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The antioxidant activity of phloretin: the disclosure of a new antioxidant pharmacophore in flavonoids

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Abstract

Phloretin is a dihydrochalcone flavonoid that displays a potent antioxidant activity in peroxynitrite scavenging and the inhibition of lipid peroxidation. Comparison with structurally related compounds revealed that the antioxidant pharmacophore of phloretin is 2,6-dihydroxyacetophenone. The potent activity of 2,6-dihydroxyacetophenone is due to stabilisation of its radical via tautomerisation. The antioxidant pharmacophore in the dihydrochalcone phloretin, i.e., the 2,6-dihydroxyacetophenone group, is different from the antioxidant pharmacophores previously reported in flavonoids.

Keywords: Phloretin; Dihydrochalcone; Flavonoid; Peroxynitrite; Lipid peroxidation

Generation of reactive oxygen species and reactive nitrogen species in vivo are involved in a wide range of human diseases, including cancer, cardiovascular, pulmonary, and neurological diseases [1]. Hence, agents with the ability to protect against these reactive species may be therapeutically useful. In line with this hypothesis is the widely accepted view that the positive health effects of flavonoids can be attributed to their antioxidant activity. Flavonoids are a group of more than 5000 different natural polyphenol substances. They are abundantly present in green vegetables, fruits, olive and soybean oils, red wine, chocolate, and tea [2]. The flavonoids are classified into different categories. Within each category there is a variation in number and arrangement of hydroxyl moieties as well as sugar groups. Several antioxidant pharmacophores in the flavonoids have been described [3–6].

Chalcones (1,3-diaryl-2-propen-1-ones) are flavonoids lacking a heterocyclic C ring. Also this category of flavonoids displays a broad spectrum of bioactivities such as anticancer, antifungal, antibacterial, antiviral, and antiinflammatory properties [7]. Dihydrochalcones which do not have α–β double bond comprise phloretin [β-(4-hydroxyphenyl)-1-(2,4,6-trihydroxypropiophenone) and its glucoside, phloridzin (phloretin 2-β-D-glucose) (Fig. 1). These compounds are abundantly present in apples [8]. The goal of the present study is to examine the antioxidant activity of phloretin and to determine the pharmacophore responsible for antioxidant activity of phloretin.

Materials and methods

Chemicals. Phloretin [β-(4-hydroxyphenyl)-1-(2,4,6-trihydroxypropiophenone], phloridzin (phloretin-2-β-D-glucose), phloroglucinol (1,3,5-trihydroxybenzene), 2,4,6-trihydroxyacetophenone, 2,4-dihydroxyacetophenone, 2,6-dihydroxyacetophenone, 2-hydroxyacetophenone, and KO_2 were obtained from Fluka. Resorcinol (1,3-dihydroxybenzene), phenol, and dihydrorhodamine-123 (DHR-123) were obtained from Sigma. 2-Hydroxy-6-methoxyacetophenone was obtained from Aldrich. Nitrogen monoxide was obtained from AGA (Hamburg, Germany) and all other chemicals were of the highest grade of purity.

Synthesis of peroxynitrite. Potassium oxoperoxonitrate (ONOOK) was produced from the reaction of solid KO_2 with NO-gas as described by Koppenol et al. [9]. Briefly, the NO-gas slowly flowed over the mixture of KO_2 and quartz-sand, which was constantly stirred and kept on ice. The mixture was poured into a cold potassium hydroxide solution. Addition of manganese dioxide removes the hydrogen peroxide that results from excess decomposition of potassium superoxide. The solution with peroxynitrite was obtained by filtering of sand and manganese dioxide. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm.

Peroxynitrite scavenging. Peroxynitrite scavenging was measured by the oxidation of DHR-123 as described by Kooy et al. [10]. In short,

