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ORIGINAL ARTICLE

3,4,4′-Trihydroxy-trans-stilbene, an analogue of resveratrol, is a potent antioxidant and cytotoxic agent

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Abstract
Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring polyphenol widely distributed in food and dietary plants. This phytochemical has been intensively studied as an efficient antioxidant and anticancer agent, and a variety of substituted stilbenes have been developed in order to improve the potency of resveratrol. In this work, we described the synthesis of 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS), an analogue of resveratrol, and studied its antioxidant and cytotoxic activity *in vitro*. 3,4,4′-THS was much more efficient than resveratrol in protecting against free radical-induced lipid peroxidation, photo-sensitized DNA oxidative damage, and free radical-induced hemolysis of human red blood cells. More potent growth inhibition in cultured human leukemia cells (HL-60) was also observed for 3,4,4′-THS. The relationship between the antioxidant efficiency and cytotoxic activity was discussed, with the emphasis on inhibition of the free radical enzyme ribonucleotide reductase by antioxidants. The result that this subtle structure modification of resveratrol drastically improves its bioactivity provides important strategy to develop novel resveratrol-based molecules.

Keywords: *free radicals, resveratrol, antioxidant, lipid peroxidation, DNA oxidation*

Introduction

The free radical-mediated autoxidation of organic molecules by molecular oxygen is a double-edged sword and has both positive and negative aspects. It has been applied for the production of chemicals such as terephthalic acid and cyclohexanol. On the other hand, the recent development of free radical biology and medicine has provided a large body of evidence that oxidative stress arising from reactive oxygen species (ROS) is implicated in a number of biomacromolecular damage and human diseases [1–3]. And small molecule antioxidants, such as vitamin C [4], and other food-derived phytochemicals [5–8] or artificial molecules [9,10], may have beneficial effects in preventing against these damage and the related diseases [11].

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, Figure 1) is a naturally occurring phytoalexin present in grapes and other dietary plants. This “magic” molecule has various biological functions [12–15]. Its presence in red wine has been suggested to link to the low incidence of heart diseases in some regions of France, the so-called “French paradox”, *i.e.*, despite high fat intake, mortality from coronary heart diseases is lower due to the regular drinking of wine [16,17]. In addition, resveratrol has recently been shown to play an important role in the prevention of cancer, and its anticancer activity is, at least partly, related to the antioxidant effect [18]. Therefore, the past several years have witnessed intense research devoted to the biological activity, especially the antioxidant and anticancer activity, of this molecule [15,18–22].

Structural modification of natural products is one efficient way to increase their activity and lower the side effects. Dozens of molecules have been developed based on the structure of resveratrol in recent years, and some hydroxystilbenes with improved activity have been discovered [21,23–30]. In our previous work, we found that the anticancer activity for hydroxystilbenes correlates well with their antioxidant

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activity [26]. In this research, we reported the synthesis of 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS, Figure 1), an analogue of resveratrol, and the antioxidant activity as well as the cytotoxicity of this molecule. Simply changing 5-hydroxy in resveratrol to 4-hydroxy efficiently increases the protective effects against free radical-induced lipid peroxidation, photo-sensitized DNA oxidative damage, and free radical-induced hemolysis of human red blood cells (HRBC). 3,4,4′-THS inhibits cultured human leukemia (HL-60) cell proliferation more efficiently than does resveratrol. The findings that this subtle structure modification drastically improves the bioactivity of resveratrol provide important strategy to develop novel resveratrol-orientated molecules.

Materials and methods

Anisole, 3,4-dimethoxyaldehyde, 2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), and 3,5-dimethoxyaldehyde were purchased from Aldrich. Methylene blue (MB), agarose, ethidium bromide and plasmid pBR322 DNA were purchased from Sigma. RPMI 1640 medium, and penicillin/streptomycin were from Invitrogen. All solvents used in organic synthesis were purified and dried by standard techniques prior to use. All other chemicals were of analytical grade and used upon received. Melting points were measured on a Kofler apparatus and were uncorrected.

Synthesis of 3,4,4′-THS and resveratrol

3,4,4′-THS and resveratrol were prepared by the method according to literature [21,28] with slight modification as shown in Scheme 1.

3,4,4′-THS: Anisole (55 ml) was dissolved in dry benzene (220 ml) and 20 g paraformaldehyde was added to the solution. Dry hydrogen chloride was bubbled into the solution with stirring over a period of 1.5 h at 0°C. After continuing for 2 h at 0°C, the reaction mixture was washed with ice-water three times, ice cold $NaHCO_3$ solution (2%) three times. The organic layer was then separated and dried over anhydrous Na_2CO_3. The 4-methoxybenzyl chloride was obtained by distilling under vacuum.

