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Polyhydroxylated 4-thiaflavans as multipotent antioxidants: Protective effect on oxidative DNA damage in vitro

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Abstract—Hydroxylated 4-thiaflavans, possessing the antioxidant features of catechol containing flavonoids and/or tocopherols, were evaluated as protective agents against oxidation damage induced in herring sperm DNA by cumene hydroperoxide (CumOOH) or by the glutathione/ferric ion (GSH/Fe^{3+}) system. Our data indicate that the effective protection exerted by some of the tested compounds is overall higher than those provided by catechin and α-tocopherol, which might be attributed both to the scavenging properties and chelation of Fe^{2+} ions.

Free radicals and reactive oxygen species (ROS) are able to induce oxidative damage to cell membranes, lipids, proteins, and DNA that is associated with aging and many degenerative diseases, such as cancer, atherosclerosis and cataracts.[1–4] Thus, inhibition of oxidative damage by supplementation with antioxidant and/or free radical scavengers might reduce the risk of these diseases.[5,6] Numerous studies have focused attention on the antioxidant and free-radical scavenger activity of different natural compounds, including polyphenols, carotenoids, resveratrol, etc., demonstrating the various beneficial effects of these products.[7] Experimental data report on the antiproliferative activity of flavonoids on human colon and breast cancer cells,[8,9] and the strong inhibition of hepatic cell proliferation provided by resveratrol,[10] while epidemiological studies showed an inverse association between the low intake of flavonoids and the risk of lung and colorectal cancer.[11] Hirvonen et al., suggested an inverse association between the intake of flavonols, flavones and the risk of cardiovascular disease[12], while Keli et al. observed a lower stroke risk for subjects with a high β-carotene intake.[13] Our previous studies demonstrated that polyphenolic compounds obtained from red wine and black tea have a protective effect against induced oxidative DNA damage in rat liver and intestine.[14,15] Moreover, 4-coumaric acid, a simple phenolic acid, is able to protect against UVB-induced oxidative DNA damage in rabbit corneal-derived cells (SIRC).[16] Recently, a new class of polyphenolic antioxidants: hydroxylated 4-thiaflavans (derivatives **1**–**5** Fig. 1) were synthesized.[17,18] What makes these compounds unique is their ability to behave like radical scavengers, using either the B ring and miming the behaviour of catechol containing flavonoids like Catechin, or the A and C rings and acting out the chroman ring of tocopherols like α-tocopherol the main component of Vitamin E (Vit E, Fig. 1).[17–19]

Thus, it was interesting to evaluate whether such compounds, with the features of two of the more important families of natural antioxidants, could exert any protection on induced oxidative damage in herring sperm DNA.

In order to clarify the mechanism of action of this new class of antioxidants, we evaluated their effects on oxidative DNA damage induced by both cumene hydroperoxide (CumOOH), a directly generating radical species,[20,21] and glutathione/ferric ions (GSH/Fe^{3+}), a system able to

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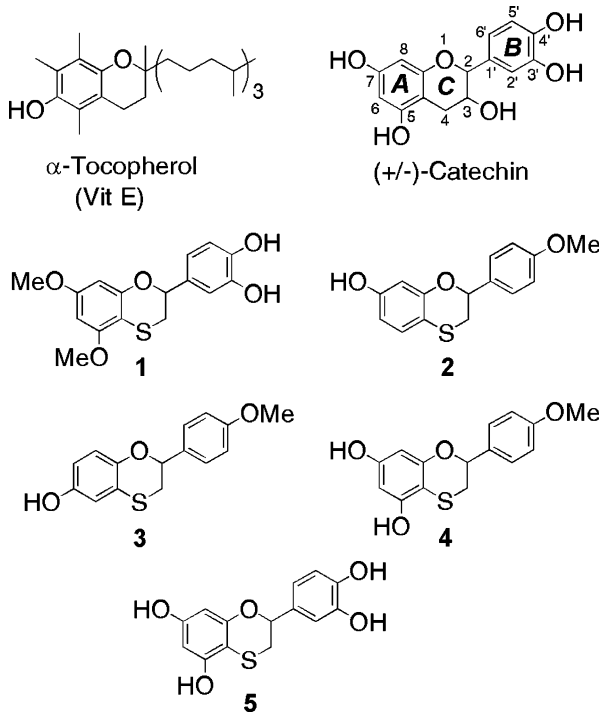

Figure 1. Structures of compounds tested in this study.

initiate the Fenton reaction. Data were reported in comparison with those obtained using Vit E and (±)-Catechin as suitable examples to verify whether any activity on the protection of DNA oxidation observed for 4-thiaflavans could be related to these two models.

