

## Nafazatrom Inhibits Peroxidation of Phosphatidylcholine Liposomes, Heart Homogenate and Low Density Lipoproteins

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**Abstract.** In order to contribute to the understanding of the biological properties of nafazatrom, an antithrombotic agent (NAF), we studied its effects on peroxidation of low density lipoproteins (LDL), lipid liposomes, heart homogenate, and its interaction with  $\alpha$ -tocopherol radical. NAF decreased the  $\text{FeSO}_4$  and  $\text{H}_2\text{O}_2$ -induced peroxidation of phosphatidylcholine liposomes and heart homogenate, and it decreased peroxidation of LDL induced by  $\text{CuSO}_4$  or 2,2'-azobis(2-amidinopropane). The antioxidant effect of NAF was about 3 times less potent than that of  $\alpha$ -tocopherol ( $\alpha$ -TOC) in phosphatidylcholine liposomes, and NAF was about 2–4 times more efficient to decrease peroxidation of LDL than  $\alpha$ -TOC. Possible interaction of NAF with  $\alpha$ -tocopherol radical ( $\alpha$ -TR) was studied by EPR spectroscopy. NAF decreased the concentration of  $\alpha$ -TR, but it was about 100-times less efficient than vitamin C. This may indicate that NAF does not interfere with  $\alpha$ -TR formation and/or reduction of  $\alpha$ -TR in biological system. The obtained results may help the explanation of biological effects of NAF.

**Key words:** Nafazatrom — Phosphatidylcholine peroxidation — Low density lipoproteins —  $\alpha$ -Tocopherol radical — Antioxidant — EPR

**Abbreviations:** AAPH, 2,2'-azobis(2-amidinopropane), BHT, butylhydroxytoluene, DHPC, diheptanoyl phosphatidylcholine, DTBP, di-tert-butylperoxide, LDL,

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low density lipoproteins, NAF, (3-methyl-1-[2-(2-naphthoxy)-ethyl]-3-methyl-2-pyrazolin-5-one), PC, egg yolk phosphatidylcholine, TBA, 2-thio-barbituric acid,  $\alpha$ -TOC,  $\alpha$ -tocopherol,  $\alpha$ -TR,  $\alpha$ -Tocopherol radical, Vit-C, vitamin C, Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol, EPR, electron paramagnetic resonance

## Introduction

Nafazatom (NAF) is an antithrombotic agent, it has been reported to reduce the size of infarction and arrhythmias in dogs, rabbits and rats subjected to period of coronary occlusion (Seuter et al 1979, Herrmann and Voigt 1984, Shea et al 1984, Fiedler 1984, Judd et al 1986) Free radicals and lipid peroxidation were suggested to play a negative role in cardiovascular diseases, e.g. atherosclerosis and thrombosis

Oxidation of low density lipoprotein (LDL) is believed to play a role in the atherogenic process (Esterbauer et al 1987, Steinberg 1988, Yla-Herttuala et al 1989) since it is not recognized by the apo B/E receptor but avidly taken up by the scavenger receptor of macrophages (Henriksen et al 1981, Steinbrecher et al 1984) Therefore, drugs which can protect LDL against oxidation may possess anti-atherogenic potency as was found for probucol (Parthasarathy et al 1986)

In order to contribute to the understanding of the biological properties of NAF we studied its effect on peroxidation of LDL, lipid liposomes, heart homogenate, and its interaction with  $\alpha$ -tocopherol radical

## Materials and Methods

### *Chemicals*

$\alpha$ -TOC and BHT were from Sigma (St. Louis, MO, USA) DHPC was from Avanti Polar Lipids TBA and DTBP were from Fluka (Switzerland) AAPH was from Polysciences, Inc., Warrington, PA NAF was from Inst. Drug Res. (Modia, Slovakia) All the other chemicals were of analytical grade

### *Lipid peroxidation*

PC solution (2.5 mg) with or without BHT, NAF or  $\alpha$ -TOC in  $\text{CHCl}_3$  /  $\text{CH}_3\text{OH}$  (2/1) was evaporated under a stream of nitrogen followed by evacuation. The dried samples were hydrated with 450  $\mu\text{l}$  of the buffer A (in mmol/l): KCl 100, Tris 5, pH 7.4. Lipid peroxidation was initiated by addition of 25  $\mu\text{l}$   $\text{H}_2\text{O}_2$  and 25  $\mu\text{l}$   $\text{FeSO}_4$ . The final concentrations of  $\text{FeSO}_4$  and  $\text{H}_2\text{O}_2$  were 0.2 and 0.1 mmol/l, respectively. The samples were incubated at 37°C for 60 min.

