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ARTICLE *in* CHEMISTRY AND PHYSICS OF LIPIDS · JANUARY 2008

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Copper-induced peroxidation of phosphatidylserine-containing liposomes is inhibited by nanomolar concentrations of specific antioxidants

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Received 21 May 2006; received in revised form 31 July 2007; accepted 14 August 2007
Available online 17 August 2007

Abstract

Copper-induced peroxidation of liposomal palmitoyllinoleoyl-phosphatidylcholine (PLPC) is inhibited by α-tocopherol at micromolar concentrations. In our previous study we found that when the liposomes contain phosphatidylserine (PS), nanomolar concentrations of Toc were sufficient to inhibit peroxidation. In an attempt to gain understanding of the origin of this extreme antioxidative potency, we tested the antioxidative potency of 36 additional antioxidants and the dependence of their potency on the presence of PS in the liposomes. The results of these studies reveal that only 11 of the tested antioxidants possess similar antioxidative potency to that of Toc. These include trolox, butylated hydroxytoluene (BHT), curcumin, nordihydroguaiaretic acid (NDGA), diethylstilbestrol (DES), 2 of the 13 tested flavonoids (luteolin and 7,3′,4′-trihydroxyflavone; T-414), α-naphthol, 1,5-, 1,6- and 1,7-dihydroxynaphthalenes (DHNs). Propyl gallate (PG), methyl syringate, rosmarinic acid, resveratrol, other flavonoids, as well as β-naphthol, 1,2-, 1,3-, 1,4-, 2,3-, 2,6-, and 2,7-DHNs were either moderately antioxidative or pro-oxidative. For liposomes made of PLPC (250 μM) and PS (25 μM) the "lag" preceding copper-induced peroxidation (5 μM copper) was doubled upon addition of 30–130 nM of the "super-active" antioxidants.

We propose that the mechanism responsible for the extreme antioxidative potency against copper-induced peroxidation in PS-containing liposomes involves replenishment of the antioxidant in a ternary PS–copper-antioxidant complex. Based on structure–activity relationship of the 37 tested antioxidants, the "super-antioxidative potency" is attributed to the recycling of relatively stable semiquinone or semiquinone-like radicals.

Keywords: Flavonoids; Naphthols; Dihydroxynaphthalenes; Phosphatidylserine; Liposomes; Copper-induced peroxidation

Abbreviations: PLPC, palmitoyllinoleoylphosphatidylcholine; POPS, palmitoyloleoylphosphatidylserine; POPE, palmitoyloleoylphosphatidylethanolamine; POPA, palmitoyloleoylphosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; OD, optical density; AAPH, 2,2′-azobis (2-methylpropionamidine) dihydrochloride; Toc, α-tocopherol; vitamin E; BHT, butylated hydroxytoluene; PG, propyl gallate; Cur, curcumin; NDGA, nordihydroguaiaretic acid; DES, diethylstilbestrol; DHN, dihydroxynaphthalene

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[1] This work was performed in partial fulfillment of the requirements for Ph.D. degree of Dr. Sigal Gal, Sackler Faculty of Medicine, Tel Aviv University, Israel.

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doi:10.1016/j.chemphyslip.2007.08.001

1. Introduction

Oxidative modifications have been implicated in 250 pathologies, including diabetes (Evans et al., 2002), cardiovascular (Witztum and Steinberg, 2001) and neurodegenerative diseases (Arlt et al., 2002). Much work has therefore been aimed at gaining understanding of the mechanisms that govern peroxidation processes. We approach this goal via a systematic study of copper-induced peroxidation of the linoleic acid at the *sn*-2 position of liposomal palmitoyl-linoleoyl-phosphatidylcholine (PLPC, Bittner et al., 2002). In addition, we have studied peroxidation in the negatively charged liposomes made of PLPC and a negatively charged phospholipid, either phosphatidic acid (PA) or phosphatidylserine (PS) (Gal et al., 2003). In both these systems, we studied the effects of both water- and lipid-soluble antioxidants (Gal et al., 2003). The most intriguing result of the latter investigation was that in the presence of PS, the antioxidative potency of α-tocopherol was dramatically enhanced (Gal et al., 2003).

The dependence of the antioxidative effects of various antioxidants on the phospholipid composition of the system is a debated issue in the literature. Most of the studies that addressed this issue were conducted in bulk oil or in organic solvents. Under the latter conditions, synergistic antioxidative effects have been demonstrated in systems containing certain antioxidants and PS (Lambelet et al., 1994; Alam et al., 1997; Saadan et al., 1998). Similar synergism was also observed in oily systems between either phosphatidylethanolamine (PE) or phosphatidylcholine (PC) and different antioxidants, including α-tocopherol (Ishikawa et al., 1984; Hamzawi, 1990; Ohshima et al., 1993; Saadan et al., 1998; Bandarra et al., 1999), flavonoids (Hudson and Lewis, 1983), isoflavones (Dziedzic and Hudson, 1983), and synthetic antioxidants such as propyl gallate (Dziedzic et al., 1986).

The synergistic antioxidative effects of various phospholipids and antioxidants have been explained by different mechanisms, including regeneration of tocopherol by the phospholipids (Lambelet et al., 1994; Dziedzic et al., 1986; Weng and Gordon, 1993) and enhancement of the radical scavenging activity of the antioxidants in the microenvironment of the oily medium (Koga and Terao, 1995; Hildebrand et al., 1984). The possibility that the synergy is a result of chelation of free metal ions (Hudson and Mahgoub, 1981), has been ruled out on the basis of stoichiometric considerations (Hudson and Lewis, 1983).

The aim of the present work was to shed light on the mechanisms responsible for the observed marked potentiation of the antioxidative effect of tocopherol by PS (Gal et al., 2003). Towards this aim, we investigated the generality of the "super-activity" observed with tocopherol in PS-containing liposomes, with respect to the effects of other antioxidants at nanomolar concentrations. Of the total of 37 compounds (including tocopherol), that were examined, only 12 exhibited such apparent "super-activity" (Scheme 1). From analysis of the structure–activity relationship of these compounds we conclude that the formation of a relatively stable radical, possibly stabilized by a semiquinone-like structure, is essential for this interaction.

2. Materials and methods

2.1. Materials

PLPC (1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine), POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) and POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL).

$CuCl_2$, EDTA, EtOH, NaCl, NaH_2PO_4, Na_2HPO_4 and curcumin were all purchased from Merck (Darmstadt, Germany).

(±) α-Tocopherol (vitamin E, Toc), (±) α-tocopherol acetate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT), *n*-propyl gallate (PG), methyl syringate, resveratrol, diethylstilbestrol, hesperetin, 1-naphthol and 2-hydroxy-1,4-naphthoquinone (lawsone) were all purchased from Sigma (St. Louis, MO).

