Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 505 (2004) 213–221

www.elsevier.com/locate/ejphar

Antioxidant properties of two novel 2-biphenylmorpholine compounds (EP2306 and EP2302) in vitro and in vivo

Anna Tavridou[a,b], Vangelis G. Manolopoulos[a,*]

[a]*Laboratory of Pharmacology, Medical School, Democritus University of Thrace, I. Kavyri 6, 68100 Alexandroupolis, Greece*
[b]*ELPEN Pharm. Co., Department of Preclinical Pharmacology, 95 Marathonos Av., 19009 Pikermi, Greece*

Received 5 July 2004; received in revised form 18 October 2004; accepted 20 October 2004

Abstract

The oxidation of low-density lipoprotein (LDL) is an important event in the development of atherosclerosis. In the present study, the antioxidant properties of two novel 2-biphenylmorpholine compounds (EP2306 and EP2302) were studied. Both compounds inhibited dose-dependently the in vitro oxidation of LDL induced by copper ions. EP2306 and EP2302 increased significantly the lag phase of the oxidation reaction at 0.1 and 10 μM, respectively, whereas they reduced the rate of the reaction at 1 and 10 μM, respectively. This inhibitory effect was not due to a free radical scavenging or copper-chelating activity of EP2300 compounds. Moreover, EP2306 and EP2302 inhibited 12-lipoxygenase activity dose-dependently with IC_{50} values of 454 and 318 μM, respectively, but had no effect on 15-lipoxygenase activity. In hyperlipidaemic rabbits treated with EP2306 for 4 weeks, there was a decrease in thiobarbituric acid-reactive substance (TBARS) levels and a significant increase in total peroxyl radical-trapping potential (TRAP) levels as compared to control animals. The present data suggest that EP2300 compounds are effective inhibitors of copper-mediated LDL oxidation in vitro. Moreover, EP2306 acts as an antioxidant in hyperlipidaemic rabbits, a property which could be beneficial in reducing atherosclerosis.

Keywords: Antioxidant; 2-Biphenylmorpholine compound; Low-density lipoprotein; Atherosclerosis; Copper-mediated oxidation; (Rabbit)

1. Introduction

In early atherosclerotic lesions, monocyte-derived macrophages accumulate cholesterol forming "foam cells" in the subendothelial space. As atherosclerosis progresses, most of these cells die, generating fatty streaks that develop into plaques (Esterbauer et al., 1993). The cholesterol that accumulates in the fatty streaks originates primarily from elevated levels of plasma LDL. Several theories have been proposed to explain the initiating factors involved in the fatty streak lesion. The oxidation hypothesis of atherosclerosis suggests that the initiating event in the development of atherosclerosis is an oxidative modification of LDL that significantly increases its uptake into the arterial intima (Steinberg et al., 1989). Oxidative modification of LDL starts in the polyunsaturated fatty acids in surface phospholipids and then propagates to core lipids, resulting in oxidative modification of the cholesterol moiety and of phospholipids as well as in modification and degradation of apolipoprotein B. The above hypothesis is supported by a number of in vitro and in vivo studies demonstrating the proatherogenic properties of oxidized LDL (Lehr et al., 1995), the occurrence of oxidatively modified LDL in atherosclerotic lesions (Yla-Herttuala et al., 1989) and the reduction of atherosclerotic events by antioxidants (Chang et al., 1995; Sparrow et al., 1992).

Metal-ion-mediated mechanisms have been the best-studied models of LDL oxidation in vitro. The metal-ion-dependent modification of LDL occurs primarily through lipid peroxidation and subsequent derivatization of apolipoprotein B lysine residues by reactive, lipid hydroperoxide-derived aldehydes (Esterbauer et al., 1992). The most common method for the initiation of LDL oxidation

* Corresponding author. Tel.: +30 2551 033445; fax: +30 2551 035248.
E-mail address: manolopoulos@med.duth.gr (V.G. Manolopoulos).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.10.047

involves incubation with copper ions (reviewed by Burkitt, 2001). The susceptibility of LDL to oxidation is assessed by determining the time before the oxidation products become detectable (lag time), the rate of oxidation, and the maximum amount of oxidation products (Esterbauer et al., 1989).

Lipoxygenases form a family of enzymes capable of mediating selective lipid oxidation. It has recently been suggested by several investigators that both 12-lipoxygenase and 15-lipoxygenase might participate in the development of atherosclerosis (Cathcart and Folcik, 2000). 15-Lipoxygenase is capable of oxidizing LDL in vitro to an atherogenic form (Belkner et al., 1998). Moreover, 15-lipoxygenase colocalizes with oxidized LDL in human atherosclerotic lesions (Hiltunen et al., 1995) and lipoxygenase products have been demonstrated in such lesions (Kuhn et al., 1997). Therefore, screening for 12/15-lipoxygenase inhibitors as potential antiatherosclerotic agents is of interest, based on the evidence that these enzymes have proatherogenic properties.

With increasing evidence that oxidation of LDL occurs in vivo, and that lipid peroxidation is associated with the development of atherosclerosis (Palinski et al., 1989; Yla-Herttuala et al., 1989), it has been hypothesized that antioxidant molecules which could slow or prevent the oxidative process may be beneficial in reducing atherosclerosis and coronary heart disease. However, clinical trials of antioxidants are controversial regarding their real clinical benefit (Steinberg, 1995). One well-known antioxidant, probucol, not only failed to reduce atherosclerosis in $LDLR^{-/-}$ mice, but actually enhanced it in a dose-dependent manner (Bird et al., 1998). Furthermore in humans, probucol lowers the high-density lipoprotein (HDL) cholesterol levels (Johansson et al., 1995) which have been shown to correlate inversely with the risk for atherosclerosis (Miller, 1987). Hypolipidaemic drugs such as statins and fibrates can reduce the increased propensity of LDL to oxidation in hypercholesterolaemic patients (Aviram et al., 1998; Hussein et al., 1997). These drugs could possess direct antioxidant properties or their effect on LDL oxidation could be indirect and result from an enhanced removal of "aged LDL" which is more prone to oxidation. Hitherto, only fluvastatin, bezafibrate and metabolites of atorvastatin and gemfibrozil have been shown to possess direct antioxidant properties.