DHPC aggregates were used to dissolve NAF and  $\alpha$ -TOC. DHPC with NAF or with  $\alpha$ -TOC at the DHPC/drug molar ratio of 3 or 1 were dissolved in chloro-

form methanol = 2:1. The solvent was evaporated by nitrogen followed by evacuation. The samples were hydrated with buffer and vortexed for 2 min.

Rat heart was homogenized (1 g/20 ml) in the buffer A (in mmol/l): KCl 100, Tris 5, pH 7.4. The homogenate (400  $\mu$ l) was incubated with or without NAF, or with 50  $\mu$ l of DHPC aggregates (prepared in buffer A) or with 50  $\mu$ l of DHPC aggregates containing NAF. Lipid peroxidation was induced by addition of 25  $\mu$ l FeSO<sub>4</sub> and 25  $\mu$ l H<sub>2</sub>O<sub>2</sub> (0.1 mmol/l and 0.05 mmol/l final concentrations). The samples were vortexed for 5 s and incubated for 30 min at 37°C.

LDL was prepared from serum obtained from fresh normal human blood (Ondiaš et al. 1994). Samples for LDL peroxidation were prepared as follows: 50  $\mu$ l dialysed LDL (0.2 mg) in 250  $\mu$ l of the buffer B (mmol/l): NaCl 150, Tris-HCl, pH 7.4, were incubated at 37°C or 23°C without and with DHPC aggregates containing NAF or  $\alpha$ -TOC. Peroxidation of the samples was induced by CuSO<sub>4</sub> (5  $\mu$ mol/l) which was added 10 min after the drug. In other experiments LDL (20  $\mu$ l, 0.037  $\mu$ g) in 250  $\mu$ l of the buffer B, were incubated at 37°C without and with DHPC aggregates containing NAF or  $\alpha$ -TOC. Peroxidation of the samples was induced by AAPH (1 mmol/l) (Sato et al. 1990) which was added 10 min after the drug. The water-soluble AAPH generates free radicals thermally at a constant rate at 37°C, and induces oxidation of polyunsaturated fatty acid chains (Niki et al. 1991) or LDL (Sato et al. 1990).

The extent of lipid peroxidation was assessed by measurement of formation of TBA-reactive products, mainly the TBA-malondialdehyde complex according to Ondiaš et al. (1989). The absorption of TBA-reactive products at 534 nm was taken as a relative value of lipid peroxidation.

#### *Nafazatrom and $\alpha$ -tocopherol radical*

$\alpha$ -TR was generated by UV decomposition of DTBP with subsequent formation of  $\alpha$ -TR in organic solution.  $\alpha$ -TOC (100 mg), benzene (2 ml) and 250  $\mu$ l of DTBP were mixed. The solution was irradiated by UV light in quartz cuvette for 4 min under xenon lamp. After the irradiation, 2 ml of *n*-butanol and 4 ml of benzene were added to the solution. For EPR measurement 300  $\mu$ l of the sample were taken and vortexed for 30 s with 10  $\mu$ l of Vit-C in ethanol (final concentrations of Vit-C were between 200 nmol/l and 200  $\mu$ mol/l) or with 10  $\mu$ l NAF in benzene (final concentrations of NAF were between 10  $\mu$ mol/l and 20 mmol/l). The EPR spectra were measured in a plastic tube (300  $\mu$ l) 2 min after vortexing the sample. The spectra were recorded by a BRUKER ER 200 D-SRC spectrometer, X-band, central field 335.7 mT, sweep 5 mT, modulation amplitude was 0.25 mT. Relative free radical concentration was estimated from the intensity of the EPR spectra.