4-Methoxybenzyl chloride (10 ml) was heated with triethyl phosphite (12 ml) under refluxing condition until the evolution of the gas had ceased. The reaction mixture was distilled under vacuum to give the diethylbenzylphosphonate. The diethylbenzylphosphonate (1.4 g) was added to 15 ml of dry dimethylformamide containing 0.5 g of sodium methoxide at room temperature. To this was added 3,4-dimethoxybenzaldehyde (0.85 g), and the reaction was allowed to proceed with stirring for 10 h at room temperature. The reaction mixture was poured into water–methanol (2:1), and the precipitated stilbene was collected by filtration and washed with water. The crude product was recrystallized from ethanol–water to give the pure 3,4,4′-trimethoxystilbene (1.25 g, 92%).

Excess pyridine hydrochloride (3 g) and 3,4,4′-trimethoxystilbene (0.5 g) were mixed and heated to 190–195°C for 4 h. The hot dark syrup was poured into 20 ml of 2 M HCl, and the reaction mixture was extracted with ether (4 × 20 ml). The ether layer was dried with anhydrous $MgSO_4$, and then ether was removed under reduced pressure. The residual was purified by column chromatography on silica gel eluted with petroleum ether-acetone (2:1) to afford 3,4,4′-trihydroxystilbene 0.20 g (43%). M. P. 242–244°C (literature 242–243°C [28]). MS (HP-5988A) m/e 228 (M^+); ^{1}H NMR (aceton-d $_6$, 400 MHz, Bruker) δ 6.80-6.83 (m, 3H), 6.88 (s, 1H), 6.89 (d, 1H, J = 16.3 Hz), 7.02 (d, 1H, J = 16.3 Hz), 7.05 (d, 1H), 7.37 (m, 2H).

Resveratrol was synthesized in the same manner as 3,4,4 -THS using 3,5-dimethoxybenzaldehyde instead of 3,4-dimethoxybenzaldehyde as a starting material. M. P. 254–257°C (literature 256–259°C [28]). MS m/e 228 (M^+); ^{1}H NMR (aceton-d $_6$, 400 MHz) δ 6.22 (t, 1H), 6.49 (d, 2H), 6.81 (m, 2H), 6.86 (d, 1 H, J = 16.4 Hz), 6.98 (d, 1H, J = 16.4 Hz), 7.37 (m, 2H).

Inhibition of free radical-induced lipid peroxidation

High performance liquid chromatography (HPLC) was used to determine the linoleic acid peroxides[21]. Linoleic acid was dissolved in t-butanol/water (3:2, v/v) and its peroxidation was initiated by addition of AAPH at 37°C. Linoleic acid hydroperoxides, which have a typical absorbance at 234 nm, were detected by HPLC analysis using a Gilson liquid chromatograph with a ZORBAX ODS reversed phase column

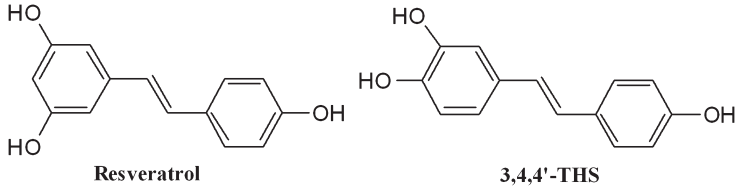
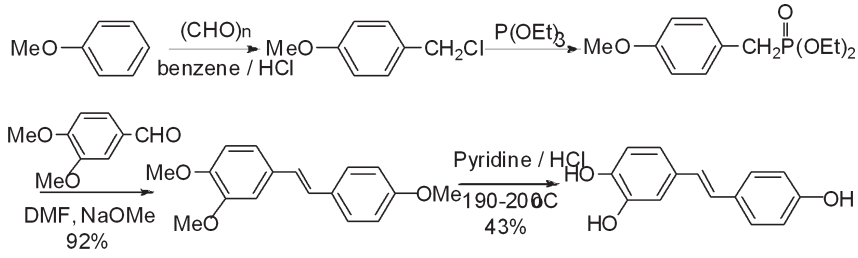


Figure 1. Chemical structures of resveratrol and 3,4,4′-THS.



Scheme 1. Synthesis of 3,4,4′-THS.

(6 × 250 mm Du Pont instruments) and eluted with methanol-propan-2-ol (3:1 v/v). A Gilson model 116 UV detector was used to monitor the total linoleic acid hydroperoxides at 234 nm. Every determination was repeated, and the experimental deviations were within ± 10%.

Determination of the antioxidants consumption

The procedure was the same as described above for determination of linoleic acid hydroperoxides, except that a Gilson model 142 electrochemical detector set at + 700 mV versus SCE was used for monitoring resveratrol, 3,4,4′-THS and α-tocopherol (TOH). The column was eluted with methanol-propan-2-ol-formic acid (80:20:1, v/v/) containing 50 mmol L^{-1} of sodium perchlorate as supporting electrolyte.

