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Amphiphilic α-tocopherol analogues as inhibitors of brain lipid peroxidation

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Abstract

Neurological disorders, such as stroke, trauma, tardive dyskinesia, Alzheimer's and Parkinson's diseases, may be partially attributed to excessive exposition of the nervous tissue to oxygen-derived radicals. A novel water-soluble α-tocopherol analogue, 2,3-dihydro-2,2,4,6,7-pentamethyl-3-(4-methylpiperazino)methyl-1-benzofuran-5-ol dihydrochloride (MDL), is a potent radical scavenger. Following subcutaneous administration to mice, MDL inhibited the lipid peroxidation induced in the 100-fold diluted brain homogenates, with an ID_{50} of 8 mg/kg. Rapid brain penetration, within 30–60 min postadministration, and even distribution into different brain areas were observed. MDL was also detected after oral administration. In brain homogenate undergoing lipid peroxidation, MDL prevented the consumption of an equal amount of α-tocopherol, while inhibiting the concomitant malondialdehyde formation. The radical scavenging capacity of MDL was superior to that of α-tocopherol, although the peak and half-peak potentials were not significantly different. However, MDL was much less lipophilic, the partition coefficient (log *P*) at the octanol/water interface being 1.91. Although it is yet unknown, whether the applied criteria sufficiently predict its usefulness, beneficial effects of MDL may be expected in the above mentioned disorders.

Keywords: α-Tocopherol analog (water-soluble); Neuroprotection; Lipid peroxidation; Antioxidant; Vitamin E; Malondialdehyde; Free radical; Cyclic voltammetry

1. Introduction

Central nervous system (CNS) tissue damage, occurring in such situations as inflammation, demyelinating disease, trauma and ischaemia, is to an unknown degree mediated by activated microglia (Banati et al., 1993). Besides secreting inflammatory cytokines and proteases, these cells produce large amounts of reactive oxygen species (Banati et al., 1994). Also in Alzheimer's disease an infiltration of phagocytotic microglia can be detected (Banati et al., 1993; McGeer et al., 1993). Another source of reactive oxygen species may be the mitochondrial electron-transport chain (Halliwell and Gutteridge, 1989; Sims et al., 1987) which, e.g. in Alzheimer's disease due to a decreased cytochrome oxidase activity (Parker et al., 1990), may accumulate reduced ubiquinone and flavin intermediates. These in turn may lead to enhanced, uncontrolled production of oxygen-derived free radicals (Partridge et al., 1994). The brain is particularly sensitive to oxidative damage because of its high concentration of polyunsaturated fatty acids which may undergo peroxidation. Furthermore, certain brain areas contain high amounts of iron, which promotes the formation of hydroxyl radicals from superoxide radicals and hydrogen peroxide (Gerlach et al., 1994). These potentially harmful molecules are detoxified by different enzymatic processes as well as low-molecular antioxidants (Halliwell and Gutteridge, 1989) which, however, have limited capacity in the CNS (Marklund et al., 1982; Adams et al., 1991). Hence, under pathological conditions the equilibrium between formation of oxidants and the antioxidant defences may be shifted towards an 'oxidative stress' situation.

An approach to cope with the increased antioxidant demand during such pathological situations, is the administration of synthetic, tissue-directed α-tocopherol analogues. For example, a hydrophilic, cardioselective 2-ethylammonium analogue was shown to protect myocardial tissue from reperfusion injury (Petty et al., 1993, 1994). Its

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ability to scavenge superoxide radicals produced by phagocytotic leukocytes (Bolkenius et al., 1991; Bolkenius, 1991), as well as its low intestinal absorption (Bolkenius et al., 1993), among other properties, provided a tissue-protective effect in a mouse model of ulcerative colitis (Murthy et al., 1994).

