Tetrahydrofolate and 5-methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore

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Abstract The presumed protective effect of folic acid on the pathogenesis of cardiovascular, hematological and neurological diseases and cancer has been associated with the antioxidant activity of folic acid. Peroxynitrite (PON) scavenging activity and inhibition of lipid peroxidation (LPO) of the physiological forms of folate and of structurally related compounds were tested. It was found that the fully reduced forms of folate, i.e. tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF), had the most prominent antioxidant activity. It appeared that their protection against LPO is less pronounced than their PON scavenging activity. The antioxidant activity of these forms of folic acid resides in the pterin core, the antioxidant pharmacophore is 4-hydroxy-2,5,6-triaminopyrimidine. It is suggested that an electron donating effect of the 5-amino group is of major importance for the antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine. A similar electron donating effect is probably important for the antioxidant activity of THF and 5-MTHF.
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1. Introduction

Folates function as cofactors in the transfer and utilization of one carbon groups. This plays a key role in the biosynthesis of purines and pyrimidines and regeneration of methionine [1]. Folic acid (FA) is made up of a pterin moiety (purine and pyrazine fused together) that is linked to the side chain containing *p*-aminobenzoic acid (pteroic acid) and glutamic acid (Fig. 1). FA exists in lactam and lactim tautomeric forms. The lactam form has a 4-OH moiety on the purine-type ring (Fig. 1). FA is in vivo reduced to dihydrofolate (DHF) and subsequently to tetrahydrofolate (THF). THF is enzymatically converted into 5-methyltetrahydrofolate (5-MTHF), the actual active form [1].

Epidemiological studies have shown that FA supplementation can significantly reduce the risk of cardiovascular and hematological diseases [2,3], neurological and neuropsychiatric disorders [4,5], neural tube defects [6] and cancer including cervical, lung, brain, pancreatic, colorectal and breast cancer [7,8]. The production of reactive oxygen species, reactive chlorine species and reactive nitrogen species is implicated in these pathologies. Therefore, the antioxidant activity of FA has been suggested to be involved in the presumed effect of FA on health [9,10]. There is no consensus about the free radical scavenging activity of FA. One study [10] suggested that the 4-OH group on the pterin ring has an important role. Another study [11] indicated that the side chain, i.e. *N*-(*p*-aminobenzoyl)-L-glutamic acid, is more important, since the products formed by nitration of FA were identified as 10-nitrofolate and 12-nitrofolate [11]. The present study was designed to further examine the antioxidant activity of FA and its physiological metabolites and to determine which part of the molecule is important for this activity.

2. Materials and methods

2.1. Chemicals

FA, DHF, 2,4-diamino-6-hydroxypyrimidine, 4-hydroxy-2,5,6-triaminopyrimidine and KO_2 were obtained from Fluka Chemie (Buchs, Switzerland). 2-Amino-4-hydroxypyrimidine, THF, 5-MTHF, 5,6,7,8-tetrahydrobiopterin (BH_4), leucopterin and dihydrorhodamine-123 (DHR-123) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade of purity available.

2.2. Isolation of rat liver microsomes

Male Lewis rats, 200–250 g, were killed by decapitation. Microsomes were prepared according to Haenen and Bast [12] with a slight modification. Livers were removed and homogenized (1:2 w/v) in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at $10\,000 \times g$ for 30 min at 4°C. Subsequently, the supernatant was centrifuged at $65\,000 \times g$ for 60 min at 4°C. The microsomal pellet was resuspended in the phosphate buffer (2 g liver tissue/ml), corresponding to 1–1.3 mg protein/ml, and stored at −80°C. Storage of microsomes at −80°C has no effect on the oxidation state of microsomes in the in vitro assay. Before use the microsomes were thawed and diluted five-fold with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl and washed twice followed by centrifugation at $65\,000 \times g$ for 40 min at 4°C. Finally, the pellet was resuspended in 4 ml Tris–HCl buffer and then used.