Assay for free radical-induced hemolysis of HRBC

The method used for determining the hemolysis of HRBC essentially followed the published procedure [31,32] with slight modification. HRBC were separated from heparinized blood that was drawn from a healthy donor. The blood was centrifuged at 2000 rpm for 10 min to separate the HRBC from plasma followed by washing the HRBC three times with PBS at 4°C.

The 5% suspension of HRBC in PBS was incubated under air atmosphere at 37°C for 5 min, into which an aqueous solution of AAPH was added to initiate hemolysis. The reaction mixture was shaken gently while being incubated at 37°C. The extent of hemolysis was determined spectrophotometrically as described previously [32]. Aliquots of the reaction mixture were taken out at appropriate time intervals, diluted with 0.15 M NaCl, and centrifuged at 2000 rpm for 10 min at 4°C. The percentage hemolysis was determined by measuring the absorbance of the supernatant at 540 nm and compared with that of complete hemolysis by treating the controlled HRBC suspension with distilled water. In case of antioxidation experiments resveratrol or 3,4,4′-THS was added and incubated before addition of AAPH. Every experiment was repeated three times, and the results were reproducible within 10% deviation

Assay for photo-sensitized oxidative DNA damage

The system for exposure of DNA to photosensitization was in principle similar to that described earlier [33,34]. Briefly, the system consisted of a 300 W Tungsten Halogen lamp which was placed 25 cm away from cuvettes containing 0.1–0.2 μg pBR322 DNA in the presence of indicated concentration of MB in 20 μl of 0.15 M phosphate buffer solution (PBS, pH 7.4). The cuvettes were kept in a water bath that was maintained at 37°C. The light source and the cuvettes were separated by a glass plate to cut off the light the wavelength of which is less than 400 nm. 3,4,4 -THS and resveratrol were added to the incubation and exposed to photosensitization under air atmosphere. Electrophoretic separation of different forms of DNA was achieved on 1.0% agarose gel using 40 mM Tris-acetate / 1 mM EDTA buffer (pH 8.3). DNA bands were stained with ethidium bromide and photographed under UV light.

MTT Assay for growth inhibition in cultured HL-60 cells

HL-60 cells were grown as suspension cultures in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and antibiotics (penicillin/streptomycin). Resveratrol and 3,4,4′-THS were dissolved in DMSO as stock solutions. Before addition to the culture medium, these stock solutions were diluted with PBS and the final concentration of DMSO in the medium is 0.1 %. To assay the antiproliferative effect of resveratrol or 3,4,4′-THS, 5000 cells in 90 μl of medium were plated to each well in 96-well plates. The medium was supplemented with 10 μl of PBS containing various concentrations of compounds. Four replicate wells were used for each concentration of both compounds. After treatment of cells at 37°C for 24 h or 48 h, 10 μl of MTT solution (2 mg/ml in PBS) was added to each well, and the plates were incubated for additional 4 h at 37°C. The plates were centrifuged and the medium within the wells was aspirated. The intracellular formazan dye crystals were dissolved by addition of 100 μl of DMSO to each well and were incubated overnight at room temperature in the dark with constant shaking. The absorbance of formazan at 562 nm was measured using a microplate

reader. The percentage of growth inhibition was calculated by comparison with control PBS-treated cells in the presence of 0.1% DMSO.

Results

Protection of linoleic acid from free radical-induced peroxidation

The primary peroxidation products of linoleic acid are hydroperoxides formed by oxygen addition at C-9 and/or C-13 positions with either *trans, cis*- or *trans, trans*-diene stereochemistry [35]. They show characteristic ultra-violet absorption at 235 nm which was used to monitor the formation of the total hydroperoxides formed during the peroxidation after separation of the reaction mixture by high performance liquid chromatography (HPLC). A set of representative kinetic curves of the total hydroperoxides formation during the peroxidation of linoleic acid in solution is shown in Figure 2A. The concentration of the hydroperoxides increased fast and linearly with time in the absence of antioxidants upon AAPH-initiation, demonstrating fast peroxidation of the substrate (line a in Figure 2A). The peroxides formation was inhibited remarkably by addition of 3,4,4′-THS (line e) with a inhibition time (t_{inh}) of around 50 min, but not by resveratrol (line b in Figure 2A) under the experimental conditions. After the inhibition period the rate of hydroperoxide formation turned faster which is close to the original rate of propagation, demonstrating the exhaustion of the antioxidant.

