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Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure–activity relationship

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Abstract

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a naturally occurring hydroxystilbene, is considered an essential antioxidative constituent of red wine possessing chemopreventive properties. However, resveratrol and even more its metabolite piceatannol were reported to have also cytostatic activities. In order to find out whether this is related to antioxidative properties of those compounds, we synthesized five other polyhydroxylated resveratrol analogues and studied structure–activity relationships between pro-/antioxidant properties and cytotoxicity. Radical scavenging experiments with $O_2^{\bullet -}$ (5,5-dimethyl-1-pyrroline-*N*-oxide/electron spin resonance (DMPO/ESR)) and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) (photometry) revealed that 3,3′,4′,5-tetrahydroxystilbene (IC_{50}: 2.69 μM; k_9: 443000 M^{-1} s^{-1}), 3,4,4′,5-tetrahydroxystilbene (IC_{50}: 41.5 μM; k_9: 882000 M^{-1} s^{-1}) and 3,3′,4,4′,5,5′-hexahydroxystilbene (IC_{50}: 5.02 μM), exerted a more than 6600-fold higher antiradical activity than resveratrol and its two other analogues. Furthermore, in HL-60 leukemic cells hydroxystilbenes with *ortho*-hydroxyl groups exhibited a more than three-fold higher cytostatic activity compared to hydroxystilbenes with other substitution patterns. Oxidation of *ortho*-hydroxystilbenes in a microsomal model system resulted in the existence of *ortho*-semiquinones, which were observed by ESR spectroscopy. Further experiments revealed that these intermediates undergo redox-cycling thereby consuming additional oxygen and forming cytotoxic oxygen radicals. In contrast to compounds with other substitution patterns hydroxystilbenes with one or two resorcinol groups (compounds **1** and **3**) did not show an additional oxygen consumption or semiquinone formation. These findings suggest that the increased cytotoxicity of *ortho*-hydroxystilbenes is related to the presence of *ortho*-semiquinones formed during metabolism or autoxidation.

Keywords: Resveratrol; Hydroxystilbenes; Antioxidants; Prooxidants; Cytotoxicity; HL-60 cells

1. Introduction

Cancer is one of the main reasons of death in both men and women, claiming over 6 million people each year worldwide. Chemoprevention in combination with anticancer treatment is therefore important to reduce morbidity and mortality [1].

One promising natural chemopreventive product is resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a phytoalexin found in grapes, which is present in concentrations of up to 10 μM in red wines and to a much lesser extent in white wines [2,3]. The anticancer activity of resveratrol was first revealed by its ability to reduce incidences of carcinogen-induced development of cancers in experimental animals [4,5]. It has since been demonstrated that it possesses chemopreventive and cytostatic properties via the inhibition of tumor initiation, promotion and progression [4]. It causes cell arrest in the S and G2 phases of the

Abbreviations: COX, cyclooxygenase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPPH$^\bullet$, 2,2-diphenyl-1-picrylhydrazyl; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; LPO, lipid peroxidation; $O_2^{\bullet -}$, superoxide radical; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; HO–Stilb–OH, hydroxystilbene; HO–Stilb–O^-, deprotonated hydroxystilbene; [HO–Stilb–O–H–$O_2^{\bullet -}$]$^{\neq}$, adduct of hydroxystilbene with $O_2^{\bullet -}$; HO–Stilb–O$^\bullet$, phenoxyl radical; O=Stilb=O, two-electron oxidation product of hydroxystilbenes; $^-$O–Stilb–O$^\bullet$, semiquinone anion; SOD, superoxide dismutase

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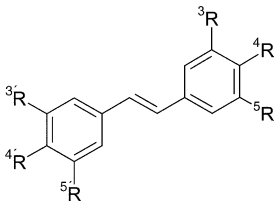
cell cycle [6] and is capable of inducing differentiation and apoptosis in a multitude of tumor cell lines, such as human leukemia, breast cancer and esophageal cells via CD95-dependent or independent mechanisms or through activation of caspase 3 or cleavage of PARP [7–9]. It has also been demonstrated that resveratrol inhibits the ribonucleotide reductase catalyzing the rate limiting step of de novo DNA synthesis [10]. While resveratrol exerts a non-selective cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibition [11], we could further show that hydroxylated resveratrol analogues are selective COX-2 inhibitors with a selectivity index (IC_{50} for COX-1/IC_{50} for COX-2) in part higher than celecoxib, a selective COX-2 inhibitor already established on the market [12]. These data are clinically important, as several lines of evidence suggest that selective COX-2 inhibitors may be beneficial both for cancer prevention and therapy.

Besides anticancer activities resveratrol also exhibits pronounced antioxidant properties by its ability to inhibit hydrogen peroxide- or lipid hydroperoxide-dependent lipid peroxidation of cellular membrane lipids [13,14]. Moreover, resveratrol reduces metal ion-dependent and independent oxidation of low-density lipoproteins [15,16], a process that is responsible for promoting atherogenesis. It also effectively protects isolated rat hearts from ischemia reperfusion injuries [17,18] reducing myocardial infarct size compared to control rat hearts [19]. Due to these experimental findings resveratrol in red wine was made responsible for the French paradox, the fact that the incidence of heart infarction in Southern France is 40% lower than in the rest of Europe despite the population's high-fat diet [3].