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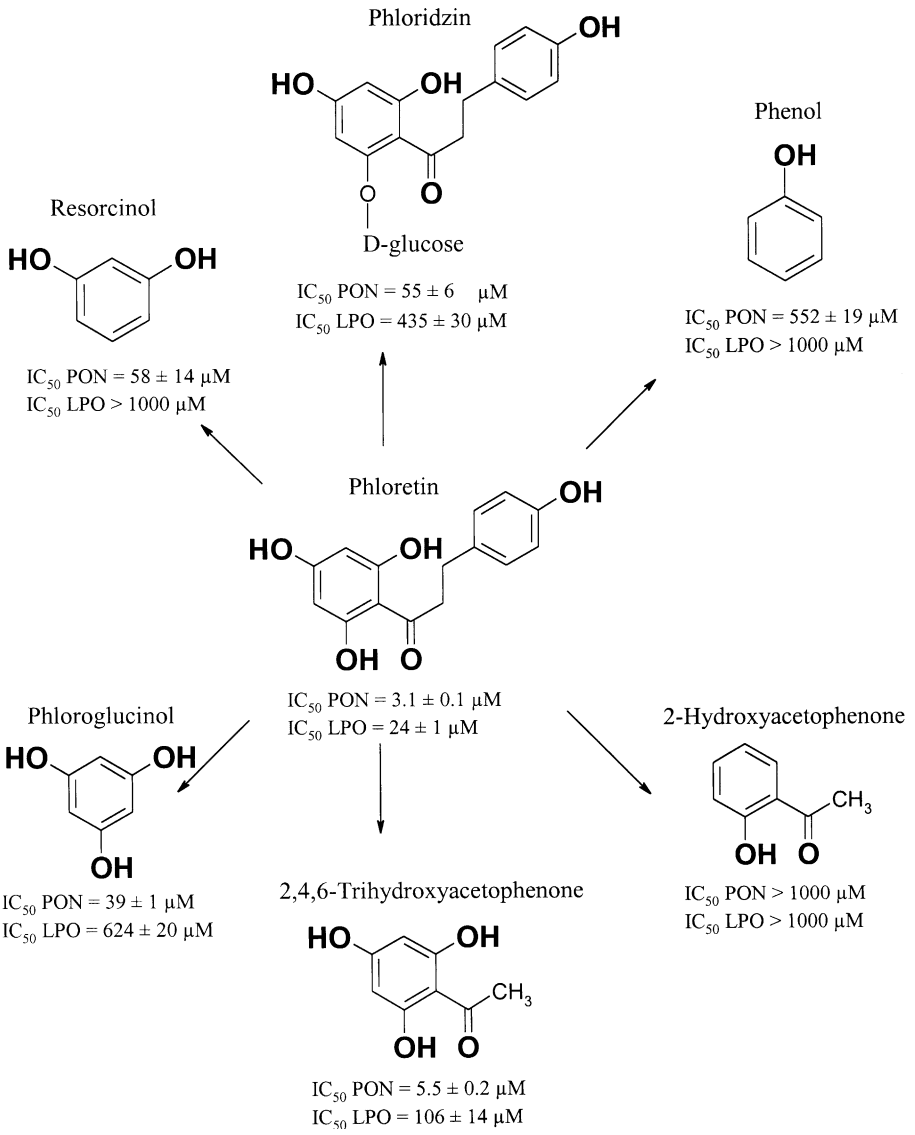

Fig. 1. The antioxidant activity of dihydrochalcones (phloretin and phloridzin) and structurally related compounds in peroxynitrite scavenging and the inhibition of lipid peroxidation. The activity is expressed as the concentration of the compound that gives 50% scavenging of the peroxynitrite (IC_{50} PON) and the concentration that is needed to inhibit 50% of the lipid peroxidation (IC_{50} LPO). Values are presented as means ± SD of at least three separate experiments.

the desired concentrations of the test compounds were added to 1 ml of 100 mM phosphate buffer at 37 °C. The final concentration of DHR-123 in phosphate buffer was 5 μM. During mixing the final concentration, 0.6 μM, of peroxynitrite was added. The fluorescent product, rhodamine-123, was measured by fluorescence detection with excitation and emission wavelengths of 500 and 536 nm, respectively. The effects are expressed as the concentration giving 50% inhibition of the oxidation of DHR (IC_{50}).

Isolation of rat liver microsomes. Male Lewis rats, 200–250 g, were killed by decapitation. Microsomes were prepared according to Haenen and Bast [11] with a slight modification. Livers were removed and homogenised (1:2 w/v) in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000*g* for 30 min at 4 °C. Subsequently, the supernatant was centrifuged at 65,000*g* for 60 min at 4 °C. The microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml), corresponding with 1–1.3 mg protein/ml, and stored at −80 °C. Before use the microsomes were thawed and washed twice, with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl, followed by centrifugation at 65,000*g* for 40 min at 4 °C. Finally, the pellet was resuspended in 4 ml Tris–HCl buffer and then used. The protein concentration was measured according to Smith et al. [12].