The present study was done in the search of α-tocopherol analogues which prevent lipid peroxidation, scavenge superoxide radicals and sufficiently penetrate the brain tissue. In a previous study MDL was selected (Grisar et al., 1995) from a series of 5-membered ring α-tocopherol analogues, because of better antioxidant properties and brain penetration as compared with 6-membered ring derivatives. Moreover, MDL was shown to be protective against superoxide and hydroxyl radical-mediated, oxidative inactivation of protease nexin-1 (glia-derived nexin), a neurotrophic and neuroprotective endogenous thrombin inhibitor possibly involved in Alzheimer's disease (Bolkenius and Monard, 1995).

2. Materials and methods

2.1. Materials

2,3-Dihydro-2,2,4,6,7-pentamethyl-3-(4-methylpiperazino)methyl-1-benzofuran-5-ol dihydrochloride (MDL) (Grisar et al., 1995) was synthesized at the Marion Merrell Dow Research Institute, Strasbourg, France. All other chemicals were from either Aldrich, St. Quentin Fallavier, France, Merck, Darmstadt, Germany or Fluka, Buchs, Switzerland. Male CD-1 mice at 22 ± 2 g were obtained from Charles River, Saint-Aubin-lès-Elbeuf, France, and were fed a standard laboratory diet.

2.2. Ex vivo lipid peroxidation

This was performed as previously described (Grisar et al., 1995). Groups of five male CD1-mice (body weight 30 g) were injected s.c. with either 20 μmol/kg of the test compound or with saline solution (control) and the animals killed at the indicated times. The intravenous administration was via the tail vein. The oral administration was done with a gavage needle, using 200 μl of 0.5% methocel per mouse as vehicle. The brains were excised quickly, frozen in liquid nitrogen and then stored at −80°C. The frozen brains were homogenized 1 + 19 (w/v) in ice-cold water.

Incubations were done in a shaking water bath with 200 μl of homogenate (10 mg tissue) plus 500 μl KH_2PO_4 (40 mM, pH 7.3; 20 mM final) and 300 μl of 467 mM KCl (0.14 M final) at 37°C for 30 min. Of each treatment group part of the samples was left unincubated to determine the basal thiobarbituric acid reactive substance content. A malondialdehyde standard was prepared by dissolving 30 μl of malondialdehyde-bis(dimethylacetal) in 10 ml of water to which 0.7% $HClO_4$ was added. From this solution a series of standard solutions ranging from 0.5 to 10 nmol per sample was prepared. Following incubation, the reaction was stopped on ice and 200 μl of 35% $HClO_4$ were added. The samples were centrifuged at 3000 × g for 5 min and 800 μl of the supernatant was mixed with 200 μl of 1% thiobarbituric acid in water. Samples were then placed in a boiling water bath for 15 min and subsequently cooled to room temperature. The thiobarbituric acid-adduct was extracted into 1 ml of *n*-butanol. The fluorescence of the supernatant was read at room temperature at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. The standard curve was fitted by second order polynomial regression and the corresponding contents of malondialdehyde equivalents (minus basal contents) calculated for all the samples. Results are expressed as mean percentages inhibition ± S.D. of ex vivo lipid peroxidation observed in the saline-treated control group. The statistical significance was analysed by one-way analysis of variance in combination with Student-Newman-Keuls multiple comparisons test. A resulting $P \leq 0.05$ was estimated as significant.

2.3. High pressure liquid chromatography determination of test compounds in the brain extracts

This was performed according to a previously published procedure (Verne-Mismer et al., 1993). Of the brain homogenates used for ex vivo lipid peroxidation, 450 μl were diluted with 1 ml methanol containing 2% (w/v) ascorbic acid and centrifuged at 1000 rpm. Separation of 50 μl of the supernatant was achieved on an Ultrabase C8 column (250 × 4.6 mm I.D., 5 μm particle size) supplied by Shandon-SFCC (Eragny, France) and isocratic elution with 0.1 M phosphate buffer containing 50% methanol. Electrochemical detection with a working potential of 0.6 V was used.