In contrast to the detailed knowledge of resveratrol activities in biological systems much less is known about the effects of higher hydroxylated stilbenes. Cai et al. compared the inhibiting activities of resveratrol and seven other hydroxylated *trans*-stilbenes with respect to an azo compound-induced peroxidation of linolic acid in vitro and to induce apoptosis in cultured HL-60 and Jurkat human leukemia cells [20]. They found that both antioxidant and apoptotic activities of the analogues containing 3,4-dihydroxyl groups namely 3,4-*trans*-dihydroxystilbene, 3,4,4′-*trans*-trishydroxystilbene and 3,4,5-*trans*-trihydroxystilbene were significantly higher than those of resveratrol and the other analogues. These data were supported by other investigators who also found free radical scavenging activity that was several times better, along with a higher growth-inhibitory activity of 3,3′,4′,5-tetrahydroxystilbene (piceatannol, astringinin) and 3,4,4′,5-tetrahydroxystilbene compared to resveratrol in tumor cells [21]. Resveratrol and its hydroxylated derivatives may be oxidized in an enzymatic or non-enzymatic manner via the one-electron pathway to a phenoxyl radical ($ArO^{\bullet}$) and subsequently yield quinone or quinone–methide type prooxidant or alkylating products. Several studies showed that the quinone products from oxidation of catecholic estrogen [22] and dopamine [23] are indeed responsible for the observed apoptotic effects of these drugs on cells.

Table 1
Structures of resveratrol (**1**) and its analogues **2–6**



Compound	^{3}R	^{4}R	^{5}R	$^{3'}R$	$^{4'}R$	$^{5'}R$
1	–OH	–H	–OH	–H	–OH	–H
2	–OH	–OH	–OH	–H	–OH	–H
3	–OH	–H	–OH	–OH	–H	–OH
4	–OH	–H	–OH	–OH	–OH	–H
5	–OH	–OH	–OH	–OH	–H	–OH
6	–OH	–OH	–OH	–OH	–OH	–OH

To clarify the possible link between antioxidant-derived radicals and cytotoxicity in cancer cells, we investigated radical production, prooxidation and radical scavenging activity of resveratrol and five synthesized hydroxylated analogues (Table 1) on the cytotoxicity of HL-60 human promyelocytic leukemia cells. Furthermore, the mechanism and structure–activity relationship of the double role as radical scavengers and prooxidants in biological systems will be discussed.

2. Materials and methods

2.1. Chemicals

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and DMSO were purchased from Sigma–Aldrich, Munich, Germany, potassium superoxide (KO_2) was obtained from Fluka, Switzerland and dibenzo-18-crown-6 (crown ether) was purchased from Merck, Germany. All other chemicals, obtained from commercial suppliers, were used as received and were of analytical grade purity.

2.2. Synthesis of hydroxylated resveratrol analogues

Hydroxylated resveratrol analogues **2–6** were synthesized using standard chemical methodologies with purity within ±0.4% of the theoretical values as previously described [12]. Resveratrol (**1**) was obtained from Sigma (Munich, Germany) with a purity of approximately 99%. Structures of synthesized compounds are presented in Table 1.

2.3. Competition of a spin trap compound and resveratrol derivatives for superoxide radicals

In order to assess the $O_2^{\bullet -}$ scavenging activity (a major aspect of the antioxidative activity) of resveratrol deriva-

tives, ESR spin trapping experiments using an ESP 300 E spectrometer from Bruker were carried out. Solutions of KO_2/crown ether (10 mM/20 mM) and hydroxylated stilbenes (5–50 mM) were prepared in dried dimethylsulfoxide (DMSO). Solutions of resveratrol derivatives (0–8 mM) and the spin trap compound DMPO (52 mM) were diluted in 1 ml DMSO and finally KO_2/crown ether (0.5 mM/1 mM) was added. The solutions were then transferred into a quartz flat cell and ESR measurements were started 2 min after mixing in a TE_{102}-cavity of the ESR spectrometer. For ESR measurements the following instrument settings were used: 9.8 GHz microwave frequency, 20 mW microwave power, 3473 G center field, 80 G sweep, 1 G modulation amplitude, 3.2×10^5 receiver gain, 57.2 G/min scan rate, 0.163 s time constant, 1 scan and 298 K temperature. The observed ESR signal exhibited a g-value of 2.006 and splitting constants of $a_N = 12.8$ G and $a_H = 10.4$ G, $a_H = 1.48$ G. Simulations with these parameters confirmed the presence of a DMPO/$^{\bullet}$OOH spin adduct [24]. The peak-to-peak intensities for each experiment were measured for calculation of the quenching activity of resveratrol derivatives. The IC_{50} parameters were calculated from the ESR intensities at various concentrations of resveratrol and its derivatives in relation to experiments without antioxidants [25].

2.4. DPPH$^{\bullet}$ assay

The radical scavenging activity of the tested compounds was determined using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$). In its radical form, DPPH$^{\bullet}$ absorbs at 515 nm but upon reduction by an antioxidant or radical species its absorption decreases. The reaction was started by the addition of 10 μl of resveratrol (compound **1**) and it analogues **2**–**6** (5–100 μM final concentration) to 3.0 ml of 0.1 mM DPPH$^{\bullet}$ in methanol. The bleaching of DPPH$^{\bullet}$ was followed using an HP 8453 diode array spectrometer equipped with a magnetically stirred quartz cell (optical path length 1 cm, kept at 25 °C by means of a thermostated water bath). Absorbance was recorded for up to 10 min, although steady-states of reaction were reached in most cases within 3 min. The reference cuvette contained up to 0.1 mM DPPH$^{\bullet}$ in 3.0 ml of methanol. Kinetic calculations were done using a second-order model described by Espin et al. [26].

2.5. Reaction with superoxide radicals—spectrophotometric measurement of intermediates

Solutions of resveratrol and its derivatives (50–200 μM in dry DMSO) were mixed with KO_2/crown ether (in dry DMSO; final concentration 10 mM/20 mM) resulting 3 ml of reaction mixtures. UV–vis spectra (180–820 nm) of resveratrol and the test compounds were recorded before and immediately after KO_2/crown ether addition in 10 s time intervals up to 5 min.