Several novel 2-biphenylmorpholine derivatives have been synthesized and shown to inhibit lipid peroxidation in a heat-inactivated rat hepatic microsomal fraction in vitro (Chrysselis et al., 2000; Chrysselis et al., 2002). The antioxidant properties of two of these compounds, 2-(4-biphenyl)-4-methyl-octahydro-1,4-benzoxazin-2-ol, hydrobromide (named EP2306) and 2-(4-biphenyl)-2-(3-nitrooxypropoxy)-4-methylmorpholine, hydrobromide (named EP2302) were further investigated. Thus, the aims of the present study were to: (a) further substantiate the in vitro antioxidant activity of EP2306 and EP2302, (b) explore the mechanism of their antioxidant activity, and (c) determine whether EP2306 shows antioxidant activity in vivo, in the hypercholesterolaemic rabbit animal model.

2. Materials and methods

2.1. Materials

2-(4-Biphenyl)-4-methyl-octahydro-1,4-benzoxazin-2-ol, hydrobromide (EP2306) and 2-(4-biphenyl)-2-(3-nitrooxypropoxy)-4-methylmorpholine, hydrobromide (EP2302) were synthesized as previously described (Chrysselis et al., 2000; Chrysselis et al., 2002). $CuSO_4$, hydrogen peroxide and ethanol were purchased from Merck (Germany). Soybean 12-lipoxygenase, sodium linoleate, eicosatetraynoic acid (ETYA), 2,2′ -diazobis (2-amidino-propane) dihydrochloride, dimethylsulphoxide, Tris, probucol, phosphate-buffered saline (PBS), α-tocopherol, 1,1 diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Germany). 2,7-Dichlorofluorescein-diacetate (DCFH-DA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Fluka Chemie (Germany). Human LDL and simvastatin were from Calbiochem (through CN Biosciences, UK). For the in vivo experiment, cholesterol was obtained from Dolder (Switzerland) and simvastatin (Zocor) from Merck. The Lipoxygenase Screening Inhibitor kit was from Cayman (through Spi-Bio, France).

2.2. Animals

Male, New Zealand White rabbits (2.5–4.0 kg) were housed individually in cages and acclimatized for a week before experiments started. They were fed a standard chow supplemented with 0.5% cholesterol and given water ad libitum. Daily records of food intake did not show any significant difference among animals. Animals were randomly divided into groups for the pharmacological treatment, which started 4 weeks after diet initiation. They were treated daily by oral gavage with 10 mg/kg EP2306 suspended in 34 mM NaCl/71 mM HCl (vehicle) for another 4 weeks. Control animals received an equal volume of vehicle only. At the end of the experiments, animals were killed by exsanguination by cardiac puncture while under phenobarbital anesthesia. The protocol of this study complies with the European Community guidelines for the use of experimental animals and it was approved by the Department of Veterinary Services of the Prefecture of East Attica (Greece).

2.3. Antioxidant assays

Lipid peroxides were determined in serum as TBARS levels and expressed as malondialdehyde equivalents (Fogelman et al., 1980).

The antioxidant capacity of the serum was measured as TRAP by the method of Niculescu et al. (2001). Briefly,

serum was incubated on ice with DCFH-DA and the reaction started by adding 2,2′ -diazobis (2-amidino-propane) dihydrochloride. The oxidation products were detected at 504 nm with a Beckman DU640 spectrophotometer. The experimental curve contains two lag phases and two propagation phases. The length of the two lag phases was calculated using the intercepts of the tangents to the two propagation phases. The TRAP values were calculated as:

$$\text{TRAP} = (T_{\text{serum}}/T_{\text{Trolox}}) \times \text{serum dilution factor} \times 2 \times [\text{Trolox}]\ (\mu\text{mol/L})$$

where, T_{serum} is the length of the first lag phase, T_{Trolox} is the length of the second lag phase (due to Trolox) and 2 is the number of the peroxyl radical molecules trapped by each Trolox molecule. After 40–50 min, the propagation phase was broken off by adding an internal standard, Trolox, an antioxidant with known activity. The addition of this compound induces a second lag phase (T_{Trolox}). The resulting TRAP values are expressed as free radicals, in μmoles, trapped by 1 L of serum.

2.4. LDL oxidation in vitro

Human LDL (50 μg protein/ml) in PBS was incubated at 37 °C in the absence or presence of various concentrations of drugs (in ethanol) from 5 min before the start of oxidation. Oxidation was initiated by the addition of a final concentration of 10 μM $CuSO_4$. The formation of conjugated dienes was determined in a Ultrospec 3100 pro Ultraviolet/Visible spectrophotometer (Amersham Pharmacia Biotech) every 10 min as the increase in the absorbance at 234 nm (Esterbauer et al., 1989). The absorption at the beginning of the reaction was set to zero. Conjugated dienes (μM) were calculated using an extinction coefficient of 29 500. In this system, the oxidation of LDL is divided into three phases, i.e., lag phase, propagation phase, and decomposition phase. The speed of oxidation was expressed by the maximum slope of the propagation phase. Lag times were determined graphically as the time point at which the tangent to the curve during the maximum slope of the propagation phase intercepted the time axis.

