PROTECTIVE ACTION OF A NEW BENZOFURAN DERIVATIVE ON LIPID PEROXIDATION AND SULPHYDRYL GROUPS OXIDATION

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SUMMARY

The antioxidant properties of a novel water-soluble antioxidant of the benzofuran family (5-hydroxy-4,6,7-trimethyl-2,3-dihydrobenzofuran-2-acetic acid, BFA) were studied. In rat liver mitochondria BFA increases the lag-time and decreases the extent of lipid peroxidation induced by ascorbate/Fe^{2+}; an IC_{50} value of about 12 μM was observed. In rat liver microsomes it inhibits the lipid peroxidation induced both by NADPH/Fe^{2+}/ADP (iron-dependent) and by cumene hydroperoxide (iron-independent), showing IC_{50} values of 25 and 30 μM respectively. The antioxidant efficiency of BFA is slightly higher than that of the congener compound Trolox C. BFA is also able to inhibit the oxidation of protein sulphydryl groups consequent to microsomal lipid peroxidation induced by NADPH/Fe^{2+}/ADP. The antioxidant properties of BFA are discussed considering its hydrophilic character and pharmacological features.

KEY WORDS: antioxidants, benzofuran, lipid peroxidation, sulphydryl groups oxidation, Trolox C.

ABBREVIATIONS: BHT, butylated hydroxytoluene; CHP, cumene hydroperoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; MDA, malondialdehyde; Tris, Tris-hydroxymethyl-aminomethane; Trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

INTRODUCTION

Vitamin E is one of the best known phenolic antioxidants playing a key role as a biological antioxidant, and many efforts were made in order to improve its

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antioxidant potency since several analogues of it were synthesized and tested [1–3].

α-Tocopherol and structurally related compounds react more rapidly with peroxyl radicals than other phenols [1, 2] and this appears to be related to the presence of the fused 6-membered heterocyclic ring that contributes to the stabilization of the developing phenoxyl radical [2]. Theoretical considerations and kinetic measurements have also demonstrated that the 5-membered rings, such as 2,3-dihydrobenzofuran, are more reactive towards hydroperoxyl radicals than the 6-membered rings (such as vitamin E) which are less planar [2]. It was also shown [3] that the benzofuran analogue 2,4,6,7-tetramethyl-2-(4',8',12'-trimethyltridecyl)-5-hydroxy-3,4-dihydrobenzofuran is more active than α-tocopherol in the rat curative myopathy assay.

In the present paper we have studied the antioxidant properties of a novel water-soluble analogue of vitamin E belonging to the benzofuran family: 5-hydroxy-4,6,7-trimethyl-2,3-dihydrobenzofuran-2-acetic acid (BFA.) The product is an original synthesis of the BF/IRFI Research Centre. Different peroxidizable substrates (mitochondria and microsomes) in the presence of different peroxidizing systems, dependent or independent of iron ions, were used.

The reported results reinforce, at subcellular level, previous *in vivo* findings showing that BFA inhibits carbon tetrachloride-induced lipid peroxidation while exerting a mucoregulatory and anti-inflammatory activity [4].

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose buffered with 15 mM Hepes/10 mM Tris at pH 7.4 essentially as described by Myers and Slater [5]. Before final resuspension mitochondria were washed with 0.125 M KCl containing 15 mM Hepes/10 mM Tris at pH 7.4. Rat liver microsomes were prepared as described by Ernster and Nordenbrand [6]. Malondialdehyde was assayed by the thiobarbituric acid procedure described by Buege and Aust [7]. Total microsomal sulphydryl groups were measured with DTNB [8] in 0.2 M Tris-HCl buffer (pH 8.1) containing 10 mM EDTA and 1% SDS to a final volume of 2.5 ml. The reaction was started by adding DTNB to a final concentration of 1 mM and the increase of absorbance was followed at 412 nm until a constant value was obtained (about 5 min). The actual concentration of sulphydryl groups was calculated by using an ε_M=13 600. The unspecific absorbance due to solubilized protein was corrected by subtracting the measurement obtained prior to the addition of DTNB. Protein was determined with the biuret method [9].

