Biomimetic chemistry on the protection of *cis* phospholipid from the thiyl radical isomerization by common antioxidants

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Dedicated to Prof. Michael Orfanopoulos on the occasion of his 67th birthday and retirement, and for being a splendid teacher, scientist and friend

DOI: http://dx.doi.org/10.3998/ark.5550190.p008.984

Abstract
In this paper the evaluation of *cis* phospholipids geometry protection from the thiyl radical isomerization in the presence of common antioxidants is studied. Thus, large unilamellar vesicles (LUVs) of glycerophospholipids containing oleate moieties were used as biomimetic model to demonstrate that hydrophilic ascorbic acid and urate, and hydrophobic all-*trans* retinol acetate and α-tocopherol, are able to inhibit *cis*-*trans* isomerization from the thiyl radicals that are known to react as efficient isomerizing species. The synergistic effect obtained by a mix of the hydrophilic and hydrophobic antioxidants at *in vivo* concentrations is also proved to exert an extraordinary protection for the integrity of *cis* lipid geometry.

Keywords: Biomimetic chemistry, lipids isomerization, thiyl radicals, antioxidants

Introduction

Lipids are a group of molecules with a wide structural diversity, classified together for their insolubility in water.[1] The primary building blocks of most cell membranes are glycerol-phosphate-containing lipids, generally referred to as phospholipids. Naturally occurring unsaturated lipids, e.g. mono- and polyunsaturated fatty acids (MUFA and PUFA), are known to have double bonds in the *cis* configuration. The isomerization of the *cis* double bond present in MUFA and PUFA residues of membrane phospholipids to the corresponding thermodynamically more stable *trans* isomers is one of the lipid structural changes that has attracted the interest of

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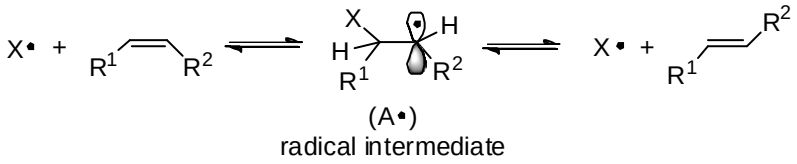
diverse research areas in the last decade. Geometric isomerism has become a topic of research involving several disciplines, such as microbiology, chemistry, biochemistry, pharmacology, nutrition, and medicine.[2-6]

Some *trans* fatty acid residues found in living organisms can only be formed through an endogenous transformation of the natural occurring *cis* conformation, and their presence has been correlated with radical stress under physiological and pathological processes.[7-9] Several free radicals, including the biologically relevant thiyl radicals (RS•)[10] and nitrogen dioxide (•NO_2),[8] are known to isomerize double bonds. Scheme 1 shows the reaction mechanism, involving reversible addition of a radical X• to the double bond to form a radical adduct (A•). The reconstitution of the double bond is achieved by β-elimination of X• which favours *trans* geometry.[5,6,11]



Scheme 1 Reaction mechanism for the *cis-trans* isomerization catalyzed by X• radical.

Based on biomimetic models, mostly using 2-mercaptoethanol (MSH) as the thiol, the isomerization has been correlated to the formation of diffusible thiyl radical (RS•), which are able to move from the aqueous compartment and reach the hydrophobic lipid bilayer.[12-15] Recently, it has been shown that biologically relevant thiols (such as glutathione) are able to induce c*is-trans* isomerization of the double bonds in unsaturated fatty acids.[12,13,16,17] Among biological relevant sulfur species, hydrogen sulfide (H_2S) is a natural occurring gas with interesting roles in nervous and cardiovascular systems and in pathological conditions such as inflammation. Chemically, the reactions of the simplest S-centered radicals formed from H_2S were studied using liposomes as biomimetic model. Indeed, the use of vesicle suspension demonstrates the potential of sulfhydryl radicals (HS•/$S^{\bullet-}$) derived from H_2S to reach the hydrophobic compartment of fatty acid and attack double bonds, thus producing phospholipids containing *trans*-fatty acid residues.[18] In humans, the relevance of lipid *cis-trans* isomerization is still to be clarified, since *trans*-fatty acids were considered only to derive from exogenous sources. In fact, *trans*-fatty acids can be taken from the diet, particularly through food like meat, milk, and partially hydrogenated fats and oils.[2,10,19] Based on the efficiency of the thiyl radical-catalyzed *cis-trans* isomerization, it was demonstrated that *trans*-phospholipids could be also formed *in vivo* under cellular stress.[12-14] The protection of the *cis* geometry is crucial, owing to the importance of keeping favourable properties of cell membranes deriving from the fluidity contribution by the bent unsaturated lipid structures.[10,11]