2.5. Free radical scavenging assay

The free radical scavenging capacity of EP2300 compounds was analyzed by the DPPH assay (Blois, 1958). Each drug (50 μM–1 mM) was mixed with 0.1 mM DPPH (in ethanol). The time course of the change in the absorbance at 517 nm was kinetically monitored for 5 min at 37 °C.

2.6. Lipoxygenase inhibition assays

The activity of 12-lipoxygenase was determined as previously described with some modifications (Taraporewala and Kauffman, 1990). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was followed by the appearance of a conjugated diene absorption at 234 nm for 3 min at room temperature. Each sample had a total volume of 1 ml, containing 100 mM Tris–HCl buffer (pH 9), 2% ethanol, 100 μM sodium linoleate, and a dilution of lyophilized 12-lipoxygenase in 0.9% NaCl to give satisfactory range of initial absorbance (150–300 IU). Freshly prepared stock solutions (100×) of EP2306, EP2302, and ETYA in 60% ethanol were added to the reaction mixture to give final concentrations as indicated. The enzymatic reaction mixture with inhibitors was compared in each case with a control containing the carrier. The reported IC_{50} values were determined graphically from three concentrations of the compounds as the mean of duplicate determinations for the number of experiments indicated.

The Lipoxygenase Screening Inhibitor kit was used according to the manufacturer's instructions to assess the inhibitory effect of EP2300 compounds on soybean 15-lipoxygenase.

2.7. Statistics

Values of P less than 0.05 were considered statistically significant. The t-test was used to compare the lag time and rate of the oxidation reaction between groups. In the rabbit experiment, comparisons between treatment groups were based on analysis of covariance (Vickers and Altman, 2001).

3. Results

3.1. Effect of EP2300 compounds on copper-induced oxidation of human LDL

The same human LDL preparation was used in all oxidation experiments. In control samples (vehicle added instead of drug), the oxidation of LDL continued for about 2.5 h until the start of the decomposition phase (Fig. 1A and B). LDL oxidation induced by 10 μM $CuSO_4$ was inhibited significantly by 0.1–50 μM EP2306 dose-dependently as shown by an increase in lag time (Table 1). In addition, there was a significant decrease in the rate of conjugated diene formation with 1–50 μM EP2306 compared to control (Table 1). EP2302 inhibited significantly the conjugated diene formation at a concentration range of 10–50 μM (Table 1). At the same range, EP2302 also showed a significant decrease in the rate of conjugated diene formation in the propagation step (Table 1). EP2306 was a more potent inhibitor of the formation of conjugated dienes than EP2302 as shown by the differences in lag time whereas the latter was more potent in decreasing the rate of the reaction. Representative curves of the oxidation reactions are shown in Fig. 1A for EP2306 and Fig. 1B for EP2302.

The antioxidant activity of EP2300 compounds was compared with the activity of the antioxidant agent probucol

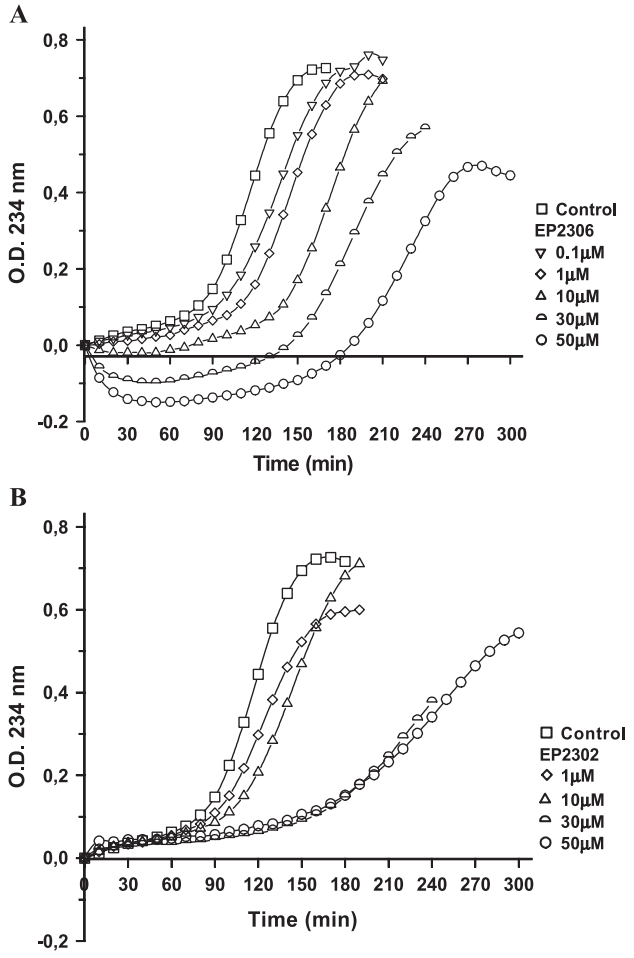

Fig. 1. The in vitro effect of EP2300 compounds on copper-induced oxidation of human LDL. Human LDL (50 μg of protein/ml) was incubated with $CuSO_4$ (10 μM) at 37 °C in the absence (control) or presence of increasing concentrations of (A) EP2306 and (B) EP2302. The increase of conjugated diene formation was measured by the absorbance at 234 nm.

and the hypolipidaemic drug simvastatin (Table 1). At a concentration of 0.1 μM, probucol increased significantly the lag time compared to control (119 vs. 82 min, $P<0.05$). At this concentration, probucol was a more potent inhibitor of conjugated diene formation than EP2306 (lag time 119 vs. 101 min). Furthermore, probucol lowered the rate of oxidation compared to control (0.0081 vs. 0.0116) but this effect was not statistically significant. At 10 μM, simvastatin did not increase the lag time of the oxidation reaction. However, it reduced significantly the rate of oxidation compared to control (0.0096 vs. 0.0116, $P<0.001$). This reduction was comparable with the one achieved by either 30 μM EP2306 or 10 μM EP2302.