RESULTS AND DISCUSSION

Mitochondrial lipid peroxidation induced by iron ions in the presence of ascorbate is reported in Fig. 1. Iron ions are able to initiate and also amplify lipid peroxidation through a branching determined by the decomposition of lipid hydroperoxides. In these processes iron (II) is oxidized to iron (III) and hence the

rate of peroxidation is increased by the addition of relatively low concentrations of ascorbic acid that cyclically reduces ferric to ferrous ions; on the contrary, high concentrations of ascorbate inhibit lipid peroxidation probably by direct reduction of some of the lipid peroxyl radicals [10, 11]. The onset of MDA formation is preceded by a lag-time of several minutes; the addition of increasing concentrations of BFA, ranging from 10 to 40 μM, markedly increases the lag-time and decreases the extent of lipid peroxidation; after 60 min of incubation an almost complete inhibition of lipid peroxidation was reached with 40 μM BFA, while at a concentration as low as 10 μM an inhibition of about 25% was obtained. In this system, the concentration of BFA needed to reach a 50% inhibition (IC_{50}) of lipid peroxidation was approximately 12 μM. Moreover, we have observed that the IC_{50} value increases with increasing times of incubation. BFA exhibits an inhibitory effect, even though to a lower extent, also when added after the onset of lipid peroxidation (not shown).

In liver microsomes lipid peroxidation can be stimulated by iron ions in the presence of NADPH or by cumene hydroperoxide; lipid peroxidation induced by NADPH/Fe^{2+}/ADP depends on the continuous reduction of the Fe^{3+}/ADP complex, mediated by the flavin of the microsomal NADPH-cytochrome P450 reductase [12]. Similarly to the above-reported iron/ascorbate system, iron is being reoxidized during lipid peroxidation. Cumene hydroperoxide-dependent lipid peroxidation is stimulated by free radicals originating from the homolytic or heterolytic cleavage of the O–O bond of the hydroperoxide at the level of cytochrome P-450 [13]; in this case lipid peroxidation is completely independent

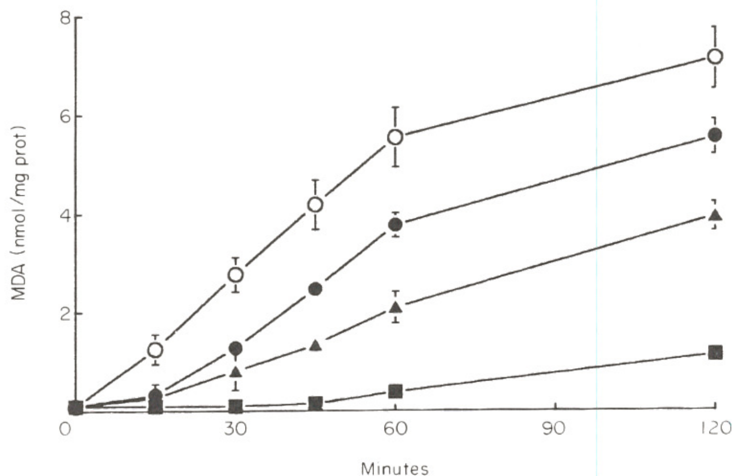

Fig. 1. Effect of increasing concentrations of BFA on MDA formation induced by Fe^{2+}/ascorbate in rat liver mitochondria. Mitochondria (1 mg/ml) were incubated at 30°C in 0.125 M KCl, 15 mM Hepes/10 mM Tris (pH 7.4). Lipid peroxidation was initiated by 20 μM $FeSO_4$/0.2 mM ascorbate and MDA was measured on 1 ml aliquots withdrawn at the indicated times. Bars represent standard deviations of the plotted values and are the average of at least four different experiments. (○—○) control, no addition; (●—●) 10 μM BFA; (▲—▲) 20 μM BFA; (■—■) 40 μM BFA.

of iron ions and takes place also in the presence of relatively high concentrations of chelating agents such as EDTA [14]. Incubation of rat liver microsomes with NADPH/Fe^{2+}/ADP or cumene hydroperoxide resulted in a MDA formation that is larger in the former case (Fig. 2a and b). BFA behaves as an antioxidant of potency comparable to that of classical antioxidants such as BHT or Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), the latter showing an antioxidant activity similar to that of α-tocopherol [15, 16]. BFA, Trolox C and

