Protective Effects of Resveratrol and its Analogues against Free Radical-Induced Oxidative Hemolysis of Red Blood Cells†

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The *in vitro* oxidative hemolysis of human red blood cells (RBCs) was used as a model to study the free radical-induced damage of biological membranes and the protective effect of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, 1) and its analogues, *i.e.*, 4-hydroxy-*trans*-stilbene (2), 3,5-dihydroxy-*trans*-stilbene (3), 3,4-dihydroxy-*trans*-stilbene (4), 4,4′-dihydroxy-*trans*-stilbene (5) and 2,4,4′-trihydroxy-*trans*-stilbene (6). The hemolysis of RBCs was induced by a water-soluble free radical initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH). It was found that addition of AAPH at 37 ℃ to the suspension of RBCs caused fast hemolysis after a short period of inhibition period, and addition of 1—6 significantly suppressed the hemolysis. Compound 4 which bears an *ortho*-dihydroxyl functionality showed much more effective anti-hemolysis activity than that of resveratrol and the other analogues.

Keywords peroxidation, antioxidation, resveratrol, red blood cell, hemolysis

Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, **1**) is a naturally occurring phytoalexin present in grapes and other plants. Its presence in red wine has been suggested to be linked 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.[1] In addition, resveratrol has recently been shown to play a role in the prevention of cancer,[2] inflammation,[3] platelet aggregation[4] and allergy.[5] Therefore, the past several years have witnessed intense research devoted to the biological activity, especially the antioxidative activity, of this compound,[3b,6] since free radical-induced peroxidation of membrane lipids and oxidative damage of DNA have been considered to be associated with a wide variety of chronic health problems, such as cancer, atherosclerosis and aging.[7] Resveratrol has been reported to be a good antioxidant against the peroxidation of low-density lipoprotein (LDL)[6c] and liposomes,[6d] a potent inhibitor of lipoxygenase,[6e] and able to protect rat heart from ischaemia reperfusion injury.[6f] However, it was reported that the antioxidant effect of resveratrol was lower than a red wine extract which contains resveratrol and other polyphenolic antioxidants.[8] These facts, coupled with our recent findings of antioxidant synergism of vitamin E with green tea polyphenols,[9] coumarins[10] and β-carotene,[11] motivated us to study the antioxidative behaviour of resveratrol and its analogues, putting emphasis on the structure/activity relationship of these compounds. This paper reports an *in vitro* study on the protective effects of resveratrol (**1**) and related *trans*-stilbene analogues, *i.e.* 4-hydroxy-*trans*-stilbene (**2**), 3,5-dihydroxy-*trans*-stilbene (**3**), 3,4-dihydroxy-*trans*-stilbene (**4**), 4,4′-dihydroxy-*trans*-stilbene (**5**) and 2,4,4′-trihydroxy-*trans*-stilbene (**6**), against free radical-initiated hemolysis of human red blood cells (RBCs) and their cooperation with vitamin E (α-tocopherol, **TOH**). RBC membranes are rich in polyunsaturated fatty acids which are

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very susceptible to free radical mediated peroxidation. 2,2′-Azobis(2-amindinopropane hydrochloride) (**AAPH**) is a water-soluble azo compound which could decompose at physiological temperature to generate alkyl radicals to initiate lipid peroxidation (Eq. 1, *vide infra*). Since **AAPH** is water-soluble and the rate of free radical generation from **AAPH** can be easily controlled and measured, it has been extensively used as a free radical initiator for biological and related studies,[8-11] and the hemolysis induced by **AAPH** provides a good approach for studying free radical-induced membrane damages.[12]