Oxidative DNA damage was quantified measuring 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels by HPLC and electrochemical detector, as we previously reported.[22] The protective effect was measured as the 8-OHdG levels in the solution of herring sperm DNA (0.5 mg/mL) incubated for 2 h at 37 °C with 20 μM solutions of derivatives **1**–**5**, Catechin or Vit E, before adding the oxidative stressors.

In a first set of experiments, we induced oxidative DNA damage with 5 mM CumOOH[22] and found that the 8-OHdG level increased about 2.8-fold compared to the control (Fig. 2). In the presence of compounds **2**, **3** and **4**, DNA oxidation was not significantly modified. On the contrary, compounds **1** and **5** exerted a protective effect on CumOOH-induced oxidative DNA damage with a similar reduction of about 30%. Catechin gave a protection of about 32%, similar to compounds **1** and **5**, while the highest protective effect was obtained in the presence of Vit E, with a reduction of 61% (Fig. 2).

In a second set of experiments, DNA was incubated, in the conditions reported above, with 3 μM $FeCl_3$ and 15 mM GSH,[22] which increased the 8-OHdG level of about 20-fold compared to the control (Fig. 3). All hydroxylated 4-thiaflavans tested, with the exception of compound **3**, exerted a certain protective effect on oxidative DNA damage induced by GSH/Fe^{3+}. The maximum protection was obtained in the presence of compound **5** with a reduction in the 8-OHdG level of about 82%, while Catechin and Vit E reduced the formation of 8-OHdG by 36% and 33%, respectively (Fig. 3).

The UV/visible spectra of compounds **1**, **4** and **5**, 20 μM in a buffered solution at pH 7 with Hepes 3 mM, registered in the absence (blue lines) and in the presence (black lines) of 3μM $FeSO_4$, are reported in Figure 4. The blue lines for compounds **1** and **5** show the catechol and resorcinol group absorption bands, with the maximum absorption at 286 and 225 nm, respectively, while only the former band appears on the spectra of derivative **4**. The black lines unambiguously show that an interaction occurred between ferrous ion and the catechol chromophore of compounds **1** and **5**, or the resorcinol chromophore of compounds **4** and **5** (Fig. 4).

Protection of thiaflavan derivatives **1**–**5** on CumOOH-induced DNA damage is in agreement with their previously reported scavenging ability.[18] Since CumOOH-induced DNA oxidation can be mainly ascribed to the formation of peroxyl radicals,[20] the protective effect of a certain compound can be related to its radical scavenging property. Accordingly, the protection observed with compounds **1** and **5**, having a catechol group on B capable of a 'catechin-like' behaviour, is similar to that offered by Catechin (Fig. 2) and lower than that found with α-tocopherol, the naturally occurring

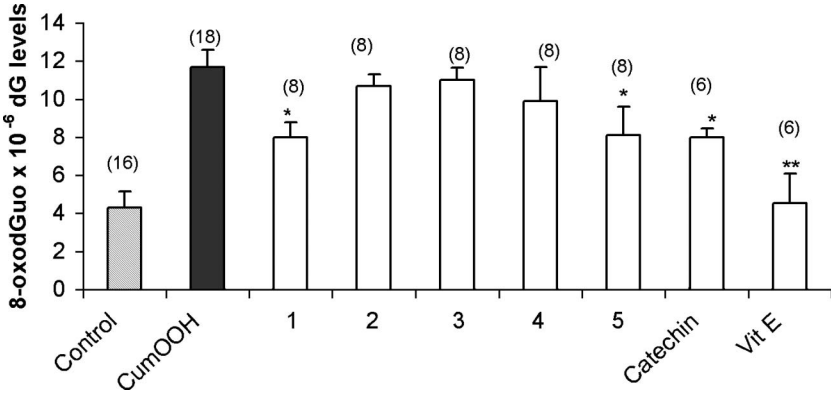

Figure 2. Effect of 20 μM solutions of thioflavans **1**–**5**, (±)-Catechin and Vit E on CumOOH induced the formation of 8-OHdG in herring sperm DNA. Values are expressed as means of (*n*) determinations ±SEM, $*P < 0.05$, $**P < 0.01$ relative to control values obtained in the absence of CumOOH.