Using biomimetic models of *cis-trans* lipid isomerization,[5,6,10] we were interested in the protection of *cis* lipid geometry from thiyl radical attack by common antioxidants. Thus,

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dioleoylphosphatidylcholine (DOPC) vesicles were prepared, while α-tocopherol (ArOH), all-*trans*-retinol acetate (retinol), ascorbic acid 6-*O*-palmitate (AAP), ascorbic acid (AH_2) and urate (UH_3) were used as thiyl radical scavengers (Figure 1). For this reason, two different methods of liposomes (LUVs) preparation were used; methanolic injection as the method of choice to incorporate defined amounts of lipophilic antioxidants (AAP, ArOH and retinol) into the liposomes, whereas with hydrophilic antioxidants (AH_2 and UH_3) both extrusion and methanolic injection methodology can be used. We observed that the ability to scavenge thiyl radicals generated by γ-radiolysis in aqueous vesicle suspensions increases along the series ArOH < retinol < UH_3 < AH_2, a different order to that observed in organic solutions or using multilamellar vesicles (MLVs) described in previous studies.[20-22] The proposed anti-isomerizing activity is based not only on the relative chemical reactivity of the antioxidants, but also on their partition coefficient, *i.e.*, the localization of the free radical scavengers in the aqueous or lipid compartment.

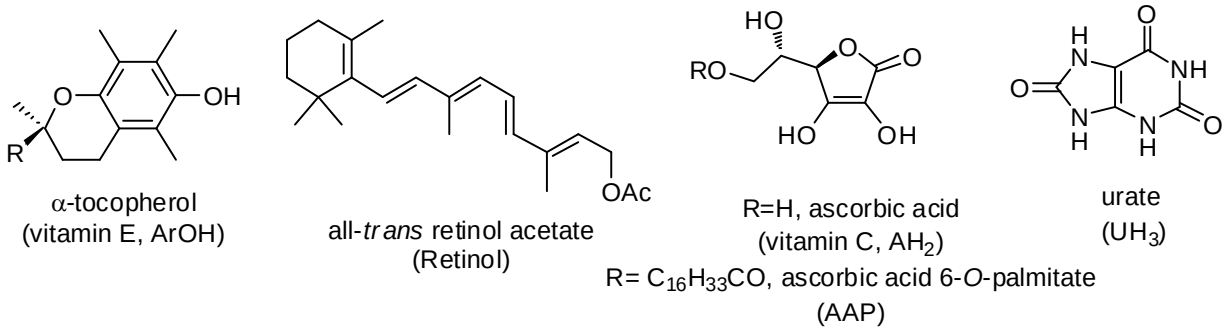

Figure 1. Hydrophobic and hydrophilic antioxidants.

Results and Discussion

The chemical process of thiyl radical-catalyzed *trans* formation of biologically relevant molecules has increasingly attracted the interest of several scientific groups in the last years. A variety of radical scavengers have been proposed to inhibit most of the hurtful free radicals. For example tocopherol, carotenes, retinoids, urate and ascorbic acid have been studied as scavenger of the lipid peroxidation process.[23-26] However, most antioxidants are known to react with thiyl radicals and generate the corresponding thiol in organic or aqueous solutions.[27-30] In a previous study, using MLVs as model moieties in which the antioxidants were incorporated, the inhibition of the isomerization process attributed to antioxidants increased in the order of α-tocopherol < ascorbate < all-*trans*-retinol acetate. In those experiments the same amount of the antioxidants was used and the thiol/antioxidant molar ratio was always 150:1.[21] Herein, the *cis-trans* isomerization of fatty acid residues in LUVs by thiyl radicals in the absence and presence of various antioxidants has been extensively studied and the data have been rationalized based on kinetic data available in the literature from pulse radiolysis.[20-22] Using the methanolic injection