2.4. α-Tocopherol determination in rat brain homogenate

Rat brain homogenate was incubated as previously described (Bolkenius et al., 1991). In short, fresh brains of adult Sprague-Dawley rats were homogenized in 40 mM potassium phosphate, pH 7.4, containing 0.14 M NaCl. The homogenate was centrifuged at 1000 × g and the supernatant diluted 1.75 times. Of the diluted homogenate 800 μl (2 mg protein) was incubated in duplicate with three different concentrations of MDL in a total volume of 1 ml at 37°C in a shaking water bath. Incubations were stopped on ice and samples were divided into halves. For the extraction, the procedure of Burton et al. (1985) was adopted. Of the incubation mixture, 500 μl was added to 100 μl 0.6% diethylenetriaminepentaacetic acid, 600 μl 100 mM sodium dodecylsulfate, 1.8 ml ethanol and 2.5 ml *n*-heptane. To each sample 1 nmol γ-tocopherol was added as an internal standard. The samples and external standards of 2 nmol α-tocopherol were extracted into the organic

phase, which was evaporated with a stream of nitrogen, and the residue taken up with 300 μl of 95% aqueous methanol. The solution was centrifuged and 100 μl were separated by high pressure liquid chromatography on a Superspher RP 18 column (150 × 4 mm I.D.; 4 μm particle size) using 96.5% aqueous methanol as the eluant at 1 ml/min and fluorescence detection (excitation: 292 nm, emission: 340 nm).

2.5. Malondialdehyde determination in rat brain homogenate

This was done according to the method published by Draper et al. (1993). To the residual 500 μl of incubated brain homogenate, 192 μl of 40% trichloroacetic acid and 48 μl of 0.005% 3,5-bis-*t*-butyl-4-hydroxytoluene were added and the samples heated at 100°C for 30 min to liberate all protein-bound malondialdehyde. The samples were centrifuged, and the supernatants and a standard made of 1 nmol malondialdehyde-bis(dimethylacetal) were allowed to react with 0.5% thiobarbituric acid at 100°C for 30 min. The chromophor was extracted into 1 ml *n*-butanol, and 500 μl of the extract diluted with 250 μl methanol and 250 μl high pressure liquid chromatography-mobile phase (5 mM phosphate buffer, pH 7, 15% acetonitrile and 0.6% tetrahydrofurane). Of this solution, 20 μl were further diluted with 1 ml mobile phase, and finally 40 μl injected onto a Superspher C-18 NC-column (250 × 3.9 mm I.D.; 4 μm particle size), and eluted at 1 ml/min. The eluted malondialdehyde-thiobarbituric acid adduct was detected by fluorescence measurement (excitation: 515 nm, emission: 550 nm).

2.6. Cyclic voltammetry measurements

These were done at ambient temperature under an argon atmosphere with 10^{-3}–10^{-4} M solutions of the compound studied, either in acetonitrile containing 0.1 M tetraethylammonium perchlorate as the electrolyte or after addition of 50% water (by addition of 2.5 ml water to ca. 3 mg of compound dissolved in 5 ml acetonitrile). A Bruker E 130 M potentio-galvanostat was used. The working electrode was a platinum button with a saturated calomel reference electrode and a platinum foil as an auxiliary electrode. Measurements were done from 0 to 1 V with a sweeping rate of 100 mV/s. Under these conditions ferrocene, used as a reference sample, had a half-wave potential Ep/2 = +400 mV.

2.7. Determination of the partition coefficient

The compound at 10^{-3} M concentration, dissolved in 5 ml of 0.1 M phosphate buffer at pH 7.4, saturated with octanol, was mixed with an equal volume of *n*-octanol in 15 ml screw-capped vials. The samples were shaken on a reciprocal shaker for 1 h at room temperature. After 10 min centrifugation at 3900 rpm, the two phases were separated and the concentration of the test compound measured in both phases by high pressure liquid chromatography analysis. An Ultrabase C_8 column (250 × 4.6 mm I.D., 5 μm particle size), supplied by Shandon-SFCC (Eragny, France), and isocratic elution at a flow rate of 1 ml/min with 0.1 M phosphate buffer, containing 50% methanol, were used. The UV absorbance at 280 nm of the eluent was measured.