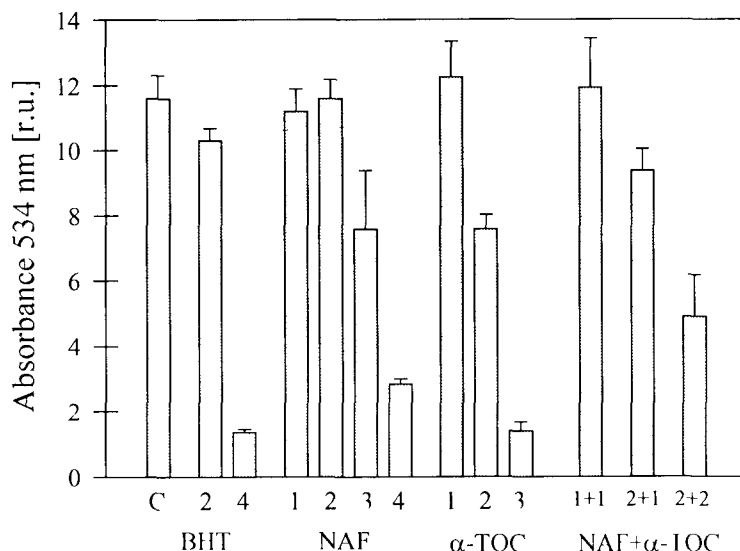
In order to measure NAF radical, NAF (1–10 mg) was added to 300  $\mu$ l of benzene without or with excess of PbO<sub>2</sub>. The mixture was vortexed for 30 s, and the EPR spectrum was recorded at 25°C, 2 min after the mixing.

## Results

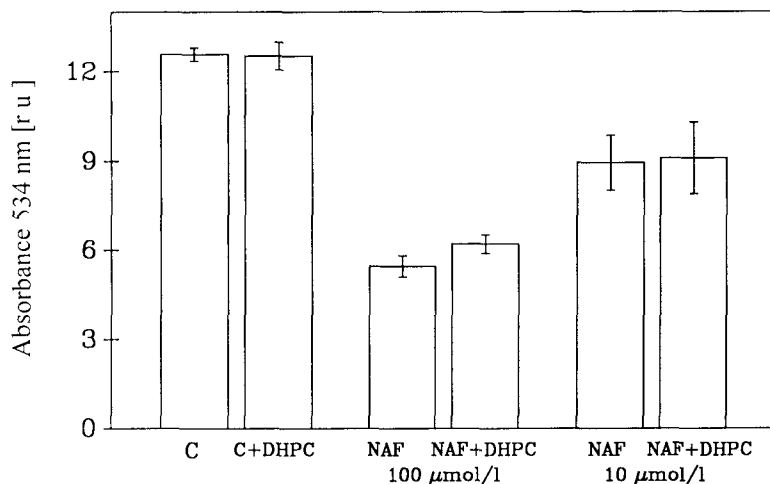
### *Nafazatrom and lipid peroxidation*

We studied the antioxidant effects of NAF in three different systems, i.e. PC liposomes, heart homogenate and LDL. The effects of NAF were compared with those of known antioxidants,  $\alpha$ -TOC and BHT. NAF decreased efficiently the  $\text{FeSO}_4$  and  $\text{H}_2\text{O}_2$ -induced formation of TBA-reactive products in PC liposomes. It was about 3 and 1.5 times less potent than  $\alpha$ -TOC or BHT, respectively (Fig. 1). Lipid peroxidation in the heart homogenate was induced by  $\text{FeSO}_4$  and  $\text{H}_2\text{O}_2$ . NAF added into the homogenate alone or in DHPC aggregates inhibited TBA-reactive products in the same extent (Fig. 2).

