

Review

Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation

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Abstract

There is growing evidence that high levels of the macular xanthophyll carotenoids lutein and zeaxanthin may be protective against visual loss due to age-related macular degeneration, but the actual mechanisms of their protective effects are still poorly understood. We have recently purified, identified and characterized a pi isoform of glutathione *S*-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye which specifically and saturably binds to the two forms of zeaxanthin endogenously found in the foveal region. In this report, we studied the synergistic antioxidant role of zeaxanthin and GSTP1 in egg yolk phosphatidylcholine (EYPC) liposomes using hydrophilic 2,2'-azobis(2-methyl-propionamidine) dihydrochloride (AAPH) and lipophilic 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) as lipid peroxy radical generators. The two zeaxanthin diastereomers displayed synergistic antioxidant effects against both azo lipid peroxy radical generators when bound to GSTP1. In the presence of GSTP1, nondietary (3*R*,3'*S*-*meso*)-zeaxanthin was observed to be a better antioxidant than dietary (3*R*,3'*R*)-zeaxanthin. This effect was found to be independent of the presence of glutathione. Carotenoid degradation profiles indicated that the zeaxanthin diastereomers in association with GSTP1 were more resistant to degradation which may account for the synergistic antioxidant effects.

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1. Introduction

Carotenoids are fat-soluble pigments that are found abundantly in dark green leafy vegetables and fruits [1]. They are thought to play critical roles in the prevention of important human diseases such as cancer, cardiovascular disease [2–4], and age-related macular degeneration (AMD) [5–7]. Over fifteen different dietary carotenoids are detectable in human serum, but only lutein [(3*R*,3'*R*,6'*R*)-β,ε-carotene-3,3'-diol] and zeaxanthin [a mixture of (3*R*,3'*R*)-β,β-carotene-3,3'-diol and (3*R*,3'*S*-*meso*)-β,β-carotene-3,3'-diol] (Fig. 1) and their metabolites are found in substantial amount in the retina of humans [8–10] and a few other primates [11]. Chromatographic

analysis of postmortem eyes shows that zeaxanthin isomers dominate over lutein in the foveal region of the human retina [12].

The Eye Disease Case-Control (EDCC) Study reported that high serum carotenoid levels and high dietary intakes of lutein and zeaxanthin are associated with lower relative risk of AMD [13,14], and subsequent studies have continued to support the potential protective role of lutein and zeaxanthin against AMD [15–18]. Lutein and zeaxanthin are likely to play important roles in combating reactive oxygen species (ROS) generated in the presence of blue light and oxygen, especially since retinal membranes are particularly rich in long-chain polyunsaturated fatty acids [6]. In some situations, these antioxidant effects of single carotenoids may be synergistic with the antioxidant effects of other carotenoids and other chemical compounds [19–21].

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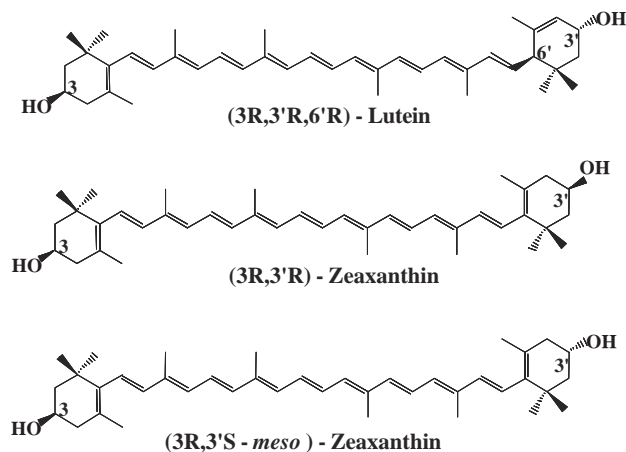


Fig. 1. The chemical structures of dietary lutein, zeaxanthin and non-dietary *meso*-zeaxanthin.