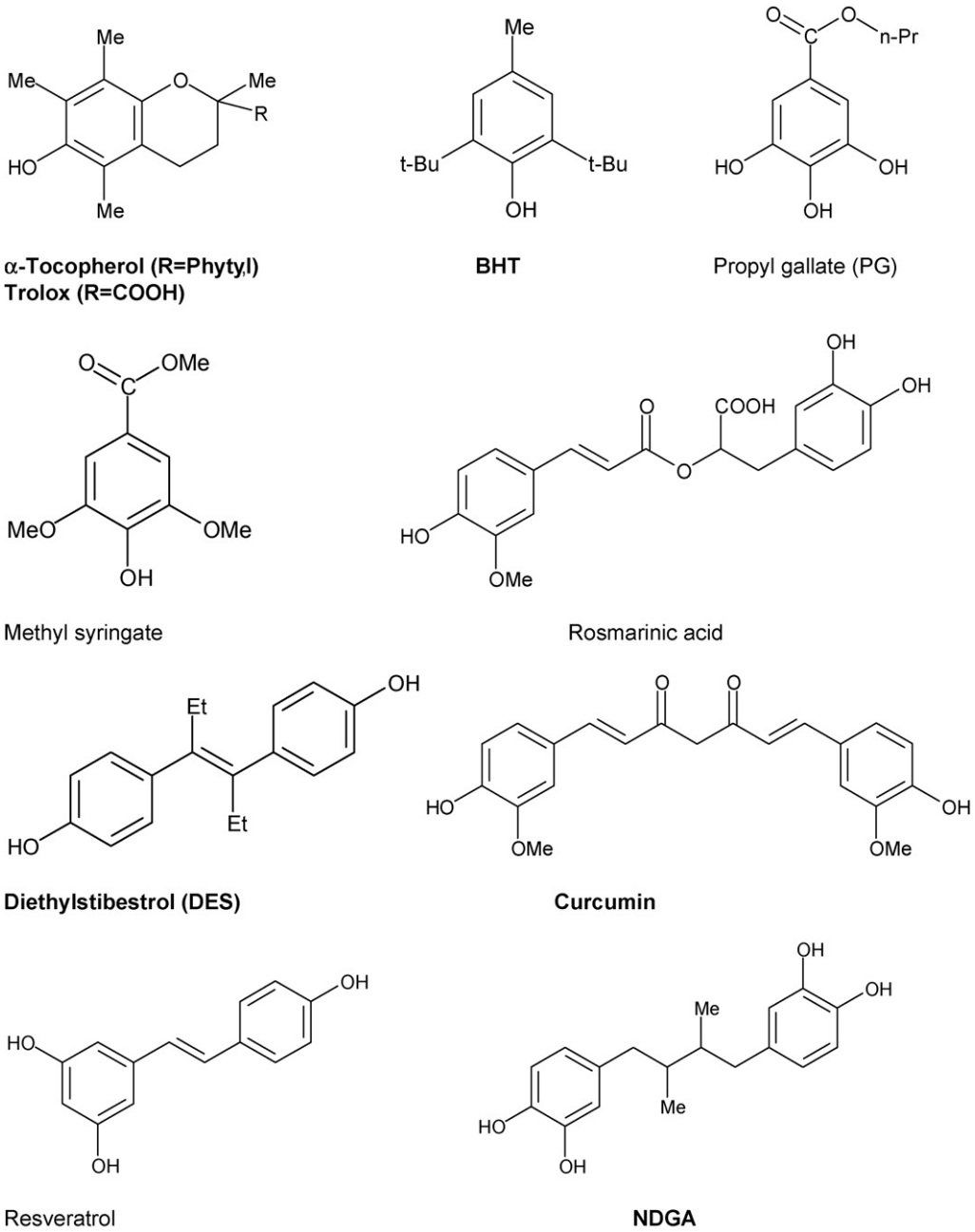
2-Naphthol, 1,7-dihydroxynaphthalene and rosmarinic acid were purchased from Aldrich (Milwaukee, WI). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride, 1,2-dihydroxynaphthalene, 1,2-naphthoquinone, 1,3-dihydroxynaphthalene, 1,4-naphthoquinone hydrate, 1,5-dihydroxynaphthalene, 1,6-dihydroxynaphthalene, 2,3-dihydroxynaphthalene, 2,6-dihydroxynaphthalene, 2,7-dihydroxynaphthalene, and 5-hydroxy-1,4-naphthoquinone (juglone) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Chloroform, nordihydroguaiaretic acid (NDGA) and 1,4-naphthohydroquinone were purchased from Fluka (Buchs, Switzerland).

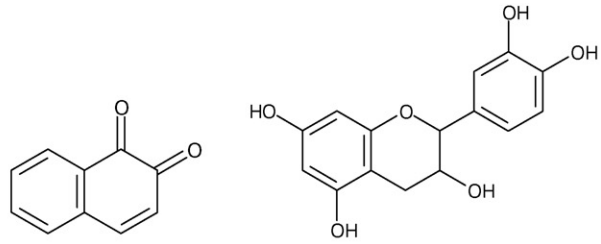
Chrysin, apigenin, T-414 (3′,4′,7-trihydroxyflavone), luteolin, kaempferol, quercetin, naringenin, taxifolin, daidzein, genistein, and catechin were products of ICC (Indofine Chemical Company, Inc. Somerville, NJ, USA).

2.2. Preparation of liposomes

Chloroformic solutions of phospholipids (PLPC with or without the appropriate volume of a solution of either POPS or POPE) were evaporated to dryness and co-lyophilized overnight, to remove traces of chloroform. Liposomes containing α-tocopherol were prepared by adding an ethanolic solution of α-tocopherol to the chloroformic solution of phospholipids, prior to removal of the solvents. The resultant films were dispersed at room temperature in a saline solution (146 mM NaCl), containing 15 μM EDTA, to minimize the peroxidation during the preparative stages. The dispersed phospholipids (3.75 mM PLPC without or with 0.375 mM POPS or 0.375 mM POPE) were mixed to homogeneity using a vortex-mixer. Liposomes were prepared by sonication

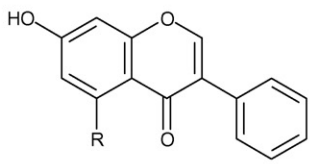


Scheme 1. Chemical structures of phenolic compounds tested for antioxidative activity. The names of "super-active" antioxidants are given in bold.

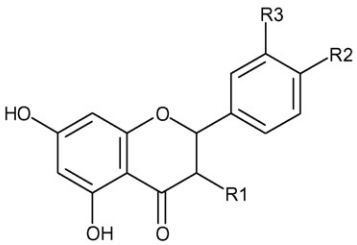


1,2-naphthoquinone

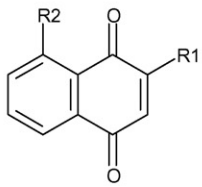
Catechin



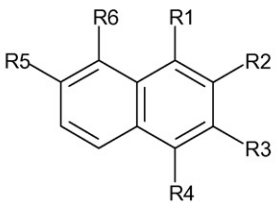
Daidzein (R=H)
Genistein (R=OH)



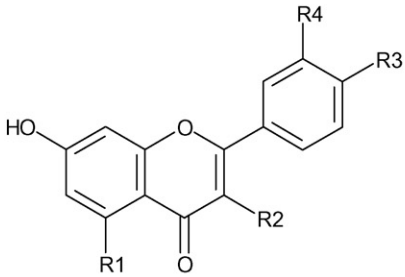
Taxifolin (R1,R2,R3=OH)
Hesperetin (R2=OMe; R3=OH; R1=H)
Naringenin(R2=OH; R1,R3=H)



1,4-naphthoquinone (R1,R2=H)
2-hydroxy-1,4-naphthoquinone (R1=OH; R2=H)
5-hydroxy-1,4-naphthoquinone (R2=OH; R1=H)



1-naphthol (R1=OH; R2,R3,R4,R5,R6=H)
2-naphthol (R2=OH; R1,R3,R4,R5,R6=H)
1,5-dihydroxynaphthalene (R4,R6=OH; R1,R2,R3,R5=H)
1,6-dihydroxynaphthalene (R3,R6=OH; R1,R2,R4,R5=H)
1,7-dihydroxynaphthalene (R2,R6=OH; R1,R3,R4,R5=H)
1,2-dihydroxynaphthalene (R1,R2=OH; R3,R4,R5,R6=H)
1,3-dihydroxynaphthalene (R1,R3=OH; R2,R4,R5,R6=H)
1,4-dihydroxynaphthalene (R1,R4=OH; R2,R3,R5,R6=H)
2,3-dihydroxynaphthalene (R2,R3=OH; R1,R4,R5,R6=H)
2,6-dihydroxynaphthalene (R3,R5=OH; R1,R2,R4,R6=H)
2,7-dihydroxynaphthalene (R2,R5=OH; R1,R3,R4,R6=H)



Luteolin (R1,R3,R4=OH; R2=H)
T414 (R3,R4=OH; R1,R2=H)
Chrysin (R1=OH; R2,R3,R4=H)
Apigenin (R1,R3=OH; R2,R4=H)
Kaempferol (R1,R2,R3=OH; R4=H)
Quercetin (R1,R2,R3,R4=OH)

Scheme 1. (*Continued*).

under nitrogen and ice cooling (Huang, 1969), using a Heat Systems Inc. XL-2020 probe-sonicator.

The mean size of the resultant liposomes was evaluated on the basis of quasi-elastic light scattering measurements, using ALV's high performance particle sizer, model ALV-NIBS/HPPS, equipped with a HeNe-laser at 632.8 nm. The mean diameter of PLPC liposomes varied between 80 and 120 nm, whereas the mean diameter for the negatively charged liposomes was somewhat smaller, varying within the range of 60–100 nm.

2.3. Kinetic studies of the peroxidation of PLPC liposomes

Prior to the peroxidation experiments, the liposomal dispersions were diluted in PBS (pH 7.4, 146 mM NaCl,

and 3.3 mM sodium phosphate). In all the kinetic experiments, the final concentration of PLPC was 250 μM (100 μL of 3.75 mM PLPC dispersion were diluted 15-fold). The final concentration of both POPS and POPE, when present in the liposomes, was 25 μM. The reaction mixtures contained 1 μM EDTA. The reported copper concentrations are the total concentrations of added $CuCl_2$. For kinetic studies of the effects of antioxidants, ethanolic solutions of the antioxidants were added to the liposomes prior to the addition of the inducer of peroxidation ($CuCl_2$ or AAPH). The antioxidants were added to the liposomes in a constant volume of 20 μL. In control experiments, the solution contained 20 μL ethanol. Solutions of copper chloride and antioxidants were freshly prepared before each experiment.