2.3. Lipid peroxidation (LPO) assay

2.3.1. Incubation conditions. LPO assay was carried out according to Haenen and Bast [12] with a minor modification. At 37°C, 450 μl of Tris–HCl/KCl buffer was added to 250 μl of microsomes, the final concentration was 0.5 g liver tissue/ml. Hundred μl of a solution containing one of the test compounds was added to achieve the desired concentration. A solution of ascorbate (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 sulfate solution giving a final con-

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centration of 10 μM. After 20 min incubation, the reaction was stopped as described in Section 2.3.2. The effects are expressed as the concentration giving 50% inhibition of LPO (LPO IC$_{50}$).

2.3.2. Spectral measurements. LPO was assayed by measuring thiobarbituric acid (TBA)-reactive material [12]. 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.

2.4. Synthesis of peroxynitrite (PON)

Potassium oxoperonitrate (ONOOK) was produced from the reaction of solid KO$_2$ with NO gas as described by Koppenol et al. [13]. 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 PON was obtained by filtering off the sand and manganese dioxide. The concentration of PON was determined spectrophotometrically at 302 nm.

2.5. Oxidation of DHR-123

PON scavenging was measured by the oxidation of DHR-123 as described by Kooy et al. [14]. In short, 100 μl of the desired concentration of the test compound was added to 0.9 ml of 100 mM phosphate buffer at 37°C. The final concentration of DHR-123 in phosphate buffer was 5 μM. During mixing 10 μl of PON (in 0.01 M NaOH) was added giving a final concentration of 0.6 μM. 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-123 (PON IC$_{50}$).
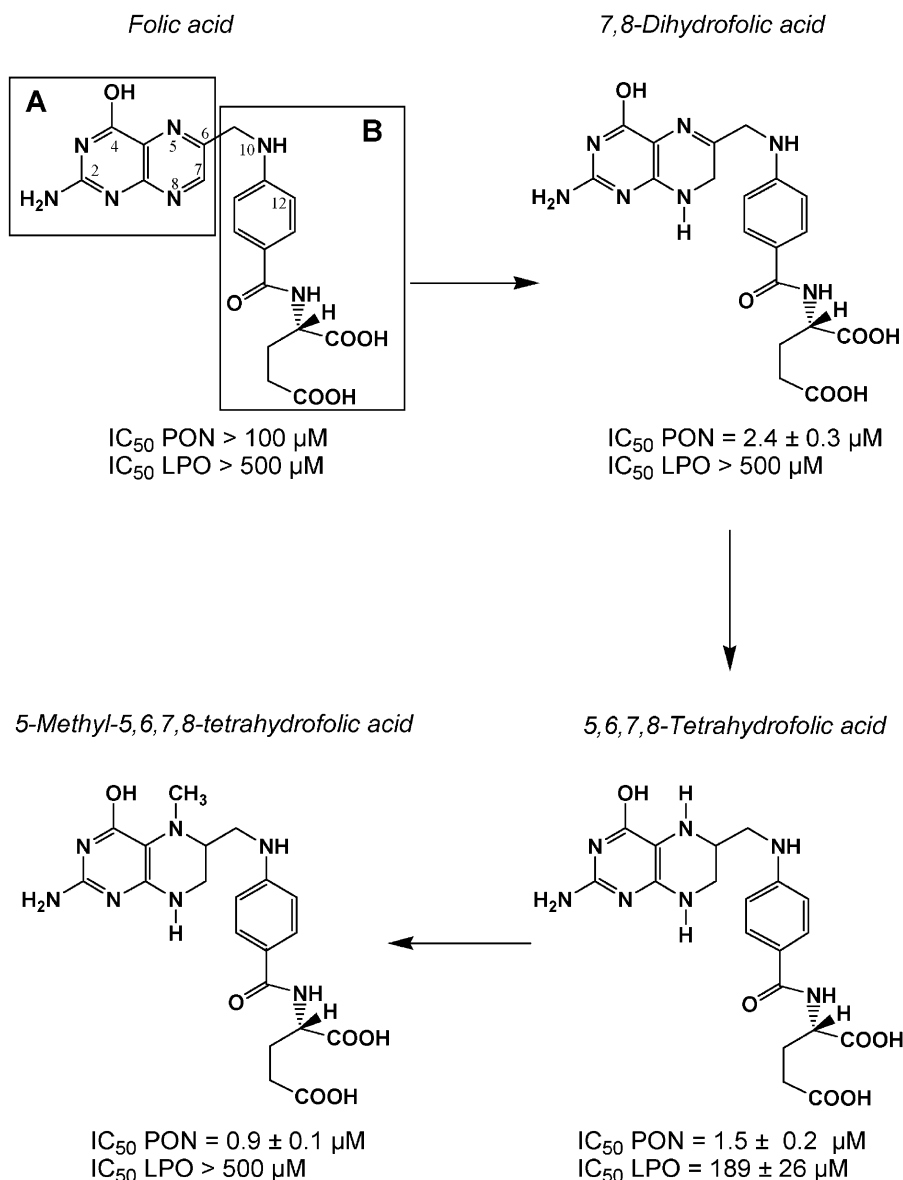