The concentrations of lutein and zeaxanthin in the Henle fiber layer of the human macula are thought to be between 0.1 and 1 mM [7]. We have previously reported that the uptake of these xanthophyll carotenoids is likely to be mediated by specific and saturable xanthophyll-binding proteins [22], and we have recently identified a pi isoform of glutathione *S*-transferase (GSTP1) as a specific and saturable binding protein for both diastereomers of zeaxanthin found in the human macula with a K_d in the range of 300–500 nM [23]. The physiological role of GSTP1 in retinal xanthophyll physiology remains unknown, however. In this study we examine whether GSTP1 enhances the antioxidant effect of zeaxanthin in a model lipid oxidation system.

2. Materials and methods

2.1. Reagents and chemicals

HPLC grade methylene chloride, methanol, and hexane were obtained from Fisher Scientific (Hampton, NH). Synthetic (3R,3'R)-zeaxanthin and (3R,3'S-*meso*)-zeaxanthin were gifts from Hoffmann-La Roche (Basel, Switzerland), and (3R,3'R,6'R)-lutein prepared from marigold flowers was a gift from Kemin Health (Des Moines, Iowa). Lipophilic and hydrophilic azo initiators 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) were obtained from Wako USA (Richmond, VA) and Sigma (St. Louis, MO) respectively. Egg yolk phosphatidylcholine (EYPC), glutathione (GSH), and recombinant human GSTP1 were also obtained from Sigma.

2.2. Preparation of liposomes

Liposomes were prepared according to a previously published methods [19,24] in which 500 μ l of 0.2 mg/ml EYPC in tricine buffer (0.1 M, pH 7.6) was sonicated on ice

in a glass tube for 10 min. In order to prepare zeaxanthin-containing liposomes, appropriate amounts of zeaxanthin were first dissolved in an ethanolic solution of EYPC prior to liposome formation. The organic solvent was evaporated under a stream of nitrogen, and liposomes were then prepared as described above. GSTP1 (0.50 μ g) was added after liposome formation. The final total concentration of zeaxanthin in each set of experiments was 2 μ M which is several-fold higher than the K_d for binding to GSTP1 [23]. In some experiments, 1 μ M glutathione was added to the reaction mixture.

2.3. Lipid peroxidation reaction

Lipid peroxidation was initiated by the addition of 1 M concentrates of the lipid peroxy radical generators AAPH or AMVN dissolved in tetrahydrofuran [THF (with 0.01% ethanol added first to AAPH to improve solubility)]. The final concentration of the initiators in the liposome suspension was 10 mM, and the final concentration of THF in the suspension was 1% (v/v). In order to stop the oxidation reaction, 5 μ l of 100 mg/ml butylated hydroxytoluene (BHT) in methanol was added to the sample at a final concentration of 0.1% (w/v). Lipid destruction was followed by the measurement of thiobarbituric acid reactive species (TBARS). Most of the experiments were carried out at 37 °C for 2 h. For kinetic studies, larger reaction volumes were used, and 0.5 ml samples were taken every 10 min for 2 h.

2.4. TBARS assay

TBARS were measured according to published methods [19,24]. BHT [0.1% (w/v)] was added to the samples before boiling to prevent formation of further TBA-reactive products. Malondialdehyde (MDA) equivalents were calculated using a published extinction coefficient: $E_{532} = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [19,24]. Absorbance at 532 nm was calculated from spectra using the formula: $\Delta A_{532} = A_{532} - (A_{515} + A_{550})/2$ to allow for baseline correction.

2.5. Zeaxanthin extraction and analysis

Fifty microliters of reaction mixture was treated with 100 μ l of acetone to disrupt lipid vesicle associations, and they were then extracted into 200 μ l of hexane at least three times. Separation between the aqueous and organic phases was promoted by the addition of 20 μ l of saturated sodium chloride. The organic layer was collected and centrifuged at 1800 \times g for 10 min. The extracts were then dried by vacuum evaporation in a Speedvac Plus (SC110, Savant, Cambridge, MA) and re-dissolved in 1 ml of HPLC mobile phase [hexane:dichloromethane:methanol:*N,N'*-di-isopropylethylamine (80:19.2:0.7:0.1 v/v)]. HPLC separation was carried out at a flow rate of 1.0 ml/min on a cyano column

(Microsorb 25 cm length×4.6 mm id, Varian, Palo Alto, CA). The column was maintained at room temperature, and the HPLC absorbance detector (Waters, Milford, MA) was operated at 450 nm. Peak identities were confirmed by co-elution with authentic standards as necessary. Peak areas were integrated and quantified with an external standardization curve.