2.6. Reaction with superoxide radicals—ESR measurement of intermediates

For ESR measurements of intermediates an ESP 300E spectrometer from Bruker was employed. Stock solutions of KO_2/crown ether (10 mM/20 mM) and resveratrol derivatives (5–50 mM) were prepared in dried dimethylsulfoxide. Both solutions were aspirated from two separate autosampler vials into the quartz flat cell, which was located in the TE_{102}-resonator of the ESR spectrometer. The mixing of both solutions was performed in the lower part of the cell prior to reaching the active zone of the cell. ESR measurements were started 20 s after mixing. The following instrument settings were used: microwave frequency, 9.73 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; center field, 3491.1 G; sweep, 25 G; modulation amplitude, 0.48 G; receiver gain, 8×10^5; scan rate, 35.77 G/min; time constant, 0.163 s; scans, 5.

2.7. Preparation of liver microsomes

The male Sprague Dawley rats were sacrificed by decapitation; livers were extracted, rinsed with buffer (150 mM KCl, 50 mM Tris, pH 7.4), minced with scissors, and homogenized on ice in a motor-driven Potter-Elvehjem homogenizer with three volumes of buffer. The crude homogenate was centrifuged at $8750 \times g$ for 15 min at 4 °C. Microsomes were sedimented from the $8750 \times g$ supernatant by centrifugation at $165{,}000 \times g$ for 38 min at 4 °C [27]. After two washing steps, microsomes were dispersed in homogenizing buffer to provide a protein concentration of approximately 30 mg/ml.

2.8. Metabolism of tested stilbenes in rat liver microsomes, ESR measurements

ESR spectra were obtained using an ESP 300 E spectrometer from Bruker. The reaction mixture was saturated with air and contained: 10 mM glucose-6-phosphate, 0.67 U/ml glucose-6-phosphate dehydrogenase, 0.38 mM $NADP^+$, 5 mM $MgCl_2$, 6 mg protein/ml microsomes and 25 mM tested compounds (final volume, 1.0 ml). Immediately after $NADP^+$ addition and mixing, the solution was transferred to a quartz flat cell inserted in a TM_{110}-cavity. Data acquisition was started 1 min after mixing. For ESR measurements, the following instrument settings were used: microwave frequency, 9.73 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; center field, 3491.1 G; sweep, 50 G; modulation amplitude, 0.48 G; receiver gain, 2×10^6; scan rate, 17.89 G/min; time constant, 0.327 s; scans, 1. ESR signals were centered at $g = 2.00$ and the maximal splitting was between 6 and 7 G. Based on the ESR experiments with $O_2^{\bullet -}$ and stilbene derivatives, the signals are compatible with the presence of the respective stilbene radical anions.

2.9. Metabolism of tested stilbenes—oxygen consumption measurements

The rate of oxygen consumption was measured continuously with a Clark-type oxygen electrode connected to an OM-4 Oxygen Meter (Microelectrodes Inc.). Analog signals from the electrode were digitalized and recorded by a PC running, the MCREC Software of own design. The electrode vessel thermostated and maintained under agitation with a magnetic stirrer contained in 560 μl air-saturated preparation buffer: 9 mM glucose-6-phosphate, 1.2 U/ml glucose-6-phosphate dehydrogenase, 0.71 mM $NADP^+$, 2.7 mg protein/ml microsomes and tested compounds 50 μM (final concentrations). The oxygen consumption by microsomes without tested stilbenes was taken as a reference and set as 100%.

2.10. Metabolism of tested hydroxystilbenes—generation of ROS

ESR spectra were obtained using an ESP 300 E spectrometer from Bruker. The reaction mixture was saturated with oxygen and contained: 10 mM glucose-6-phosphate, 0.67 U/ml glucose-6-phosphate dehydrogenase, 0.38 mM $NADP^+$, 1.5 mg protein/ml microsomes, 5 mM DTPA, 100 mM DMPO and 250 μM tested compounds (final volume, 1.0 ml). Immediately after $NADP^+$ addition and mixing, the solution was transferred to a quartz flat cell inserted in a TM_{110}-cavity. Data acquisition was started 1 min after mixing. For ESR measurements the following instrument settings were used: microwave frequency, 9.73 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; center field, 3491.1 G; sweep, 80 G; modulation amplitude, 0.96 G; receiver gain, 5×10^5; scan rate, 28.6 G/min; time constant, 0.163 s; scans, 1.

2.11. Cell culture

The human promyelocytic leukemia HL-60 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI 1640 medium (Gibco, Paisely, Scotland) supplemented with 10% heat inactivated fetal calf serum (FCS) (GIBCO, Grand Island Biological Co., Grand Island, NY, USA), 1% L-glutamine and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO_2. Logarithmically growing cells were seeded in 25 cm^2 Nunc tissue culture flasks at a density of 0.1×10^6 ml^{-1} and exposed to various concentrations of resveratrol and its analogues 0–100 μM. Cell count and IC_{50} values for these compounds were determined after 72 h of incubation using a CC-108 microcellcounter (SYSMEX, Kobe, Japan). Viability of cells was determined by staining with trypan blue. Results were calculated as numbers of viable cells. Gallic acid acted as a positive control.

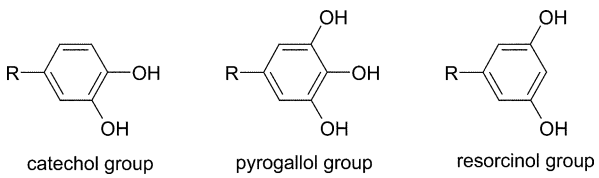
2.12. Statistical analysis

All experiments were performed at least three times. Results are expressed as mean ± S.D. Statistical differences were evaluated using the Student's *t*-test. The threshold of significance was $p < 0.05$.