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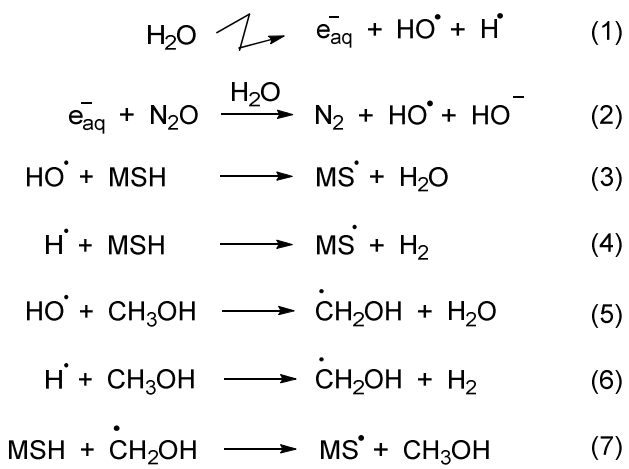
method, it is possible to affect the liposome size[31] and in our experiments the preparation of vesicles was performed to reproduce the same size of vesicles obtained by the extrusion technique, ascertained by Dynamic Light Scattering (DLS) measurements.[32] It was gratifying to find that similar dose profiles of the *cis–trans* isomerization by $HOCH_2CH_2S\bullet$ radicals (MS•) were observed for extruded DOPC vesicle suspensions added with 1.2 M MeOH and vesicle suspensions obtained by an appropriate methanolic DOPC injection in water (dashed line in Figure 2). The methanolic injection was used as the method of choice to incorporate defined amounts of lipophilic antioxidants in the liposomes. With hydrophilic antioxidants, both techniques can be used since the antioxidant is added after liposome formation, and gave similar results (see Experimental Section and Supporting Information).

The use of an amphiphilic thiol, such as 2-mercaptoethanol (MSH), eliminated the concern about the partition of this substrate between hydrophobic and hydrophilic regions. A 2.2 mM DOPC vesicle suspension in phosphate buffer (pH 7) containing 1 mM $HOCH_2CH_2SH$ (MSH) in the presence or absence of an antioxidant was saturated with N_2O prior to γ-radiolysis. Radiolysis of neutral water led to the transient species shown in eq 1.[33] The presence of N_2O efficiently transformed hydrated electrons into the HO• radical (eq 2, k_2 9.1×10^9 $M^{-1}s^{-1}$). Hydrogen abstraction from 2-mercaptoethanol by HO• and H• directly produced thiyl radicals (eqs 3 and 4, k_3 6.8×10^9 $M^{-1}s^{-1}$ and k_4 1.7×10^9 $M^{-1}s^{-1}$). Both HO• and H• species can also react with methanol (eqs 5 and 6, k_5 9.7×10^8 $M^{-1}s^{-1}$ and k_6 2.6×10^6 $M^{-1}s^{-1}$) increasing finally the amount of thiyl radical via a hydrogen abstraction from the resulting α-hydroxy methyl radical and the thiol (eq 7).[34] In the presence of an antioxidants thiyl radicals (MS•) can be trapped or reduced to the corresponding anion/protonated form, as described below (eqs 8-11). After irradiation, lipid isolation and derivatization to the corresponding fatty acid methyl esters, followed by GC analysis, allowed the *cis*/*trans* ratio to be determined.[12]



(1) (2) (3) (4) (5) (6) (7)

All the reactions were monitored by GC and quantitative determination was performed so that the *trans* isomer (elaidic acid) was ascertained as the only product of the reaction under the conditions used. The disappearance of oleate residue (corresponding to the formation of the

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elaidic residue) *vs* dose of irradiation in the absence of antioxidant at 40 min irradiation is shown in Figure 2 (dashed line). In analogous experiments in the presence of different concentrations of vit-A (red lines) and vit-E (blue lines), a significant inhibition on the *cis-trans* isomerization was observed based on the *cis* residue disappearance. For both antioxidants, two different concentrations have been used 0.02 mM (▲,Δ) and 0.06 mM (●,○). As shown in Figure 2, the inhibition is strongly dependent on the concentration of each antioxidant, with the retinol to be more efficient scavenger than the ArOH for the thiyl radical.