Lipid peroxidation assay. At 37 °C, 250 μl of microsomes was added to 540 μl of Tris–HCl/KCl buffer. Ten μl of the test compounds with different concentrations were added. Ascorbate solution (125 μl) was added giving a final concentration of 0.2 mM. The reaction was started by adding 75 μl of a freshly prepared ferrous sulphate solution giving a final concentration of 10 μM. Lipid peroxidation was assayed by measuring thiobarbituric acid (TBA) reactive material. The incubation mixture (1 ml) was stopped by adding 2 ml of an ice-cold TBA–trichloroacetic acid–HCl–butylhydroxytoluene (BHT) solution. The mixture was heated for 15 min at 90 °C and then centrifuged for 5 min. The absorbance was determined at 535 nm vs 600 nm. The TBA–trichloroacetic acid–HCl–BHT solution was prepared by dissolving

41.6 mg TBA/10 ml trichloroacetic acid (16.8 w/v in 0.125 N HCl). To 10 ml TBA–trichloroacetic acid–HCl 1 ml BHT (1.5 mg/ml ethanol) was added. The added chemicals did not interfere with the assay in the concentrations used.

Results

Phloretin is a potent antioxidant in peroxynitrite scavenging and the inhibition of lipid peroxidation. The concentration of phloretin needed to scavenge 50% of the peroxynitrite (IC_{50}) is 3.1 μM and the concentration of phloretin needed to inhibit 50% of the lipid peroxidation is 24 μM (Fig. 1). Occupation of 2-OH by glucose decreased the antioxidant activities of phloridzin 18 times in comparison to phloretin. The hydroxyl groups of the sugar moiety have no role in the antioxidant activity of phloridzin, since the IC_{50} of glucose is more than 1000 μM for either peroxynitrite scavenging or inhibition of lipid peroxidation.

It has been shown that the antioxidant activity of flavonoids resides in the aromatic OH groups [6]. To determine the role of the different functional groups in phloretin, structurally related compounds were tested. Phenol is a very poor antioxidant. Introducing more OH groups in *meta* position, similar to the A-ring of phloretin, gives resorcinol or phloroglucinol. Resorcinol and phloroglucinol have a substantial higher antioxidant activity compared to phenol, however, it is still far less than that of phloretin. This points toward a substantial contribution of the carbonyl group in phloretin. However, 2-hydroxyacetophenone is a very poor antioxidant, its activity is even lower than that of phenol (Fig. 1). Remarkably, 2,4,6-trihydroxyacetophenone has an activity that is similar to that of phloretin. Surprisingly, the antioxidant activity of 2,6-dihydroxyacetophenone is comparable to that of 2,4,6-trihydroxyacetophenone, whereas 2,4-dihydroxyacetophenone is a very poor antioxidant (Fig. 2). 2-Hydroxy-6-methoxyacetophenone displayed virtually no antioxidant activity.

Discussion

Phloretin is a relatively potent antioxidant in peroxynitrite scavenging and inhibition of lipid peroxidation. The potent antioxidant activity of phloretin has also been found in other assays, e.g., hydroxyl radical scavenging [3,13] and 1,1-diphenyl-2-picrylhydrazyl radical scavenging [7]. In comparison with structurally related compounds it was found that the activity of phloretin does not reside only in the three hydroxyl groups of ring A. Their activity is enhanced by the carbonyl group. Remarkably, introduction of a carbonyl group in phenol (giving 2-hydroxyacetophenone) reduces the antioxidant activity, whereas introduction of the same group in phloroglucinol (giving 2,4,6-trihydroxyacetophenone) increases the antioxidant activity. The activity of 2,4,6-trihydroxyacetophenone is comparable to that of phloretin.

Based on NMR data, it has been reported that in 2-hydroxyacetophenone the hydroxyl group forms an intramolecular hydrogen bridge with the adjacent double bound oxygen [14]. This will drastically reduce radical scavenging by means of hydrogen donation, explaining the very poor antioxidant activity of 2-hydroxyacetophenone. In 2,4,6-trihydroxyacetophenone there is evidence that no intramolecular hydrogen bridge between the carbonyl group and one of the aromatic hydroxyl group is formed [14]. Moreover, an intramolecular hydrogen bridge can only be formed with one of the aromatic hydroxyl groups, indicating that the carbonyl group cannot reduce the activity of all aromatic hydroxyl groups in 2,4,6-trihydroxyacetophenone. This can explain why the carbonyl group cannot effectively block the antioxidant activity of 2,4,6-trihydroxyacetophenone as in 2-hydroxyacetophenone.

Remarkable is also the difference in activity between 2,6-dihydroxyacetophenone, with an activity identical to 2,4,6-trihydroxyacetophenone, and 2,4-dihydroxyacetophenone, with virtually no antioxidant activity. This indicates that the effect of the carbonyl group is not due to electron donation via the aromatic ring, since this effect is comparable for the 4 and 6 positions.