Fig. 1. The antioxidant activity of FA and its physiological metabolites measured by PON scavenging and inhibition of LPO. The activity is expressed as the concentration of the compound that gives 50% scavenging of PON (IC$_{50}$ PON) and the concentration that is needed to inhibit 50% of LPO (IC$_{50}$ LPO). FA is made up of two parts, viz. a pterin moiety (A) and an *N*-(*p*-aminobenzoyl)-L-glutamic acid part (B). The numbering of the most important atoms is also shown in the chemical structure of FA. Values are presented as median ± half of the range of at least three separate experiments.

2.6. The trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay modified by Arts et al. [15] was used. ABTS˙ stock solution was prepared by dissolving 30 mg ABTS in 7.8 ml of 2.46 mM potassium peroxodisulfate. The ABTS˙ stock solution was diluted with 100 mM phosphate buffer (pH 7.4) to give an absorbance at 734 nm between 0.68 and 0.73 at 37°C. The concentration of ABTS˙ was determined using a molar extinction coefficient of 1.5×10^4 M^{-1} cm^{-1}. For measuring the antioxidant capacity, a fixed concentration of the test compounds in this experiment, 5 μM, was mixed with different concentrations (0–46 μM) of the ABTS˙ solution. Absorbance was monitored at 734 nm after 6 min incubation at 37°C. The total amount of ABTS˙ scavenged by the antioxidant, i.e. a in the formula, was calculated using the exponential formula $y = a(1-e^{(-bx)})$ (Sigma Plot, version 7). In this formula y is the reduction of the ABTS˙ concentration after 6 min and x is the initial ABTS˙ concentration. The TEAC value is determined using the equation $TEAC_{test\ compound} = a/(5 \times 1.9)$, where 5 is the concentration of the test compound in μM used in the experiment and 1.9 is the average concentration of ABTS˙ that is scavenged per μM trolox.

3. Results

Fig. 1 gives an overview of the antioxidant activities of FA and its physiological metabolites. The concentration of FA needed to prevent the PON-mediated oxidation of DHR-123 by 50% (IC$_{50}$ PON) is higher than 100 μM and the concentration of FA needed to inhibit LPO by 50% (IC$_{50}$ LPO) is higher than 500 μM. Reduction of FA to DHF increased the PON scavenging activity (IC$_{50}$ PON = 2.4 μM), whereas the concentration needed to inhibit LPO by 50% was still above 500 μM. Reduction of DHF to THF resulted in a more than three-fold increase in the ability to protect against LPO and also the ability to scavenge PON was increased. The PON scavenging activity of 5-MTHF compared to that of THF was higher (the IC$_{50}$ values were 0.9 μM and 1.5 μM, respectively) and the protective effect against LPO was lower (the IC$_{50}$ values were above 500 μM and 189 μM, respectively). The TEAC values of THF and 5-MTHF were equivalent to 1.8 ± 0.1 and 0.7 ± 0.1 μM trolox, respectively (Fig. 2).