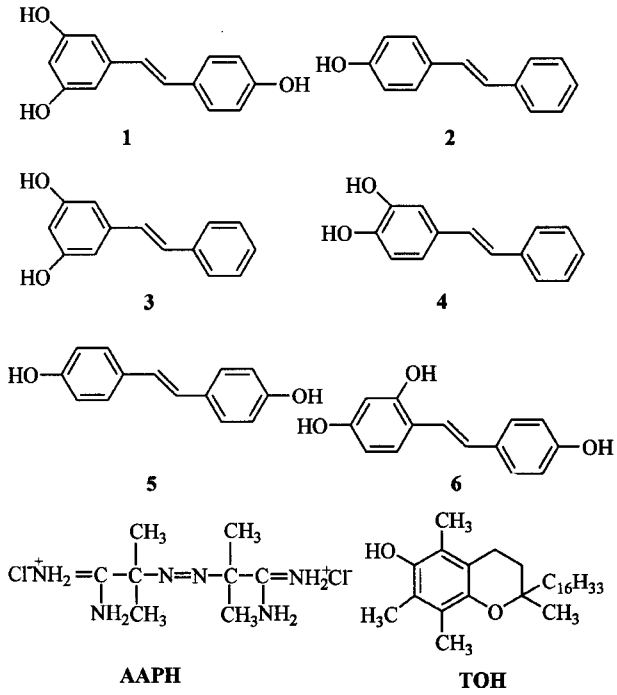


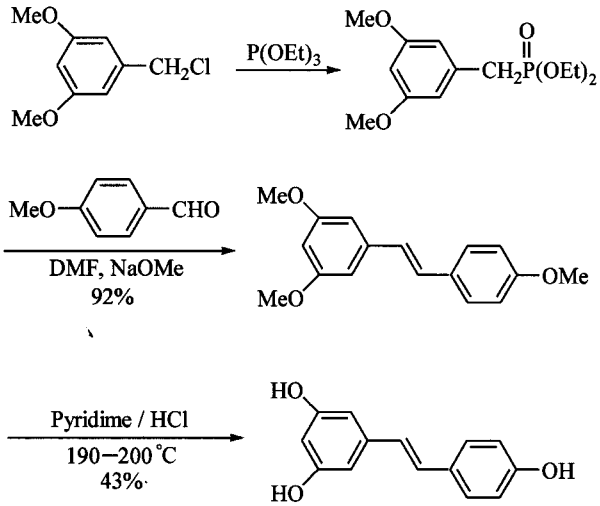
Fig. 1 Molecular structures.

Experimental

Materials

Resveratrol and its analogues **1**—**6** were synthesized with reference to the modified Wittig reaction[13] using diethylbenzylphosphonate, which was prepared from methoxyl substituted benzyl chloride and triethyl phosphite, reacted with methoxyl substituted benzaldehyde and followed by removing the methyl protecting group with pyridine hydrochloride as exemplified in Scheme 1. This procedure gave exclusively the *trans*-isomer and the good to moderate yields depending predominantly on the demethylation reaction. Their structures were fully identified with ^{1}H NMR and EI-MS and the data are consistent with those reported in the literature.[13] The purity of the compounds was checked by HPLC.

Scheme 1 Synthesis of **1**



2,2′-Azobis(2-amidinopropane hydrochloride) (**AAPH**) and *dl*-α-tocopherol (Biochemical reagent, >99.9%) were purchased from Aldrich and Sigma, respectively. All other chemicals used were of analytical grade.

RBC *preparation*

Human red blood cells were separated from heparinized blood that was drawn from a healthy donor. The blood was centrifuged at 2000 r/min for 10 min to separate the RBCs from plasma, then the RBCs were washed three times with phosphate-buffered saline (PBS) at pH 7.4. During the last washing the cells were centrifuged at exactly 2000 r/min for 10 min to obtain a constantly packed cell volume.

Assay for hemolysis

The 5% suspension of RBCs in PBS (pH 7.4) was incubated under air atmosphere at 37 ℃ 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 ℃. The extent of hemolysis was determined spectrophotometrically as described method previously.[14] Aliquots of the reaction mixture were taken

out at appropriate time intervals, diluted with NaCl (0.15 mol/L), and centrifuged at 2000 r/min for 10 min to separate the RBCs. 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 same RBC suspension with distilled water. In case of antioxidation experiments resveratrol or its analogues was added and incubated before addition of **AAPH**. Every experiment was repeated three times and the results were reproducible within 10% deviation.