2.6. Statistical analysis

All experimental sets were repeated five times, and values are expressed with the standard deviation of the means. Unpaired *t*-tests with a significance level of 0.05 were performed.

3. Results

The protective effects of both forms of ocular zeaxanthin on EYPC lipid peroxidation induced by the azo initiators AAPH and AMVN are summarized in Tables 1 and 2, respectively. AMVN is a non-polar, lipid soluble azo initiator that is known to penetrate into the hydrophobic interior of the phospholipid bilayer where it forms peroxy radicals which oxidize the unsaturated phospholipids. In all cases, AMVN was observed to be more damaging to lipid membranes than water soluble AAPH.

Dietary (3*R*,3'*R*)-zeaxanthin in the absence of added GSH or GSTP1 was significantly more protective than non-dietary (3*R*,3'*S*-*meso*)-zeaxanthin with either initiator ($P < 0.005$). For AAPH the protective effects offered by both forms of zeaxanthin were $38 \pm 4\%$ and $33 \pm 3\%$, respectively, whereas for AMVN the effects were $49 \pm 3\%$ and $45 \pm 2\%$, respectively. Assuming a linear antioxidant response, a borderline synergistic antioxidant effect was seen when the two xanthophylls were present at equimolar concentrations in the presence of AMVN, but not in the presence of AAPH.

In association with GSTP1, (3*R*,3'*S*-*meso*)-zeaxanthin became a significantly better antioxidant than dietary (3*R*,3'*R*)-zeaxanthin in protecting against AAPH or AMVN induced peroxidation ($P < 0.005$). Considering the fact that GSTP1 alone was minimally effective in preventing the peroxidation of lipids, both (3*R*,3'*R*)-zeaxanthin and (3*R*,3'*S*-*meso*)-zeaxanthin exhibited synergistic protective effects in the presence of GSTP1.

Glutathione *S*-transferases (GSTs) are members of a super family of phase II enzymes known to be involved in the detoxification of various metabolites generated by oxidative processes in the body. Since most of the detoxification activities are glutathione dependent, we also studied the antioxidant effect of GSH in the presence and absence of zeaxanthin and/or GSTP1. One micromolar concentrations of GSH showed excellent antioxidant effects, but in the presence of GSTP1 and/or either form of ocular zeaxanthin, the effects were never synergistic. The origin of

Table 1
Protection of AAPH-induced lipid peroxidation by antioxidants

Experimental groups	Experimental combinations	TBARS (mean±S.D., <i>n</i> =5)	
		nmol/mg phospholipid	Percent protection ^a
A1 ^b	EYPC control	3.18±0.17	0
A2	GSTP1 (0.5 µg)	2.96±0.03	7
A3	GSH (1 µM)	1.63±0.16	49
A4	GSTP1+GSH	1.45±0.12	54 (56)
B1 ^c	(3 <i>R</i> ,3' <i>R</i>)-Zeaxanthin (2 µM)	1.96±0.13	38
B2	Zeaxanthin+GSTP1	1.60±0.07	50 (45)
B3	Zeaxanthin+GSH	1.19±0.18	63 (87)
B4	Zeaxanthin+GSH+GSTP1	1.08±0.17	66 (92)
C1 ^d	(3 <i>R</i> ,3' <i>S</i> - <i>meso</i>)-Zeaxanthin (2 µM)	2.14±0.12	33
C2	<i>meso</i> -Zeaxanthin+GSTP1	1.40±0.86	56 (40)
C3	<i>meso</i> -Zeaxanthin+GSH	1.16±0.16	64 (82)
C4	<i>meso</i> -Zeaxanthin+GSH+GSTP1	1.00±0.13	69 (87)
D1 ^e	Zeaxanthin (1 µM)+ <i>meso</i> -Zeaxanthin (1 µM)	2.08±0.22	35 (36)
D2	Zeaxanthin+ <i>meso</i> -Zeaxanthin+GSTP1	1.35±0.10	58 (42)
D3	Zeaxanthin+ <i>meso</i> -Zeaxanthin+GSH	1.03±0.07	67 (84)
D4	Zeaxanthin+ <i>meso</i> -Zeaxanthin+GSH+GSTP1	0.89±0.10	72 (89)

^a Percent protection values were recorded with the EYPC control as 0%, and the remaining values were expressed with reference to this value. Values in parentheses show the predicted additive effects of the mixtures. GSTP1+GSH (54% protection) was used as the base to calculate the expected additive effects for the multi-component mixtures. Bold entries highlight synergistic effects that were greater than or equal to the typical precision of the lipid peroxidation assay ($\pm 10\%$).