3.2. Free radical scavenging and copper-chelating activity of EP2300 compounds

The inhibitory effect of EP2300 compounds on LDL oxidation may be related to a free radical scavenging activity. On using the DPPH assay, there was no time-dependent reduction in the absorbance at 517 nm by either EP2306 or EP2302 (50 μM–1 mM) (Fig. 2). In the same assay, 20 μM of vitamin E resulted in 66% reduction in the optical density, suggesting that unlike vitamin E, EP2300 compounds do not act as free radical scavengers.

In order to analyze whether the inhibitory effect of EP2300 compounds on LDL oxidation is related to their copper-chelating activity, we monitored LDL oxidation induced by either 10 or 50 μM $CuSO_4$ in the presence of either EP2306 (10 μM) or EP2302 (30 μM) (Fig. 3). The lag time of oxidation was decreased on using 50 μM $CuSO_4$ compared to 10 μM $CuSO_4$ both in the absence (control) and presence of the compounds studied. However, EP2300 compounds increased the lag time of oxidation compared to control to a similar extent at both concentrations of $CuSO_4$. Therefore, it appears that the antioxidant activity of EP2300 compounds is not mediated by chelation of metal ions.

3.3. Effect of EP2300 compounds on lipoxygenase activity

One possible explanation for the observed inhibition of LDL oxidation by EP2300 compounds could be through the inhibition of either 12-lipoxygenase or 15-lipoxygenase. We investigated this possibility and found that there was no significant inhibition of 12-lipoxygenase activity by both EP2306 and EP2302 at concentrations up to 100 μM. At higher concentrations, both compounds inhibited 12-lipoxygenase activity dose-dependently as shown in Fig. 4 (four experiments in total). The IC_{50} values were 454 μM for EP2306 and 318 μM for EP2302. The nonspecific lipoxygenase inhibitor ETYA was used as a positive control. ETYA inhibited 12-lipoxygenase activity by 49% and 68% at 25 and 50 μM, respectively.

There was no effect of EP2306 (100 nM–300 μM) or EP2302 (10–500 μM) on 15-lipoxygenase activity (data not shown). However, ETYA inhibited 15-lipoxygenase activity by 65% and 68% at 25 and 50 μM, respectively.

3.4. Effect of EP2306 on oxidative status in hyperlipidaemic rabbits

In animals treated for 4 weeks with EP2306 (10 mg/kg), TBARS levels were reduced (7.0 vs. 5.3 nmol eq malondialdehyde/ml, before vs. after treatment) compared with control animals (6.4 vs. 6.3 nmol eq malondialdehyde/ml, before vs. after treatment) (Table 2, Fig. 5). However, this difference did not reach significance marginally ($P=0.057$). It appears that upon treatment with EP2306 the reduction of TBARS levels is positively associated with the pretreatment TBARS values as indicated by the intersection of the two fitted lines towards low levels (Fig. 5).

There was a significant difference ($P=0.015$) in TRAP levels between animals treated with EP2306 and control animals (Table 2, Fig. 6). Treatment with EP2306 resulted in

Table 1
Effect of EP2300 compounds, probucol, and simvastatin on the lag time and the rate of conjugated diene formation

	n	Lag time (min)	S.E.M.	% Control	Rate	S.E.M.	% Control
Control	9	82	1.8		0.0116	0.0002	
EP2306 (μM)							
0.1	3	101[a]	9.8	123	0.0117	0.0007	101
1	3	108[b]	4.9	132	0.0109[c]	0.0001	94
10	3	135[b]	3.5	165	0.0108[c]	0.0001	93
30	4	150[a]	9.7	183	0.0096[b]	0.0002	83
50	3	179[c]	12.1	218	0.0092[b]	0.0003	79
EP2302 (μM)							
1	3	88	4.7	107	0.0087	0.0018	75
10	3	101[b]	2.9	123	0.0096[b]	0.0001	83
30	1	150		183	0.0042		36
50	2	157[b]	6.5	191	0.0042[b]	0.0002	36
0.1 μM Probucol	3	119[c]	11	145	0.0081	0.0015	70
10 μM Simvastatin	2	85	0.5	104	0.0096[b]	0.0002	83

[a] $P<0.01$.
[b] $P<0.001$.
[c] $P<0.05$.

a 7.5% increase in TRAP values (Table 2). This effect is independent of TRAP levels before treatment, as indicated by the two parallel lines (Fig. 6).

4. Discussion

Oxidation of LDL gives rise to modifications of the molecule that render it atherogenic (Steinberg et al., 1989). These changes include the formation of oxidized lipids that act as chemotactic and mitogenic agents and the modification of the charge on apolipoprotein B creating a ligand for the scavenger receptors on macrophages. The results of this study suggest that EP2300 compounds are not only potent inhibitors of LDL oxidation in vitro but also act as antioxidants in vivo.