Following the addition of the inducer of peroxidation, the solutions were mixed with a pasteur pipette and the peroxidation of the liposomes was monitored at 37 °C by continuous recording of the absorbance at several wavelengths (234, 245, 250, 268, 300, and 338 nm), using a Kontron (Uvikon 933) double-beam spectrophotometer equipped with a 12-position automated sample changer. Measurements were conducted in quartz cuvettes containing a final volume of 1.5 mL with an optical pathway of 1 cm. Typically, the reaction was monitored for approximately 18 h with intervals of 11 min between measurements. Each reported kinetic profile is typical of at least three experiments conducted with different liposomal preparations.

Data analysis was performed by the standard procedures provided by Microsoft Excel XP and Microcal Origin 5.0 software.

2.4. Determination of tocopherol concentration

Tocopherol concentration was evaluated on the basis of its fluorescence intensity at 334 nm following excitation at 298 nm (Ramos-Lledo et al., 2001). The fluorescence intensity was measured using the ISS K2 multifrequency cross-correlation phase and modulation fluorometer (ISS, Champaign, IL), with excitation slits of 1 nm and emission slits set to 2 nm. All the measurements were conducted in quartz cuvettes.

2.5. Evaluation of the parameters used to characterize the kinetics of peroxidation

Initial optical density (OD) values were recorded immediately after the addition of the peroxidation-inducing agent. The presented time-dependencies of absorbance were corrected by subtracting the initial OD from later time points.

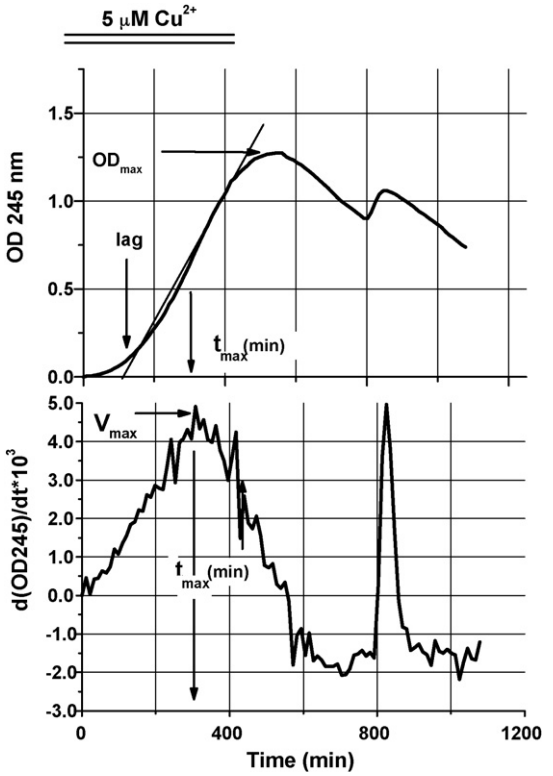


Fig. 1. Characterization of the kinetics of peroxidation. The kinetic parameters of copper-induced peroxidation of PLPC liposomes containing 9% POPS, as monitored by continuous recording of UV absorption of oxidation products at 245 nm, are defined in this figure. The upper panel demonstrates a typical time course of absorbance (i.e. of product accumulation). It was observed during peroxidation of PLPC (250 μM) with 9% POPS induced by $CuCl_2$ (5 μM). The lower panel depicts the first derivative of this time course, namely the time-dependence of the rate of accumulation of the absorbing products.

The major contribution to the time-dependent increase of optical density at 245 nm is that of the conjugated dienic lipid hydroperoxides (Pinchuk et al., 1998). Fig. 1 depicts a typical kinetic profile of absorbance at 245 nm. In many experiments, the time-dependence of absorbance is characterized by a "lag phase" followed by a "propagation phase" of faster accumulation of intermediate reaction products (mostly hydroperoxides). During the propagation phase, the rate of accumulation of hydroperoxides becomes maximal (V_{max}). The time at which V_{max} was achieved (denoted t_{max}) has been previously shown to correlate with the "lag time" (Ramos et al., 1995). The kinetic profile reaches a maximal optical density (OD_{max}) when the rate of hydroperoxide accumulation becomes equal to their decomposition rate. Thereafter, the rate of hydroperoxide decomposition exceeds the rate of their production and the optical density decreases. In many cases, this "decomposition

phase" was followed by another phase of relatively fast increase in the OD and a subsequent decrease. This phenomenon was observed in our previous work and attributed to size growth and/or aggregation of the oxidized liposomes (Bittner et al., 2002).

The ratio between the t_{max} observed in the presence of an antioxidant and the t_{max} observed in its absence is defined as the relative t_{max}. As shown below, the dependencies of the relative t_{max} on the concentration of the added antioxidant were linear. The potencies of the various studied antioxidants were expressed in terms of C_{2t}, which is the concentration of a given antioxidant needed to double t_{max}. For each of the studied compounds, the value of C_{2t} was evaluated from the linear regression of the dependence of the t_{max} on the concentration of antioxidant. For each antioxidant the concentration dependence of the lag was studied at least three times using different liposomal preparations. The average values for C_{2t} and their standard deviations are presented in Table 1 (see Section 3).

Notably although C_{2t} is a convenient factor for quantitative evaluation of the potency of antioxidants, it does not fully characterize the effect of an antioxidant on the kinetics of peroxidation. Specifically, during the antioxidants-induced "lag phase", autoxidation may still occur without affecting t_{max}. Hence C_{2t} cannot be regarded an exact measure of the effect of the antioxidants on the initial stages of peroxidation.

3. Results

3.1. Peroxidation of both PLPC and tocopherol in PS-containing liposomes is very slow

The upper panel of Fig. 2 confirms the results of our previous studies (Bittner et al., 2002; Gal et al., 2003). It shows that tocopherol, co-sonicated with PLPC to form tocopherol-containing liposomes (dashed lines) acted as a "mild" antioxidant, prolonging the lag phase and slightly reducing the maximal rate of accumulation of hydroperoxides (V_{max}), whereas in POPS-containing liposomes (solid lines), Toc prevented peroxidation for many hours. The kinetics of consumption of Toc, as monitored fluorometrically (Fig. 2, lower panel) show that in Toc-containing PLPC liposomes, essentially all the Toc (1 μM) was consumed in about 5 h (lower panel), as compared to the much slower peroxidation of PLPC (upper panel). By contrast, in POPS-containing liposomes, the Toc levels did not decrease appreciably within the monitored time range (Fig. 2, lower panel). In other words,

Table 1
Potency of selected antioxidants, as expressed by the concentration C_{2t} which is needed to double t_{max}, in liposomes made of 250 μM PLPC and in liposomes made of PLPC (250 μM) and POPS (25 μM)

Antioxidant	C_{2t} in POPS-containing PLPC liposomes	C_{2t} in PLPC liposomes
Toc	**38 ± 14 nM**	**Pro-oxidant 0.1 μM < Toc < 50 μM**
Trolox	**53 ± 14 nM**	**Pro-oxidant 1 μM < Trolox < 5 μM**
BHT	**33 ± 10 nM**	**>0.5 μM**
Methyl syringate	>1000 nM	n.d.
Propyl gallate (PG)	Pro-oxidant 100 nM < PG < 5000 nM	Pro-oxidant 0.1 μM < PG <5 μM
Curcumin	**47 ± 19 nM**	**>1.5 μM**
Rosmarinic acid	Pro-oxidant 100 nM < Ros < 1000 nM	n.d.
NDGA	**125 ± 40 nM**	**>1.2 μM**
Diethylstilbestrol (DES)	**126 ± 20 nM**	**>0.9 μM**
Resveratrol	Pro-oxidant 100 nM < Res < 1000 nM	n.d.
Luteolin	**41 ± 8 nM**	**>5 μM**
T-414	**75 ± 35 nM**	**>5 μM**
1-Naphthol	**58 ± 10 nM**	**>8 μM**
2-Naphthol	>600 nM	>3 μM
1,5-Dihydroxynaphthalene	**69 ± 24 nM**	**>5 μM**
1,6-Dihydroxynaphthalene	**63 ± 15 nM**	**>2.5 μM**
1,7-Dihydroxynaphthalene	**73 ± 20 nM**	**>3.5 μM**
1,2-Dihydroxynaphthalene	Pro-oxidant 250 nM < 1,2-DHN < 1000 nM	Pro-oxidant 0.25 μM < 1,2 DHN < 5 μM
1,3-Dihydroxynaphthalene	>250 nM	n.d.
1,4-Dihydroxynaphthalene	Pro-oxidant 10 nM < 1,4-DHN < 250 nM	Pro-oxidant 0.25 μM < 1,4-DHN < 5 μM
2,3-Dihydroxynaphthalene	Pro-oxidant 200 nM < 2,3-DHN < 1000 nM	>2 μM
2,6-Dihydroxynaphthalene	>800 nM	>3 μM
2,7-Dihydroxynaphthalene	>1000 nM	>4 μM

When the antioxidant in fact promotes peroxidation, the studied concentration range was given in the table. n.d.: not determined.