3. Results

3.1. Inhibition of ex vivo lipid peroxidation

Subcutaneous administration of MDL (20 mg/kg) to mice was followed by a rapid and significant ex vivo inhibition of thiobarbituric acid reactive substance formation in the brain homogenates within 30 min (Fig. 1). Maximal inhibition, achieved at 30–60 min, was between 60–70%, as compared with saline-treated controls. The maximum was followed by a restoration period of about 90 min, during which time a residual 20% inhibition was attained. This small inhibition observed up to at least 4 h, however, was not significant. The corresponding tissue content of MDL was measured by high pressure liquid chromatography (Fig. 1); it decreased as the ex vivo lipid peroxidation returned to normal. The inhibition measured

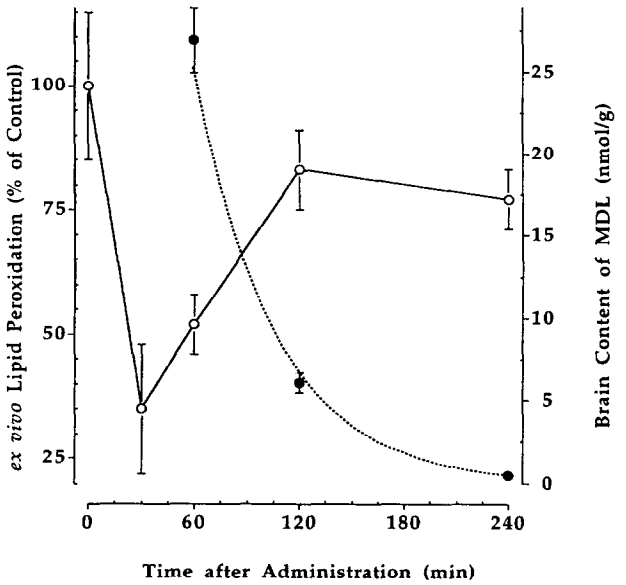

Fig. 1. Time course of inhibition of ex vivo lipid peroxidation in mouse brain homogenates by subcutaneous MDL. Mice were administered MDL (20 mg/kg). The brains were collected at the times indicated and the diluted homogenates analysed for thiobarbituric acid reactive substance formation (○) as described. In order to show that both parameters were in accordance, some of the brain homogenates were also analysed by high pressure liquid chromatography for their content of MDL (●). Values represent means ± S.D. of five mice. Inhibition of lipid peroxidation was significant ($P \leq 0.05$) at 30 and 60 min.

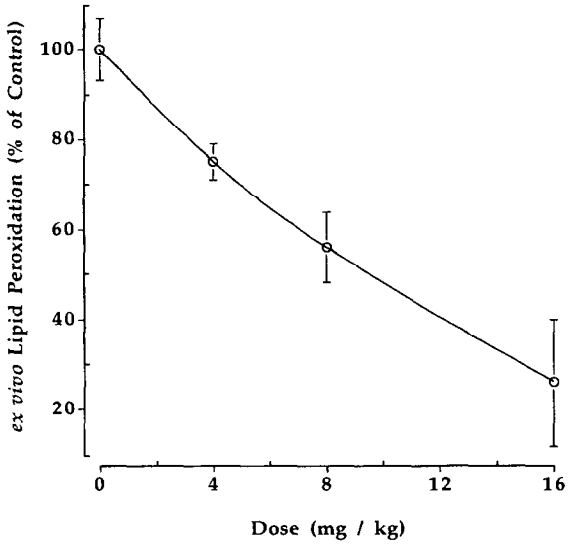

Fig. 2. Dose-response relationship of inhibition of ex vivo lipid peroxidation in mouse brain homogenates by subcutaneous MDL. The time of collection of brains after subcutaneous administration was 30 min. Values represent means ± S.D. of five mice. The inhibition was statistically significant ($P \leq 0.05$) at all doses. Same conditions as for Fig. 1.