^b Comparative statistical tests for Group A: A2, A3, and A4 were compared with A1, and all *P* values were < 0.001 ; A4 was compared with A2 and A3, and both *P* values were < 0.001 .

^c Comparative statistical tests for Group B: B2, B3, and B4 were compared with B1, and all *P* values were < 0.001 ; B4 was compared with B2 and B3, and both *P* values were < 0.002 ; B1 was also compared with A1, and the *P* value was observed to be < 0.005 .

^d Comparative statistical tests for Group C: C2, C3, and C4 were compared with C1, and all *P* values were < 0.001 ; C4 was compared with C2 and C3, and both *P* values were < 0.001 ; C1 was also compared with A1 and B1 and the *P* values were observed to be < 0.05 ; C2 was compared with B2 and the *P* values were observed to be < 0.05 .

^e Comparative statistical tests for Group D: D2, D3, and D4 were compared with D1, and all *P* values were < 0.001 ; D4 was compared with D2 and D3, and both *P* values were observed to be < 0.005 ; D1 was also compared with A1, B1, and C1, and *P* values were observed to be < 0.008 , 0.24, and 0.40 respectively.

the antagonistic interaction of GSH and zeaxanthins will require further investigation.

The stability of the two forms of ocular zeaxanthin to azo-initiated lipid peroxidation was studied in the presence and absence of GSTP1 (Fig. 2). In all cases, zeaxanthin degradation in the presence of GSTP1 was slower than the control during the first hour. It has been reported that zeaxanthin is more stable than β -carotene against various lipid peroxy radical generators [25], and our results indicate

Table 2
Protection of AMVN-induced lipid peroxidation by antioxidants

Experimental groups	Experimental combinations	TBARS (mean±S.D., n=5)	
		nmol/mg phospholipid	Percent protection ^a
A1 ^b	EYPC control	14.23 ± 1.39	0
A2	GSTP1 (0.5 µg)	13.58 ± 1.77	5
A3	GSH (1 µM)	6.73 ± 0.85	53
A4	GSTP1+GSH	6.24 ± 0.76	56 (58)
B1 ^c	(3 <i>R</i> ,3' <i>R</i>)-Zeaxanthin (2 µM)	7.27 ± 0.45	49
B2	Zeaxanthin+GSTP1	5.69 ± 0.98	60 (54)
B3	Zeaxanthin+GSH	4.72 ± 0.31	67 (>100)
B4	Zeaxanthin+GSH+GSTP1	4.47 ± 0.26	69 (>100)
C1 ^d	(3 <i>R</i> ,3' <i>S</i> - <i>meso</i>)-Zeaxanthin (2 µM)	7.77 ± 0.88	45
C2	<i>meso</i> -Zeaxanthin+GSTP1	4.41 ± 0.50	69 (50)
C3	<i>meso</i> -Zeaxanthin+GSH	3.63 ± 0.29	74 (98)
C4	<i>meso</i> -Zeaxanthin+GSH+GSTP1	3.56 ± 0.18	75 (>100)
D1 ^e	Zeaxanthin (1 µM)+ <i>meso</i> -Zeaxanthin (1 µM)	6.29 ± 0.54	52 (47)
D2	Zeaxanthin+ <i>meso</i> -Zeaxanthin+GSTP1	4.37 ± 0.75	69 (57)
D3	Zeaxanthin+ <i>meso</i> -Zeaxanthin+GSH	3.50 ± 0.21	75 (>100)
D4	Zeaxanthin+ <i>meso</i> -Zeaxanthin+GSH+GSTP1	3.44 ± 0.19	76 (>100)

^a Percent protection values were recorded with the EYPC control as 0%, and the remaining values were expressed with reference to this value. Values in parentheses show the predicted additive effects of the mixtures. GSTP1+GSH (56% protection) was used as the base to calculate the expected additive effects for the multi-component mixtures. Bold entries highlight synergistic effects that were greater than or equal to the typical precision of the lipid peroxidation assay ($\pm 10\%$).