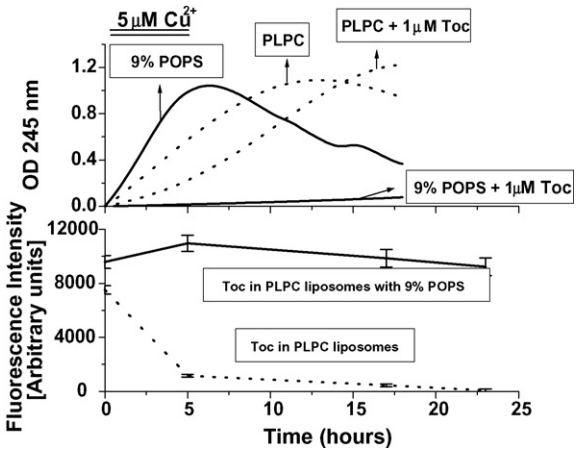


Fig. 2. Effect of Toc and PS on the kinetics of copper-induced peroxidation of PLPC liposomes and on the simultaneous consumption of Toc. $CuCl_2$ (5 μM) was added at time zero to PLPC liposomes (250 μM; dashed lines) or to PLPC liposomes containing 9% POPS (solid lines) with or without co-sonicated Toc (1 μM), as indicated. Peroxidation was monitored at 37 °C by recording the time-dependence of absorbance at 245 nm (upper panel). Toc consumption was monitored fluorometrically by recording the fluorescence at 334 nm following excitation at 298 nm (lower panel). All the reaction mixtures contained EDTA (1 μM).

in liposomes containing PS and Toc, the peroxidation of PLPC was inhibited by Toc, while the concentration of Toc did not change markedly (lower panel).

3.2. Tocopherol acetate does not influence peroxidation in the presence of PS

In our previous study we found that liposomal Toc (co-sonicated with PLPC) inhibited the peroxidation of PLPC, whereas externally added tocopherol promoted peroxidation. In PS-containing liposomes, tocopherol was a very efficient antioxidant even when externally added at nanomolar concentrations (Gal et al., 2003 and Table 1). In order to test whether the influence of tocopherol on the physical properties of the liposomes may contribute to this effect, we have studied the effect of the redox-inactive tocopherol acetate on the peroxidation of PLPC in POPS-containing liposomes. In fact, the addition of tocopherol acetate (0.1–5 μM) had no influence on the peroxidation induced by $CuCl_2$ (5 μM) of either PLPC liposomes (250 μM) or PLPC liposomes containing POPS (results not shown), in agreement with the results of Mora et al. (2000) which showed that tocopherol acetate is 500 times less potent than tocopherol against peroxidation induced either by xanthine/xanthine oxidase or by ADP–Fe(III).

3.3. Potentiation of specific antioxidants by PS

In an attempt to test whether the "super-activity" observed is unique to Toc, we have studied the concentration-dependence of the antioxidative effect of an additional 36 antioxidants on the kinetics of copper-induced peroxidation of PLPC in both pure PLPC liposomes and POPS-containing liposomes. The potencies of the different antioxidants expressed as C_{2t} (see Section 2) are given in Table 1. Those antioxidants for which C_{2t} was lower than 150 nM are (arbitrarily) defined as being "super-active" antioxidants. Twelve out of the 37 studied antioxidants (including Toc) were "super-active" in of PS-containing liposomes. The latter 12 antioxidants, appear in Table 1 in bold.

The antioxidants have been chosen for our studies so as to present structurally related compounds within several families. As obvious from Table 1, in most of the groups of compounds, some antioxidants were "super-active" but other compounds, whose structure was only slightly different, were either poor antioxidants or promoters of peroxidation. A rough estimation of the potency of each of the various antioxidants on the peroxidation of pure PLPC liposomes (250 μM), induced by 5 μM copper, is also expressed in terms of C_{2t}, in a semi-quantitative fashion (Table 1).

The data presented in Table 1 reveal several interesting clues regarding the mechanism of action of the "super-active" antioxidants. Special attention is given to the following findings:

1. In terms of its effect on the lag, trolox is an efficient, dose-dependent inhibitor of copper-induced peroxidation of POPS-containing liposomes, whereas V_{max} is only slightly affected (Fig. 3).
2. BHT is a commonly used synthetic antioxidant. In PLPC liposomes, it is a rather potent antioxidant; and in PS-containing liposomes it is "super-active" (Table 1). By contrast, propyl gallate accelerated lipid peroxidation of PLPC throughout the studied range of concentrations in both PLPC and PS-containing liposomes. Notably, methyl syringate which is a structurally related derivative of PG acted as an antioxidant at micromolar concentrations in the PS-containing liposomes (Table 1).
3. Similar to BHT, curcumin inhibited copper-induced peroxidation in both PLPLC liposomes (at micromolar concentrations) and PS-containing liposomes (at nanomolar concentrations). By contrast, the naturally occurring antioxidant, rosmarinic acid promoted the copper-induced peroxidation of PLPC in PS-containing liposomes (Table 1).

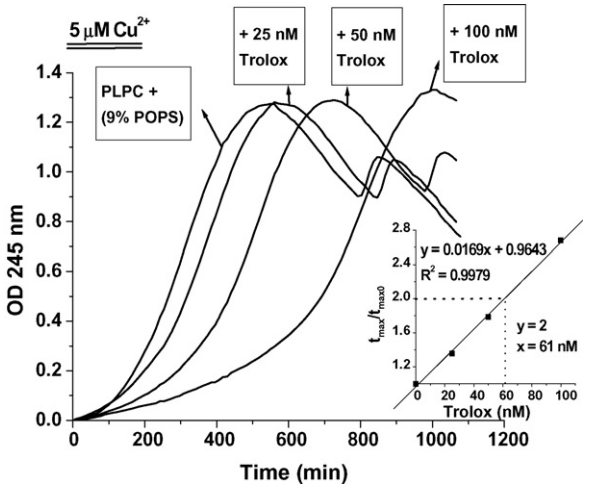


Fig. 3. Effect of Trolox on the kinetics of copper-induced peroxidation POPS-containing liposomes. Different concentrations of Trolox (0–100 nM, as indicated) and $CuCl_2$ (5 μM) in the presence of 1 μM EDTA, were added to PLPC liposomes (250 μM) containing 9% POPS. The absorbance at 245 nm was continuously monitored at 37 °C. The inset depicts the dependence of the relative prolongation of t_{max} on the concentration of added Trolox. From this linear regression the C_{2t} value is calculated, in this case 61 nM (see text for details).

4. Similar to both BHT and curcumin, the natural product, NDGA, inhibited copper-induced peroxidation at micromolar concentrations in PLPC liposomes, whereas in POPS-containing liposomes its activity is an order of magnitude higher than in the absence of POPS (Table 1).
5. The natural antioxidant resveratrol did not inhibit copper-induced peroxidation, in fact, it promoted it (Table 1). By contrast, diethylstilbestrol (DES) which is a member of the same stilbene family, inhibited copper-induced peroxidation, of PLPC at micromolar concentrations and in PS-containing liposomes, its antioxidative potency was an order of magnitude higher (Table 1).
6. The naturally occurring polyphenols denoted flavonoids are believed to be potent antioxidants (Rice-Evans et al., 1996; Brown et al., 1998; Fuhrman and Aviram, 2001; Mira et al., 2002; Vaya et al., 2003). In our investigation only two flavonoids, namely luteolin and T-414, out of the 12 tested flavonoids were "super-active" antioxidants, in PS-containing liposomes. In pure PLPC liposomes, both these antioxidants inhibited peroxidation only at micromolar concentrations (Table 1). The other tested flavonoids were either slightly antioxidative (kaempferol), or pro-oxidative (catechin) or ineffective (chrysin, apigenin, quercetin, naringenin, hesperetin, taxifolin, daidzein, and genistein) (results not shown).
7. Of special interest is the difference between the activities of the two closely related derivatives of naphthalene; the 1- and 2-naphthols. Specifically, 1-naphthol inhibited the copper-induced peroxidation of PS-containing liposomes at nanomolar concentrations whereas 2-naphthol exhibited antioxidative effect only at much higher concentrations (Table 1). Notably, in pure PLPC liposomes, 2-naphthol was almost three times more potent than 1-naphthol (Table 1).