The involvement of metal ions in atherosclerosis has been questioned since there is no convincing evidence for free metal ions in plasma or the arterial wall. However, samples from atherosclerotic lesions have been shown to contain both iron and copper in forms that can catalyze free-radical formation (Smith et al., 1992) and cell-mediated LDL oxidation appears to be metal-dependent (Garner et al., 1997). Furthermore, it has been shown that copper bound to ceruloplasmin may promote LDL oxidation (Mukhopadhyay et al., 1997). The ability of several compounds to prolong the lag phase or to decrease the rate of accumulation of conjugated dienes during oxidation of LDL has been used as a marker for their possible benefit as antiatherogenic agents (O'Leary et al., 1996). In the present study, the production of conjugated dienes was expressed

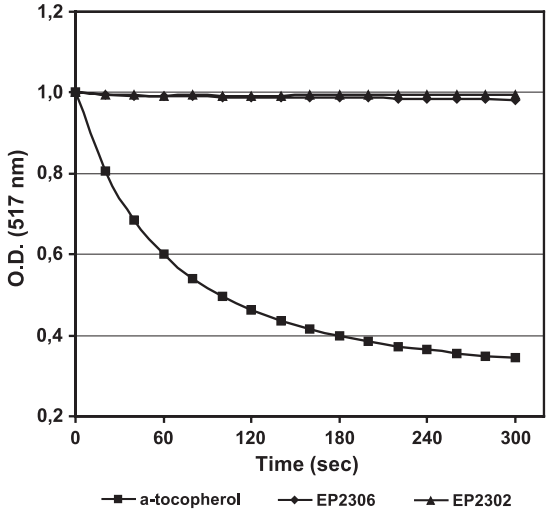

Fig. 2. Free radical scavenging activity of EP2300 compounds. DPPH (0.1 mM) was mixed with 50 μM–1 mM of either EP2306 or EP2302. a-Tocopherol (20 μM) was used as positive control. The time course of the change in the optical density was monitored at 517 nm. A representative experiment is presented.

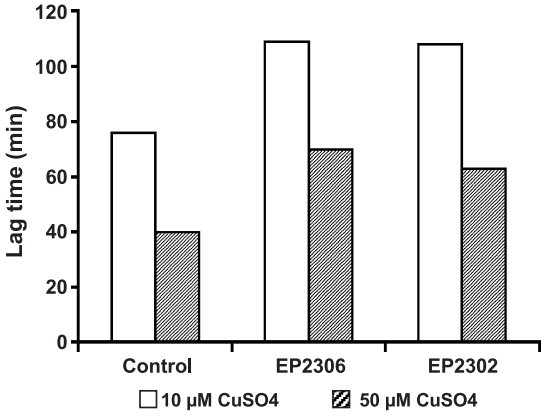

Fig. 3. Copper-chelating activity of EP2300 compounds. Human LDL (50 μg of protein/ml) was incubated with 10 μM (open bars) or 50 μM (hatched bars) of $CuSO_4$ in the absence (control) or presence of either 10 μM EP2306 or 30 μM EP2302. A representative experiment is presented.

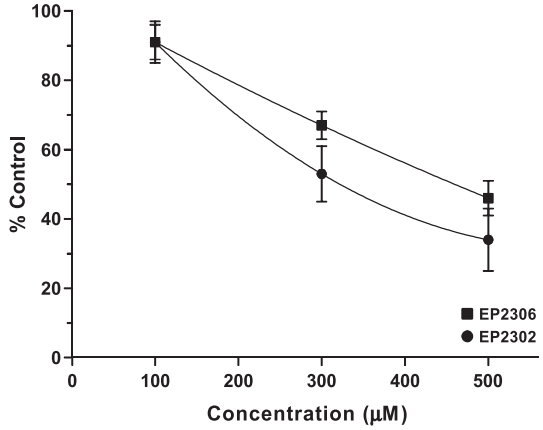

Fig. 4. Inhibition of 12-lipoxygenase activity by EP2306 and EP2302. 12-Lipoxygenase activity was determined in the absence and presence of increasing concentrations of EP2306 and EP2302. The enzyme assay was performed in duplicate determinations in four experiments (bars indicate S.E.M.). The activity is expressed as percentage of control values.

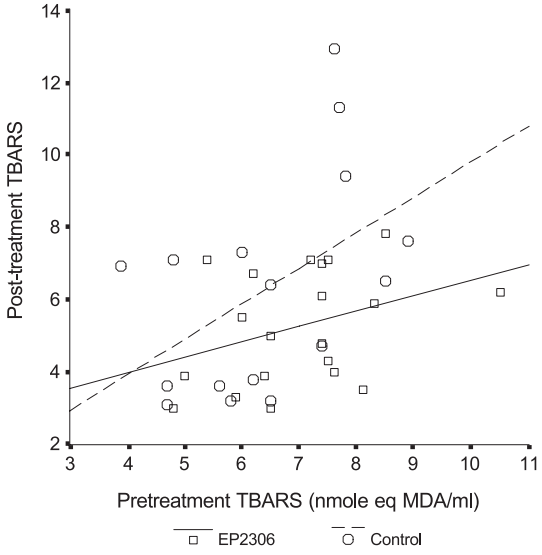

Fig. 5. Effect of EP2306 on TBARS values in serum of hyperlipidaemic rabbits. Pretreatment and post-treatment TBARS values showing fitted lines in hyperlipidaemic rabbits. Animals were treated with vehicle (n=16) or EP2306 (n=20) for 4 weeks before sacrifice.

by the absorbance at 234 nm. This index of LDL oxidation correlates highly with the TBARS production during the reaction (Kleinveld et al., 1992). We found that EP2300 compounds are potent antioxidants even at a concentration of 10 μM. During copper-induced oxidation of LDL, the lag time is a reflection of the endogenous antioxidant content of LDL. These antioxidants are gradually consumed along with added phenolic antioxidants (e.g., a-tocopherol, probucol) extending the lag time of oxidation but once consumed they do not reduce the rate of oxidation during the propagation phase. EP2300 compounds not only increased significantly the lag time of the oxidation reaction dose-dependently but also reduced the rate of the reaction in the propagation step suggesting a different antioxidant mechanism.