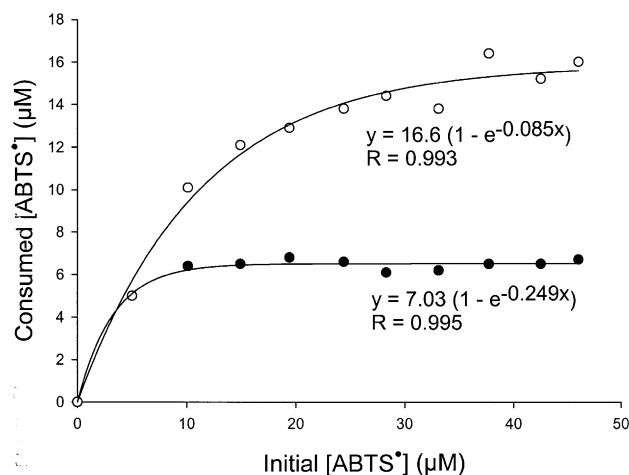FA can be split into two parts, viz. an N-(p-aminobenzoyl)-L-glutamic acid part and a pterin moiety. The IC$_{50}$ of N-(p-aminobenzoyl)-L-glutamic acid was higher than 500 μM for either PON scavenging or inhibition of LPO (data not shown). As shown in Fig. 3, the antioxidant activity of BH$_4$ was comparable to that of THF (IC$_{50}$ LPO = 86 ± 1 μM, IC$_{50}$ PON = 0.5 ± 0.2 μM). The antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine was also comparable to that of THF. Removing the 5 amino group or the 5 and the 6 amino groups of 4-hydroxy-2,5,6-triaminopyrimidine drastically reduced the antioxidant activity. Pterin also displayed no antioxidant activity, whereas leucopterin has a moderate PON scavenging activity.

4. Discussion

An antioxidant effect of FA and its physiological metabolites might be involved in some of the positive health effects. The rationale for the present study was to determine the antioxidant activity of the various endogenous folates and to elucidate which moiety of the molecule determines this activity. FA has a poor antioxidant activity in PON scavenging and LPO inhibition. Its physiological metabolites are more active. Joshi et al. [10] reported that 500 μM of FA induced a 37% inhibition of LPO, which corresponds to the protection we found, i.e. IC$_{50}$ LPO > 500 μM. Based on this partial inhibition by a relatively high concentration, Joshi et al. [10] speculated on a physiological relevance of FA as an antioxidant. We think that the minor protective effect of FA on LPO is physiologically not relevant. The marginal antioxidant effect of FA is also seen from its poor PON scavenging activity.

Reduction of FA to DHF results in a more prominent PON scavenging activity than that of FA, whereas the IC$_{50}$ for inhibition of LPO is still higher than 500 μM. Further reduction to THF and conversion to 5-MTHF enhanced the PON scavenging activity even more. The fully reduced forms, i.e. THF and 5-MTHF, were the most active compounds in inhibiting LPO. 5-MTHF had a relatively lower activity in the protection against LPO than THF (500 μM of 5-MTHF induced 46% LPO inhibition), whereas the PON scavenging activity of 5-MTHF was superior to that of THF. Compared to other compounds, the protective effect of the folates against LPO seems less relevant than their PON scavenging activity. For example, the flavonoid quercetin is much more potent in inhibiting LPO (IC$_{50}$ of quercetin = 9 μM) [16] than the folates, whereas its PON scavenging activity is in the same order of magnitude (IC$_{50}$ of quercetin = 0.53 μM) [17]

To determine the pharmacophore which is responsible for the antioxidant activity of the reduced forms of FA, structurally related compounds were tested. The minor importance of the side chain for the antioxidant activity is demonstrated by (i) the absence of antioxidant activity of N-(p-aminobenzoyl)-L-glutamic acid, (ii) the large difference in activity of the various folates which all contain the same side chain and (iii) the antioxidant activity of BH$_4$ (which lacks the side chain) which is comparable to that of THF. Apparently, the antioxidant activity resides in the pterin moiety. An optimal antioxidant activity is achieved when the pterin ring is in its reduced form. By comparing structurally related compounds (Fig. 3), it was found that the antioxidant pharmacophore of the fully reduced form of folates, i.e. THF, is 4-hydroxy-2,5,6-triaminopyrimidine.