The antioxidative activity of dihydroxynaphthalenes (DHNs), against copper-induced peroxidation of liposomal PLPC depended also on the liposome composition: In pure PLPC vesicles, micromolar concentrations of 1,5-, 1,6-, 1,7-, 2,3-, 2,6-, and 2,7-DHNs, were inhibitory whereas 1,2 and 1,4-DHNs were pro-oxidative. By contrast, in PS-containing liposomes, 1,5-, 1,6-, and 1,7-DHNs were "super-active" antioxidants, 1,3-, 2,6-, and 2,7-DHNs were less potent antioxidants, and the other tested DHNs (1,2-, 1,4-, and 2,3-) were pro-oxidative (Table 1).

In other words, 1-hydroxynaphtol is "super-active", while a second hydroxyl group in position 2,3 or 4 (i.e. in the same ring) interferes with this activity whereas a second hydroxyl group at position 5,6 or 7 does not. Interestingly, all the tested naphtoquinones (1,2-, and 1,4-naphthoquinones) and hydroxyl naphthoquinones (5-hydroxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone), promoted copper-induced peroxidation in PS-containing liposomes (not shown). Thus, all these naphthoquinones are not likely to participate in the mechanism of "super-activity".

3.4. Dependence of the observed "super-activity" on the inducer of peroxidation and its concentration

First, we note that unlike the influence of PS on the inhibition of copper-induced peroxidation by "super antioxidants", PS does not enhance the antioxidative potency of antioxidants against AAPH-induced peroxidation. In fact, both externally added and co-sonicated Toc slightly inhibited the AAPH-induced peroxidation of POPS-containing PLPC liposomes (Bittner et al., 2002). Similarly, no "super-activity" was observed with either Trolox, BHT, curcumin, luteolin, or 1,5-DHN against AAPH-induced peroxidation (results not shown).

Next, we tested whether phospholipids other than PS enhance the antioxidative potency of the studied antioxidants. Similar to POPS, POPE also contains a pri-

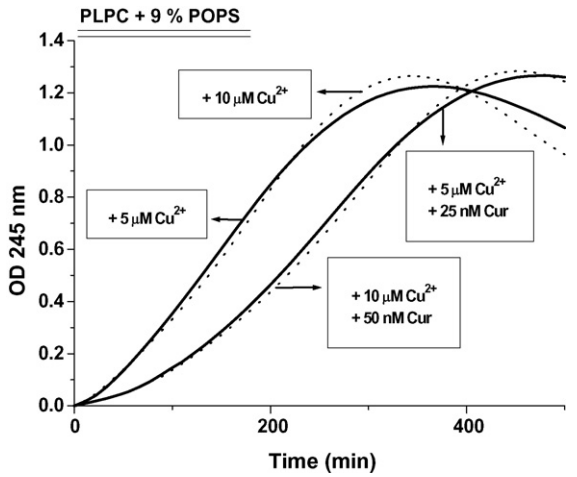


Fig. 4. Dependence of the effect of "super-active" antioxidants on the copper concentration. Peroxidation of PLPC liposomes (250 μM) containing 9% POPS was induced by either 5 μM $CuCl_2$ (solid lines) in the absence or presence of 25 nM curcumin, or 10 μM $CuCl_2$ (dotted lines) in the absence or presence of 50 nM curcumin, as indicated. The absorbance at 245 nm was recorded at 37 °C. The reaction mixtures contained 1 μM EDTA.

mary amine group. Similar to POPS, POPA is negatively charged. Yet, in our previous study (Gal et al., 2003) we found that tocopherol is "super-active" in PS containing liposomes but not in liposomes containing either POPA or a mixture of POPA and POPE. Likewise, Trolox, curcumin, BHT, luteolin and T-414 at a concentration of 100 nM did not affect the copper-induced peroxidation in the presence of POPE (results not shown).

In relating to the mechanism responsible for the"super-antioxidative" effect, it is important to note that the observed "super activity" against copper-induced peroxidation in PS-containing liposomes depends on the copper concentration, as shown in Fig. 4. In fact, increasing the copper concentration from 5 to 10 μM did not influence the peroxidation profile substantially (Fig. 4), in accordance with our previous findings (Gal et al., 2003). However, upon increasing the concentration of copper, "super-active" antioxidants became less potent. As an example, the inhibitory effect of 50 nM curcumin (Cur) against peroxidation induced by 10 μM copper was identical to that of 25 nM curcumin against peroxidation induced by 5 μM copper (Fig. 4). A similar trend was observed with Toc, as well as with 1,5- and 1,7-DHNs (results not shown).

4. Discussion

As stated above, the present investigation aimed at gaining understanding of the main finding of our previous study, namely that in POPS-containing PLPC liposomes tocopherol inhibits peroxidation at nanomolar concentrations (Gal et al., 2003). Towards this end, we have investigated the generality of this phenomenon by studying the dose-dependent effects of 37 antioxidants on the kinetics of peroxidation, induced either by copper ions or by AAPH, either in liposomes made of PLPC or in liposomes made of PLPC with POPS or POPE.

Out of the 37 tested compounds (including tocopherol) only 12 exhibited marked antioxidative potency against copper-induced peroxidation of PS-containing liposomes. Prior to relating to the common attributes of these 12 "super-active" antioxidants, we will discuss in general terms the possibility of "sub-stoichiometric" activity of antioxidants. In this discussion, we hypothesize that the mechanism responsible for the activity of Toc and other "super-active" antioxidants involves regeneration of these antioxidants. We propose a specific mechanism of regeneration, via a stabilized semiquinone-type radical formed upon oxidation of the antioxidant. We also present a scheme that explains the apparent extraordinary antioxidative potency of these antioxidants in PS-containing liposomes.

4.1. The mode of action of antioxidants

As described in Fig. 2, when PS-containing liposomes contained Toc, its level, as monitored fluorometrically, did not decrease substantially upon exposure of the liposomes to copper ions for more than 23 h. At the same time, no hydroperoxide accumulation was recorded (Fig. 2). In other words, tocopherol inhibited peroxidation without being consumed. To explain this finding, we first consider the various mechanisms by which antioxidants can inhibit copper-induced peroxidation (Pinchuk and Lichtenberg, 2002):

i. *Inhibition of free radical production*: either by reduction of the binding of copper to the liposomes or by complexation of the bound copper, rendering the copper redox inactive. This possibility can be ruled out on the basis of stoichiometric considerations. Specifically, in most of our experiments, the model system contained 25 μM PS and 5 μM copper, most of which is probably bound to PS (Gal et al., 2003). Nanomolar concentrations of antioxidants are not likely to affect this binding.
ii. *Inhibition of the propagation through non-radical decomposition of hydroperoxides*: This possibility can also be refuted on the basis of stochiometric considerations because the nanomolar concentrations of the "super-active" antioxidants are insufficient to

vary the accumulation of hydroperoxides via non-radical reactions in a system containing much higher concentrations of hydroperoxides.

iii. *Alteration of the physical properties of the liposomal bilayers to the extent that propagation is inhibited*: Under certain conditions even low concentrations of "super-active" antioxidants may induce physical changes to the liposomes, such as lateral phase separation (Sanchez-Migallon et al., 1996; Wang and Quinn, 1999), and by that inhibit peroxidation. However, the possibility that such an effect is responsible for the "super-activity" can be ruled out because structurally similar but redox-inactive compounds (e.g. tocopherol acetate) did not affect the peroxidation at the relevant concentrations (not shown).

iv. *Quenching of free radicals*: Phenolic antioxidants inhibit peroxidation of PUFA via the following sequence of steps:

- quenching of a radical ($X^\bullet$) by a phenolic antioxidant (AH)

$$AH + X^\bullet \rightarrow XH + A^\bullet \quad \text{(fast reaction)} \quad (R1)$$

- quenching of a second radical by the antioxidant-derived radical ($A^\bullet$)

$$A^\bullet + X^\bullet \rightarrow AX \quad \text{(very fast reaction)} \quad (R2)$$

- Through this series of two reactions ((R1) and (R2)) each molecule of antioxidant quenchs two $X^\bullet$ radicals other reactions may reduce this potency. These include biradical quenching of $A^\bullet$ (R3)

$$A^\bullet + A^\bullet \rightarrow AA \quad (R3)$$

- It also includes the possible propagation of peroxidation of lipids (LH) mediated by $A^\bullet$ ((R4); Frankel, 1998, pp. 129–135):

$$A^\bullet + LH + O_2 \rightarrow LO_2^\bullet + AH \quad (R4)$$

The well-established theory of inhibition via reactions (R1)–(R3) (Frankel, 1998, pp. 129–135) gives a quantitative estimate for the prolongation of the lag, i.e. for the time (t) of consumption of the antioxidant (Frankel, 1998, pp. 13–21):

$$t = \frac{n[AH]}{R_i} \quad (1)$$

where [AH] represents the concentration of the antioxidant, R_i is the rate of production of free radicals (initiation rate) and n is a coefficient (usually in the range 1–2 for compounds containing one phenolic group) that depends on the ratio of the rate constants of reactions (R2) and (R3) (Denisov and Denisova, 2000, p. 207).