An explanation for the potent antioxidant activity of 2,6-dihydroxyacetophenone might be found in the possible stabilisation of the radical that is formed after hydrogen abstraction. As shown in Fig. 3, the free electron that is generated due to hydrogen abstraction of one of the hydroxyl groups can be delocalised over the three oxygen atoms present in the molecule. This involves a keto–enol transformation of the carbonyl group, and the transfer of an α-hydrogen atom of the carbonyl group to the oxygen radical. In this way the unpaired electron is transferred to the carbonyl group. The unpaired electron can then be transferred to the other aromatic hydroxyl group by a hydrogen transfer. Such a stabilisation of the radical is not possible in the radical of 2,4-dihydroxyacetophenone, which explains the poor antioxidant activity of 2,4-dihydroxyacetophenone.

Our explanation is confirmed by the poor antioxidant activity of 2-hydroxy-6-methoxyacetophenone (Fig. 2). The radical of this compound can also not be stabilised in the same way as 2,6-dihydroxyacetophenone. In phloridzin, one of the two hydroxyl groups adjacent to the carbonyl group has been substituted by a sugar moiety. Subsequently, also in this compound stabilisation of its radical as in the radical of 2,6-dihydroxyacetophenone cannot be achieved. This explains the relatively poor antioxidant activity of phloridzin compared to phloretin.

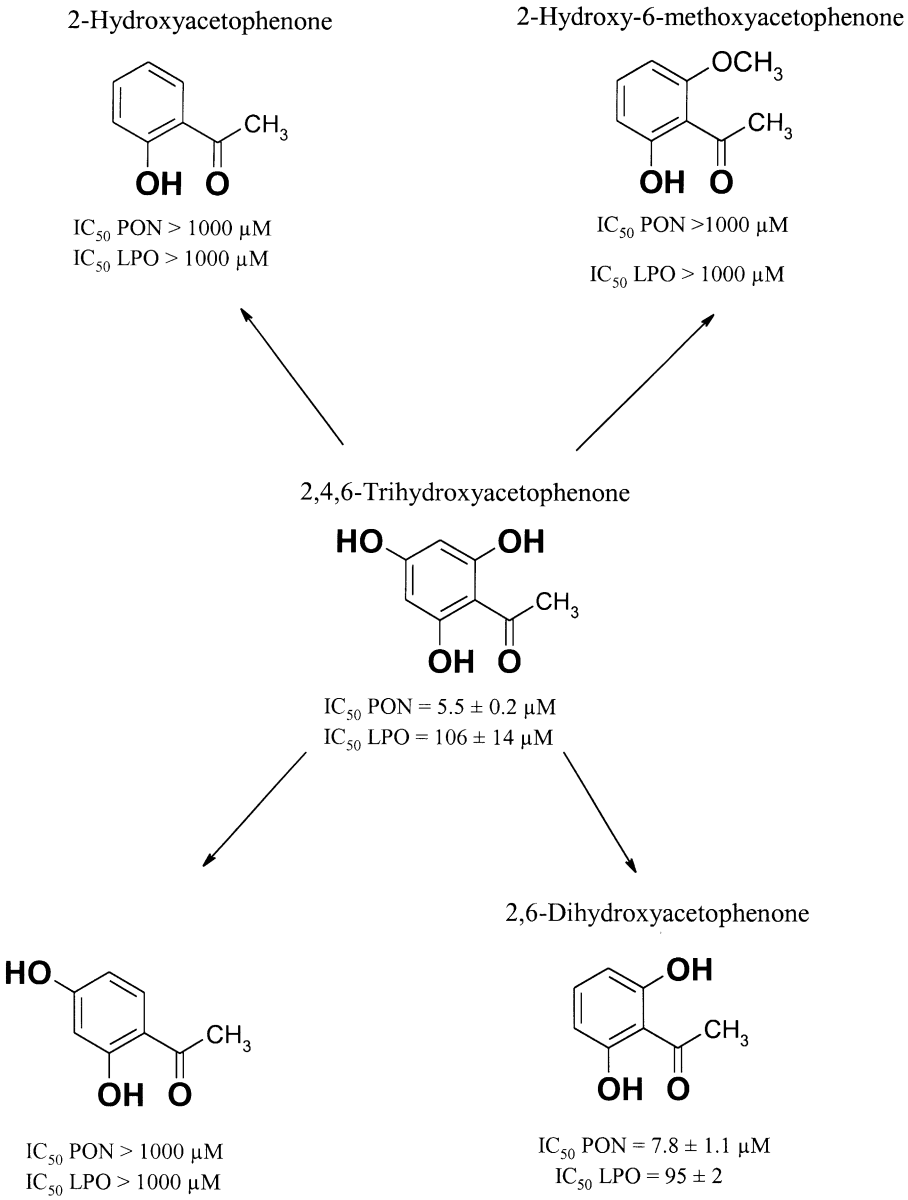

Fig. 2. The antioxidant activity of 2,4,6-trihydroxyacetophenone and structurally related compounds in peroxynitrite scavenging and the inhibition of lipid peroxidation. The activity is expressed as the concentration of the compound that gives 50% scavenging of the peroxynitrite (IC_{50} PON) and the concentration that is needed to inhibit 50% of the lipid peroxidation (IC_{50} LPO). Values are presented as means ± SD of at least three separate experiments.