Results and discussion

AAPH-*induced* RBC *hemolysis*

Thermal decomposition of **AAPH** in the aqueous dispersion of RBCs produces an initiating radical ($R^{\cdot}$) which can attack the polyunsaturated lipids (LH) in RBC membranes to induce lipid peroxidation [Eqs. (1)—(5)]. The initiation rate of **AAPH** at 37 ℃ in aqueous dispersions has been determined to be 1.3×10^{-6} [AAPH]/s.[15] Since the lipid peroxidation is a free radical chain reaction and one initiating radical could induce up to twenty propagation reactions,[9b,10b,15] the RBC membrane is quickly damaged, leading to hemolysis. On the other haod, if antioxidants (AHs), such as vitamin E, vitamin C and resveratrol, are present or added to RBCs they would react with the chain propagating peroxyl radicals to stop the peroxidation [Eq. (6)], hence inhibit hemolysis.

$$\text{Initiation:}\quad R{-}N{=}N{-}R \longrightarrow 2R^{\cdot} + N_2 \qquad (1)$$

$$R^{\cdot} + LH \longrightarrow RH + L^{\cdot} \qquad (2)$$

$$\text{Propagation:}\quad L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \qquad (3)$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \qquad (4)$$

$$\text{Termination:}\quad LOO^{\cdot} + LOO^{\cdot} \longrightarrow \text{molecular products} \qquad (5)$$

$$\text{Antioxidation:}\quad LOO^{\cdot} + AH \longrightarrow LOOH + A^{\cdot} \qquad (6)$$

Fig. 2 shows the **AAPH**-induced RBC hemolysis in the air. In the absence of **AAPH** the RBCs were stable and little hemolysis took place within 4 h. Addition of **AAPH** induced, after an inhibition period, fast hemolysis. Since no inhibition period was observed in micellar model systems after the addition of **AAPH**,[9a,10a] this inhibited hemolysis must stem from the action of native bioantioxidants, *e.g.*, vitamin E and/or ubiquinol-10 presented in biomembranes.[7d] We have observed similar inhibited peroxidation in **AAPH**-induced peroxidation of human low-density lipoprotein.[9b,10b] It is seen from Fig. 2 that the rate of hemolysis and the inhibition time are correlated dose-dependently with the concentration of **AAPH**. The inhibition time was (91 ± 5) min when the concentration of **AAPH** was 51.6 mmol/L.

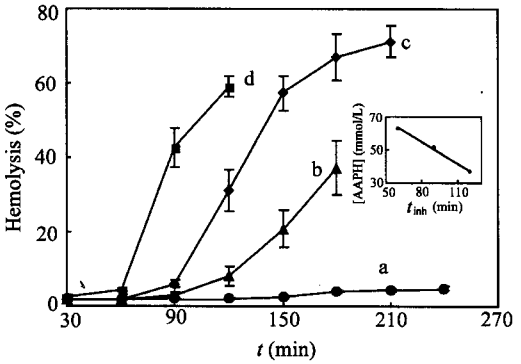


Fig. 2 **AAPH**-induced hemolysis of 5% human RBCs in PBS (0.15 mol/L, pH 7.4) under air atmosphere at 37 ℃. The initial concentrations of **AAPH**: (a) 0; (b) 37.2 mmol/L; (c) 51.6 mmol/L; (d) 62.9 mmol/L. The inset shows the relationship between the inhibition time, t_{inh}, and the initial concentration of **AAPH**. Data are expressed as mean of 3 RBC samples.

Inhibition of RBC *hemolysis by resveratrol and its analogues*

Addition of resveratrol and its analogues (**ArOHs**) into the RBC suspension significantly increased the intrinsic inhibition time of the RBCs. The inhibition time produced by **1** and **ArOHs** depended on the concentration of **1** as illustrated in Fig. 3 and those of the specific **ArOHs** used as shown in Fig. 4. When initiated with **AAPH** (51.6 mmol/L) the inhibition time produced by **1**—**6** (15 μmol/L) were (134 ± 6), (119 ± 7), (110 ± 8), (192 ± 8), (141 ± 4) and (144 ± 11) min, respectively. It corresponds to the additional inhibition time produced by these antioxidants being 43, 28, 19, 101, 50 and 53 min, respectively, giving a sequence of anti-hemolysis efficiency as $\mathbf{4} \gg \mathbf{6} \approx \mathbf{5} \approx \mathbf{1} > \mathbf{2} \approx \mathbf{3}$. This sequence is similar to the activity sequence of these **ArOHs** against linoleic acid peroxidation in micelles we observed recently,[16] suggesting that the anti-hemolysis activity of these **ArOHs** is due to their antioxidative effect against

free radical initiated lipid peroxidation of the RBC membranes.

