82%) and **10** (93%).

Biological procedures

Cell culture

Cell culture media were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, U.K.). 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 Thermo Scientific™ BioLite. All tested compounds were dissolved in DMSO at a concentration of 10 µg/mL and stored at –20°C until use.

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.

Cytotoxicity assays

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 (*49*). Some 5 x 10^3 cells of HT-29, MCF-7 or HEK-293 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.

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.

RT-qPCR analysis

HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated with combretastatin derivatives in DMSO (see Figs. 2 and 3) 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.

Genes were amplified by use of a thermal cycler and StepOnePlus ™ Taqman ® probes. TaqMan ® Gene Expression Master Mix Fast containing the appropriate buffer for the amplification conditions, dNTPs, thermostable DNA polymerase enzyme and a passive reference probe was used. To amplify each of the genes the predesigned primers were used and sold by Life Technologies TaqMan ® Gene Expression Assays, Hs99999903-m1 (β-actin), Hs00900055-m1 (VEGF), Hs00972646-m1 (hTERT) y Hs00153408-m1 (c-MYC).

Literature

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