Eq. (1) implies that the effect of an antioxidant on the lag preceding peroxidation of liposomal lipids should depend only on the concentration of the antioxidant and on the rate of production of free radicals (R_i). We do not have a numerical estimate of the relevant R_i values, yet in our previous studies we found that the maximal rate of uninhibited peroxidation of liposomal PLPC, in liposomes containing POPA is similar to the maximal rate observed in POPS-containing liposomes. This means that the rate of production of free radicals in these two systems is similar (Frankel, 1998, p.17) and we could have therefore expected that for a given concentration of antioxidant(s), the lag time preceding peroxidation in the latter two systems will be of the same order of magnitude.

Our experimental results are inconsistent with this expectation. Specifically, for the given antioxidant, the lag depends critically on the presence of PS in the liposomes and in PS-containing liposomes, the lag depends critically on the nature of the antioxidant.

Hence, the possibility that the "super-activity" is due to the "conventional" quenching mechanisms, can be ruled out. We think that the most likely explanation of "super-activity" is that in PS-containing liposomes, the "super-active" antioxidants are regenerated, as discussed below.

4.2. Attributes of the "super-active" antioxidants

In an attempt to understand the latter mechanism, we searched for the specific chemical moiety associated with this phenomenon. Towards this end, we have studied 37 compounds of different groups of antioxidants and found that 12 of them exhibited antioxidative potency at nanomolar concentrations in PS-containing liposomes and that minor structural modifications eliminate this activity.

Accordingly, we have compared the physicochemical properties of the various antioxidants, including the acid–base dissociation constants, pK_a, the partition coefficients of the antioxidants between octanol and water at pH 7 ($\log D$), and the electrochemical potentials of the compounds. None of these properties differentiates the 12 "super-active" compounds from the other studied compounds.

Notably, much data are available in the literature for the various flavonoids. We used this data to search for what distinguishes luteolin and T-414 from the other flavonoids. No substantial difference was found either in the redox potential (Hodnick et al., 1998), or in the Fe(III)-reducing capabilities (Sugihara et al., 2003), or in the Cu(II)-reducing capabilities (Mira et al., 2002), or in

the copper-chelating properties (Brown et al., 1998) or in the free radical scavenging reactivity (Rice-Evans et al., 1996; Pannala et al., 2001; McPhail et al., 2003; Butkovic et al., 2004), or in the calculated bond dissociation energies (BDE; Leopoldini et al., 2004), or in the calculated stabilities of the radicals derived from flavonoids (van Acker et al., 1996; Vaya et al., 2003). Hence, we had to look for another property that will explain the correlation between the structure of the different antioxidants and their activity.

4.3. Specific structure–activity relationship

Irrespective of the (presumable replenishment) mechanism, we note that unlike the less active antioxidants, the 12 "super-active" compounds possess the ability to form quinones, whereas the other antioxidants do not. It should, however, be clarified that the quinones formed from these antioxidants are not the chemical moiety that directly undergo recycling, as evident from the finding that the studied quinones (1,2-, 1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone) were not "super-active" (see Section 3). We propose that the "super-active" antioxidants or their reaction intermediate products are hydroquinones, which upon oxidation form relatively stable semiquinone-like radicals and that such radicals can interact with PS and copper to reform the active antioxidant.

Another striking common characteristic of part of the "super-active" antioxidants is the high symmetry of their structures (e.g. curcumin, BHT dimers, NDGA and DES), which may be responsible for the stabilization of their intermediate structures, including free radicals. In the following discussion we present evidence for the proposed mechanism of the "super antioxidative effect" described above. Specifically, we show that in each of the groups of the studied antioxidants, only those compounds that can form semiquinone-like radicals are "super-active".

4.3.1. Toc and Trolox

Tocopheryl quinone is a well-known metabolite of tocopherol (Frankel, 1998; pp. 139–147). Its presence has been demonstrated in human plasma under physiological conditions (Mottier et al., 2002) and its conversion back to tocopherol has been demonstrated in vivo (Moore and Ingold, 1997). The antioxidative action of the reduced form of the quinone, i.e. of tocopheryl hydroquinone, has been studied extensively (Neuzil et al., 1997; Shi et al., 1999; Gille et al., 2001). The analogous quinone of Trolox is formed in the two electron oxidation reactions of Trolox both by peroxynitrite (Hogg et al., 1994; Priyadarsini et al., 2001) and by lipoxygenase (Delicado et al., 1997).

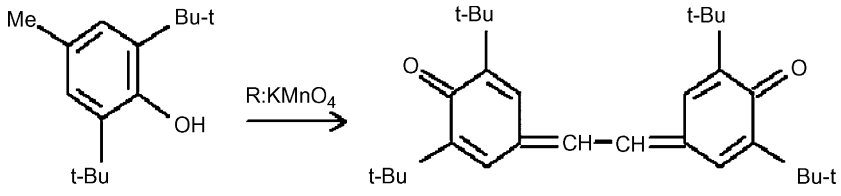
4.3.2. Butylated hydroxytoluene, propyl gallate and methyl syringate

Amongst these synthetic antioxidants, BHT is the only one with known quinone or quinone-methide chemistry (Lewis et al., 1996; Rabin et al., 1998; Oikawa et al., 1998; Reed et al., 2001; Thompson et al., 2001; Kupfer et al., 2002).

Both the "super-activity" of the quinone-forming BHT ($C_{2t} = 33$ nM) and the lack of such activity of PG and methyl syringate accord with our hypothesis that the "super-activity" of antioxidants is due to regeneration of semiquinone radicals. Interestingly, several studies reported on a dimeric structure of the BHT quinone-methide (Scheme 2; Benjamin et al., 1978; Henderson et al., 1999). The structure of this proposed dimeric metabolite of BHT closely resembles the structure of the diethylstilbestrol quinone (see below). It is possible that radicals derived from this dimer are the actual active structures that undergo recycling.

4.3.3. Curcumin and rosmarinic acid

Curcumin is a "super-active" antioxidant, showing "sub-stoichiometric" antioxidative effects in the presence of PS. Several studies have shown that under certain conditions curcumin may form complex quinone-methide dimers (Masuda et al., 1999, 2002; Schaich et al., 1994). We hypothesize that such complex semiquinone-like structures are responsible for the "super-activity" of curcumin. This hypothesis is strengthened by the results of a recent publication that showed that a synthetic Cu(II)–curcumin complex can be fully regenerated under different experimental con-



Scheme 2. A possible oxidation pathway of BHT by potassium permanganate (Benjamin et al., 1978).

ditions (Barik et al., 2005). The proposed mechanism included mainly the reversible redox reactions within the Cu^{II}/Cu^{I} couple in the complex. Alternatively, radicals could cause oxidation of the phenolic moiety, producing phenoxyl radicals, which reacted with reduced copper ions of the complex and caused regeneration of the complex.

In our experiments, Rosmarinic acid acted as a pro-oxidant. Theoretically, rosmarinic acid can form *ortho*-quinones, although, investigation of this possibility yielded conflicting results (Johnson et al., 2001; Nakazawa and Ohsawa, 1998). We do not fully understand why in our model system rosmarinic acid does not act as a "super-active" antioxidant. One possibility that will have to be further investigated is that the lack of activity is due to the relatively low yield of the *ortho*-quinone formed upon oxidation of rosmarinic acid.

4.3.4. Nordihydroguaiaretic acid (NDGA)

The di-catechol structure of NDGA is known to produce reactive *ortho*-quinones (Gati et al., 1990; Koob and Hernandez, 2002; Koob, 2002), which may explain its potency as a "super-active" antioxidant.