Among statins, fluvastatin sodium has been shown to have a similar effect on LDL oxidation in vitro (Suazumura et al., 1999). In ex vivo copper-induced oxidation of LDL in hypercholesterolaemic subjects, both pravastatin and simvastatin decreased the rate and the total amount of dienes produced during the reaction (Kleinveld et al., 1993). In the present in vitro study, simvastatin at a high dose (10 μM) did not prolong the lag phase of the reaction whereas in agreement with the study by Kleinveld et al. (1993) it caused a decrease in the rate of the reaction which was similar with the decrease achieved by EP2300 compounds. Probucol was more potent than EP2300 compounds in increasing the lag time of oxidation probably due to its incorporation into the LDL molecule and its free radical scavenging activity but its effect on the rate of oxidation was not clear. EP2300 compounds seem to have an additive antioxidant activity with the endogenous antioxidants in the initiation step of oxidation but part of them may remain even after the endogenous antioxidants are exhausted and thereafter diminish the rate of the reaction.

It has been hypothesized that lipoprotein oxidation occurs primarily in the arterial wall following the influx of lipoproteins into the subendothelial space and the intima layer. Antioxidants incorporated within the lipoprotein particle itself would be expected to be more effective in protecting against oxidative modification of lipoproteins (Steinberg et al., 1989). Under our experimental conditions, preincubation of human LDL with EP2300 compounds for 90 min did not show an increase in the lag phase of the reaction compared to the one observed in control samples (data not shown). It is, therefore, possible that EP2300 compounds are not incorporated into the LDL molecule. Under oxidative stress, LDL oxidation involves the action of reactive oxygen species (Steinberg et al., 1989). How-

Table 2
The effect of EP2306 on the antioxidant status in vivo

Treatment	TBARS (nmol eq malondialdehyde/ml)			TRAP (μM)		
	n	Pretreatment	Post-treatment	n	Pretreatment	Post-treatment
Control	16	6.4±0.4	6.3±0.7	18	762±12	792±10
EP2306	20	7.0±0.3	5.3±0.4	25	755±8	812±7

Hyperlipidaemic rabbits were treated with vehicle (control) or EP2306 for 4 weeks before sacrifice.
Values are mean±S.E.M.

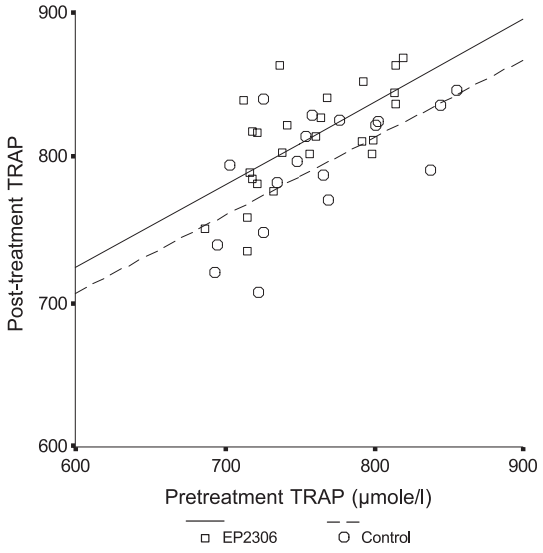

Fig. 6. Effect of EP2306 on TRAP values in serum of hyperlipidaemic rabbits. Pretreatment and post-treatment TRAP values showing fitted lines in hyperlipidaemic rabbits. Animals were treated with vehicle (n=18) or EP2306 (n=25) for 4 weeks before sacrifice.

ever, based on our results, EP2300 compounds did not act as antioxidants through a free radical scavenging activity. In addition, increased copper ion concentration did not abolish the inhibitory effect of EP2300 compounds on LDL oxidation suggesting that these compounds do not act as metal ion chelators. Other mechanism(s) should be responsible for the protective effect of EP2300 compounds on LDL oxidation.

Copper is believed to coordinate to the apolipoprotein B component of LDL, with histidinyl residues serving as ligands (Burkitt, 2001). It has been shown that interfering with the interactions between apolipoprotein B and the lipid moiety of LDL modifies the conformation of LDL and, as a consequence, hinders copper binding to apolipoprotein B and reduces oxidizability of LDL (Abuja et al., 1999). It is possible that EP2300 compounds do not have a direct antioxidant effect but their inhibitory effect on LDL oxidation in vitro may be attributed to a mechanistic protection of LDL due to binding and conformational changes of the molecule. The different effect of EP2306 and EP2302 on the lag time and the rate of the oxidation reaction could point to differences in binding of these molecules to the apolipoprotein B moiety of LDL. Based on the structural differences of the two molecules studied, one could hypothesize that the hydroxyl group of EP2306 forms a complex with LDL more rapidly than EP2302 but is rather unstable whereas the side chain of EP2302 ($–CH_2CH_2CH_2ONO_2$) forms a complex with LDL more slowly but once formed is more stable. This hypothesis could explain the rapid action of EP2306 in extending the lag time but reducing less efficiently the rate of the oxidation reaction and the slower action of EP2302 which is less effective in increasing the lag time but reduces more efficiently the rate of the reaction.

The detailed mechanisms of cell-mediated oxidation of LDL are unknown. Oxidative modification of LDL by rabbit endothelial cells (Parthasarathy et al., 1989), human monocytes (McNally et al., 1990), and mouse peritoneal macrophages (Rankin et al., 1991) was prevented by high concentrations of nonspecific lipoxygenase inhibitors suggesting a role of the lipoxygenase pathway in this process. In the present study, based on the high concentrations of EP2306 and EP2302 required for inhibition of 12-lipoxygenase activity in vitro, none of these compounds can be characterized as a potent inhibitor of this enzyme. Moreover, 15-lipoxygenase was not inhibited by either of the compounds studied. The lack of inhibition of 15-lipoxygenase by EP2300 compounds is not unexpected since there are several compounds that inhibit 5- and 12-lipoxygenase, but only a few of them can also inhibit 15-lipoxygenase. It is unlikely that EP2300 compounds would inhibit lipoxygenase-mediated LDL oxidation in a biological system.