No synergistic effect of resveratrol and 3,4, 4′-THS in the presence of α-tocopherol

α-Tocopherol (TOH), the most abundant and active form of vitamin E, is a well-known and the principal lipid-soluble chain-breaking antioxidant in plasma and erythrocytes. Its synergistic antioxidative effect with other antioxidants has been well documented [36]. Therefore, we want to know if TOH could also interact synergistically with resveratrol or 3,4,4′-THS in solution. TOH showed typical antioxidant behavior against linoleic acid peroxidation (line c in Figure 2A) as reported previously with a t_{inh} of around 30 min [21]. Addition of 3,4,4′-THS together with TOH remarkably prolonged the inhibition period with a t_{inh} of around 80 min (line f in Figure 2A), which almost equals to the sum of the inhibition times caused by TOH and 3,3,4′-THS individually, and showed no synergistic antioxidation effect. Addition of resveratrol together with TOH has little effect on the inhibition period of the latter (line d in Figure 2A), confirming resveratrol is not a efficient antioxidant in homogenous solution.

Consumption of antioxidants

In order to rationalize the antioxidant mechanism under the inhibiting the linoleic acid peroxidation, the decay of antioxidants was determined by HPLC separation of the reaction mixture followed by electrochemical detection of resveratrol, 3,4,4′-THS, and TOH. Representative results are illustrated in Figure 2B. The decay of TOH was approximately linear in the absence of other antioxidants (line a in Figure 2B), in accordance with the previous reported kinetic behavior for antioxidation reactions. When resveratrol was added, the decay of TOH didn't change (line b in Figure 2B). While 3,4′4′-THS was added, the decay of TOH became slower, but was still linear (line c). Resveratrol didn't decay either with or without TOH

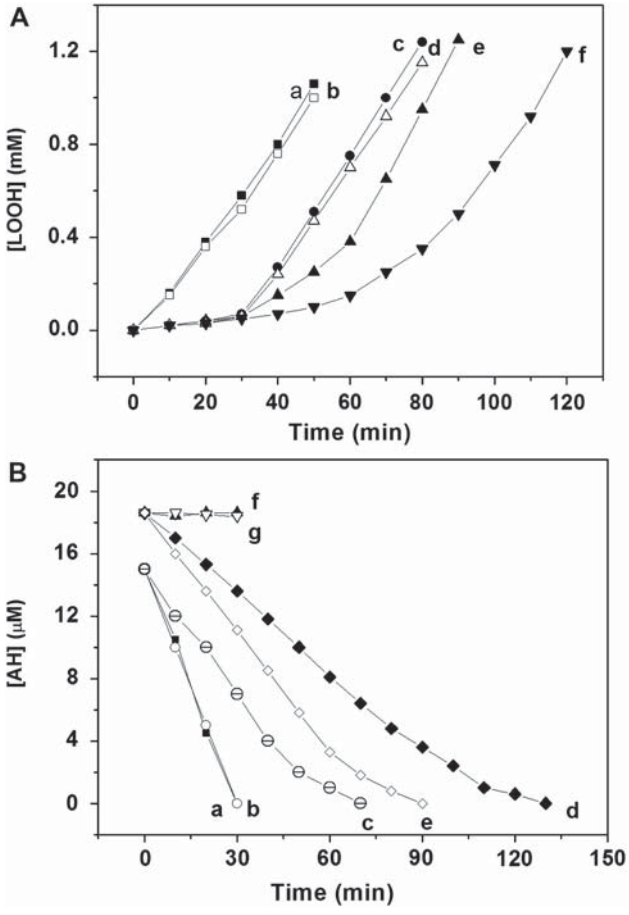


Figure 2. AAPH-induced formation of lipid hydroperoxides (LOOH) and inhibition by antioxidants. (A). The peroxidation of linoleic acid (LH, 0.1 M) was initiated by addition of AAPH (10 mM) in *tert*-butyl alcohol-water (3:2 v/v) at 37°C (line a), and inhibited by resveratrol (18.6 μM, line b), TOH (15.0 μM, line c), resveratrol plus TOH (line d), 3,4,4′-THS (18.6 μM, line e), and 3,4,4′-THS plus TOH (line f). (B). Consumption of antioxidant (AH) during the prevention of AAPH (10 mM)-induced linoleic acid (0.1 M) peroxidation in *tert*-butyl alcohol-water (3:2 v/v) at 37°C. $[TOH]_0$ = 15 μM $[AH]_0$ = 18.6 μM. a: decay of TOH; b: decay of TOH in the presence of resveratrol; c: decay of TOH in the presence of 3,4,4′-THS; d: decay of 3,4,4′-THS in the presence of TOH; e: decay of 3,4,4′-THS; f: decay of resveratrol in the presence of TOH; g: decay of resveratrol.

(lines g and f), supporting that it's not an efficient antioxidant under the experimental condition. The decay of 3,4,4′-THS in the presence of TOH was slower compared that without TOH ((lines d and e in Figure 2B).