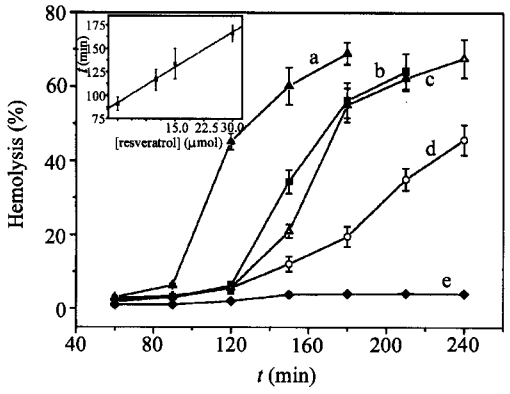


Fig. 3 Inhibition of **AAPH**-induced hemolysis of human RBCs by resveratrol (**1**). The experimental conditions were the same as described in the legend of Fig. 1 with $[AAPH]_0 = 51.6$ mmol/L. The initial concentrations of **1**: (a) 0; (b) 10 μmol/L; (c) 15 μmol/L; (d) 30 μmol/L; (e) control ($[AAPH]_0 = 0$). The inset shows the relationship between the inhibition time, t_{inh}, and the initial concentration of **1**. Data are expressed as mean of 3 RBC samples.

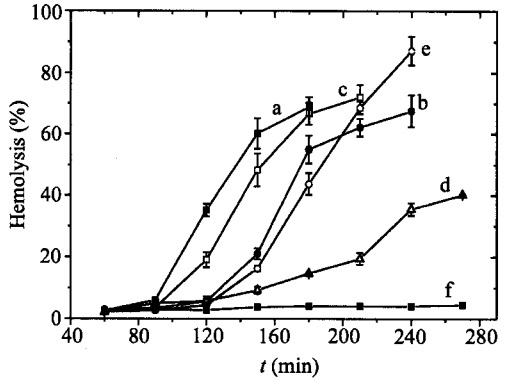
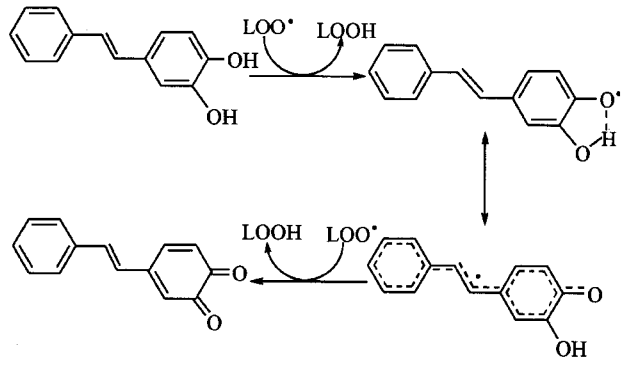


Fig. 4 Inhibition of **AAPH**-induced hemolysis of human RBCs by resveratrol analogues (**ArOHs**). The experimental conditions were the same as described in the legend of Fig. 2 with $[AAPH]_0 = 51.6$ mmol/L and $[ArOH]_0 = 15$ μmol/L. (a) native RBCs; (b) inhibited with **1**; (c) inhibited with **3**; (d) inhibited with **4**; (e) inhibited with **6**; (f) control ($[AAPH]_0 = 0$). Lines for **2** and **5** are not shown for clarity.