TBA-reactive products in LDL treated with  $5 \mu\text{mol/l}$   $\text{CuSO}_4$  or  $1 \text{ mmol/l}$  AAPH increased pronouncedly with the incubation time in comparison with samples without  $\text{CuSO}_4$  or AAPH treatment. NAF or  $\alpha$ -TOC in DHPC aggregates decreased the formation of TBA-reactive products in LDL treated with  $\text{CuSO}_4$  or AAPH (Figs. 3 and 4). A comparison of the antioxidant effects of NAF and  $\alpha$ -TOC on LDL oxidation induced by  $5 \mu\text{mol/l}$   $\text{CuSO}_4$  is shown in Fig. 3. After 4 hours of incubation of the samples, DHPC aggregates at higher concentrations ( $570 \mu\text{mol/l}$ ) showed a slightly protective effect on LDL peroxidation. NAF and  $\alpha$ -TOC



**Figure 1.** Inhibition effects of BHT, NAF,  $\alpha$ -TOC and of a combination of NAF +  $\alpha$ -TOC on the formation of TBA-reactive products (ABS 534 nm) in PC liposomes induced by  $\text{FeSO}_4$  ( $0.2 \text{ mmol/l}$ ) and  $\text{H}_2\text{O}_2$  ( $0.1 \text{ mmol/l}$ ). Drug/PC molar ratios: 1/3162 (1), 1/1000 (2), 1/316 (3), 1/100 (4).



**Figure 2.** Inhibition effects of DHPC aggregates (0.3 mmol/l) NAF, and NAF in DHPC aggregates on the formation of TBA-reactive products (ABS 534 nm) in heart homogenate induced by  $\text{FeSO}_4$  (0.1 mmol/l) and  $\text{H}_2\text{O}_2$  (0.05 mmol/l). The DHPC/NAF molar ratio was 3. C = control.

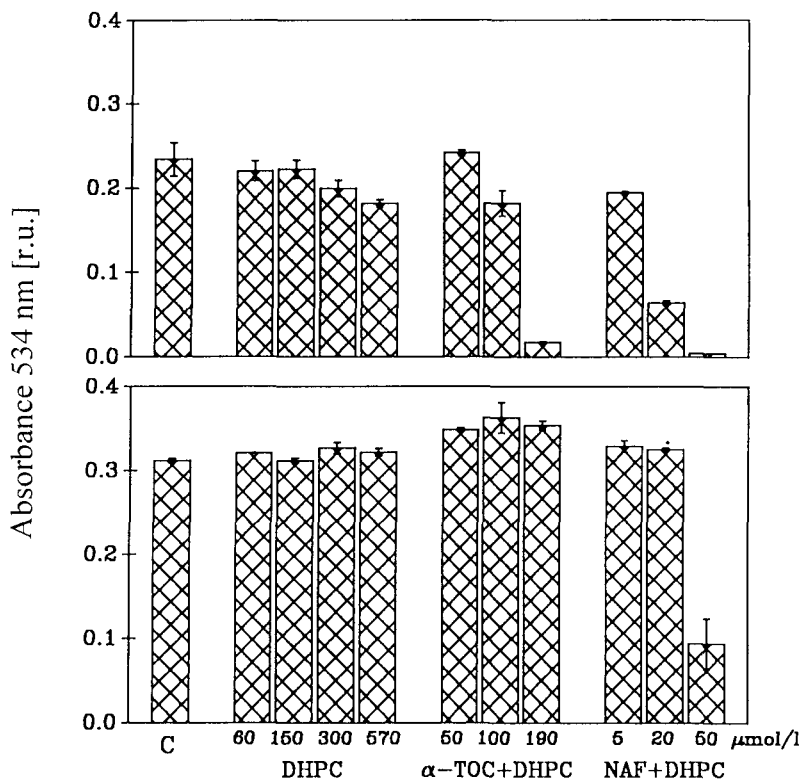
in DHPC aggregates were applied at the drug/lipid molar ratio of 1/3. NAF was more efficient ( $> 4\times$ ) in decreasing LDL peroxidation than  $\alpha$ -TOC (upper panel). After prolonged incubation of the samples (bottom panel) only NAF at 50  $\mu\text{mol/l}$  was effective in protecting LDL from peroxidation.

Fig. 4 shows a comparison of the antioxidant effects of NAF and  $\alpha$ -TOC on LDL oxidation induced by 1 mmol/l AAPH. NAF and  $\alpha$ -TOC in DHPC aggregates were applied at the drug/lipid molar ratio of 1. After 5 hours of incubation of the samples at 37°C, DHPC aggregates at higher concentrations (200  $\mu\text{mol/l}$ ) showed a slight protective effect on the LDL peroxidation. NAF was about  $\geq 2\times$  more efficient in decreasing LDL peroxidation than  $\alpha$ -TOC.