Protection of HRBC from free radical-induced hemolysis

AAPH decomposes at 37°C to produce initiating free radicals [21]. Since the lipid peroxidation is a free radical chain reaction, and one initiating radical can induce up to twenty propagation reactions [21], the HRBC membrane is quickly damaged, and eventually leading to hemolysis. As shown in Figure 3, in the absence of AAPH the HRBC were stable, and little hemolysis took place within 4 h (line d). Addition of AAPH induced, after an inhibition period, fast hemolysis (line a). This inhibition stems from the action of native antioxidants, *e.g.* vitamin E and/or ubiquinol-10 presented in the HRBC membranes. Addition of resveratrol or 3,4,4′-THS into the HRBC suspension significantly increased the intrinsic inhibition time of the HRBC (lines b and c). 3,4,4′-THS gives much longer inhibition time than does resveratrol (line c), indicating that 3,4,4′-THS is a more efficient free radical scavenger.

Protection of DNA from photosensitized oxidative damage

MB is a photosensitizer for generating ROS and has been commonly used to investigate cellular and genotoxic effects in a variety of organisms. Singlet oxygen ($^1O_2$) is one of the major ROS generating in this system [34]. Figure 4A shows a photograph resulting from agarose gel electrophoresis of plasmid pBR322 DNA exposed to light in the presence of MB under air atmosphere. The plasmid DNA was easily oxidative damaged induced by photosensitization, showing a dose-dependent manner. Exposure of the plasmid DNA to MB and light increased the relative intensity of the open circular form (Form II) and decreased that of supercoiled form (Form I) (lanes 1 and 2). When the concentration of MB reached to 100 μM under a 30-min photosensitization, the supercoiled form of DNA completely disappeared (lane 7).

Figures 4B and C show protective effects of resveratrol and 3,4,4′-THS against photosensitized oxidative DNA damage, and quantitative results were shown in Figure 4D based on the band density from 3 independent experiments. As seen from the figures, both 3,4,4′-THS and resveratrol can protect plasmid pBR322 DNA from oxidative damage with different

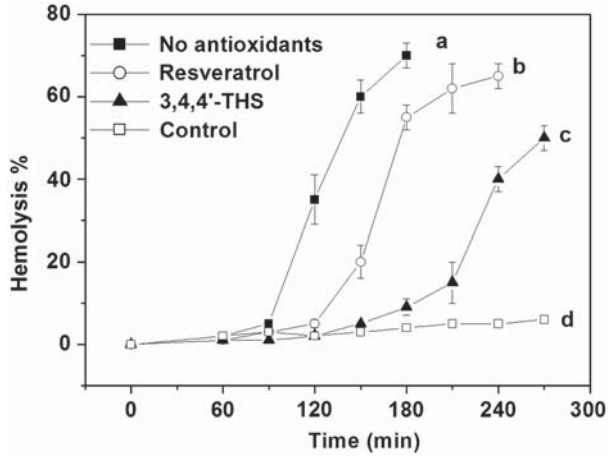


Figure 3. Inhibition of AAPH-induced hemolysis of HRBC by resveratrol or 3,4,4′-THS. The hemolysis was initiated by addition of 51.6 mM AAPH (a) and inhibited by addition of 10 μM resveratrol (b), or 10 μM 3,4,4′-THS (c). There was no hemolysis of HRBC in the experimental conditions with out AAPH (d). Every experiment was repeated three times and dada were expressed as mean ± SE.