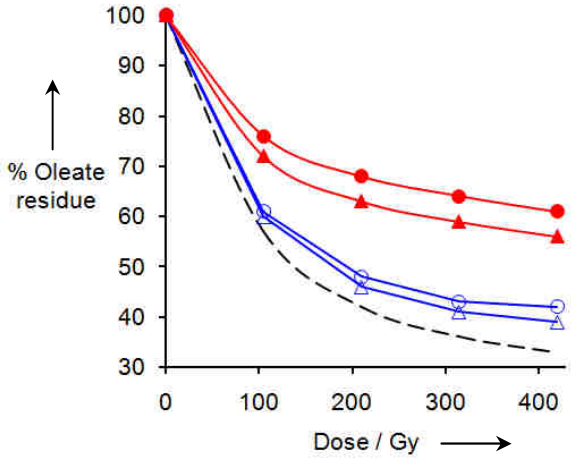

Figure 2 Disappearance of methyl oleate residue in DOPC vesicles under Gamma-radiolysis (10.2 Gy/min) with 1 mM mercaptoethanol, in the absence (dashed line) and in the presence of a) all-*trans* retinol acetate: 0.06 mM (●), 0.02 mM (▲); b) vit-E: 0.06 mM (○), 0.02 mM (Δ); the lines are interpolated from the data.

As far as the model membranes are concerned, DOPC large unilamellar vesicles were prepared by both methodologies (extrusion and methanolic injection), to whom the two hydrophilic antioxidants vit-C (AH_2) and urate (UH_3) were added. Radiolysis experiments were carried out as described in the previous section, and the results are shown in Tables and Figures S2 and S3 in Supporting Information. Again, the inhibition was strongly dependent on the concentration of each antioxidant, with AH_2 more efficient scavenger than the UH_3 for the thiyl radical, especially at low radiation doses (see Tables and Figures S6 and S7, in Supporting Information). Based on the amount of the *cis-trans* isomerization at initial reaction times, both hydrophilic antioxidants were more active than the lipophilic ArOH and retinol (see Supporting Information). This indicates that thiyl radicals generated in the aqueous phase by the primary water radicals from mercaptoethanol (MS•) are indeed efficiently scavenged at this level, limiting the species able to diffuse into the lipid compartment and catalyze the isomerization of the double bonds. Under these biomimetic conditions, the inhibition of the isomerising process due to antioxidants increased following the order of vit-C > urate ≈ retinol > α-tocopherol, in contrast to that observed in solution.[21] This behavior, compared also with the results of hydrophobic antioxidants (vit-A and vit-E), also evidences that the protection is based not only

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on the chemical reactivity of each species, but also on the localization of each antioxidant in the two-phases system represented by liposomes and mimics of the cellular compartments.

Moreover, the water soluble vitamin C,[35] because of the first pK_a value of 4.04,[36] exists at neutral pH as ascorbate monoanion (HA^-), which is generally accepted to act as a strong antioxidant compared to the protonated form of ascorbic acid (H_2A).[35-37] Until now, long-lived tocopheroxyl and persistent nitroxyl radicals have been employed as probes to assess the interaction of oxidizing species at liposome interface by using transient absorption and EPR spectroscopy. In parallel, Fluorazophore-L, an azoalkane moiety has been used as a fluorescent probe for reactive radicals in membrane systems for the determination of the vit-C role as antioxidant of the lipid radicals.[37] Herein, we used *cis-trans* lipid isomerization of DOPC vesicles as biomimetic model for the evaluation of *cis* phospholipids geometry protection from the thiyl radicals by vit-C in different pH values. For this reason separated experiments were carried out at different pH values (pH 3, 5, 7 and 9), in the presence of 2.2 mM DOPC, 1 mM MSH and 60 μM of vit-C. As shown in Figure 3 (A), the efficiency of isomerization of the DOPC vesicles by thiyl radical (MS•) in the presence of vit-C was influenced by the pH conditions: it increased slightly when the pH value increased from pH 3 (●,○), to pH 5 (▲,Δ) and to 7 (■,□), and was substantially higher at pH 9 (♦,◊). The dashed line corresponds to the average values taken at different pH values (see Supporting Information). The slight decrease of vit-C inhibition of the *cis-trans* lipid isomerization observed at pH 3 for DOPC liposomes, demonstrated that the protonated form AH_2 (pKa 4.04) is less active than the corresponding monoanion AH^-, which predominated at pH 5 and 7).