in the brains at 30 min after s.c. administration, was dose-dependent (Fig. 2), following a quasi linear slope up to 16 mg/kg. The dose that inhibited lipid peroxidation to 50% of the control value (ID_{50}) was 8 mg/kg body weight. Oral administration of MDL (50 mg/kg) produced a delayed and prolonged inhibition as compared to subcutaneous administration. However, the maximal inhibition

Table 1
Time course of inhibition of ex vivo lipid peroxidation in mouse brain homogenates by orallly administered MDL

Time after administration (min)	% of control [a]
0	100 ± 11
30	100 ± 9
60	81 ± 5 [b]
120	78 ± 14 [b]
240	92 ± 12

[a] Values represent means ± S.D. ($n = 5$); [b] different from time 0 min (ANOVA, $P \leq 0.05$).

Table 2
Distribution of MDL in different mouse brain areas 1h after intravenous administration (via the tail vein) of different doses, measured by high pressure liquid chromatography as described in the experimental section

	Intravenous dose, μmol/kg (mg/kg)		
	5 (2)	10 (4)	20 (8)
Cortex	2.9 ± 1.1	7.0 ± 0.6	9.9 ± 2.2
Midbrain	2.3 ± 0.8	5.5 ± 0.9	7.8 ± 2.5
Cerebellum	2.3 ± 0.7	5.3 ± 0.6	8.0 ± 2.9
Medulla	2.3 ± 0.5	5.9 ± 1.0	8.0 ± 2.1

Values represent means ± S.D. (nmol/g tissue), $n = 4$.

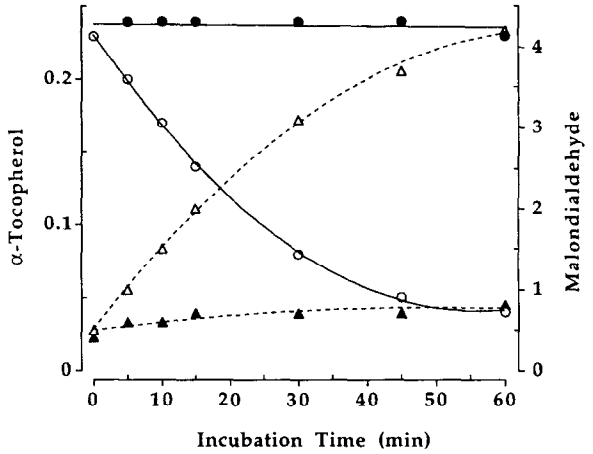

Fig. 3. Formation of malondialdehyde and consumption of endogenous α-tocopherol during lipid peroxidation in diluted rat brain homogenate. Lipid peroxidation was induced by incubation at 37°C. α-Tocopherol (○) and malondialdehyde (△) were measured by high pressure liquid chromatography at the times indicated. MDL protected at 0.125 nmol/0.5 ml (closed symbols). All concentrations are given in nmol/0.5 ml; values represent means of duplicate determinations with ≤ 10% scatter.

observed was only about 20% under these circumstances (Table 1). When intravenously administered, similar brain contents of MDL were reached as after subcutaneous administration (Table 2 and Fig. 2, respectively). An even distribution between different brain areas was measured 1 h after i.v. administration.

3.2. Preservation of endogenous α-tocopherol and inhibition of malondialdehyde formation in rat brain homogenate

Formation of thiobarbituric acid reactive substances in brain homogenate during in vitro incubation, as shown in