3. Results

3.1. HL-60 cytotoxicity

The cytotoxicity of resveratrol and its hydroxylated analogues **2**–**6** to human promyelocytic leukemia HL-60 cells are shown in Fig. 1. Resveratrol (**1**) demonstrated a higher growth inhibition than gallic acid, a commonly used reference anti-leukemic compound (IC_{50}-values: 12.1 ± 0.17 and 18.3 ± 0.23 μM). Hydroxylation of resveratrol in position 3′, 4 and 5′(compounds **2**, **4**–**6**) even further increased cytotoxicity (Fig. 1). The most active compound in this experiment was analogue **6** (IC_{50}: 4.2 ± 0.09 μM) followed by analogues **4**, **2** and **5** (IC_{50}-values: 9.0 ± 0.11, 9.1 ± 0.28 and 10.3 ± 0.16 μM, respectively). The IC_{50} value for compounds **3**, a metabolized resveratrol molecule without catechol or pyrogallol groups, was 12.5 ± 0.19 μM. Calculating IC_{90}-values, the differences between analogues with pyrogallol and catechol groups and compounds with only one or two resorcinol group (analogues **1** and **3**, respectively) were even more pronounced. This was also the case for resveratrol and analogue **3** (IC_{90}-values >100 μM), while compounds **2**, **4**–**6** required between 18 and 25 μM for IC_{90}.



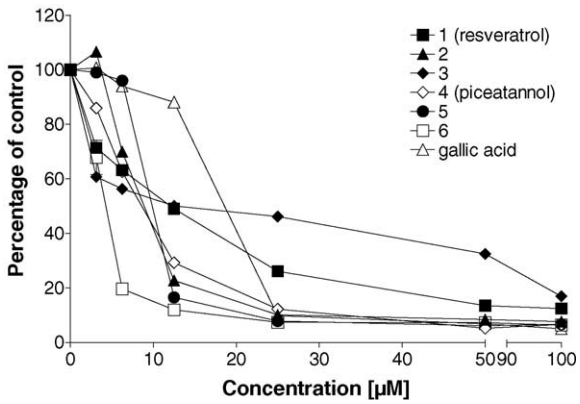


Fig. 1. Cytotoxicity of resveratrol (**1**) and its hydroxylated analogues **2**–**6** to human leukemia HL-60 cells. Data are means of three or more determinations. Standard deviations were within 5%.

3.2. Chemical properties of hydroxystilbenes interfering in oxidative stress

3.2.1. Scavenging of radicals

3.2.1.1. Superoxide radicals. It has been demonstrated in several studies that resveratrol and related compounds exhibit a high radical scavenging activity in biological membranes undergoing lipid peroxidation (LPO). In order to clarify whether these compounds have an antioxidative effect on superoxide radicals, representing precursors of radicals involved in LPO, we performed competition experiments between a spin trap compound DMPO and resveratrol analogues using KO_2 as $O_2^{\bullet -}$ source in DMSO.

The removal of $O_2^{\bullet -}$ by hydroxystilbenes (HO–Stilb–OH) from the solution can be expected to proceed via concerted H-atom abstraction (reactions (1)–(3)) or by catalyzing the dismutation of $O_2^{\bullet -}$ (reactions (4)–(7)) [28,29]. Superoxide radicals that escape from the antioxidative activities (reactions (1)–(7)) were trapped as DMPO spin adduct (reaction (8)) giving rise to the ESR signal shown in Fig. 3.

$$O_2^{\bullet -} + HO{-}Stilb{-}OH \rightarrow [HO{-}Stilb{-}O{-}H{-}O_2^{\bullet -}]^{\neq} \quad (1)$$

$$[HO{-}Stilb{-}O{-}H{-}O_2^{\bullet -}]^{\neq} \rightarrow HO{-}Stilb{-}O^{\bullet} + HO_2^- \quad (2)$$

$$HO_2^- + H^+ \rightarrow H_2O_2 \quad (3)$$

$$O_2^{\bullet -} + HO{-}Stilb{-}OH \rightarrow HO_2^{\bullet} + HO{-}Stilb{-}O^- \quad (4)$$

$$HO_2^{\bullet} + O_2^{\bullet -} \rightarrow HO_2^- + O_2 \quad (5)$$

$$HO_2^- + H^+ \rightarrow H_2O_2 \quad (6)$$

$$HO{-}Stilb{-}O^- + O_2 \rightarrow HO{-}Stilb{-}O^{\bullet} + O_2^{\bullet -} \quad (7)$$

$$O_2^{\bullet -} + DMPO + H^+ \rightarrow DMPO/^{\bullet}OOH \quad (8)$$

The observed ESR signal exhibited a g-value of 2.006 and splitting constants of $a_H = 12.8$ G, $a_H = 10.4$ G and $a_H = 1.48$ G, confirming the presence of a DMPO/$^{\bullet}$OOH spin adduct [30].

The ESR signal height of this spin adduct once formed was not sensitive to further additions of resveratrol analogues in the time scale of the experiment. By variation of the amount of antioxidant, keeping DMPO and KO_2 concentrations constant, the concentration of a decline of the ESR signal by 50% was obtained (Fig. 2).

Experimental data revealed that most hydroxylated analogues exhibited stronger scavenging activities than resveratrol and the model antioxidant α-tocopherol. Compounds **4** (piceatannol) and **6** were most potent with up to 1250-fold higher antiradical activity than resveratrol (IC_{50}: 2.69 and 5.02 μM, respectively). IC_{50} values indicated that there is no simple correlation between the number of HO groups in the molecule and its $O_2^{\bullet -}$ scavenging potency. Fig. 3 shows the representative decrease of the ESR signal by increasing concentrations of compound **6**. Interestingly increasing concentrations of compound **6** showed a lower decline of ESR signals at concentration higher than 50 μM (Fig. 3). The effect was also observed for resveratrol