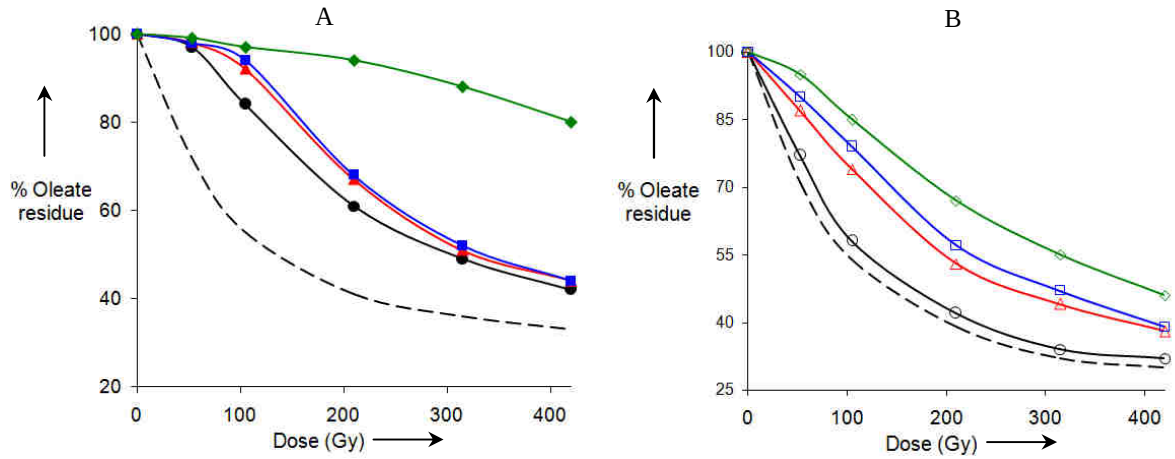

Figure 3. Disappearance of methyl oleate residue in DOPC vesicles (2.2 mM) under gamma-radiolysis (10.2 Gy/min) with 1mM mercaptoethanol as the thiyl radical source, in N_2O-saturated flushed phosphate buffer solution, in the absence (dashed lines) and in the presence of 0.06 mM of (A): vit-C at pH 9 (♦), 7 (■), 5 (▲) and 3 (●), and (B): urate at pH 9 (◊), 7 (□), 5 (Δ) and 3(○).

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To evaluate the influence of urate under the same reaction conditions, DOPC vesicle suspensions were irradiated in the presence of MSH and UH_3. Figure 3 (B) shows the dose profiles of methyl oleate disappearance in LUVs in the absence (dashed line), as well as in the presence of 60 μM of UH_3 at different pH values. The inhibition of the isomerization process owing to presence of UH_3 is similar to that observed with previous hydrophilic antioxidant AH_2. However, the substantially low inhibition effect of urate on the *cis-trans* lipid isomerization observed at pH 3, demonstrated that the protonated form of UH_3 (pK_{a1} 5.4 and pK_{a2} 9.8 for the UH_3)[38,39] is less active than the corresponding deprotonated form UH_2^- which predominates at pH 7 and 9. Our results suggest that deprotonated moieties of vit-C and urate (AH^- and UH_2^-) essentially form a protective barrier for the diffusion of reactive free radicals through the lipid surfaces of liposomes.

This finding may also be important to understand the paradox that polar antioxidants (AH_2 and UH_3) are more active in bulk oil systems, whereas nonpolar antioxidants, for example ascorbic acid 6-*O*-palmitate, are more active in emulsion systems.[40] This observation can also be supported by the direct determination of the association constant or partition coefficient of antioxidants. Thus, the inhibition of the isomerization process in the presence of hydrophobic ascorbic acid 6-*O*-palmitate (AAP) was investigated under the same conditions. The antioxidant activity of the hydrophobic compound (AAP) is substantially less than the corresponding one observed with hydrophilic ascorbic acid (AH_2). Figure 4 shows the dose profiles of methyl oleate disappearance in liposomes in the absence (dashed line), as well as in the presence of 60 μM of AAP (Δ) and AH_2 (□), with the same concentration (60 μM) and pH value (pH 7). It is clear that, at initial irradiation doses (105 Gy) the hydrophilic vit-C inhibited effectively the *cis-trans* isomerization process compared to the corresponding hydrophobic analogous ascorbic acid 6-*O*-palmitate. This result indicates a correlation between the antioxidant activity and the antioxidant partition coefficient.