^b Comparative statistical tests for Group A: A2, A3, and A4 were compared with A1, and all *P* values were <0.001; A4 was compared with A2 and A3, and both *P* values were <0.001.

^c Comparative statistical tests for Group B: B2, B3, and B4 were compared with B1, and all *P* values were <0.001; B4 was compared with B2 and B3, and both *P* values were <0.03; B1 was also compared with A1, and the *P* value was observed to be <0.005.

^d Comparative statistical tests for Group C: C2, C3, and C4 were compared with C1, and all *P* values were <0.001; C4 was compared with C2 and C3, and both *P* values were <0.006; C1 was also compared with A1 and B1, and the *P* values were observed to be <0.005; C2 was also compared with B2, and the *P* values were observed to be <0.005.

^e Comparative statistical tests for Group D: D2, D3, and D4 were compared with D1, and all *P* values were <0.001; D4 was compared with D2 and D3, and both *P* values were observed to be >0.05; D1 was also compared with A1, B1, and C1, and all of the *P* values were observed to be <0.05, >0.05, >0.05, respectively.

that GSTP1 further stabilizes its zeaxanthin ligand, thereby enhancing its antioxidant activity.

4. Discussion

The human retina is a well oxygenated tissue that is rich in long-chain polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid, that are quite vulnerable to lipid

peroxidation. High-energy short-wavelength visible light promotes the formation of reactive oxygen species (ROS) which can initiate lipid peroxidation in the macula and elsewhere. The macular carotenoids are thought to combat light-induced damage mediated by ROS by absorbing the most damaging incoming wavelengths of light prior to the formation of ROS and by chemically quenching ROS once they are formed.

Chromatographic analysis of the central fovea and the peripheral regions of the human retina has revealed that the zeaxanthin to lutein ratio in the fovea is about 2:1, while in the periphery lutein predominates [8,9]. More recently, it was determined that foveal zeaxanthin is actually an equimolar mixture of dietary (3*R*,3'*R*)-zeaxanthin and non-dietary (3*R*,3'*S*-*meso*)-zeaxanthin [9]. It is likely that dietary lutein is the metabolic precursor to *meso*-zeaxanthin [11,12]. Along with these three major macular carotenoids, several of their oxidation products are also detectable in substantial amounts, indicating that the macular carotenoids are likely to be acting as antioxidants [8].

Xanthophyll carotenoids have poor aqueous solubility, and therefore in biological tissues such as the human macula they are present in the lipid membrane bilayers or associated with proteins. The polar hydroxyl groups at each end of the xanthophyll molecules encourage a membrane spanning configuration in lipid bilayers, especially in the case of zeaxanthin [24,25]. Their presence in biological membranes is ideal if they are to act as lipid antioxidants, but lipid associations alone cannot explain the extraordinarily specific uptake of carotenoids into the macula. Thus, xanthophyll-binding proteins are likely to be involved in the specific uptake, stabilization, and physiological functions of lutein and zeaxanthin in the retina, but until recently relatively little has been known about xanthophyll-binding proteins in any vertebrate system. Initially, we found that tubulin in the cone axons of the Henle fiber layer was a likely site for the deposition and stabilization of the high concentrations of xanthophyll carotenoids found there [26]. In follow up studies, we demonstrated that there was an additional high affinity xanthophyll-binding activity present in membrane fractions derived from human retina and macula [22]. Recently, we identified the protein responsible for this binding activity as a pi isoform of glutathione *S*-transferase (GSTP1) [23]. Recombinant human GSTP1 binds both forms of macular zeaxanthin with high affinity (300–500 nM), while lutein binds poorly. GSTP1's physiological role in the transport, stabilization, and metabolism of carotenoids is currently under investigation in our laboratory.