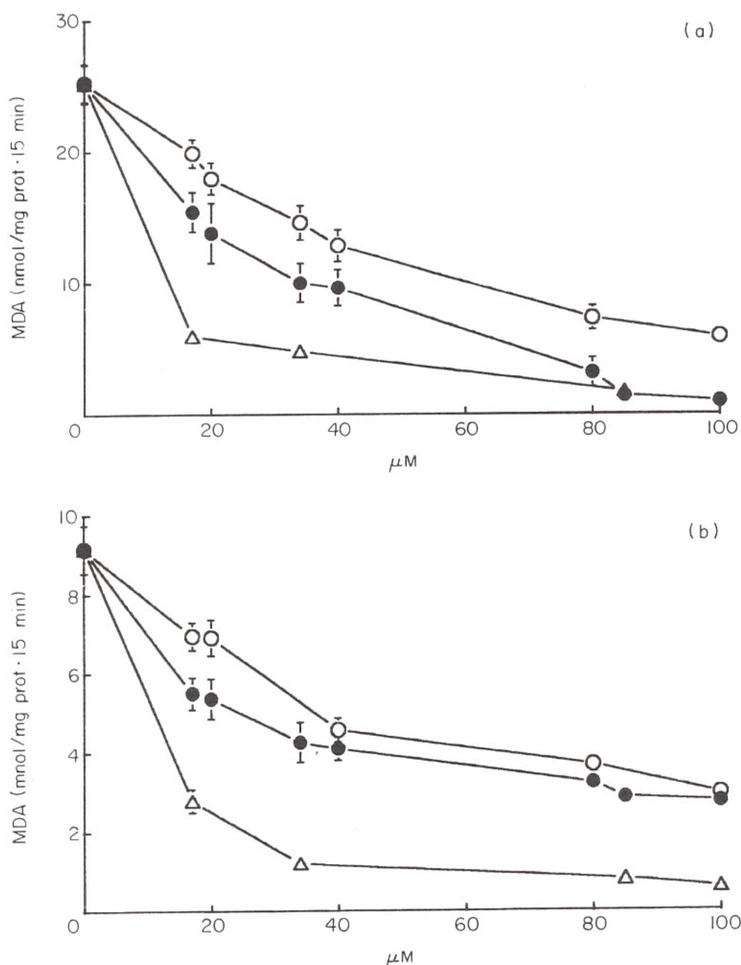

Fig. 2. Effect of increasing concentrations of BFA, Trolox C and BHT on MDA formation induced in rat liver microsomes by NADPH/Fe^{2+}/ADP (a) and by cumene hydroperoxide (b). The assay system, consisting of 0.125 M KCl, 15 mM Hepes/10 mM Tris (pH 7.4) contained 1 mg/ml of microsomal protein. Lipid peroxidation was initiated by 0.1 mM NADPH, 10 μM $FeSO_4$ and 0.1 mM ADP (a) or by 0.5 mM cumene hydroperoxide (b). Bars represent standard deviations of the plotted values and are the average of at least four different experiments. Incubations were performed at 30°C for 15 min. BFA (●—●), Trolox C (○—○) and BHT (△—△) were added at the indicated concentrations.

BHT exhibit a strong inhibitory effect on lipid peroxidation (Fig. 2a and b). In accordance with other studies on the antioxidant properties of tocopherol analogues [2, 3] BFA is slightly more efficient than Trolox C in inhibiting lipid peroxidation and this results from the comparison between the IC_{50} values calculated from the data of Fig. 2a and b, i.e. 24 and 40 μM with NADPH/Fe^{2+}/ADP and 30 and 40 μM with CHP respectively for BFA and Trolox