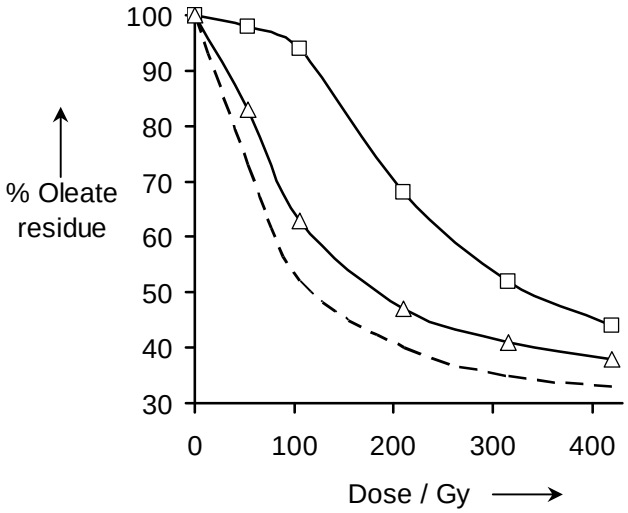

Figure 4 Disappearance of methyl oleate residue in DOPC vesicles (2.2 mM) under gamma-radiolysis (10.2 Gy/min) with 1 mM mercaptoethanol as the thiyl radicals source, in N_2O-

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saturated flushed phosphate buffer solution (pH 7), in the absence (dashed lines) and in the presence of ascorbic acid 0.06 mM (□) and ascorbic acid 6-*O*-palmitate 0.06 mM (Δ).

To prove further the protection role of the common antioxidants of the lipid isomerization catalyzed by thiyl radical, we studied this process in the presence of a mixture of all antioxidants. Figure 5 shows the dose profile of *cis*-*trans* isomerization in the presence of two lipophilic and two hydrophilic antioxidants. In particular, 60 μM *α*-tocopherol (▲), 5 μM all-*trans* retinol acetate (■), 180 μM urate (♦) and 60 μM L-ascorbic acid (●). The concentrations correspond to the average *in vivo* values.[41] For dose profiles of different antioxidant concentrations see Supporting Information. In a N_2O-saturated solution (~0.02 M of N_2O), e_{aq}^- are transformed into HO• (eq 2), affording a *G*(HO•) 0.55 μmol/J, *i.e.*, HO• radicals and H• atoms account for 90 and 10%, respectively, of the reactive species. In the presence of MSH, hydrogen abstraction by HO• and H• directly produces thiyl radicals (eqs 3 and 4).[34] The inhibition of the isomerization process due to the antioxidants can be rationalized as follows. The lipophilic *α*-tocopherol (eq 8) has a weaker inhibition effect than all-*trans* retinol acetate (eq 9), although the latter concentration is 12 times smaller.[28,29] At pH 7, the hydrophilic urate and ascorbic acid are in their deprotonated forms AH^- and UH_2^- (pK_{a1} 5.4 and pK_{a2} 9.8 for the UH_3)[38,39] and show much higher anti-isomerizing activity (eqs 10 and 11),[12,28,29] ascorbic acid being the most efficient even at low concentrations. Indeed, the MS• radical is a moderately strong one-electron oxidant [*E*(MS•, H^+/MSH) 1.3 V] and should oxidize ascorbate to yield ascorbyl radicals [*E*($A^{\bullet -}$, H^+/AH^-) 0.3 V] or urate to yield UH_2•/UH^{-}• radicals [*E*($UH^{\bullet -}$, H^+/UH_2^-) 0.59 V] as it was found.[38,39]

$$MS^{\bullet} + ArOH \rightarrow MSH + ArO^{\bullet} \quad (8)$$

$$MS^{\bullet} + Retinol \rightarrow MS\text{-}adduct^{\bullet} \quad (9)$$

$$MS^{\bullet} + AH^- \rightarrow MS^- + A^{-\bullet} + H^+ \quad (10)$$

$$MS^{\bullet} + UH_2^- \rightarrow MS^- + UH^{-\bullet} + H^+ \quad (11)$$

The behavior of the compounds in Figure 5 shows once again that the anti-isomerising activity toward diffusible thiyl radicals is based not only on the chemical reactivity, but also on the partition coefficient and localization of the radical scavengers in the aqueous or lipid compartment. Thus, the ability of thiyl radical scavenging in aqueous solution under *γ*-radiolysis increases along the series *α*-tocopherol < all-*trans* retinol acetate < urate < ascorbic acid.[42] Mixing all antioxidants at the above reported concentrations has an extraordinary protective effect for the *cis* vesicle. This result is explained as a synergistic effect between hydrophilic and lipophilic antioxidants, with additive effects of chemical reactivity and partition coefficients, which can also occurs *in vivo* and has a crucial role in the protection of the *cis* geometry towards diffusible thiyl radicals.