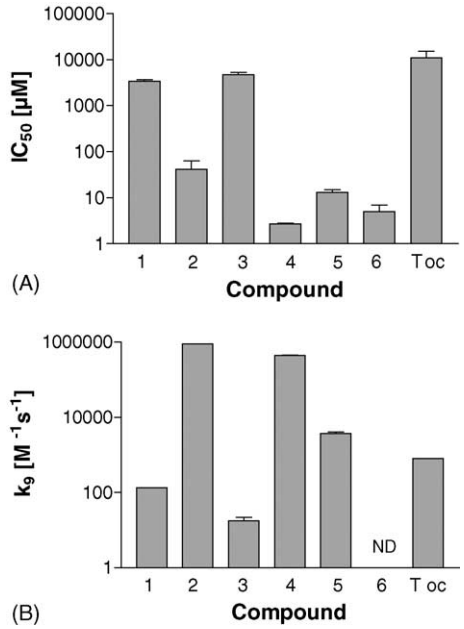


Fig. 2. Antioxidant activity of resveratrol (**1**) and hydroxylated analogues (**2**–**6**). (A) IC_{50}: concentrations of resveratrol and its analogues causing 50% quenching of DMPO/$^{\bullet}$OOH signal in the ESR experiments. (B) k_9: second-order rate constants for the reaction of abstraction of H-atoms from hydroxystilbenes by DPPH$^{\bullet}$. Toc: α-tocopherol; ND: not determined because of the formation of strongly colored intermediates.

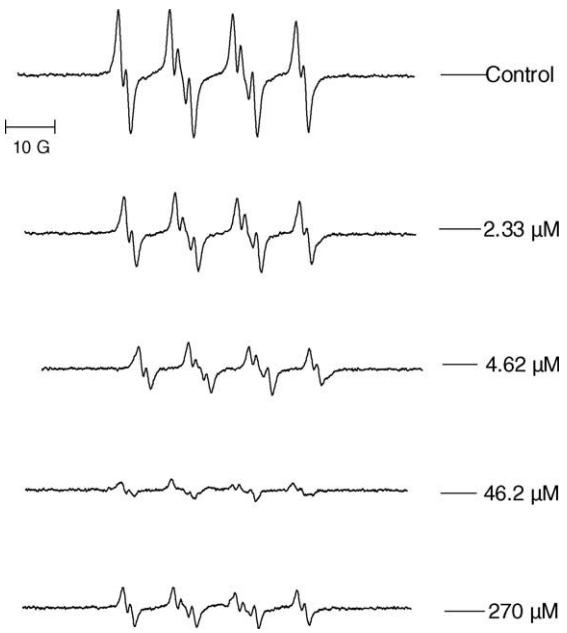


Fig. 3. ESR spectra obtained during the competition reaction of the spin trap compound DMPO (52 mM) and resveratrol analogue **6** (concentrations are indicated in the figure) for superoxide radicals. The control experiment was performed in the absence of antioxidants. The splitting patterns of the ESR signals indicate in all cases the presence of DMPO/$^{\bullet}$OOH spin adducts.

analogues with a catechol group (compound **4**) or a pyrogallol group (compounds **2** and **5**).

This could be indicative of counteracting mechanism in those compounds due to the antioxidant-derived prooxidant formation.

ESR spin trapping experiments with $O_2^{\bullet-}$ and hydroxystilbenes revealed the possible presence of antioxidant-derived prooxidants at higher concentrations of compounds **2**, **4–6** containing a catechol or pyrogallol group. Possible candidates for this prooxidative activity are antioxidant-derived radicals. Therefore, we studied the oxidation of these hydroxystilbenes by $O_2^{\bullet-}$ in DMSO in the absence of the spin trap compound. During oxidation of the resveratrol analogues **2**, **4–6** by superoxide radicals, ESR signals were observed indicating the formation of radicals (Fig. 4A). Based on the low resolution of the ESR spectra a detailed analysis of the hyperfine structure was not possible. ESR signals of intermediates were centered around $g = 2.00$ and the sum of hyperfine splittings were between 6 and 7 G. Quantum chemical calculations by Gaussian 98 software (Gaussian Inc., Pittsburgh, PA, 1998) of hydroxystilbene radicals and corresponding radical anions using the semiempirical PM3/UHF method for geometry optimization and the ab initio UB3LYP/6-31G(d) method for spin distributions revealed that experimental ESR signals are compatible with radical anions. In contrast neutral stilbene radicals would exhibit a significantly larger maximal splitting. In analogy to our compounds pyrogallol semiquinones are present as radical anions at pH values above 6.5 [31,32].

For resveratrol and compound **3** containing only resorcinol groups in the molecule ESR signals were absent (Fig. 4B). Thus, our data suggest that compounds containing catechol and/or pyrogallol groups generate *ortho*-semiquinone intermediates during reaction with superoxide radicals, which could be responsible for the prooxidative effects following the interaction with $O_2^{\bullet-}$ radicals.

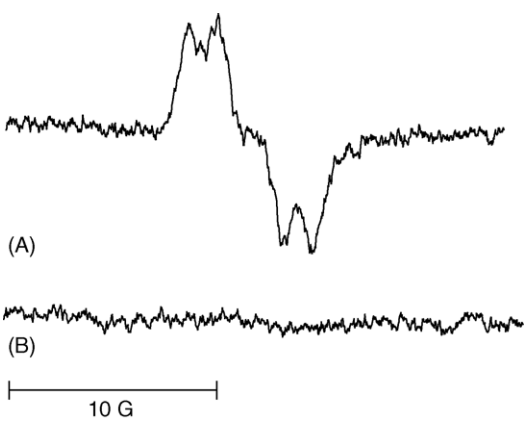


Fig. 4. ESR spectra obtained from the reaction of superoxide radicals with hydroxystilbenes in the absence of spin trap compound: (A) with compound **5**; (B) in the presence of resveratrol (**1**).

When the same reactions with compounds **2**, **4–6** were monitored by UV–vis spectroscopy the existence of intermediates absorbing at 440 nm was observed. This is in accordance with observations during the oxidation of other stilbenes, which exhibit absorptions of semiquinones at 450 nm [33].