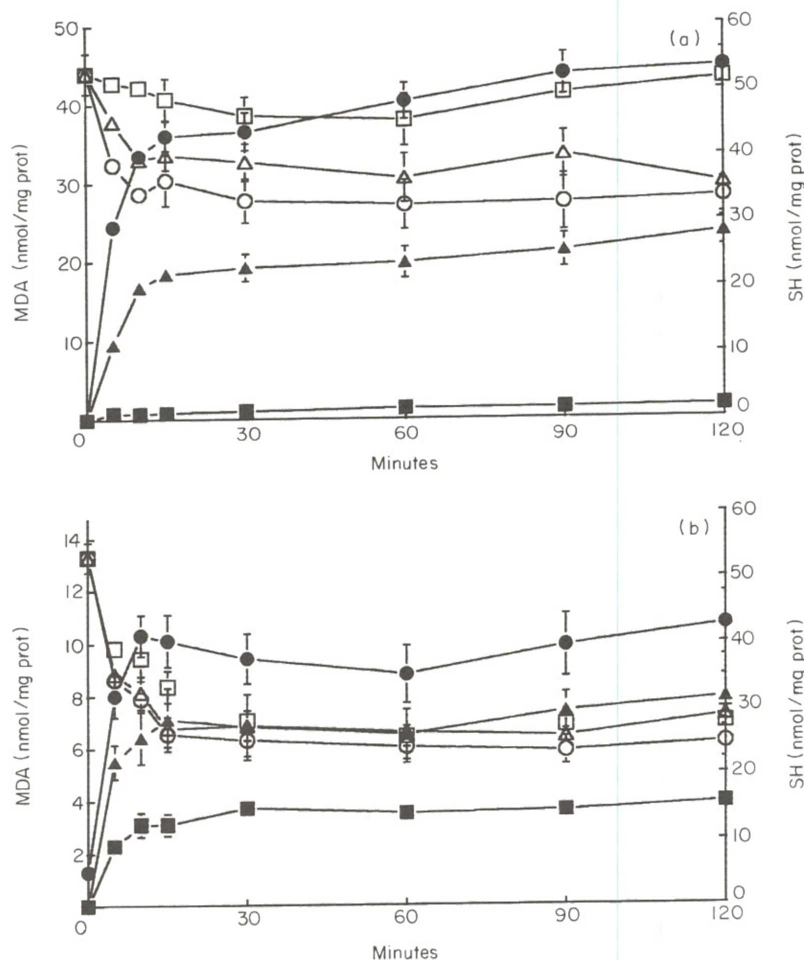

Fig. 3. Correlation between lipid peroxidation and sulphydryl groups oxidation in rat liver microsomes peroxidized with NADPH/Fe^{2+}/ADP (a) or with cumene hydroperoxide (b). Microsomes (1 mg/ml) were incubated at 30°C in 0.125 M NaCl, 15 mM Hepes/10 mM Tris (pH 7.4). Lipid peroxidation was initiated by 0.1 mM NADPH, 10 μM $FeSO_4$ and 0.1 mM ADP or by 0.5 mM cumene hydroperoxide. MDA and total sulphydryl groups were measured on 1 ml aliquots withdrawn at the indicated times. Bars represent standard deviations of the plotted values and are the average of at least four different experiments. MDA (●—●) control, no addition; (▲—▲) 20 μM BFA; (■—■) 80 μM BFA; sulphydryl groups (SH): (○—○) control, no addition; (△—△) 20 μM BFA; (□—□) 80 μM BFA.

C. On the other hand BFA *in vitro* is less effective than BHT that shows an IC_{50} of 12 μM with NADPH/Fe^{2+}/ADP and 13 μM with cumene hydroperoxide (Fig. 2a and b).

While vitamin E is able to trap peroxyl radicals present in the lipid phase or at the interface between lipid and aqueous phase, hydrophilic antioxidants (ascorbate, urate and glutathione) can trap peroxyl radicals present in the aqueous phase. During lipid peroxidation the hydroperoxides formed inside the membrane can be expelled outside in a more hydrophilic environment; consequently, water-soluble peroxyl radicals might be better reduced by a water-soluble antioxidant than by a hydrophobic one.

It is well known that lipid peroxidation causes the oxidation of protein sulphydryl groups even though the resulting products remain to be determined [17]. As shown in Fig. 3 (a and b), MDA formation in microsomes is accompanied by a decrease in titrable sulphydryl groups that levels off at about 40% of the oxidation, indicating that part of the sulphydryl groups are oxidized to the corresponding disulphides. BFA is able to inhibit the oxidation of sulphydryl groups consequent to lipid peroxidation when the peroxidizing system is NADPH/Fe^{2+}/ADP (Fig. 3a) and Trolox C acts in a similar manner (not shown); on the contrary, when lipid peroxidation is elicited by CHP no protection on sulphydryl groups is afforded by BFA (Fig. 3b) that, on the other hand, strongly inhibits the peroxidative process. This result can be referred to the different ways by which lipid peroxidation is initiated: in the first case by the perferryl ion ($[FeO_2]^{2+}$), and in the second by the free radical formed after interaction of cumene hydroperoxide with cytochrome P-450; probably only the latter is able to directly influence sulphydryl groups that do not appear to be sufficiently protected by the antioxidant agents. It should also be noted that, in turn, the oxidation of sulphydryl groups stimulates an increase of lipid peroxidation probably by producing changes in protein conformation which expose the unsaturated fatty acids of phospholipids to the catalysts of lipid peroxidation [18]. The increased mucus production induced by BFA [4] might be referred, at least in part, to its protective effect of the integrity of sulphydryl groups.