Animal models of atherosclerosis have demonstrated a beneficial effect of antioxidant vitamins that correlates with delayed progression or regression of atherosclerosis. In cholesterol-fed rabbits, it has been shown that supplementation with antioxidants such as vitamin E and butylated hydroxytoluene retards atherogenesis (Bjorkhem et al., 1991; Wilson et al., 1978). Therefore, this animal model was used to assess the in vivo antioxidant effect of EP2306. It is not known whether the concentrations of EP2306 that inhibit LDL oxidation in vitro correspond to the pharmacological levels achieved in rabbits treated with this agent. However, this compound decreased circulating lipid peroxides and increased the capacity of serum to resist attacks by free radicals indicating that EP2306 is an effective physiological antioxidant. Whether this effect is solely due to the antioxidant properties of EP2306 or it is also due to a reduced residence time of LDL in the plasma awaits further investigation. In addition, it remains to be established whether EP2302 acts in a similar way to EP2306 in vivo.

Our findings with cholesterol-fed rabbits should be tested in humans before any conclusions are drawn regarding the potential clinical relevance of EP2306. Results from clinical trials of vitamin E are controversial (for review, see Witztum and Steinberg, 2001) and antioxidants so far have shown little or no clinical efficacy in preventing or reversing atherosclerotic disease in man. Explanations proposed for the discrepancy in results between animal models and humans include differences in patient characteristics, the antioxidant content of their diets, and dose selection.

In conclusion, the present data suggest that EP2300 compounds are effective inhibitors of copper-mediated LDL oxidation in vitro. Moreover, EP2306 acts as an antioxidant in hyperlipidaemic rabbits, a property which could be beneficial in reducing atherosclerosis in vivo.

Acknowledgements

We thank Drs. L. Kaklamanis, A. Charalambus, and D. Roukounas for helpful discussions, Drs. M. Simionescu, A. Sima, and L. Niculescu (ICBP, Bucharest) for help with the TBARS and TRAP measurements, and Dr. A. Papalois, Mr. T. Grigoriou, Ms. E. Karabela, Ms. A. Papadopoulou, and Mr. G. Sifnios for excellent technical assistance with the in vivo experiments.

References

Abuja, P.M., Lohner, K., Prassl, R., 1999. Modification of the lipid–protein interaction in human low-density lipoprotein destabilizes ApoB-100 and decreases oxidizability. Biochemistry 38, 3401–3408.

Aviram, M., Rosenblat, M., Bisgaier, C.L., Newton, R.S., 1998. Atorvastatin and gemfibrozil metabolites, but not the parent drugs, are potent antioxidants against lipoprotein oxidation. Atherosclerosis 138, 271–280.

Belkner, J., Stender, H., Kuhn, H., 1998. The rabbit 15-lipoxygenase preferentially oxygenates LDL cholesterol esters, and this reaction does not require vitamin E. J. Biol. Chem. 273, 23225–23232.

Bird, D.A., Tangirala, R.K., Fruebis, J., Steinberg, D., Witztum, J.L., Palinski, W., 1998. Effect of probucol on LDL oxidation and atherosclerosis in LDL receptor-deficient mice. J. Lipid Res. 39, 1079–1090.

Bjorkhem, I., Henriksson-Freyschuss, A., Breuer, O., Diczfalusy, U., Berglund, L., Henriksson, P., 1991. The antioxidant butylated hydroxytoluene protects against atherosclerosis. Arterioscler. Thromb. 11, 15–22.

Blois, M.S., 1958. Antioxidant determination by the use of a stable free radical. Nature 189, 1199–1200.

Burkitt, M.J., 2001. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. Arch. Biochem. Biophys. 394, 117–135.

Cathcart, M.K., Folcik, V.A., 2000. Lipoxygenases and atherosclerosis: protection versus pathogenesis. Free Radic. Biol. Med. 28, 1726–1734.

Chang, M.Y., Sasahara, M., Chait, A., Raines, E.W., Ross, R., 1995. Inhibition of hypercholesterolemia-induced atherosclerosis in the non-human primate by probucol: II. Cellular composition and proliferation. Arterioscler. Thromb. Vasc. Biol. 15, 1631–1640.

Chrysselis, M.C., Rekka, E.A., Kourounakis, P.N., 2000. Hypocholesterolemic and hypolipidemic activity of some novel morpholine derivatives with antioxidant activity. J. Med. Chem. 43, 609–612.

Chrysselis, M.C., Rekka, E.A., Siskou, I.C., Kourounakis, P.N., 2002. Nitric oxide releasing morpholine derivatives as hypolipidemic and antioxidant agents. J. Med. Chem. 45, 5406–5409.

Esterbauer, H., Striegl, G., Puhl, H., Rotheneder, M., 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Radic. Res. Commun. 6, 67–75.

Esterbauer, H., Gebicki, J., Puhl, H., Jurgens, G., 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic. Biol. Med. 13, 341–390.

Esterbauer, H., Wag, G., Puhl, H., 1993. Lipid peroxidation and its role in atherosclerosis. Br. Med. Bull. 49, 566–576.

Fogelman, A.M., Shechter, I., Seager, J., Hokom, M., Child, J.S., Edwards, P.A., 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. Proc. Natl. Acad. Sci. U. S. A. 77, 2214–2218.