#### *Nafazatrom and $\alpha$ -tocopherol radical*

UV irradiated DTBP in benzene decomposes and generates free radicals. In our system some of the radicals were reduced by  $\alpha$ -TOC and so  $\alpha$ -TR was formed. The formation of  $\alpha$ -TR after UV irradiation was detected by EPR spectroscopy. The EPR spectrum of  $\alpha$ -TR is shown in Fig. 5A. The half-time decay of the EPR signal was  $> 40$  min, and was not studied in detail.

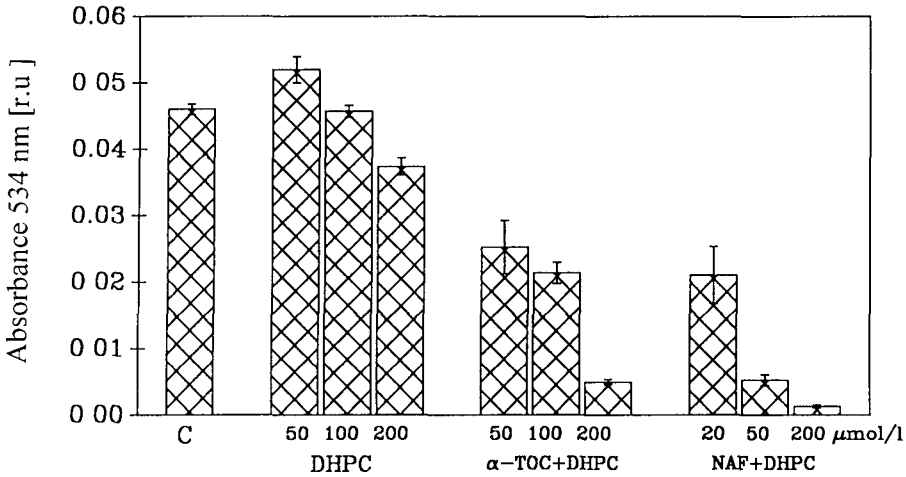
The EPR signal of  $\alpha$ -TR decreased when NAF or Vit-C was added to the samples containing  $\alpha$ -TR (Figs. 5B and 5C). During the experiments, we did not observe EPR spectra of Vit-C or NAF radicals. The EPR spectrum of NAF radical



**Figure 3.** Effects of drugs on the formation of TBA-reactive products (ABS 534 nm, 0.5 cm) induced by 5  $\mu\text{mol/l}$   $\text{CuSO}_4$  in LDL after incubation for 4 hours at 37°C (upper panel) and after incubation for 6 hours at 37°C followed by incubation for 6 hours at 23°C (bottom panel). C - control LDL, DHPC at concentrations 60, 150, 300 and 570  $\mu\text{mol/l}$ ,  $\alpha$ -Tocopherol added in DHPC aggregates (1:3 molar ratio) at concentrations of 50, 100 and 190  $\mu\text{mol/l}$ , NAF added in DHPC aggregates (1:3 molar ratio) at 5, 20 and 50  $\mu\text{mol/l}$ .

was obtained when NAF in benzene was oxidised by  $\text{PbO}_2$  (Fig. 5D). The EPR spectrum had a triplet shape indicating that an unpaired electron was localised on the nitrogen atom ( $a_N = 0.7 \pm 0.03$  mT) of NAF. Since hyperfine splitting constants were not obtained when oxygen was removed from the samples, details concerning the localisation of the unpaired electron were not determined.

A comparison of Vit-C and NAF effects on the decrease of the EPR signal of  $\alpha$ -TR formed by UV decomposition of DTBP is shown in Fig. 6. NAF was about 100 times less potent in decreasing  $\alpha$ -TR concentration than Vit-C.