The potency of substituted phenols for PON scavenging

Fig. 2. Determination of the TEAC of THF and 5-MTHF. The reaction of 5 μM THF (○) and 5 μM of 5-MTHF (●) with various concentrations of ABTS˙ was assessed. The concentration of ABTS˙ that is consumed by the antioxidant in 6 min is plotted against the initial concentration of ABTS˙. The TEAC of THF is 1.8 ± 0.1 and that of 5-MTHF is 0.7 ± 0.1.

4-Hydroxypyrimidine

IC$_{50}$ PON > 500 μM
IC$_{50}$ LPO > 500 μM

4-Hydroxy-2-aminopyrimidine

IC$_{50}$ PON > 500 μM
IC$_{50}$ LPO > 500 μM

Pterin

4-Hydroxy-2,5,6-triaminopyrimidine

4-Hydroxy-2,6-diaminopyrimidine

IC$_{50}$ PON > 500 μM
IC$_{50}$ LPO > 500 μM

IC$_{50}$ PON = 0.8 ± 0.3 μM
IC$_{50}$ LPO = 55 ± 2 μM

IC$_{50}$ PON = 143 ± 18 μM
IC$_{50}$ LPO > 500 μM

Leucopterin

5,6,7,8-Tetrahydro-L-biopterin

IC$_{50}$ PON = 27 ± 8 μM
IC$_{50}$ LPO > 500 μM

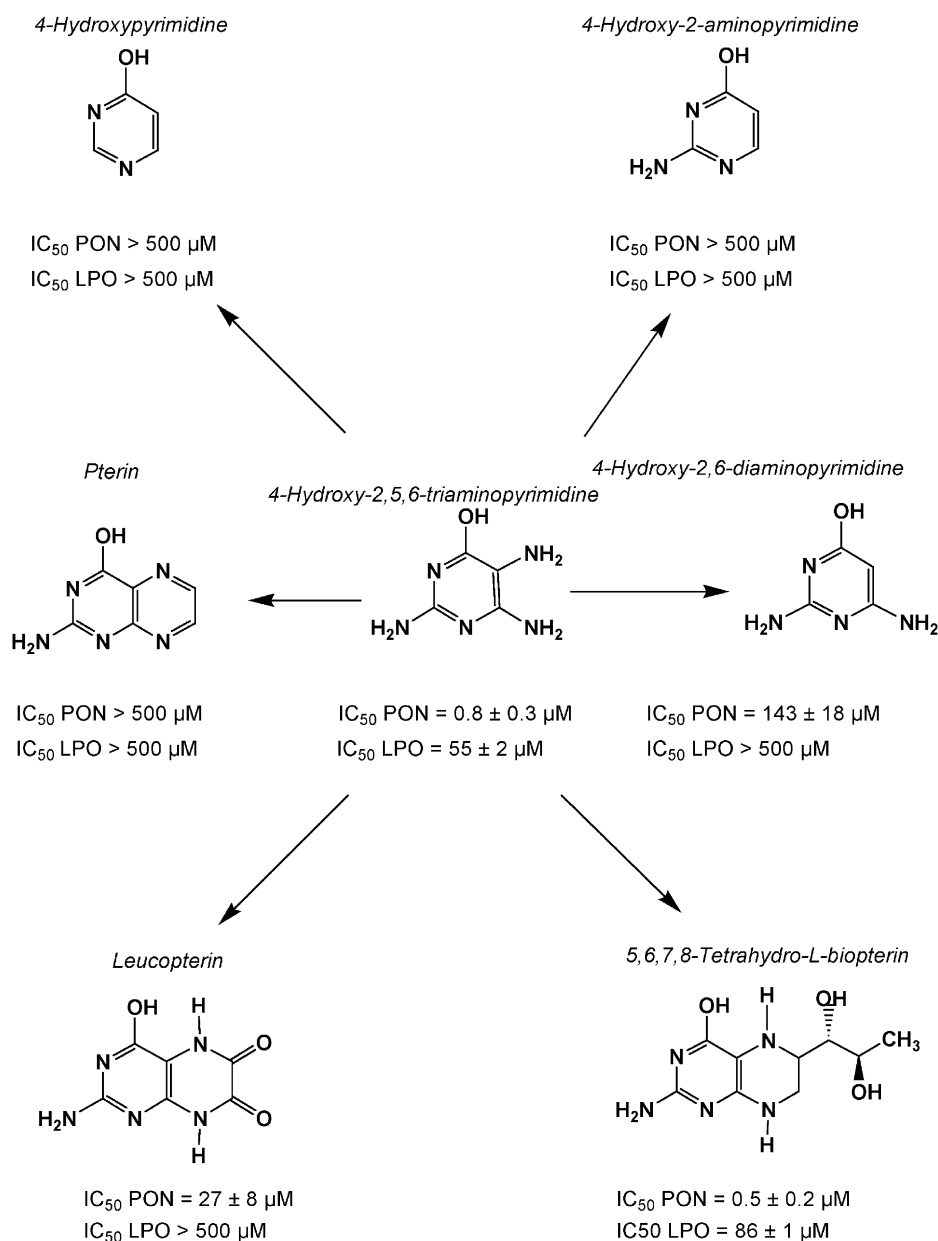IC$_{50}$ PON = 0.5 ± 0.2 μM
IC50 LPO = 86 ± 1 μM