3.2.1.2. DPPH$^{\bullet}$ as a model for lipid peroxyl radicals. Diphenylpicrylhydrazyl is widely used for assessing the ability of polyphenols to transfer labile H-atoms to radicals, a common mechanism of antioxidant protection [34]. The kinetics of H-atom transfer, however, may be even more important than the total H-atom-donating capacities normally evaluated. The scavenging of DPPH$^{\bullet}$ radicals by antioxidants can be summarized as [35]:

$$\mathrm{DPPH}^{\bullet} + (\mathrm{AH})_n \xrightarrow{k_9} \mathrm{DPPH{-}H} + (\mathrm{A}^{\bullet})_n \quad (9)$$

The newly formed radical ($A^{\bullet}$) can undergo radical disproportionation or other reactions thereby forming stable molecules. The second-order rate constants, k_9, calculated from the reaction of DPPH$^{\bullet}$ with resveratrol and its analogues was also the highest for compounds **2** and **4**. These derivatives were over 6600- and 3300-fold more active than resveratrol, respectively (Fig. 2). Compound **3**, however, showed only 14% of the anti-radical property of resveratrol indicating that the antioxidant activity of hydroxystilbenes are not only determined by the number of hydroxyl groups but also by their positions in the two aromatic rings. Substitution of resveratrol (**1**) at position 3′, 4 and 5′ increases the electron density of the hydroxyl groups and decreases the dissociation energy of the oxygen–hydrogen bond. This also correlates with a stronger antioxidant activity. Because of strongly colored intermediates formed during the reaction interfering with the DPPH$^{\bullet}$ assay at 515 nm, no k_9-value for compound **6** could be obtained.

3.3. Metabolism of hydroxystilbenes and oxidative stress

3.3.1. Formation of radical intermediates by microsomes

The question of whether radical intermediates can be formed under metabolic conditions was studied in rat liver microsomes, which are known to activate xenobiotics via cytochrome b_5 and/or cytochrome P450 reductase [27]. In order to protect paramagnetic species from reduction the experiments were performed using an NADPH-generating system consisting of glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$. When microsomes were incubated with this system in the presence of hydroxystilbenes **2**, **4–6** but not with compounds **1** and **3**, ESR signals centered at $g = 2.00$ were observed. Fig. 5A and C shows the representative ESR spectra for compounds **5** and **1**, respectively. These ESR signals were not observed in the absence of microsomes or the NADPH-generating system. Even the resolution of the signals was lower than in the

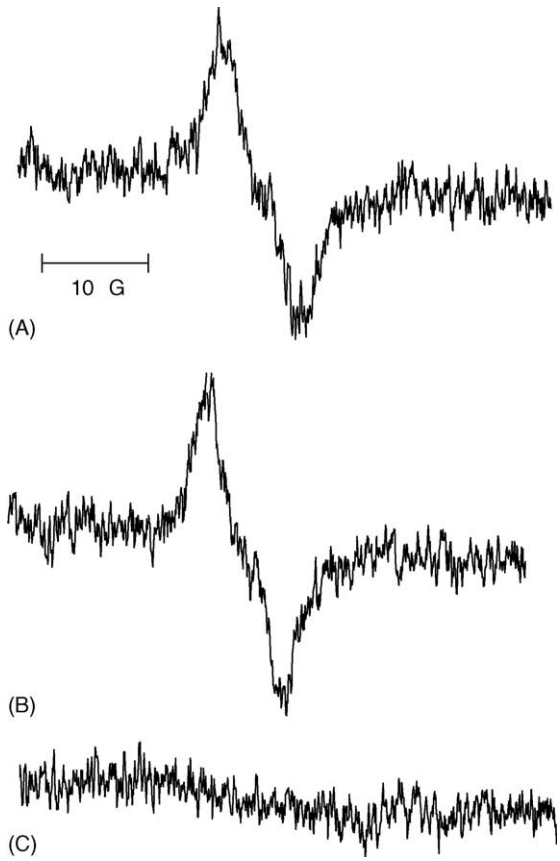


Fig. 5. ESR spectra observed during the metabolism of compound **5** (A) possessing a pyrogallol group and resveratrol (**1**) (C) containing a resorcinol group in rat liver microsomes (6 mg protein/ml) in the presence of a NADPH-generating system and 25 mM compounds **1** and **5**. Even after addition of 20 U of SOD (B), the signal from compound **5** does not disappear, suggesting a direct interaction with microsomal electron carriers.

previous chemical experiments (Fig. 4). However, similar maximal splitting values suggest the presence of *ortho*-semiquinones. These ESR signals could be also observed when 20 U of superoxide dismutase was added to the reaction mixture indicating a direct interaction of the compounds with microsomal electron carriers and not an indirect generation via microsomal $O_2^{\bullet -}$ production (Fig. 5B).

3.3.2. Additional consumption of oxygen by microsomes

The presence of *ortho*-semiquinones during the metabolism of certain hydroxystilbenes raises the question of whether such compounds could contribute to oxidative stress by autoxidation. Irrespectively of whether the final product of this reaction is $O_2^{\bullet -}$ or other reactive oxygen species one would expect an additional O_2 consumption by redox-cycling of semiquinones. Therefore, we measured O_2 consumption driven by the NADPH-generating system. Only compounds exhibiting ESR signals consumed additional oxygen which supports the hypothesis that hydroxystilbenes with pyrogallol and/or catechol groups (compounds **2**, **4**–**6**) induce oxidative stress by redox-cycling of *ortho*-semiquinone structures (Fig. 6).

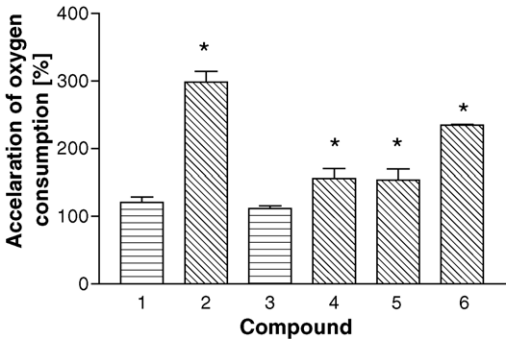


Fig. 6. Acceleration of oxygen consumption in rat liver microsomes caused by tested compounds. Reaction mixture contained 1 ml of air-saturated buffer, 50 μM compounds **1**–**6** and 1.5 mg/ml microsomal protein. In order to prevent the reduction of possible radicals, an NADPH-generating system was used. Consumption of oxygen in control samples (DMSO alone) was taken as 100%. Data are means ± S.D. of six samples. (*) Significant difference from compounds **1** and **3** ($p < 0.05$).