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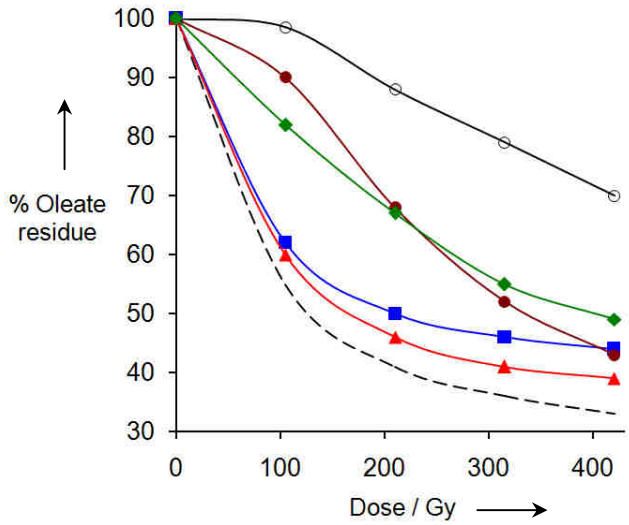

Figure 5 Gamma-radiolysis (10.2 Gy/min) of DOPC was carried out in the presence of mercaptoethanol as source of thiyl radicals. The graph shows the disappearance of methyl oleate residue in DOPC vesicles (2.2 mM) with 1 mM mercaptoethanol in N_2O-saturated flushed phosphate buffer solution at pH 7, in the absence (dashed lines), and in the presence of vit-C 0.06 mM (●), urate 0.18 mM (♦), all-*trans* retinol 0.005 mM (■), vit-E 0.06 mM (▲), and mixture of them with the above concentrations (○).

Conclusions

Biomimetic studies for the evaluation of *cis* phospholipids geometry protection from the thiyl radical isomerization in the presence of common antioxidants were performed. Thus, using large unilamellar vesicles (LUVs) of glycerophospholipids containing oleate moieties we demonstrated that hydrophilic ascorbic acid and urate, and hydrophobic all-*trans* retinol acetate and α-tocopherol, are able to inhibit *cis*-*trans* isomerization catalyzed by the thiyl radical species. In all cases, taking into account that the generation of radicals occurred by γ-radiolysis in the water compartment, the hydrophilic antioxidants showed better anti-isomerizing activity compared to the hydrophobic compounds, results that amplify the correlation between the antioxidant activity and the partition coefficient between lipid and aqueous phase. The synergistic effect obtained by mixing both hydrophilic and hydrophobic antioxidants at *in vivo* concentrations was also proved to exert an extraordinary protection for the integrity of *cis* lipid vesicles. Due to the need of the lipid *cis* geometry for eukaryote membranes, it is important to evidence the synergic protection given by common antioxidants at *in vivo* concentrations, which becomes crucial for inhibiting lipid isomerization since an alternative enzymatic repair is not known.

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Experimental Section

General. β-Mercaptoethanol ($HOCH_2CH_2SH$), all-*trans* retinal acetate, urate, α-tocopherol, ascorbic acid, ascorbic acid 6-*O*-palmitate, and dioleoylphosphatidylcholine (DOPC) were commercially available from Aldrich (Milwaukee, WI, USA), Fluka (Buchs, Switzerland), or Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. Chloroform, methanol and isopropanol were purchased from Merck (Darmstadt, Germany) (HPLC grade) and used without further purification.

GC analysis for the determination of the isomeric ratio of the unsaturated fatty acids was performed at different reaction times. For this reason an Agilent 5860 gas chromatograph equipped with a flame ionization detector and a DB-23 column (90% biscyanopropyl-10% phenylcyanopropylpolysiloxane capillary column; 60 m, 0.25 mm i.d., 0.25-mm film thickness) was used. Temperature started from 190 °C held for 15 min, followed by an increase of 30 °C/min up to 220 °C, held for 15 min, followed by a second increase of 30 °C/min up to 250 °C. Methyl esters of oleic and elaidic acids were identified by comparison with the retention times of commercially available authentic samples. When necessary, GC/MS spectra were recorded on a Hewlett-Packard GC 5890 (series II) coupled to a Hewlett-Packard mass selective detector model 5971A.