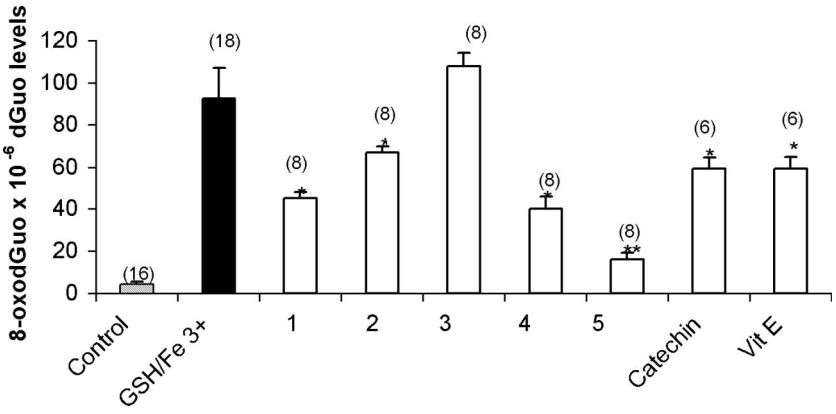

Figure 3. Effect of 20 μM solutions of thioflavans **1**–**5**, (±)-Catechin and Vit E on GSH/Fe^{3+}-induced the formation of 8-OHdG in herring sperm DNA. Values are expressed as means of (*n*) determinations ±SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ relative to control values obtained in the absence of GSH/Fe^{3+}.

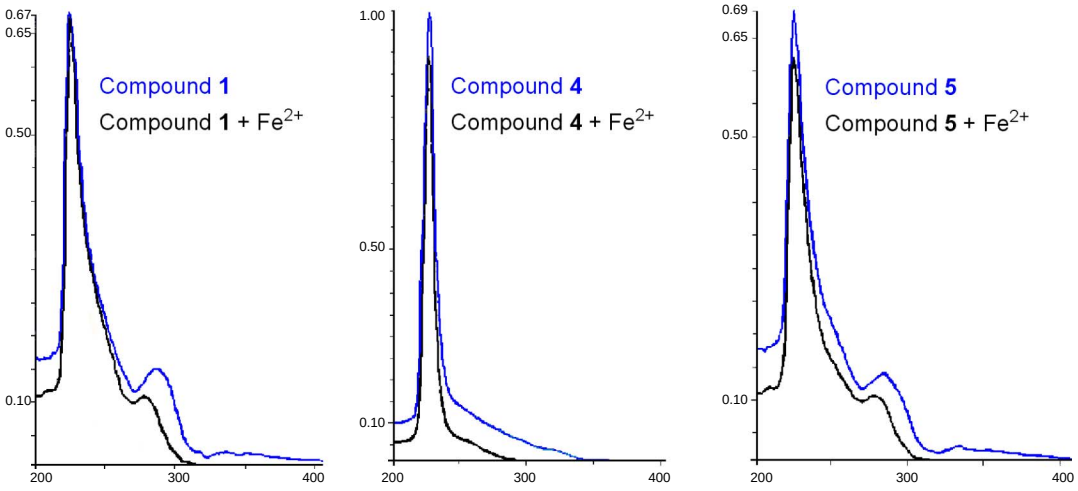

Figure 4. UV/visible spectra of compounds **1**, **4**, and **5** in the absence (blue lines) and in the presence (black lines) of $FeSO_4$.

most efficient chain-breaking antioxidants known.[23] These data indicate that the 'tocopherol-like' mechanism, operative in thiaflavans **2**–**5**,[18] independent of the number and the position of OH groups on the A ring, has a minor protective effect on CumOOH-induced damage of DNA, compared to the related activity of Vit E.

Data obtained by measuring the ability of thiaflavan derivatives **1**–**5** to work against DNA oxidation induced by GSH/Fe^{3+} are less obvious yet more interesting. Indeed, the redox reaction of GSH with ferric ions allowed the formation of Fe^{2+} cations, able, in turn, to promote oxidative damage through Fenton chemistry. In these conditions, a mixture of highly reactive and dangerous oxidants, like perferryl ion species (FeO^{2+} and FeO^{3+}) and hydroxyl radical ($HO^{\cdot}$), is formed in solution. In such a context, the radical scavenging ability of an antioxidant will play its role by blocking secondary free radicals generated, for example, from $HO^{\cdot}$, while the ability of blocking Fe^{2+} ions by chelation will directly prevent the formation of the dangerous oxidizing cocktail. As a matter of fact, we previously demonstrated[22] that the addition of EDTA, as an iron chelator, stopped the oxidative DNA damage induced by GSH/Fe^{3+}. In this light we can argue that the observed protective effect offered by α-tocopherol (Fig. 3), which is unable to chelate any cation, is mostly due to the scavenging of the secondary free radicals. On the other hand, the ability of flavonoids, particularly those bearing a catechol group, like Catechin, to act as metal ion chelators is well known.[24–26] Thus, the fairly similar protective effect observed for Catechin and derivative **1** is possibly derived from their ability to act as radical scavenger and as metal chelator (Fig. 3). Amazingly, compound **4**, which resulted as poorly efficient in the protection against CumOOH-induced DNA damage (see Fig. 2), showed an activity that was even better than that of **1** or Catechin (Fig. 3). To rationalize this result, we supposed that compound **4** might in fact be able to chelate Fe^{2+} ions using the 5-OH group on the A ring and sulfide sulfur on the C ring. This observation seems soundly corroborated by the remarkable protection (82%) offered by compound **5** (Fig. 3), which can be explained considering an association, for this compound, of the previously verified radical scavenging attitude (Fig. 2), with its ability to perform as an efficient dual metal chelating polyphenol by means of both the catecholic B ring and the A/C ring arrangement (Fig. 5).