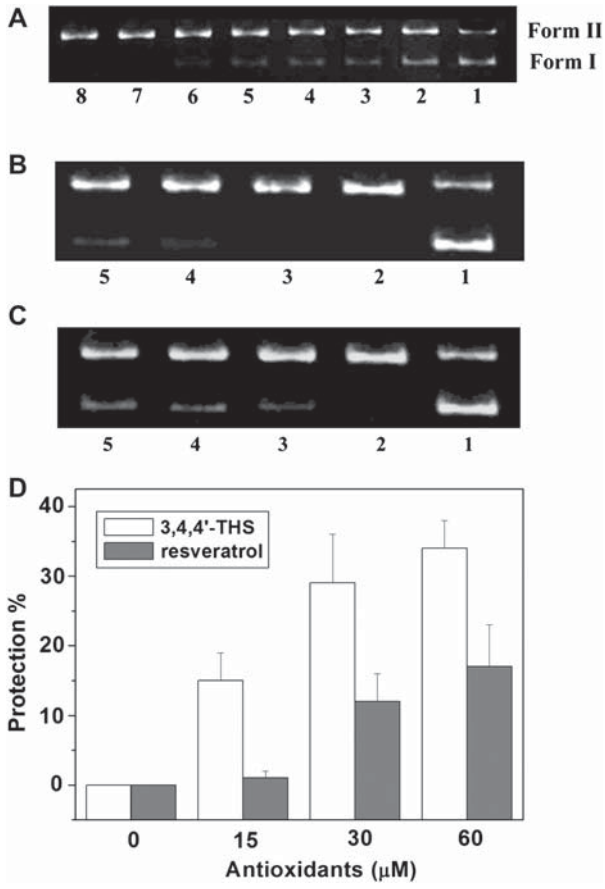


Figure 4. Protection of plasmid DNA from singlet oxygen-induced oxidative damage by resveratrol and 3,4,4′-THS. (A) Agarose gel electrophoretic pattern showing strand breaks in plasmid pBR322 DNA as a function of MB concentrations. DNA was exposed to light for 30 min. Lane 1′control (without MB); Lanes 2–8: 3.12, 6.25, 12.5, 25, 50, 100, 200 μM MB. Protection against MB (100 μM) plus light-induced plasmid pBR322 DNA strand breaks by resveratrol (B) or 3,4,4′-THS (C). The DNA was exposed to light for 30 min. Lane 1: control; Lanes 2–5: 0, 15, 30, 60 μM resveratrol. (D) Quantitative analysis of protective effects by resveratrol and 3,4,4′-THS. Band density from panel B and panel C was quantified by densitometry, and was expressed as the percentage of the control (mean ± SD).

levels. As antioxidant concentration accrues, the protective effect increases. Although 15 μM of resveratrol has little effect, the same concentration of 3,4,4′-THS can significantly protect DNA from the photosensitized oxidative damage (lane 3 in Figures 4B and C). Under other concentrations used in the experiments, 3,4,4′-THS is also more efficient than resveratrol to protect plasmid pBR322 DNA from damage.

Inhibition of cancer cells growth

We previously reported that the induction of cell apoptosis by hydroxystilbenes is correlated well with their antioxidant potency in different cancer cell lines [26]. Therefore, we compared the anticancer activity of these two molecules by measuring their ability to inhibit HL-60 cells proliferation. As shown in Figure 5, both resveratrol and 3,4,4′-THS, time- and dose-dependently inhibit HL-60 cells growth under the experimental conditions. 3,4,4′-THS, with IC_{50} around 30 μM after 48 h incubation, is more potent than resveratrol to prevent cancer cells proliferation.

Discussion

3,4,4′-THS and resveratrol were synthesized with reference to the modified Wittig reaction. Diethylbenzylphosphonate, which was prepared from methoxy substituted benzyl chloride and triethyl phosphite, reacts with methoxy substituted benzaldehyde to afford methoxylstilbene. The methyl-protected resveratrol and 3,4,4′-THS further reacted with pyridine hydrochloride, a demethylating reagent, to yield the desired compounds. The synthesis route was exemplified in Scheme 1. This procedure gives exclusively the *trans*-isomer, and the yield depends predominantly on the demethylation reaction.

Peroxyl radicals can be generated *in vivo* due to the presence of lipid hydroperoxides (LOOH, the primary products of lipid peroxidation). For example, metal ions can decompose LOOH to generate lipid peroxyl radicals following the reaction shown in equation 1:[1]

$$LOOH + Fe^{3+} \rightarrow LOO^{\cdot} + Fe^{2+} + H^{+} \quad (1)$$

To mimic the effect of peroxyl radicals *in vivo*, AAPH, a water-soluble azo compound, is widely used as an artificial source of peroxyl radicals [21,37–39]. Thermal decomposition of AAPH in the aqueous solution produces an initiating radical (R·) which can attack the polyunsaturated lipids (LH) to induce lipid peroxidation (Eqs. 2–6) [21]. On the other hand, if antioxidant (AH), such as vitamin E, are present, it would react with the chain propagating peroxyl radicals to stop the peroxidation (Eq. 7), hence inhibiting lipid peroxidation.

$$\text{Initiation: } AAPH \rightarrow 2R^{\cdot} + N_2 \quad (2)$$
$$R^{\cdot} + LH \rightarrow RH + L^{\cdot} \quad (3)$$
$$\text{Propagation: } L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (4)$$
$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (5)$$
$$\text{Termination: } LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{molecular products} \quad (6)$$
$$\text{Antioxidation: } LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot} \quad (7)$$

MB is a photo sensitizer for generating ROS and has been routinely used to investigate cellular and genotoxic effects in a variety of organisms. Upon irradiation by light, singlet oxygen ($^1O_2$) is generated as one of the major ROS in this system. Singlet oxygen is evolved in mammalian cells under normal and various pathophysiological conditions [1,40]. Due to its relatively long half-life, in the range of 10 to 50 μs in aqueous systems, $^1O_2$ is capable of travelling appreciate distance in the cellular milieu causing considerable damage to the cellular molecules.