Continuous radiolysis was performed at room temperature (22 ± 2 °C) using a ^{60}Co-Gammacell (Atomic Energy of Canada Ltd., Ontario, Canada) at different dose rates. The exact absorbed radiation dose was determined with the Fricke chemical dosimeter by taking G(Fe^{3+}) 1.61 μmol/J.[43]

Preparation and isomerization of DOPC large unilamellar vesicles (LUV)

Extrusion methodology. A solution DOPC (31 mg, 0.04 mmol) in chloroform (2 mL) was evaporated to a thin film in a test tube under an argon stream and dried under vacuum for 30 min. Degassed milliQ water (1 mL) was added, and multilamellar vesicles were formed by vortex stirring for 7 min under an argon atmosphere. To obtain large unilamellar vesicle by extrusion technique (LUVET), the lipid emulsion was transferred into a LiposoFast (produced by Avestin, Inc.) and extruded through two polycarbonate filters with a pore diameter of 100 nm.[13,44] In each irradiation experiments, an aliquot of the stock PC suspension (40 mM) was added to a phosphate buffer (PB) solution, affording liposome dispersion with a final lipid concentration of 2.2 mM. 1 mL of this final suspension was added into a vial equipped with an open top screw cap and a Teflon-faced septum and saturated with N_2O prior to γ-irradiation. $HOCH_2CH_2SH$ (MSH) (1 mM) and MeOH (40 mM) were consecutively added and the suspension was irradiated. Aliquots of 100 μL were withdrawn and processed at different times, by partitioning between chloroform/methanol (2:1) and brine, extraction and collection of the organic phases dried (Na_2SO_4), and evaporation of the solvent under vacuum at room temperature. The residue containing the phospholipids was treated with 0.5 M KOH/MeOH (1 mL), for 10 min at room temperature and then poured into the brine and extracted with *n*-hexane. The organic layer

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containing the corresponding fatty acid methyl esters was examined by GC analysis in comparison with the retention times of authentic samples.

In the presence of hydrophilic inhibitors, such as vitamin C and urate, a suspension of DOPC (1 mL) was prepared as described previously and an exact amount of the stock solution containing the inhibitors was added. The final hydrophilic inhibitors concentrations were in the range of 20 to 180 μM. The addition volume of the inhibitors solution was not higher than 25 μL, in order that the final concentrations of lipids and MSH were kept constant. For this reason, a stock solution of vitamin C and urate in phosphate buffer (pH 7) were prepared in higher than 2 mM concentration for each one. At lower pH or in water, the urate was soluble in max of 1 mM concentration. It is interesting to note that at pH 9 urate was also soluble in higher than 2 mM concentration, however it was decomposed even after one day, followed by HPLC.

Methanolic Injection. The injection method[31,32,45,46] was used to prepare DOPC liposomes containing defined amounts of the lipophilic *α*-tocopherol, all-*trans* retinol acetate and L-ascorbyl-6-palmitate, as inhibitors. Stock solutions were prepared freshly and based on the methanol density by weighting the solvent and preventing further evaporation. Antioxidants (vitamins) were weighted and the chloroform solutions were stored in vials at –20 °C for the duration of each set of experiments. 50 μL of chloroform containing various amount of antioxidants were added to 100 μL of a methanolic stock solution (68 mM DOPC). The mixture was evaporated under vacuum for 20 min and then methanol (150 μL) was added. This mixture (corresponding to 40 mM DOPC) was injected with a Hamilton syringe into 3 mL of magnetically stirred 10 mM phosphate buffer solution at 40 °C affording a liposome dispersion with a final lipid concentration of 2.2 mM and an overall methanol amount of 4.7% (or 1.2 M). The final concentrations of the hydrophobic compounds enclosed in the vesicles were in the range of 5 to 60 μM, therefore with a lipid/vitamin ratio from 440:1 to ~ 35:1. The suspensions were left under stirring for 15 min at 40 ^{o}C and another 15 min at room temperature.

Acknowledgements

The sponsorship of the COST Action CM1201 “Biomimetic Radical Chemistry” is gratefully acknowledged.

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