Garner, B., van Reyk, D., Dean, R.T., Jessup, W., 1997. Direct copper reduction by macrophages. Its role in low density lipoprotein oxidation. J. Biol. Chem. 272, 6927–6935.

Hiltunen, T., Luoma, J., Nikkari, T., Yla-Herttuala, S., 1995. Induction of 15-lipoxygenase mRNA and protein in early atherosclerotic lesions. Circulation 92, 3297–3303.

Hussein, O., Schlezinger, S., Rosenblat, M., Keidar, S., Aviram, M., 1997. Reduced susceptibility of low density lipoprotein (LDL) to lipid peroxidation after fluvastatin therapy is associated with the hypocholesterolemic effect of the drug and its binding to the LDL. Atherosclerosis 128, 11–18.

Johansson, J., Olsson, A.G., Bergstrand, L., Elinder, L.S., Nilsson, S., Erikson, U., Molgaard, J., Holme, I., Walldius, G., 1995. Lowering of HDL2b by probucol partly explains the failure of the drug to affect femoral atherosclerosis in subjects with hypercholesterolemia. A probucol quantitative regression Swedish trial (PQRST) report. Arterioscler. Thromb. Vasc. Biol. 15, 1049–1056.

Kleinveld, H.A., Hak-Lemmers, H.L., Stalenhoef, A.F., Demacker, P.N., 1992. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein. Clin. Chem. 38, 2066–2072.

Kleinveld, H.A., Demacker, P.N., De Haan, A.F., Stalenhoef, A.F., 1993. Decreased in vitro oxidizability of low-density lipoprotein in hypercholesterolaemic patients treated with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors. Eur. J. Clin. Investig. 23, 289–295.

Kuhn, H., Heydeck, D., Hugou, I., Gniwotta, C., 1997. In vivo action of 15-lipoxygenase in early stages of human atherogenesis. J. Clin. Invest. 99, 888–893.

Lehr, H.A., Frei, B., Olofsson, A.M., Carew, T.E., Arfors, K.E., 1995. Protection from oxidized LDL-induced leukocyte adhesion to microvascular and macrovascular endothelium in vivo by vitamin C but not by vitamin E. Circulation 91, 1525–1532.

McNally, A.K., Chisolm III, G.M., Morel, D.W., Cathcart, M.K., 1990. Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. J. Immunol. 145, 254–259.

Miller, N.E., 1987. Associations of high-density lipoprotein subclasses and apolipoproteins with ischemic heart disease and coronary atherosclerosis. Am. Heart J. 113, 589–597.

Mukhopadhyay, C.K., Mazumder, B., Lindley, P.F., Fox, P.L., 1997. Identification of the prooxidant site of human ceruloplasmin: a model for oxidative damage by copper bound to protein surfaces. Proc. Natl. Acad. Sci. U. S. A. 94, 11546–11551.

Niculescu, L., Stancu, C., Sima, A., Toporan, D., Simionescu, M., 2001. The total peroxyl radical trapping potential in serum—an assay to define the stage of atherosclerosis. J. Cell. Mol. Med. 5, 285–294.

O'Leary, V.J., Tilling, L., Fleetwood, G., Stone, D., Darley-Usmar, V., 1996. The resistance of low density lipoprotein to oxidation promoted by copper and its use as an index of antioxidant therapy. Atherosclerosis 119, 169–179.

Palinski, W., Rosenfeld, M.E., Yla-Herttuala, S., Gurtner, G.C., Socher, S.S., Butler, S.W., Parthasarathy, S., Carew, T.E., Steinberg, D., Witztum, J.L., 1989. Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. U. S. A. 86, 1372–1376.

Parthasarathy, S., Wieland, E., Steinberg, D., 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. Proc. Natl. Acad. Sci. U. S. A. 86, 1046–1050.

Rankin, S.M., Parthasarathy, S., Steinberg, D., 1991. Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. J. Lipid Res. 32, 449–456.

Smith, C., Mitchinson, M., Aruoma, J.O., Halliwell, I.B., 1992. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. Biochem. J. 286, 901–905.

Sparrow, C.P., Doebber, T.W., Olszewski, J., Wu, M.S., Ventre, J., Stevens, K.A., Chao, Y.S., 1992. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant *N*,*N*′ -diphenyl-phenylenediamine. J. Clin. Invest. 89, 1885–1891.

Steinberg, D., 1995. Clinical trials of antioxidants in atherosclerosis: are we doing the right thing? Lancet 346, 36–38.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L., 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. 320, 915–924.

Suzumura, K., Yasuhara, M., Tanaka, K., Suzuki, T., 1999. Protective effect of fluvastatin sodium (XU-62-320), a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on oxidative modification of human low-density lipoprotein in vitro. Biochem. Pharmacol. 57, 697–703.

Taraporewala, I.B., Kauffman, J.M., 1990. Synthesis and structure–activity relationships of anti-inflammatory 9,10-dihydro-9-oxo-2-acridine-alkanoic acids and 4-(2-carboxyphenyl)aminobenzenealkanoic acids. J. Pharm. Sci. 79, 173–178.

Vickers, A.J., Altman, D.G., 2001. Statistics notes: analysing controlled trials with baseline and follow up measurements. Br. Med. J. 323, 1123–1124.

Wilson, R.B., Middleton, C.C., Sun, G.Y., 1978. Vitamin E, antioxidants and lipid peroxidation in experimental atherosclerosis of rabbits. J. Nutr. 108, 1858–1867.

Witztum, J.L., Steinberg, D., 2001. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? Trends Cardiovasc. Med. 11, 93–102.

Yla-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L., Steinberg, D., 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest. 84, 1086–1095.