Our data demonstrate that 3,4,4′-THS protects biomolecules from either peroxyl radicals- or $^1O_2$-induced oxidative damage more efficiently than does resveratrol. The only structural difference between the two molecules is one hydroxyl position on the benzene ring (Figure 1). This subtle structure modification remarkably improves the antioxidant potency of resveratrol, and thus provides information to develop novel antioxidants with higher activity derived from resveratrol. Resveratrol has no effect against free radical-induced lipid peroxidation under the experimental conditions. In stark contrast, 3,4,4′-THS inhibits lipid peroxidation efficiently. This difference should be due to the lower reactivity of resveratrol toward free radicals [21,27]. In line with the above results, 3,4,4′-THS decays gradually during the process of lipid peroxidation, while resveratrol doesn't (Figure 2B). In our previous work, 3,4,4′-THS synergistically prevents free radical-induced lipid peroxidation with TOH in micelles [21], however, no such effect was observed in homogeneous solution. The synergistic antioxidant effect between polyphenols and TOH is believed due to the regeneration of TOH radicals by polyphenols, and thus retards the consumption of TOH [21,41,42]. We reasoned that the lipid peroxidation in homogeneous solution is a pure chemical reaction, which is quite different from that in micelles, where the TOH radicals is fairly stable and accumulate with polyphenols in a certain microenvironment, and hence the TOH regeneration reaction could took place [21]. However, there is no such a microenvironment in homogeneous solution, and thus no TOH regeneration happens. To verify this, we monitored the decay of antioxidants in the process of lipid peroxidation. As shown in Figure 2B, although the decay of TOH in the presence of 3,4,4′-THS is slower than that without 3,4,4′-THS, there is no obvious lag phase, which means no TOH regeneration. We also noticed the same pattern for the decay of 3,4,4′-THS, *i.e.* its consumption became

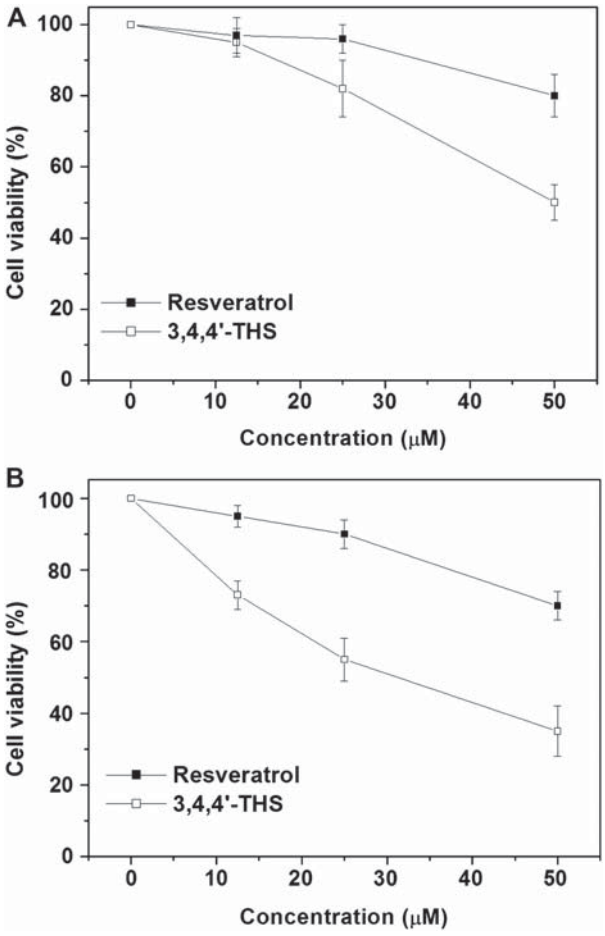


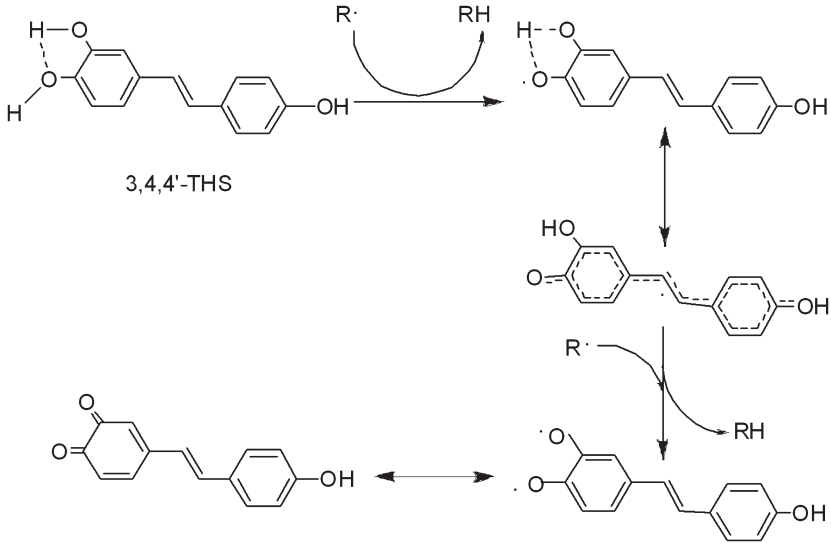
Figure 5. Cytotoxicity of resveratrol and 3,4,4′-THS in inhibition of HL-60 cells proliferation. HL-60 cells were treated with either resveratrol or 3,4,4′-THS for 24 h (panel A) or 48 h (panel B), and the cell viability was determined using the MTT assay as described in materials and methods. Values are the mean ± SD.