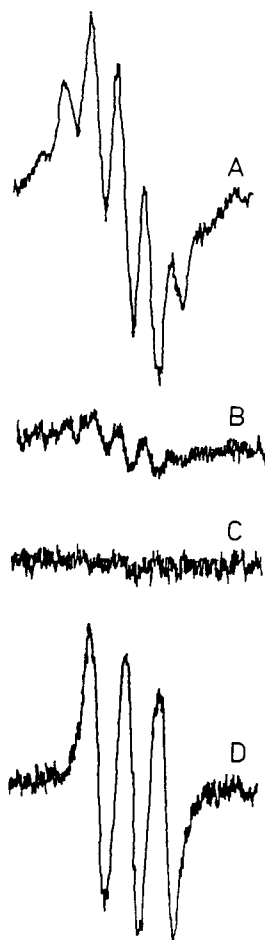
**Figure 4.** Effects of drugs on the formation of TBA-reactive products (ABS 534 nm 0.5 cm) induced by 1 mmol/l AAPH in LDL after incubation for 5 hours at 37°C. C control LDL, DHPC at concentrations 50, 100, 200 μmol/l, α-Tocopherol added in DHPC aggregates (1:1 molar ratio) at concentrations of 50, 100 and 200 μmol/l, NAF added in DHPC aggregates (1:1 molar ratio) at 20, 50 and 200 μmol/l.

## Discussion

### *Nafazatrom and α-tocopherol radical*

The EPR spectra of α-TR obtained in our experiments were similar to those found for α-TR in membranes or in solutions (Bascetta et al 1983, Rousseau-Richard et al 1988, Motoyama et al 1989, Hiramatsu et al 1990). NAF was found to be easily oxidised by Br<sub>2</sub><sup>-</sup> radicals (Sevilla et al 1983, Jovanovic et al 1985) and to form phenoxyl radical in water solution after UV photolysis at 77 K (Sevilla et al 1983). In our study, NAF was oxidised in benzene containing PbO<sub>2</sub> and formed free radicals (Fig 5D). The EPR spectrum, measured at room temperature, had a triplet shape indicating that an unpaired electron was localised on the nitrogen atom. It indicates that NAF can transfer an electron to PbO<sub>2</sub> and can form radicals. NAF was reported to be electron donor for the reduction of hydroperoxy fatty acids to hydroxy fatty acids by the peroxidase activity of prostaglandin H synthase (Marnett et al 1984). Our results support the electron donor properties of NAF.

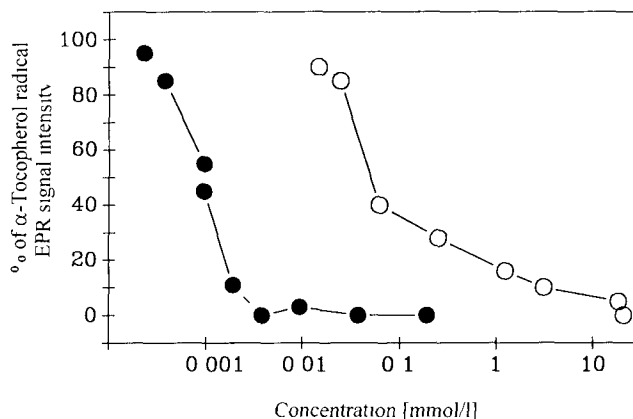
Being a natural antioxidant in biomembranes, α-TOC can trap one peroxy radical to generate α-TR. In the presence of reducing agents, e.g. Vit-C, α-TR may be reduced to regenerate α-TOC (Sato et al 1990). Since α-tocopherols are not synthesised *in vivo*, compounds which regenerate α-TR to α-TOC can be beneficial.