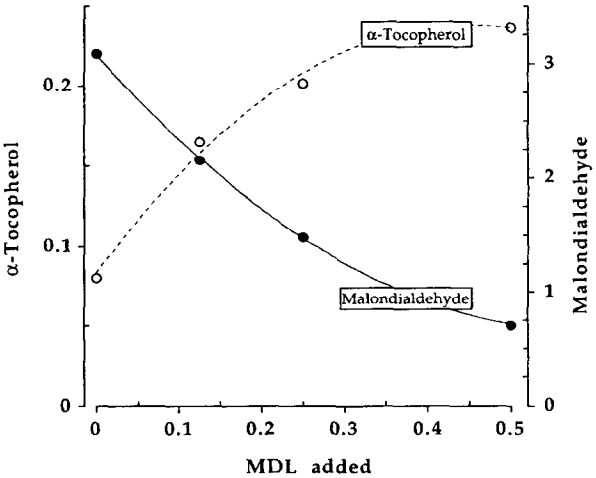

Fig. 4. Protective effect of MDL on alterations of malondialdehyde and endogenous α-tocopherol during lipid peroxidation in rat brain homogenate. Conditions are as for Fig. 3 with 30 min incubation. All concentrations are given in nmol/0.5 ml; values represent means of duplicate determinations with ≤ 10% scatter.

Table 3
Cyclic voltammetry measurements of peak (Ep) and half-peak potentials (Ep/2) of α-tocopherol and MDL (the free amine of MDL was used)

Compound	(CH_3CN) [a]		(CH_3CN:H_2O = 2:1) [a]	
	Ep	Ep/2	Ep	Ep/2
α-Tocopherol	0.84	0.78	0.51	0.42
MDL	0.79	0.64	0.51	0.43

[a] Solvent used. Means of three determinations (V) (S.D. = +0.05).

Fig. 1, was closely paralleled by a time-dependent formation of malondialdehyde, as followed by high pressure liquid chromatography (Fig. 3). A concomitant decrease of the endogenous α-tocopherol content was also evident under these conditions. When MDL was added to the incubation mixture at 0.5 nmol/0.5 ml, the production of malondialdehyde was suppressed, while the endogenous content of α-tocopherol was preserved (Fig. 3). In the same experiment at 30 min incubation time, the presence of MDL enabled a concentration-dependent attenuation of both parameters (Fig. 4). MDL prevented the loss of an equal amount of endogenous α-tocopherol.

3.3. Electrochemical behaviour

The peak and half-peak potentials of MDL were determined by cyclic voltammetry and compared with the parent α-tocopherol (Table 3). The value observed for α-tocopherol in acetonitrile was in agreement with the previously determined value (Nagaoka et al., 1992). Although in acetonitrile a slightly lower oxidation potential was observed for the 5-membered ring derivative MDL, in accordance with the theoretical considerations of Burton and Ingold (1986), this may need further study. The addition of water had a lowering effect on the measured values, however, due to its insolubility in that medium, the value obtained with α-tocopherol may be somewhat underestimated.

3.4. Partition coefficient

To determine the degree of lipophilicity of MDL, the partition coefficient between *n*-octanol and phosphate buffer (pH 7.4) was measured. No difference was observed, whether isotonic sodium chloride was present or not. A log $P = 1.91 \pm 0.04$ ($n = 6$) was measured, indicating an intermediate lipophilicity, i.e. amphiphilic behaviour.

4. Discussion

A strong radical scavenging effect in vitro is clearly a prerequisite if one intends to select an antioxidant for the treatment of neurological disorders. In addition, sufficient tissue penetration, or rather tissue specificity, as well as prolonged duration of action would be further criteria for a potential therapeutic efficacy. Measurement of inhibition of ex vivo lipid peroxidation allows an estimate of the relative brain penetration of the compounds as active antioxidants. In the present experiments, the actual in vivo effect, however, is expected to be substantially more pronounced, because the measurement was done with 100-fold diluted homogenates.