Fig. 3. The antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine and structurally related compounds measured by PON scavenging and the inhibition of LPO. The activity is expressed as the concentration of the compound that gives 50% scavenging of PON (IC$_{50}$ PON) and the concentration that is needed to inhibit 50% of LPO (IC$_{50}$ LPO). Values are presented as median ± half of the range of at least three separate experiments.

and protection against LPO is governed by the electron donating effect of the substituent [16,17]. The higher the electron donating effect, the higher the antioxidant activity. The electron donating effect of a substituent depends on the nature as well as the position of the substituent. For example, the activity of a strong electron donating group, such as the hydroxyl group, on the *ortho* and *para* positions is much higher than that on the *meta* position. This may explain the potent antioxidant activity of catechol (hydroxyl group at the *ortho* position; IC$_{50}$ LPO = 10 μM, IC$_{50}$ PON = 2.7 μM) [16,17] and hydroquinone (hydroxyl group at the *para* position; IC$_{50}$ LPO = 156 μM, IC$_{50}$ PON = 1.8 μM) [16,17] compared to that of resorcinol (hydroxyl group at the *meta* position; IC$_{50}$ LPO > 1000 μM, IC$_{50}$ PON = 42.5 μM) [16,17]. For com-

parison, for phenol the IC$_{50}$ LPO is > 1000 μM and the IC$_{50}$ PON is 263 μM [16,17]. An amino group has an even higher electron donating effect than a hydroxyl group. For 2-aminophenol, the IC$_{50}$ LPO is 5 ± 0.5 μM and the IC$_{50}$ PON is 0.6 ± 0.1 μM. Similarly, the IC$_{50}$ LPO of 4-aminophenol is 12 μM and the IC$_{50}$ PON is 0.9 μM [16,17]. In analogy with the difference in activity due to the position of the substituent given above, it is suggested that the 5 amino group of 4-hydroxy-2,5,6-triaminopyrimidine is of major importance. The location of this potent electron donating group at the *ortho* position of the 4 hydroxyl group will boost the activity of the aromatic hydroxyl group, and may explain the potent activity of 4-hydroxy-2,5,6-triaminopyrimidine to a great extent.