It is worth noting that the antihemolysis efficiency of **4**, which bears an *ortho*-dihydroxyl functionality, is remarkably higher than that of resveratrol and molecules bearing no such functionality. This is understood because the *ortho*-hydroxyl would make the oxidation intermediate, *ortho*-hydroxyl phenoxyl radical, more stable due to the intramolecular hydrogen bonding interaction as reported recently from both experiments[17] and theoretical calculations.[18] The theoretical calculation showed that the intramolecular hydrogen bond in *ortho*-OH phenoxyl radical is *ca.* 16.7 kJ/mol stronger than that in the parent molecule catechol and the bond dissociation energy (BDE) of catechol is 38 kJ/mol lower than that of phenol and 36.8 kJ/mol lower than that of resorcinol.[18] In addition, *ortho*-OH phenoxyl radical and/or *ortho*-semiquinone radical anion shall be easier be further oxidized to form the final product *ortho*-quinone[17] (Scheme 2). Compounds **5** and **6** which possess *para*-dihydroxyl groups are also more active because the unpaired electron in the oxidation intermediate phenoxyl radical can delocalize to the whole molecule through the *trans*-stilbene skeleton, and they are also easier to form the final product *para*-quinone.

Scheme 2 Antioxidation mechanism of **4**



Anti-hemolysis effect of resveratrol and its analogues in the presence of external α-tocopherol

It is well-known that α-tocopherol (**TOH**), the most abundant and active form of vitamin E is a principal lipid-soluble chain-breaking antioxidant in plasma and RBCs.[19] Its synergistic antioxidative effect with other antioxidants, such as *L*-ascorbic acid (vitamin C)[20] and green tea polyphenols,[9,11] has been well documented. We have found recently that the resveratrol analogues, especially **4**, could act synergistically with **TOH** to inhibit the peroxidation of linoleic acid in micelles.[16] That is, **4** could reduce α-tocopheroxyl radical (TO·) and regener-

ate **TOH**. Hence the inhibition time produced by **4** and **TOH** when they were used in combination was longer than the sum of the inhibition times when the two antioxidants were used individually. Therefore, it is desirable to see if the **ArOHs** and **TOH** possess similar synergistic anti-hemolysis effect. A representative result is shown in Fig. 5. It can be seen that addition of **TOH** (15 μmol/L) produced an additional inhibition time of 39 min. Addition of **1** (15 μmol/L) produced an additional inhibition time of 43 min. When the two antioxidants were used together the additional inhibition time was 84 min that is approximately the sum of the inhibition times when **1** and **TOH** were used individually. Other **ArOHs** including **4** gave similar results (figures not shown). That is, no synergistic interaction presents between these **ArOHs** and **TOH** in their anti-hemolysis action. This is probably due to the fact that **ArOHs** are water-soluble and resides in the bulk water phase, while **TOH** is highly lipophilic and tightly anchored inside the RBC membrane. Therefore, **ArOHs** could not directly react with TO˙ to regenerate **TOH** as it does in micelles.

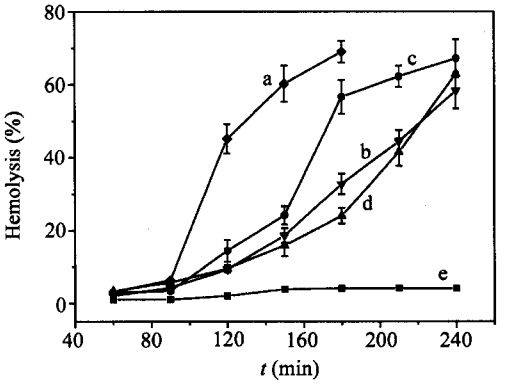


Fig. 5 Inhibition of **AAPH**-induced hemolysis of human RBCs by **TOH** and **1**. The experimental conditions were the same as described in the legend of Fig. 2. (a) native RBCs; (b) inhibited with **TOH** (15 μmol/L); (c) inhibited with **1** (15 μmol/L); (d) inhibited with **TOH** (15 μmol/L) and **1** (15 μmol/L); (e) control ($[AAPH]_0 = 0$). Data are expressed as mean of 3 RBC samples.

Conclusion

Resveratrol and its analogues **1**—**6** are effective antioxidants which can protect human red blood cells from free radical induced oxidative hemolysis. The observation that *trans*-stilbene compounds bearing *ortho*-dihydroxyl and/or *para*-dihydroxyl functionalities possess remarkably higher anti-hemolysis activity than the ones bearing no such functionalities gives us useful information for antioxidant drug design.

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