slower when TOH is present. Together, these results indicate that TOH and 3,4,4′-THS counteract lipid peroxyl radicals ($LOO^{\cdot}$, Eq. 7) independently in the solution, and there is no chance for TOH regeneration.

Why 3,4,4′-THS is a more powerful antioxidant than resveratrol? The recent works from our group as well as other groups reported that *trans*-stilbene compounds bearing *ortho*-dihydroxyl and/or *para*-dihydroxyl functionalities possess remarkably higher antioxidant activity than those bearing no such functionalities [21,24,26,27,43,44]. These are understood as the follows. First, molecules bearing *ortho*-dihydroxyl group have lower bond energy for H-O, which would make them to react easily with ROS to form *ortho*-semiquinone radical intermediates through direct hydrogen-abstraction; Secondly, the *ortho*-dihydroxy would make the *ortho*-semiquinone intermediates fairly stable via an intramolecular hydrogen bond and the 4′-hydroxyl group can stabilize the semiquinone radical intermediate by resonance through the *trans*-double bond; And thirdly, this *ortho*-semiquinone intermediate is easily to be further oxidized to finally stable product *ortho*-quinone [27]. Resveratrol, not like 3,4,4 -THS, has a *meta*-dihydroxy structure, which makes it unfavorable reacting with free radicals. The proposed antioxidant mechanism for scavenging peroxy radicals by 3,4,4′-THS is illustrated in Scheme 2. Singlet oxygens, unlike peroxy radicals, have low reactivity towards phenolic compounds. Quenching singlet oxygen back to triplet by unsaturated molecules like β-carotene and lycopene is a general pathway to detoxify singlet oxygen [45,46]. And thus, the protective effect against singlet oxygen-induced DNA damage by resveratrol and 3,4,4′-THS likely follows the same mechanism. This is further supported by the recent work by Celaje et al. [47], which demonstrated that physical quenching of singlet oxygen by resveratrol accounts for 75% of the total removal of singlet oxygen.

The results described in this paper support our previous hypothesis that the cytotoxic potency of hydroxystilbenes is correlated well with their antioxidant activity, *i.e.* compounds with higher antioxidant activity can suppress cancer cells proliferation more efficiently. Resveratrol was reported an inhibitor of DNA synthesis in mammalian cells via inhibition of the ribonucleotide reductase (RNR) [48]. This inhibition is caused due to the scavenging tyrosyl free radicals within RNR. The tyrosyl radical is a key intermediate in reduction of ribonucleotides into the corresponding deoxyribonucleotides [49], which are the basic building blocks for DNA synthesis. In this work, our results demonstrated that 3,4,4′-THS is a more potent antioxidant than resveratrol in preventing ROS-induced oxidative damage. So it is rational to expect that 3,4,4′-THS is a stronger inhibitor for RNR than resveratrol. This hypothesis is supported by the recent publications where resveratrol analogues were reported to inhibit deoxyribonucleoside triphosphates synthesis and direct inhibitors of RNR [50,51]. Since the proliferation of cancer cells is crucially dependent on the constant DNA synthesis, the better inhibitor of RNR should suppress DNA synthesis more severely. This accounts for the better activity of 3,4,4′-THS towards cancer cells than resveratrol, and might be applied to understand that why dietary antioxidants usually have anticancer activity. Taken together, inhibition of RNR by antioxidants through quenching tyrosyl radicals is a possible pathway to explain the anticancer property of antioxidants, and it still needs much more evidence to evaluate and understand the relationship between antioxidant activity and anticancer effect.

In summary, we demonstrated that simple structural modification of resveratrol by changing the 5-hydroxy group to the 4-position remarkably increases its antioxidant activity and cytotoxicity. These findings contribute new data to enrich the structure-activity relationships of hydroxystilbenes, and shed lights in modifying natural products for better activity.



Scheme 2. Proposed antioxidative mechanism of 3,4,4′-THS.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by the National Natural Science Foundation of China (21002047), the Ministry of Education of China (20100211110027) and Lanzhou University (the Fundamental Research Funds for the Central Universities, lzujbky-2009-73).

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