In leucopterin, the electron withdrawing effect of the carbonyl groups next to the 5 and 6 amino groups will reduce the electron donating effect the 4-hydroxyl group receives. This may explain the lower antioxidant activity of leucopterin compared to 4-hydroxy-2,5,6-triaminopyrimidine and BH_4.

A difference in electron donating effect may also explain the higher PON scavenging activity of 5-MTHF compared to THF. The methyl group at the 5 amino group in 5-MTHF will slightly enhance the electron donating effect of the 5 amino group. Based on the TEAC, the number of radicals that can be scavenged by 5-MTHF is lower than that of THF. Possibly, this may explain the low activity of 5-MTHF in the protection against LPO compared to that of THF. The actual antioxidant effect of folates in vivo might depend on both reduction by the enzyme 5,10-methylenetetrahydrofolate and consumption which is related to vitamin B_{12} and homocysteine levels.

In conclusion, FA is a poor antioxidant. The more FA is reduced, the higher its antioxidant activity becomes: THF and 5-MTHF are the folates with the most prominent antioxidant activity. The antioxidant pharmacophore of THF and 5-MTHF, i.e. 4-hydroxy-2,5,6-triaminopyrimidine, resides in the pterin moiety. It is suggested that an electron donating effect of the 5 amino group is of major importance in the antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine. A similar electron donating effect is probably involved in the antioxidant activity of THF and 5-MTHF.

References

[1] Stanger, O. (2002) Curr. Drug Metab. 3, 211–223.
[2] Verhaar, M.C., Stroes, E. and Rabelink, T.J. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 6–13.
[3] Lindenbaum, J. and Nath, B.J. (1980) Br. J. Haematol. 44, 511–513.
[4] Manzoor, M. and Runcie, J. (1976) Br. Med. J. 1, 1176–1178.
[5] Alpert, J.E. and Fava, M. (1997) Nutr. Rev. 55, 145–149.
[6] Daly, L.E., Kirke, P.N., Molloy, A., Weir, D.G. and Scott, J.M. (1995) J. Am. Med. Assoc. 274, 1698–1702.
[7] Duthie, S.J., Narayanan, S., Brand, G.M., Pirie, L. and Grant, G. (2002) J. Nutr. 132, 2444S–2449S.
[8] Akoglu, B., Faust, D., Milovic, V. and Stein, J. (2001) Nutrition 17, 652–653.
[9] Nakano, E., Higgins, J.A. and Powers, H.J. (2001) Br. J. Nutr. 86, 637–639.
[10] Joshi, R., Adhikari, S., Patro, B.S., Chattopadhyay, S. and Mukherjee, T. (2001) Free Radic. Biol. Med. 30, 1390–1399.
[11] Nakamura, M., Nagayoshi, R., Ijiri, K., Nakashima-Matsushita, N., Takeuchi, T. and Matsuyama, T. (2002) Biochem. Biophys. Res. Commun. 297, 1238–1244.
[12] Haenen, G.R.M.M. and Bast, A. (1983) FEBS Lett. 159, 24–28.
[13] Koppenol, W.H., Kissner, R. and Beckman, J.S. (1996) Methods Enzymol. 269, 296–302.
[14] Kooy, N.W., Royall, J.A., Ischiropoulos, H. and Beckman, J.S. (1994) Free Radic. Biol. Med. 16, 149–156.
[15] Arts, M.J.T.J., Dallinga, J.S., Voss, H.-P., Haenen, G.R.M.M. and Bast, A. (submitted).
[16] Heijnen, C.G., Haenen, G.R., Oostveen, R.M., Stalpers, E.M. and Bast, A. (2002) Free Radic. Res. 36, 575–581.
[17] Heijnen, C.G.M., Haenen, G.R.M.M., Vekemans, J.A.J.M. and Bast, A. (2001) Environ. Toxicol. Pharmacol. 10, 199–206.