3.3.3. ROS generation during metabolism of hydroxystilbenes in microsomes

In order to evaluate as to how the above observations influence the total balance of oxygen radicals in cells, we performed ESR spin trapping experiments in the microsomal system in the presence of resveratrol analogues using DMPO as the spin trap compound. The control experiment (Fig. 7A) without resveratrol analogues shows superimposed ESR signals arising from DMPO/$^{\bullet}$OOH (a_N=14.2G, a_H = 11.0 G) and DMPO/$^{\bullet}$OH (a_N = 15.2 G, a_H = 14.6 G) due to the native microsomal $O_2^{\bullet -}$ formation.

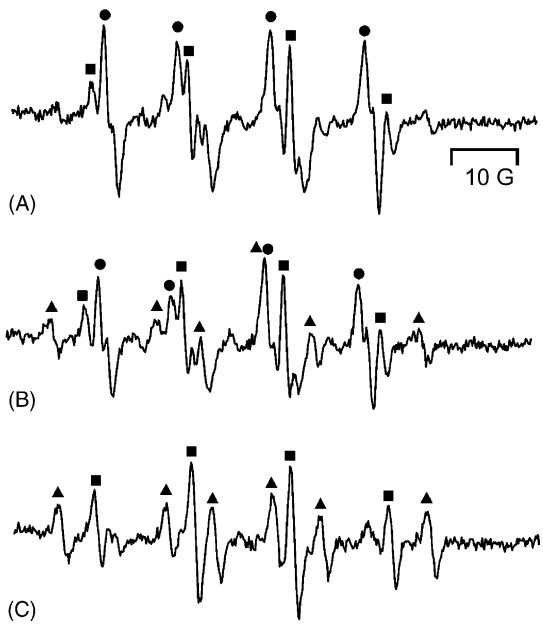


Fig. 7. ESR spectra obtained during the metabolism of hydroxystilbenes (compound **1** and **5**) in the presence of the spin trap compound DMPO. The reaction mixture contained again an NADPH-generating system, 1.5 mg microsomal protein/ml, 5 mM DTPA, 100 mM DMPO and 250 μM tested compounds. (A) ESR signals were identified as superimposed spectra of (●) DMPO/$^{\bullet}$OOH, (■) DMPO/$^{\bullet}$OH, (▲) DMPO/$^{\bullet}$CR spin adducts. (A) Control, (B) compound **1** (resveratrol), (C) compound **5**.

The presence of DMPO/$^{\bullet}$OH adducts in this experiment is most likely due to a degradation of the DMPO/$^{\bullet}$OOH adducts, since the existence of free HO$^{\bullet}$ radicals in the presence of DMSO would lead to the formation of carbon-centered radicals and finally to DMPO/$^{\bullet}$CR adducts. DMSO, which was used as solvent of resveratrol analogues, did not interfere in the detection of $O_2^{\bullet-}$ radicals by this method. In the presence of compound **1** (resveratrol; Fig. 7B) the DMPO/$^{\bullet}$OOH signal was only slightly diminished due to its poor antioxidant activity. In addition small amounts of carbon centered radicals DMPO/$^{\bullet}$CR were detected (a_N =16.4 G, a_H = 23.5 G). However, in the presence of compound **5** (Fig. 7C) the DMPO/$^{\bullet}$OOH adduct completely disappeared and strong signals of DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$CR became visible. This suggests that this type of compounds effectively reduces or dismutates $O_2^{\bullet-}$ to H_2O_2 but, obviously, its radical intermediates can cleave H_2O_2 to HO$^{\bullet}$ radicals. These are detected after attacking DMSO as carbon centered radical DMPO/$^{\bullet}$CR adducts.

4. Discussion

Natural polyphenols are considered chemopreventive agents able to prevent tumor initiation caused by reactive oxygen species targeting not only lipids and proteins but also DNA and RNA in living cells. Free radicals react with phenolic compounds much faster than with lipids or DNA. Therefore, phenols protect lipids and DNA from oxidative damage. In addition, polyphenols also decrease cancer growth and tumor promotion by scavenging free radicals which activate signal-transduction pathways including transcriptional induction of the growth-stimulating protooncogenes c-FOS, c-JUN and c-MYC [11,36]. Following this concept a downregulation of free radical levels is expected to be beneficial in chemoprevention and cancer treatment. This conclusion, however, contrasts with some clinically established anti-cancer treatment concepts using doxorubicin, mitomycin C, or radiation to destroy cancer tissues by increased levels of ROS. In order to elucidate this contradiction we studied the cytotoxicity and anti-/prooxidant properties of hydroxylated stilbenes.

In our experiments, resveratrol analogues with hydroxyl groups in positions 3′, 4 and 5′ (compounds **2**, **4**–**6**) showed an even higher cytotoxicity in HL-60 cells than resveratrol indicating an increased antitumor activity (Fig. 1). A similar pattern of compound properties was observed for their antioxidant activities with respect to $O_2^{\bullet-}$ and DPPH$^{\bullet}$ (Fig. 2). Our data are consistent with the findings of Cai et al. who also found that 3,4-dihydroxyl groups are important for enhanced antioxidant and anti-cancer activities of *trans*-resveratrol analogues [20]. The question of why polyhydroxylated stilbenes with pyrogallol- and catechol-groups have higher antioxidant capacities and exert more pronounced antiproliferative activities than resveratrol remains unclear so far.

Since antioxidant reactions are linked to the formation of antioxidant-derived radicals possible prooxidant properties of such intermediates have to be considered.