4.3.5. Resveratrol and diethylstilbestrol

In our studies, DES is a "super-active" antioxidant in PS containing liposomes, whereas the structurally related Resveratrol in fact promoted lipid peroxidation (Table 1). We think that these results are consistent with our hypothesis that quinone-like intermediates are required for "super-activity" of antioxidants. Specifically, it is known that oxidation of DES yields 4′,4″-diethylstilbestrol quinone (DES-quinone), through an intermediate DES semiquinone structure (Scheme 3). Both these reactions are reversible, unlike the subsequent spontaneous rearrangements of the DES-quinone into (*Z*,*Z*)-diensterol (*Z*,*Z*-DIES; Scheme 3). Reduction of DES-quinone back to the hydroquinone form has been demonstrated (Liehr et al., 1983), and the redox cycle DES ⇔ DES-quinone has been implicated in the inactivation of hydroperoxides (Roy et al., 1992).

In our system, DES may be oxidized by free radicals, and the resultant semiquinone may then be reduced back to DES, which can react with another free radical, as described below. No such intermediates are known for Resveratrol.

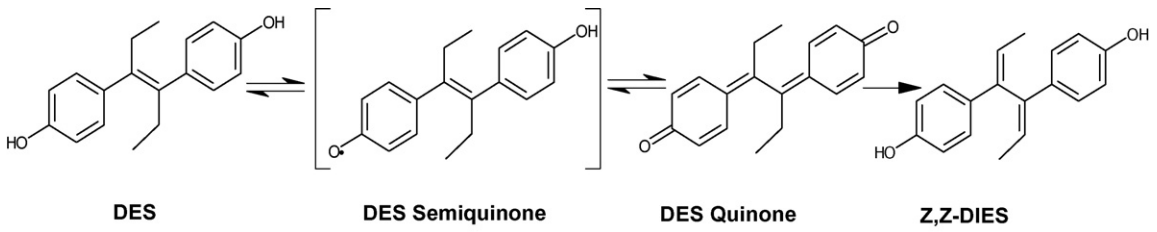
4.3.6. Flavonoids

Out of the 12 flavonoids that were screened in this study, only luteolin and T-414 were found to be "super-active" antioxidants in PS-containing liposomes. In pure PLPC liposomes, these compounds were much less efficient antioxidants (Table 1).

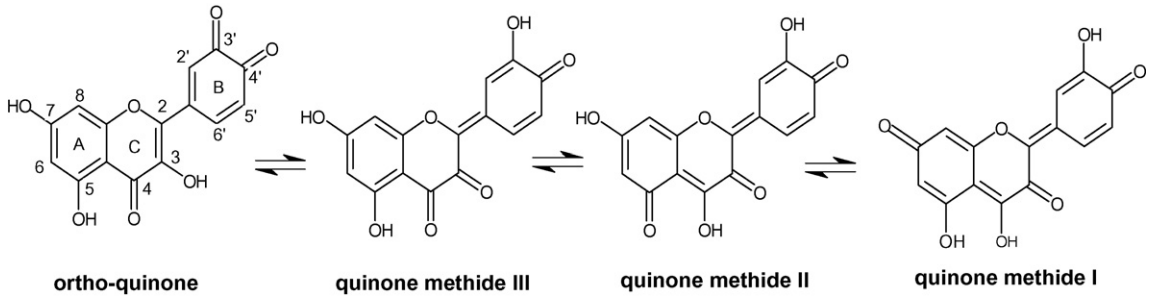
Analysis of the organic chemistry of these compounds revealed what differentiates T-414 and luteolin from the other studied flavonoids is that they can form restricted *ortho*-quinones (Awad et al., 2001; Galati et al., 2001). Formation of such *ortho*-quinone requires the 3′,4′ hydroxyl groups on the B ring as in T-414, luteolin quercetin, catechin and taxifolin (Scheme 1). However, an OH group in position 3 of the C-ring (as indicated in Fig. 4 for quercetin) may result in further tautomerization of the *ortho*-quinone to quinone-methides (Scheme 4; Awad et al., 2001; Galati et al., 2001), which may explain the lack of "super-activity" of both quercetin and catechin (Awad et al., 2001). The relatively low activity of both catechin and taxifolin may be attributed to the lack of extended conjugation, due to saturation of the C2–C3 bond (Pannala et al., 2001).

The activity of T-414 also indicates that the 5-OH group on the A ring is not essential for the "super-activity". We attribute the "super-activity" to replenishment of "semiquinone-like" radicals, and not of quinones (see below). As previously proposed by Pannala et al. (2001) and by McPhail et al. (2003), semiquinone radicals, are stabilized by the quinoid structure, which results in modification of the antioxidative activity of flavonoids (Pannala et al., 2001; McPhail et al., 2003).

Further support for the enhanced stability of the radicals derived from luteolin can be found in a study by Bors and Saran (1987), who found that radicals derived



Scheme 3. Oxidation of DES to DES-quinone and its rearrangement to *Z*,*Z*-DIES (Liehr et al., 1983).



Scheme 4. Quinone–quinone-methide isomerization of quercetin (Awad et al., 2001).

from luteolin and taxifolin decay considerably slower than the radicals derived from other flavonoids. Nonetheless, the relevance of this finding to our observation is not clear because of two reasons. First, the latter measurements were conducted at pH 11.5, and secondly, in our study taxifolin was not a "super-active" antioxidant. Further investigations are therefore required to clarify the interrelationship between "super-activity" and radical stability.

4.3.7. 1-Naphthol, 2-naphthol and dihydroxynaphtalenes

The physical properties of the two studied naphthols (1- and 2-naphthol) are similar; they have identical partition coefficients and similar pK_a values. Yet, 1-naphthol is a "super-active" antioxidant, whereas 2-naphthol is not. We attribute the large difference of the antioxidative potencies of the two naphthols to the higher stability of the radicals derived from 1-naphthol (but not 2-naphthol), as hypothesized for 1,5-DHN (Feiser, 1930). This interpretation is consistent with the results obtained more recently, which have shown that 1-naphthol has a much higher tendency to form *ortho*- and *para*-quinones than 2-naphthol (Karthikeyan and Chorover, 2000; Preuss et al., 2003). We speculate that these semiquinone-like radicals can undergo redox cycling in the presence of PS and by that inhibit peroxidation at nanomolar concentrations.

The antioxidative potency of the dihydroxynaphthalenes observed in PS-containing liposomes depends on their pattern of substitution. In general, DHNs are "super-active" antioxidants only if they contain at least one hydroxyl in the 1-position and another hydroxyl in the other aromatic ring. This is the case for 1,5-, 1,6-, and 1,7-DHNs. An additional hydroxyl substituent on the same ring of the active 1-naphthol interferes with its activity. Compounds with no hydroxyl group in position-1 are not "super-active" antioxidants.

It is of interest to note that the order of reactivity found in the present work is similar to that observed for the relative rates of reaction of the various DHNs with singlet oxygen (Croux et al., 1990; Luiz et al., 1996). Notably, in their study, Croux et al. (1990) concluded that DHNs with an OH group in position 1 react relatively fast with singlet oxygen and produce naphthoquinones, whereas DHNs without an OH group in position 1 react slowly with singlet oxygen and do not produce naphthoquinones (Croux et al., 1990; Luiz et al., 1996; Amat-Guerri et al., 1998).

An explanation for the relative activity of the various DHNs can be based on the "critical oxidation potentials" defined by Feiser (1930). The ability of a dihydroxyl derivative of aromatic hydrocarbons to yield a quinone appears to be reflected in its "critical oxidation potential" (Feiser, 1930). In this context, it was argued that the lower "critical oxidation potential" of 1,5-DHN, relative to its methoxy derivative, is indicative of the formation of a quinone. The possibility of quinone formation was ruled out for 2,3- and 2,7-DHNs on the basis of similar rationale (Feiser, 1930). An apparent discrepancy exists between the relatively low "critical oxidation potential" for 2,6-DHN, as reported by Feiser (1930) and our results, which showed that this compound is not a "super-active antioxidant". This discrepancy may result from the formation of a highly unstable amphi-naphthoquinone (Feiser, 1930), which may be too unstable to undergo recycling in our model system (see below).

Both 1,2-, and 1,4-DHNs promoted the copper-induced peroxidation of PLPC either in the absence or in the presence of PS in the liposomes. The oxidation potentials of these two compounds are considerably lower than those of the other DHNs (Fueno et al., 1959), possibly because the 1,2- and 1,4-DHNs can form extremely stable semiquinone radicals. These radicals may initiate lipid peroxidation chain reactions, either directly or through their reactions with copper. Further research is required to elucidate this possibility.