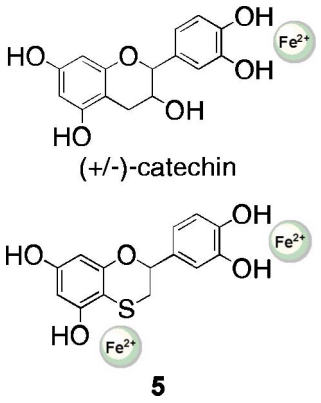

Figure 5. Possible chelating modes of Catechin and thiaflavan derivative **5**.

The presence of the sulfur atom on the C ring is clearly crucial for the chelating ability of derivatives **4** and **5**. Indeed, a qualitative yet convincing validation of these results arose from the UV/visible analyses of compounds **1**–**5**. In the presence of Fe^{2+} ions, added at concentrations used for oxidative DNA damage, we observed a hypochromic effect for the catechol band of compounds **1** and **5**, and for the resorcinol band of thiaflavans **4** and **5** (Fig. 4) as the result of a possible chelation phenomenon (Fig. 4). Such a hypochromic effect is indeed removed by adding EDTA to the solution. No modification of the UV/visible spectra was observed when ferrous ions were added to compounds **2** and **3** (data not shown). Since compounds **2** and **3** possess limited scavenging activity (Fig. 2) and are unable to chelate ferrous ions, we expected poor protection towards GSH/Fe^{3+}-induced DNA damage. Indeed this was the case of compound **3,** while thiaflavan **2**, with a sulfide sulfur conjugated to the hydroxyl group, was surprisingly more efficient (Fig. 3). At the moment, we cannot easily rationalize this result and a more detailed study on the effect of conjugated sulfide on the redox potential of phenols will be necessary.

In conclusion, our data indicate that properly synthesized polyhydroxylated 4-thiaflavans can be considered multipotent[27] protective agents for their ability to behave as antioxidants due to the 'catechin-like' and/or 'tocopherol-like' radical scavenging attitude, as well as the metal chelating capability. A more detailed SAR is under study for the preparation of 4-thiaflavan derivatives suitable for in vivo testing.

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22. Herring sperm DNA, GSH, CumOOH, α-tocopherol (Vit E) and Catechin were commercially available. Hydroxylated 4-thiaflavans **1**–**5** were synthesized following as previously reported.[18] Herring sperm DNA (1 mg/mL) was incubated in air for 2 h at 37 °C in the dark in a buffered, pH 7 (Hepes 3 mM) solution with and without the test substances, in the presence of 6 mM CumOOH or 3 μM $FeCl_3$/15 mM GSH. All potential antioxidants were tested at concentration of 20 μM. After incubation, DNA was precipitated by ethanol in the presence of 20% 10 M ammonium acetate, washed with 70% ethanol, dried, dissolved in 10 mM Tris–HCl, pH 7.3, and hydrolysed following the procedure recommended by ESCODD, with nuclease P1 (15 IU) and alkaline phosphatase (10 IU). The hydrolysed mixture was filtered by Micropure-EZ enzyme remover (Amicon, Bedford, MA, USA) and 50 μl was injected into an HPLC apparatus. The nucleosides were separated by C18 reverse-phase column (Supelco, 5 μm, I.D. 0.46×25 cm). The eluting solution was 50 mM KH_2PO_4, pH 5.5, containing 8% methanol at a flow rate of 0.85 ml/min. The 8-OHdG and dG were detected using an ESA Coulochem III electrochemical detector in line with a UV detector as previously reported: Lodovici, M.; Guglielmi, F.; Meoni, M.; Dolara, P. *Food Chem. Toxicol.* **2001**, *39*, 1205.
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