Goldman et al. [37] reported that phenols undergo redox-cycling catalyzed by myeloperoxidase which increases their cytotoxic activities. Even for well recognized antioxidants, like ubiquinones, the formation of prooxidant metabolites was suggested [25]. The structure–activity relationship of another class of polyphenolic compounds (flavonoids) was intensively investigated and described by Rice-Evans et al. [38], van Acker et al. [39], Heim et al. [40] and Silva at al. [41]. These authors reported that the most effective antioxidant configuration in ring B of flavonoids is an *ortho*-dihydroxy structure, which confers higher stability to the radical form and participates in electron delocalization. Although flavonoids were found to be excellent radical scavengers, there are also reports describing their prooxidative properties [42,43]. For example, the *ortho*-hydroxy substituted flavonoid quercetin has been found to be a mutagen in some experimental in vitro systems [43]. This cytotoxic effect of quercetin is believed to be the result of its prooxidant ability caused by the formation of oxidation products.

For hydroxystilbenes, however, most publications favor the exceptional antioxidant potency of additionally hydroxylated resveratrol-derivatives [44]. Based on our experimental results structural consideration of hydroxystilbenes with a different number of hydroxyl-groups and different substitution patterns revealed that the test compounds can be grouped into molecules that can either form quinoid systems upon two electron oxidation (compounds **2**, **4**–**6**) or are unable to form such structures (compounds **1** and **3**). The first group of compounds exhibits a several-fold higher radical scavenging activity ($O_2^{\bullet-}$ and DPPH$^{\bullet}$) as compared to the second group of compounds and α-tocopherol (Vitamin E). Interestingly this coincides with the ability of such compounds to chelate Fe^{2+} (data not shown), which contributes to the overall antioxidative activity. Obviously neighboring hydroxyl groups in the resveratrol molecule are necessary not only for binding of multivalent metal cations but also for the formation of *ortho*-quinoid structures. ESR experiments clearly revealed that upon one-electron oxidation only compounds of the first group gave detectable signals. Spectral properties of the ESR signals indicate the presence of *ortho*-semiquinones arising from the oxidation of hydroxystilbenes. In contrast, resveratrol and compound **3**, which are expected to form phenoxyl radicals, gave no detectable ESR signals during the one-electron oxidation by superoxide radicals. This difference is based on the stabilized semiquinone structure as compared to phenoxyl radicals, which only can be observed by ESR at room temperature if the radicals are stabilized by additional *tert*-butyl substituents [45]. Furthermore, deprotonation of semiquinones leading to semiquinone anions

makes them resistant to disproportionation and dimerization due to charge repulsion. Since pK_a values for similar *ortho*-semiquinone structures were reported to be below pH 7 [31] under physiological conditions, the presence of semiquinone anions is also likely (reaction (10)). In contrast, molecules which can form only neutral phenoxyl radicals, decay rapidly by disproportionation (reaction (11)) or dimerization (reaction (12)). This is well known for resveratrol which preferably forms the dimer *trans*-ε-vineferin and oligomers upon oxidation [46,47].

$$HO{-}Stilb{-}O^{\bullet} + O_2{}^{\bullet -} \rightarrow {}^{-}O{-}Stilb{-}O^{\bullet} + HO_2{}^{\bullet} \quad (10)$$

$$HO{-}Stilb{-}O^{\bullet} + HO{-}Stilb{-}O^{\bullet} \rightarrow HO{-}Stilb{-}OH + O{=}Stilb{=}O \quad (11)$$

$$HO{-}Stilb{-}O^{\bullet} + HO{-}Stilb{-}O^{\bullet} \rightarrow \text{dimers} \quad (12)$$

However, even if prooxidative intermediates such as semiquinone anions are formed during antioxidative reactions, it does not convert hydroxystilbenes to prooxidants. As can be seen from Fig. 3 at higher concentrations of compound **6**, the antioxidative activity declines while it still scavenges radicals. Similar effects were observed for compounds **2**, **4** and **5**. Since the prooxidative side effects are by far counterbalanced by the antioxidative capacity this effect cannot contribute to the observed increased cytotoxicity.

This does, however, not exclude the involvement of *ortho*-semiquinones ($^{-}$O–Stilb–O$^{\bullet}$) arising from oxidation products of hydroxystilbenes in cytotoxic activities. Following this concept we were able to demonstrate that these intermediates are also formed in microsomal suspensions (reaction (13)) being again absent during the metabolism of resveratrol and compound **3**. In order to induce cytotoxicity, a continuous generation of reactive oxygen species will be required at the expense of oxygen and reducing equivalents possibly transferred via cytochrome b_5. In the case of *ortho*-hydroxyl-substituted stilbenes in the microsomal system additional oxygen consumption was observed indicating a redox-cycling leading to the formation of reactive oxygen species (reaction (14)).

$$O{=}Stilb{=}O + e^{-} \xrightarrow{\text{microsomes}} {}^{-}O{-}Stilb{-}O^{\bullet} \quad (13)$$

$${}^{-}O{-}Stilb{-}O^{\bullet} + O_2 \rightleftharpoons O{=}Stilb{=}O + O_2{}^{\bullet -} \quad (14)$$

ESR spin trapping experiments in the systems have shown that ROS formation does not stop at the level of superoxide radicals or hydrogen peroxide but in the end leads to hydroxyl radical formation.

In conclusion, our data show that resveratrol and even more its hydroxylated analogues with *ortho*-hydroxyl groups exhibit pronounced antioxidant activity. The observed radical scavenging properties correlate with growth inhibition in HL-60 leukemia cells due to the formation of antioxidant-derived prooxidants. The formation of reactive oxygen species of resveratrol analogues **2**, **4**–**6** in human liver cannot be excluded and needs further in vivo evaluations. A prooxidant action, however, particular at higher concentrations, could support their use as novel anticancer agents but not as antioxidants.

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