4.4. Mechanistic aspects

In this study we have demonstrated potentiation of the antioxidative effects of several antioxidants by PS. Such potentiation of the effects of antioxidants by various phospholipids has been previously shown in autoxidation processes in both bulk oil and organic solutions. In spite of the similarity between these effects, they are distinctly different in several respects, so that different mechanisms may be responsible for the observation in the two systems. First, in bulk oil or in organic solutions, such effects were observed for phosphatidylethanolamine (Weng and Gordon, 1993; Ohshima et al., 1993; Dziedzic et al., 1986; Bandarra et al., 1999; Lambelet et al., 1994; Dziedzic and Hudson, 1983; Hudson and Lewis, 1983), phosphatidylserine (Lambelet et al., 1994; Alam et al., 1997; Saadan et al., 1998) and phosphatidylcholine (Koga and Terao, 1995; Bandarra et al., 1999; Hudson and Lewis, 1983). By contrast, in our model system such effects were only observed in the presence of PS. Secondly, several antioxidants, including propyl gallate (Dziedzic et al., 1986) and flavonoids (Hudson and Lewis, 1983) that showed synergism in bulk oil were not potentiated by PS in our model system. Third, in the presence of PE, tocopherylquinone was antioxidative (Weng and Gordon, 1993), whereas in our model system quinones had no such effect. The mechanistic implications of these differences require further investigation.

As discussed above, the "super-active" antioxidants studied in this work probably exert "sub-stoichiometric" inhibition of the free radical chain reactions involved in lipid peroxidation. This phenomenon requires reformation of the antioxidants from their corresponding radicals. We attribute the "super-activity" to the replenishment of these antioxidants via the formation of a ternary complex of $Cu(PS)_2$ with the specific "super-active" antioxidants. Several lines of indirect evidence support this hypothesis. First, no "sub-stoichiometric" effects were observed for the antioxidants in AAPH-induced peroxidation (results not shown). Secondly, "super-activity" was observed only with specific phenolic antioxidants and only when the liposomes included POPS, and not POPE (results not shown) or POPA (Gal et al., 2003). This indicates that the primary amine group by itself is not sufficient to enhance the antioxidative potency of the "super-active" antioxidants.

Based on the dependence of the antioxidative potency on the copper concentration, we propose (see below) that the alleged relevant ternary complex has the stoichiometry of PS:copper 2:1. The potency of the various antioxidants, as expressed in terms of the dose required for doubling t_{max} (C_{2t}), depends on the concentration of copper as follows. Increasing the copper concentration within the range of 5–10 μM had little effect, if any, on the kinetic profile of peroxidation of liposomes made of PLPC and 9% POPS (Fig. 4 and Gal et al., 2003), probably because the rate of initiation of free radicals remained about constant. Nonetheless, in the presence of 10 μM copper, the antioxidants appeared to be less active than at 5 μM copper (Fig. 4). We think that these results relate to a change in the binding characteristics of copper to PS, which can change from a 1:2 (copper/PS) complex (Shirane et al., 1984) to a possible 1:1 complex (Gal et al., 2003). Our interpretation of these results is that $Cu(PS)_2$ is capable of replenishing the "super-active" antioxidants, whereas the Cu(PS) complex is not. At relatively low copper/PS ratios the $Cu(PS)_2$ is likely to be predominant, whereas at higher copper concentrations, more Cu(PS) exists, on the expense of the $Cu(PS)_2$. According to our interpretation, this can be expected to result in lower recycling capability, if any. Hence, higher concentrations of the "super-active" antioxidants are required to obtain similar antioxidative effect.

As discussed above, the structure–activity relationship of the 37 studied antioxidants indicates that formation of a relatively stable semiquinone-like radical is a prerequisite for "super-activity". Accordingly, we explain the "sub-stoichiometric" inhibition in terms of the following sequence of reactions.

(i) Binding of the phenolic antioxidant QH_n (where n is the number of phenolic groups, coordinated by copper) to a $Cu(PS)_2$ complex on the surface of the liposome and subsequent ionization of the antioxidant:

$$QH_n + Cu^{II}(PS)_2 \rightarrow (Q^{n-})Cu^{II}(PS)_2 + nH^+ \quad \text{(R5)}$$

(ii) When a phenolic antioxidant binds to a $Cu(PS)_2$ complex, it may form a relatively stable semiquinone-like radical, stabilized either by delocalization of charge within the complex or even by redox isomerism (valence tautomerism) of the type described for several copper–catechol complexes (reviewed in Pierpont, 2001; Kaim et al., 2002):

$$(Q^{n-})Cu^{II}(PS)_2 \rightleftharpoons (Q^{\bullet 1-n})Cu^{I}(PS)_2 \quad \text{(R6)}$$

(iii) Once peroxyradicals $X^\bullet$ are produced (either directly via copper-catalyzed decomposition of preformed hydroperoxides or via sequence of reactions, including oxidation of phenolic species by

Cu^{II} and reaction (R4)), both these isomeric forms may undergo a rapid interaction with free radicals, similar to the proposed sequential proton loss electron transfer (SPLET) mechanism (Foti et al., 2004; Litwinienko and Ingold, 2003, 2004). This results in quenching of an external radical $X^{\bullet}$:

$$X^{\bullet} + (Q^{n-})Cu^{II}(PS)_2 \rightarrow X^{-} + (Q^{\bullet 1-n})Cu^{II}(PS)_2 \quad \text{(R7)}$$

$$X^{\bullet} + (Q^{\bullet 1-n})Cu^{I}(PS)_2 \rightarrow X^{-} + (Q^{\bullet 1-n})Cu^{II}(PS)_2 \quad \text{(R8)}$$

(iv) The resultant radical complex, $(Q^{1-n\bullet})Cu^{II}(PS)_2$, is not stabilized by redox isomerization. As a consequence, it tends to be reduced back to the form stabilized by the redox isomerization (reaction (R6)), which is equivalent to reduction of Cu^{II} to Cu^{I}.

We have no conclusive evidence for reduction of such a complex, nor do we know which reductant is responsible for such recovery of the antioxidant. A possible reduction mechanism involves abstraction of hydrogen from radical $X^{\bullet}$ by the semiquinone-like radical, with subsequent rearrangement, that stabilizes hydrogen-depleted X^{-H} as described for "cyclic inhibition" mechanisms (Denisov and Denisova, 2000, pp. 207–220).

However, in our view, the most straightforward mechanism for the reduction involves abstraction of hydrogen from the amino group of the serine headgroup of PS, available in large excess to the "super-active" antioxidant (25 μM PS versus up to 150 nM antioxidant).

$$(Q^{\bullet 1-n})Cu^{II}(NH_3{}^{+}\text{–PS})_2 \rightarrow (Q^{\bullet 1-n})Cu^{I}(NH_2{}^{\bullet}\text{–PS})(NH_3{}^{+}\text{–PS}) + H^{+} \quad \text{(R9)}$$

In other words, the PS headgroups are "sacrificed" to replenish the phenolic antioxidants. A similar reaction has been described by Lambelet et al. (1994).

This mechanism implies that the apparent "substoichiometric" activity of phenolic antioxidants is due to stoichiometric oxidation of the serine headgroups of PS. This should result in a decrease of the concentration of PS. Our evaluation, based on tocopherol sparing, have shown that the expected loss of PS-groups constitutes merely 2% of total PS content (about 0.5 μM). However, we have no quantitative evidence for a decrease of the PS-concentration, probably because the TNBS assay (Barenholz et al., 1977), used in our studies, was not sufficiently accurate to discover the consumption of a small fraction of PS (results not shown). Further work will have to be conducted to evaluate these and/or other possibilities.

The physiological significance of our findings is not clear at the present time. Phosphatidylserine (PS) is an aminophospholipid found mainly in the inner leaflet of plasma membranes (reviewed in Balasubramanian and Schroit, 2003). It plays a central role in apoptosis and in cell signaling (Mozzi et al., 2003). The content of PS in plasma membranes varies considerably (e.g. in the brain, it varies between 2.1% in neuroblatoma cells and 11.1% in glial cells; Mozzi et al., 2003). In LDL particles, the PS constitutes only 0.36% of the total phospholipid content (Deguchi et al., 2000).

In summary, we found that the antioxidative potency of specific compounds against copper-induced peroxidation of liposomal PLPC is markedly increased by liposomal PS. We attribute this behavior to reformation of the antioxidants by the phosphatidylserine headgroup via a redox-active semiquinone-type radical. The possibility that PS can act as a reducing agent in vivo and by that influence the oxidant/antioxidant status of biological systems containing "super-active" antioxidants requires further investigation.

Acknowledgments

We acknowledge the Lady Davis chair of Biochemistry and Israel Science Foundation for financial support (research grant 362/02-18.1) and Prof. Vaya of the Migal Galilee Technology Center for helpful discussions and for his gift of 11 different flavonoids (products of ICC).

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