**Figure 5.** EPR spectra of  $\alpha$ -TR obtained upon UV irradiation of DTBP and  $\alpha$ -TOC in benzene-*n*BuOH solution. *A* – control  $\alpha$ -TR. *B* –  $\alpha$ -TR+3 mmol/l NAF. *C* –  $\alpha$ -TR+40  $\mu$ mol/l Vit-C. *D* – EPR spectra of NAF radical obtained upon NAF (10 mg/ml) being oxidised by excess  $\text{PbO}_2$  in benzene. Spectral width 5 mT. Temperature 25 °C.

in conditions in which free radicals play a deleterious role. We previously reported that NAF accelerated the decay rate of  $\alpha$ -TR generated in the reaction of  $\alpha$ -TOC with 1,1-diphenyl-2-picrylhydrazyl in *n*-butanol (Ondriaš et al. 1993). In the present work Vit-C and NAF decreased the concentration of  $\alpha$ -TR generated by UV irradiation of DTBP in benzene. Vit-C, used in our experiments as a control drug, is known to reduce  $\alpha$ -TR and thus regenerate  $\alpha$ -tocopherol (Bascetta et al. 1983; McCay 1985; Niki 1987). In our study Vit-C was very efficient in decreasing  $\alpha$ -TR concentrations. NAF also decreased the concentrations of  $\alpha$ -TR in organic solutions, but it was about 100-times less efficient than Vit-C. By analogy with Vit-C and since NAF was found to transfer an electron and formed free radicals (Fig 1D), we may speculate that in our model systems NAF could have reduced  $\alpha$ -TR or interfered with  $\alpha$ -TOC oxidation. However, since NAF was about 100-





**Figure 6.** Concentration dependence of the effects of Vit-C (●) and NAF (○) in decreasing concentrations of  $\alpha$ -TR, obtained upon UV irradiation of DTBP and  $\alpha$ -TOC. Temperature 25°C

times less efficient than Vit-C we may suggest that NAF does not interfere with  $\alpha$ -TR formation and/or reduction of  $\alpha$ -TR in biological systems

#### *Nafazatrom and lipid peroxidation*

Previously we reported that nifedipine and nimodipine solubility increased in the presence of DHPC (Giertlová and Ondriaš 1992). Since NAF and  $\alpha$ -TOC are sparingly soluble in water we also used DHPC aggregates (micelles or liposomes) to solubilize NAF and  $\alpha$ -TOC. Since NAF and  $\alpha$ -TOC incorporated in DHPC aggregates effectively inhibited lipid peroxidation in liposomes and LDL, we suppose that the aggregates are suitable for the NAF and  $\alpha$ -TOC delivery into them.

NAF has been reported to protect cardiac lipids from oxidative injury (Janero et al 1989), it was able to interrupt the propagation of an on-going peroxidation reaction in which the antioxidant profiles resembled to that of  $\alpha$ -tocopherol. We found in our study that NAF effectively inhibited lipid peroxidation in lipid liposomes, heart homogenate and LDL. To rule out the possibility that NAF may complex copper ions and inhibit LDL peroxidation, we also used AAPH to initiate LDL peroxidation. NAF and  $\alpha$ -TOC were effective in both systems. The antioxidant effects of NAF may be partly explained by its electron donor properties. However, we do not know whether the stronger effect of NAF as compared to  $\alpha$ -TOC in inhibiting LDL peroxidation is a result of its higher partitioning into the LDL or whether NAF has better antioxidant potential with respect to LDL than  $\alpha$ -TOC. The effect of  $\alpha$ -TOC was several times weaker in our study than reported

by Breugnot et al (1990) The different results may have been caused by different drug/LDL ratios and assay systems used

Lipid peroxidation in LDL was found to be linked with the vitamin E content, and it was suggested that oxidation of LDL is preceded by destruction of vitamin E (Esterbauer et al 1987) It has been supposed that Vit-C might regenerate  $\alpha$ -tocopherol in LDL (Sato et al 1990) Several drugs such as probucol, a hypocholesterolemic drug (Parthasarathy et al 1986), 17 beta estradiol, an antiatherosclerotic drug (Huber et al 1990), and phenothiazines (Breugnot et al 1990) were found to protect LDL against lipid peroxidation, and the known antioxidant butylated hydroxytoluene showed an antiatherosclerosis potential (Bjorkhem et al 1991) Ardhe et al (1987) found that oxidised LDL was more reactive with platelets than native LDL, and that it causes aggregation of platelets These observations suggest that oxidized LDL-induced platelet aggregation may play a role in the pathogenesis of atherosclerosis or thrombosis

The present results support the hypothesis that the biological potency of NAF may be explained, at least partly, from its antioxidant effect on LDL and lipid membranes

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