In the study reported here, we have investigated whether or not GSTP1 can enhance zeaxanthin's well known ability to protect polyunsaturated lipid membranes from oxidation. We found that GSTP1 did indeed synergistically enhance zeaxanthin's antioxidant properties, while the addition of GSTP1's other known ligand, GSH, led only to additive effects at best. Our quantitative

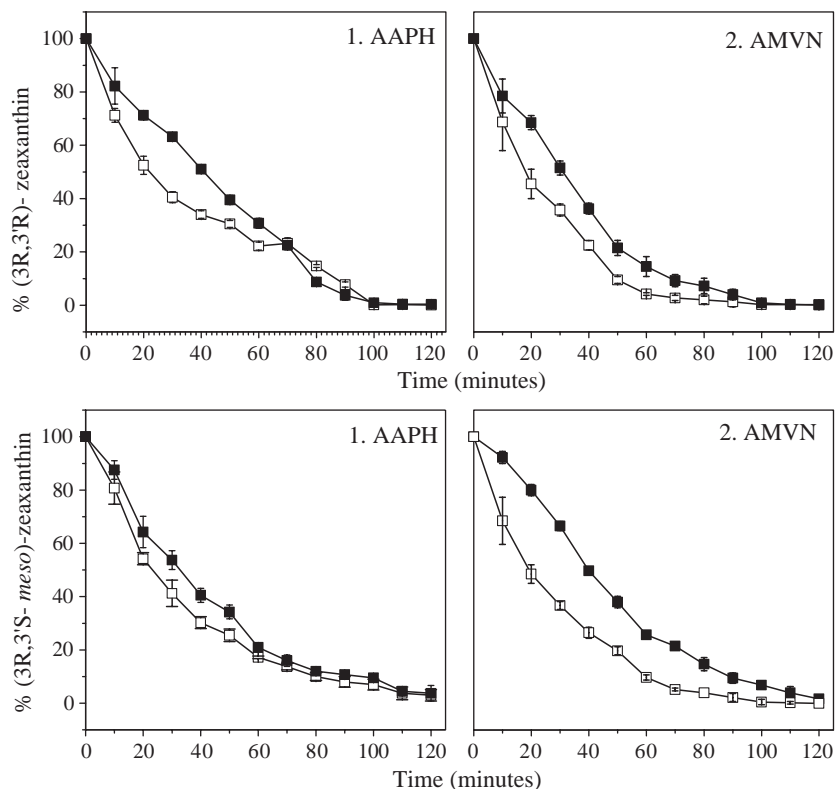


Fig. 2. Degradation profile of macular zeaxanthins in the absence (open squares) and presence (closed squares) of recombinant human GSTP1 during assays of AAPH- and AMVN-induced peroxidation of EYPC liposomes. Zeaxanthin levels at the starting point of each experiment were set to 100%. Error bars represent mean \pm S.D. for $n=4$.

degradation studies indicate that part of GSTP1's synergistic enhancement of zeaxanthin's antioxidant activity stems from its ability to bind and shield dietary (3R,3'R)-zeaxanthin and non-dietary (3R,3'S-*meso*)-zeaxanthin from irreversible oxidative degradation.

Synergistic antioxidant enhancements in liposome models have been displayed previously between two different antioxidant vitamin [27] and carotenoid [19] complexes, and four different isoforms of GST have been reported to bind and protect protoporphyrins from non-enzymatic autoxidation in *Zea mays* L. [28]. In previous photophysical studies, we have observed that the S_1 - S_n decay kinetics of *meso*-zeaxanthin bound to partially purified macular xanthophyll-binding protein were dominated by a decay component much slower than expected for unbound carotenoids with 11 conjugated double bonds, suggesting that stabilizing changes in the structure of *meso*-zeaxanthin occur when it is in association with specific binding proteins [29].

The experiments reported here are just the beginning of our efforts to understand the potential antioxidant role of macular xanthophylls in association with binding proteins. Assay protocols still need to be optimized, and other proteins and ocular carotenoids such as lutein need to be examined under similar conditions. Increased knowledge of the functions of lutein and zeaxanthin and their ocular binding proteins should yield important insights into their

potential role as protectants against age-related macular degeneration.

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