MDL has the advantage of carrying a positive charge at physiological pH. Charge interactions with membranes of polyunsaturated lipids are able to control the protective effect of an antioxidant against lipid peroxidation, as was recently demonstrated by Barclay and Vinqvist (1994). Another amino analogue, 3,4-dihydro-2-(2-dimethylaminoethyl)-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol hydrochloride (benzopyran-MDL), was thus previously found superior to an anionic α-tocopherol analogue, Trolox, to inhibit the lipid peroxidation induced in rat brain homogenate (Bolkenius et al., 1991); this is explained by the composition of brain tissue as a mixture of negatively charged lipids and lipids devoid of a net charge. A similar charge effect has been demonstrated by Bisby and Parker (1995), showing that ascorbic acid was able to reduce the tocopheroxyl radical of benzopyran-MDL over twice as rapidly than the corresponding Trolox radical. The fast scavenging of lipoperoxyl as well as hydroxyl and superoxide radicals is probably the reason why benzopyran-MDL protected rat brain tissue against the damage, caused by permanent middle cerebral artery plus 10 min carotid artery occlusion, followed by 24 h reperfusion, as previously reported (Bolkenius et al., 1992). As demonstrated in Fig. 2 and Table 2, respectively, the 5-ring α-tocopherol analogue MDL penetrated mouse brains after low dose subcutaneous as well as intravenous administration. The ex vivo inhibition data were in accordance with the tissue concentrations measured by high pressure liquid chromatography (Fig. 1). MDL was also active in mouse brain after oral administration, remaining there for at least 3 h (Table 1). Although a delayed onset of its inhibitory effect was observed under this condition, the activity extended over a prolonged time period as compared to the subcutaneous treatment (Fig. 1).

MDL had a vitamin E-sparing effect in rat brain homogenate in vitro while inhibiting the concomitant formation of malondialdehyde (Fig. 3). Thus, under these conditions, MDL very probably scavenges the intermediate lipoperoxyl radicals in replacement of the endogenous α-tocopherol. It is evident that the natural α-tocopherol content was not sufficient to control the enhanced lipoperoxyl radical formation under the test conditions, because it was unable to induce a 'lag phase' in the formation of malondialdehyde. MDL prevented the loss of an equal amount of endogenous α-tocopherol, as well as slowing down the malondialdehyde formation (Fig. 4). This is in accordance with our previous finding (Bolkenius et al., 1991; Grisar et al., 1995) that amino analogues are better

inhibitors of lipid peroxidation than the parent compound. Unsignificantly small differences in the peak and half-peak oxidation potentials of MDL and α-tocopherol were observed (Table 3). This does, however, not totally exclude the possibilty, that MDL regenerates the tocopheroxyl radical in a way similar to ascorbic acid (Bisby and Parker, 1995). Nevertheless, the result of this experiment suggests that MDL, as yet to be shown, has the potential to conserve the tissue α-tocopherol content in vivo in an oxidative stress situation. Furthermore, probably due to reaction of the tocopheroxyl radical with the lipoperoxyl radical intermediates, the former was consumed during incubation (Burton and Ingold, 1986; Yamauchi et al., 1994), whereas the α-tocopheryl quinone was not a major product (F. Bolkenius, unpublished observation).

Synthetic α-tocopherol analogues such as MDL are expected to protect the nervous tissue in situations, in which damage by oxygen-derived free radicals is involved, such as stroke, trauma, tardive dyskinesia, Alzheimer's and Parkinson's diseases. It is not yet clear, however, whether the applied criteria are sufficiently predictive for a potential therapeutic usefulness in these pathologies. Besides lipid peroxidation as a general tissue destructive process, other potential targets of free radical damage, e.g. certain indispensible proteins, have to be considered. For example such endogenous serine protease inhibitors like α_1-proteinase inhibitor (Carrell and Travis, 1985; Bolkenius, 1991) or protease nexin-1 (glia-derived nexin) (Bolkenius and Monard, 1995), are prone to oxidative inactivation. While the negative consequences of protease inhibitor inactivation have so far been documented for α_1-proteinase inhibitor during peripheral inflammation (Weiss, 1989), this has possibly a more common part in the concerted microbicidal action of macrophages, also in the CNS. Oxidative protease inhibitor inactivation entails an amplification of the tissue damage, and specific targeting of radical scavengers for their protection could be useful. The results of further pharmacological testing of MDL in neurological disorders by Petty et al. (submitted) and Cowley et al. (submitted) will be published elsewhere.

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