The use of analogues of vitamin E appears particularly promising since antioxidants other than vitamin E frequently are not able to reach the site where lipid peroxidation occurs. For instance, at variance with vitamin E, the synthetic antioxidant BHT is able to prevent lipid peroxidation *in vitro* but is completely ineffective in inhibiting lipid peroxides when administered *in vivo* [19]. Similar conclusions were reached by Scuri *et al.* [4] since BFA *in vivo* appears to be more effective than BHT or butylated hydroxyanisole (BHA) on the CCl_4-induced intoxication. In conclusion, BFA appears to be a useful tool to compare the antioxidant properties of various hydrophilic analogues of α-tocopherol since studies in aqueous systems are hampered by the poor solubility of α-tocopherol; the novel antioxidant also has a potential pharmacological interest because of its hydrosolubility, anti-inflammatory and mucoregulatory properties [4].

REFERENCES

1. Burton GW, Ingold KU. Autoxidation of biological molecules. I. The antioxidant activity

of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J Am Chem Soc* 1981; **103:** 6472–7.

2. Burton GW, Hughes L, Ingold KU. Antioxidant activity of phenols related to vitamin E. Are there chain-breaking antioxidants better than α-tocopherol? *J Am Chem Soc* 1983; **105:** 5950–1.
3. Ingold KU, Burton GW, Foster DO, *et al*. A new vitamin E analogue more active than α-tocopherol in the rat curative myopathy bioassay. *FEBS Lett* 1986; **205:** 117–20.
4. Scuri R, Giannetti P, Paesano A. 2-(2,3-Dihydro-5-acetoxy-4,6,7-tribenzofuranil)acetic acid (IRFI 016): a new antioxidant mucoactive drug. *Drug Exptl Clin Res* 1990; **12:** 649–56.
5. Myers DK, Slater EC. The enzymatic hydrolysis of adenosine triphosphate by liver mitochondria. *Biochem J* 1957; **67:** 558–72.
6. Ernster L, Nordenbrand K. Microsomal lipid peroxidation. *Methods Enzymol* 1967; **10:** 574–80.
7. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; **52:** 302–10.
8. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; **82:** 70–7.
9. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 1949; **177:** 751–6.
10. Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*. 2nd ed. Oxford: Clarendon Press, 1989.
11. Wills ED. The role of dietary components in oxidative stress in tissues. In: Sies H, ed. *Oxidative stress*. New York: Academic Press, 1985: 197–218.
12. Hochstein P, Nordenbrand K, Ernster L. Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and mitochondria. *Biochem Biophys Res Commun* 1964; **14:** 323–8.
13. Cadenas E, Sies H, Graf H, Ullrich V. Oxene donors yield low-level chemiluminescence with microsomes and isolated cytochrome P-450. *Eur J Biochem* 1983; **130:** 117–21.
14. Bindoli A, Cavallini L, Jocelyn P. Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate. *Biochim Biophys Acta* 1982; **681:** 496–503.
15. Cort WM, Scott JW, Araujo M, *et al*. Antioxidant activity and stability of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. *J Am Oil Chem Soc* 1975; **52:** 174–8.
16. Davies MJ, Forni LG, Willson RL. Vitamin E analogue Trolox C. *Biochem J* 1988; **255:** 513–22.
17. Little C, O'Brien PJ. Products of oxidation of a protein thiol group after reaction with various oxidizing agents. *Arch Biochem Biophys* 1967; **122:** 406–10.
18. Bindoli A, Cavallini L, Siliprandi N. Effect of thiols oxidation on lipid peroxidation in rat liver mitochondria. *Chem-Biol Interactions* 1977; **19:** 383–6.
19. Hammer CT, Willis ED. The role of lipid components of the diet in the regulation of the fatty acid composition of the rat liver endoplasmic reticulum and lipid peroxidation. *Biochem J* 1978; **174:** 585–93.