It has been reported that in another group of flavonoids, i.e., the flavonols, two different pharmacophores can be found, i.e., on the B-ring or the AC-ring (Fig. 4) [5,6]. In ring B this is the catechol moiety and the activity of one of the hydroxyl groups is enhanced by the electron donating effect of the other one. In the AC-ring the hydroxyl group at position 3 is the most reactive one and its activity is enhanced by the electron donating effect of the hydroxyl groups at positions 5 and 7. In the dihydrochalcone phloretin another pharmacophore is present, i.e., the 2,6-dihydroxyacetophenone group. It is proposed that delocalisation of the unpaired electron in the radical that is obtained after hydrogen abstraction is essential for its antioxidant activity.

One of the pharmacophores in flavonols, i.e., the AC-ring, has a strong chemical resemblance with the pharmacophore in phloretin, i.e., the 2,6-dihydroxyacetophenone group. In both pharmacophores there are two aromatic hydroxyl groups with a carbonyl group in between and an intramolecular hydrogen bridge can be formed with either hydroxyl group. Methylation of one of the aromatic hydroxyl groups in 2,6-dihydroxyacetophenone abolishes the activity of the compound. In contrast, 2′,3′,5,7-tetra-*O*-methyl-quercetine still has a

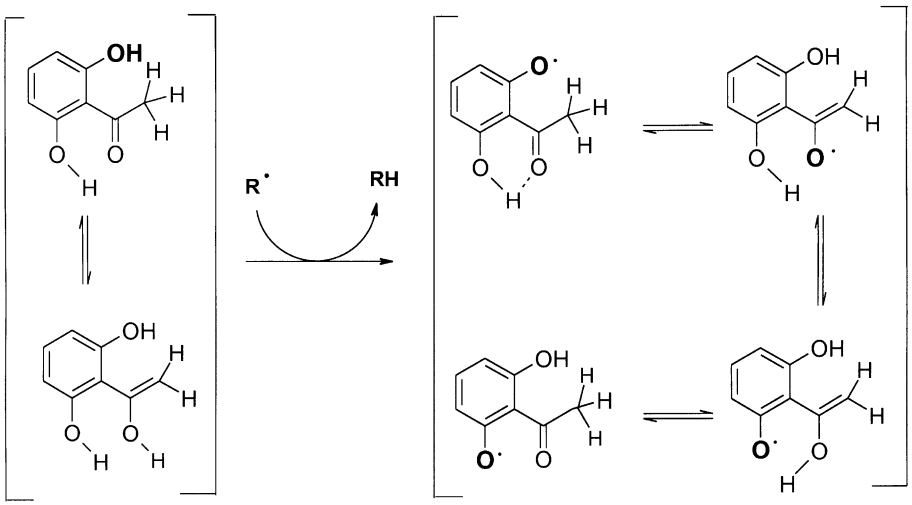

Fig. 3. The proposed mechanism for the antioxidant activity of 2,6-dihydroxyacetophenone.

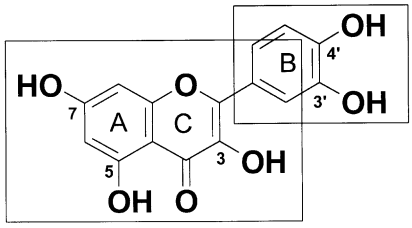

Fig. 4. The antioxidant pharmacophores in flavonols. Two different pharmacophores have been proposed, i.e., the catechol group in ring B and the three hydroxyl groups in ring AC. The activity of the 3-OH group is enhanced by an electron donating effect of the hydroxyl groups at positions 5 and 7.

potent antioxidant effect [6]. This points toward a fundamental difference between both pharmacophores. In the AC-ring an electronic effect is important, while in 2,6-dihydroxyacetophenone tautomerisation is proposed to play a pivotal role. This indicates that within the chemically heterogeneous group of flavonoids different pharmacophores with different characteristics can be found in the various categories.

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