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Peridinin Is an Exceptionally Potent and Membrane-Embedded Inhibitor of Bilayer Lipid Peroxidation

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Abstract

Antilipoperoxidant protein dysfunction is associated with many human diseases, suggesting that bilayer lipid peroxidation may contribute broadly to pathogenesis. Small molecule inhibitors of this membrane-localized chemistry could in theory enable better understanding and/or treatment of such diseases, but currently available compounds have important limitations. Many biological questions thus remain unanswered, and clinical trials have largely been disappointing. Enabled by efficient, building block-based syntheses of three atypical carotenoid natural products produced by microorganisms that thrive in environments of extreme oxidative stress, we found that peridinin is a potent inhibitor of nonenzymatic bilayer lipid peroxidation in liposomes and in primary human endothelial cells. We also found that peridinin blocks monocyte-endothelial cell adhesion, a key step in atherogenesis. A series of frontier solid-state NMR experiments with a site-specifically ¹³C-labeled isotopolog synthesized using the same MIDA boronate building block-based total synthesis approach revealed that peridinin is completely embedded within and physically spans the

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ASSOCIATED CONTENT

Supporting Information

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Supplementary figures, general experimental details and procedures, synthetic procedures, compound characterization data, and NMR spectra for all new compounds (PDF)

Notes

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hydrophobic core of POPC membranes, maximizing its effective molarity at the site of the targeted lipid peroxidation reactions. Alternatively, the widely used carotenoid astaxanthin is significantly less potent and was found to primarily localize extramembranously. Peridinin thus represents a promising and biophysically well-characterized starting point for the development of small molecule antilipoperoxidants that serve as more effective biological probes and/or therapeutics.

Graphical Abstract

INTRODUCTION

Proteins, such as glutathione *S*-transferase (GST),¹ glutathione peroxidase (GPx4),² and peroxiredoxin (Prdx),³ mitigate and/or eliminate byproducts of the peroxidation of polyunsaturated phospholipids occurring in the hydrophobic core of lipid bilayers. Loss-of-function mutations in GST have been associated with increased incidence or severity of several human diseases, including atherosclerosis.^{4–7} GPx4 overexpression in apolipoprotein E-deficient mice results in an attenuation of atherogenesis,⁸ and Prdx6 deficiency increased atherogenesis in mice with certain genetic backgrounds.⁹ These observations suggest that antilipoperoxidant protein dysfunction may contribute to some aspects of human disease pathogenesis. However, it remains unclear whether unchecked free radical lipid peroxidation is a causative factor.

In the case of atherosclerosis, it is proposed that oxidation of membrane-localized polyunsaturated fatty acyl chains of phospholipids leads to the generation of truncated and hydrophilic lipid moieties, which are reoriented from the hydrophobic bilayer core into the extracellular space.¹⁰ In this “lipid whisker model,” the exposed oxidized lipids act as ligands for scavenger receptors on circulating monocytes leading to monocyte-endothelial cell binding, a key step in atherogenesis.¹¹

This model predicts that inhibition of the bilayer-localized chemical reaction should mitigate atherosclerosis, particularly in patients with deficiencies of antilipoperoxidant protein function. However, extensive clinical trials targeting the reduction of cardiovascular disease with small molecules thought to inhibit bilayer lipid peroxidation, including the apolar carotenoid β -carotene (**1**) (Figure 1a) and vitamin E, have largely been disappointing.¹² It has remained unclear if these failures reflect a lack of importance of bilayer lipid peroxidation in promoting atherogenesis or limitations of currently available small molecule antilipoperoxidants. Supporting the latter hypothesis, β -carotene was found to actually

promote lipid peroxidation under certain conditions,^{13,14} which we confirmed (Figure S1). Vitamin E is only a weak inhibitor in abiotic model membrane systems (Figure S2, S3), suggesting that the antilipoperoxidant activity of vitamin E observed in cells and animals may be primarily attributable to its other biological effects.^{15,16} Thus, more effective small molecule antilipoperoxidants are needed to help clarify and potentially mitigate the role of bilayer lipid peroxidation in disease pathogenesis.

In this vein, polar oxygen-containing carotenoids (xanthophylls) have shown more promise relative to their apolar counterparts.^{17,18} Multiple modes of radical scavenging reactivity have been demonstrated for such polar carotenoids, including radical addition to the polyene core, electron transfer from the polyene to the radical, and radical-mediated hydrogen atom abstraction from activated carotenoid C–H bonds.¹⁹ In particular, the ketocarotenoid astaxanthin (**2**) has undergone preclinical testing for the treatment of atherosclerosis,^{20,21} ischemia/reperfusion injuries,²² and cancer^{23,24} for over a decade and is sold as an antioxidant nutraceutical, with a global market valued at nearly half a billion US dollars in 2014.²⁵ Evidence in the literature^{13,26} and our own results (*vide infra*), however, have shown that astaxanthin has limited potency as demonstrated by the requirement for high doses or lack of activity in biophysical experiments,^{13,26} human cells,²⁷ animals models,^{20,21} and initial clinical trials.^{28,29} The explanations for this lack of potency remain enigmatic, thus precluding rational optimization of the antilipoperoxidant activity of astaxanthin.

We therefore sought to identify a more effective carotenoid-based inhibitor of bilayer lipid peroxidation. Such a compound could help illuminate the underpinnings of the targeted antilipoperoxidant activity, advance our understanding of the specific role(s) lipid peroxidation plays in the pathogenesis of atherosclerosis and other diseases, and potentially serve as a starting point for the development of small molecule replacements for missing or dysfunctional antilipoperoxidant proteins.^{30,31}

Since the “target” in this case is a bilayer-localized nonenzymatic chemical reaction rather than a protein or other macromolecule, and the basis for the lack of potency of astaxanthin is unknown, identifying a highly effective inhibitor is a challenging problem. We thus questioned whether context-specific selective pressures may have driven the evolution of natural products with exceptional antilipoperoxidant capacities in microorganisms that thrive in environments of extreme oxidative stress. We specifically sought to characterize the antilipoperoxidant activity of three structurally atypical polar carotenoids: peridinin (**3**), synechoxanthin (**4**), and chlamydaxanthin (**5**) (Figure 1b).

Peridinin (**3**) is an allenic norcarotenoid produced by photosynthetic dinoflagellates, including *Amphidinium carterae*.³² This small molecule is localized to the peridinin-chlorophyll *a*-protein complex in the producing organisms, where it plays important roles in protection from photo-mediated oxidation and light harvesting.^{33,34} The aromatic dicarboxylate carotenoid synechoxanthin (**4**) is produced by the marine cyanobacterium *Synechococcus* sp. PCC 7002, which is tolerant to high light intensities and other environmental stressors.³⁵ Attenuation of synechoxanthin production via genetic modification of its biosynthetic pathways renders the cyanobacterium significantly more susceptible to oxidative stress.³⁶ The bis-glycolipidated carotenoid di[6-*O*-oleoyl]- β -D-

glucopyranosyl)oxy]-astaxanthin (chlamydaxanthin, **5**) is produced by the snow alga *Chlamydomonas nivalis*. This alga is responsible for the “watermelon snow” phenomenon in which patches of snow become dark red as a result of increased carotenoid production during periods of peak light intensity.^{37,38} Notably, *Amphidinium carterae*, *Synechococcus* sp. PCC 7002, and *Chlamydomonas nivalis* have all been shown to possess polyunsaturated lipids.^{39–41}

For all three of these complex carotenoid natural products, isolation from the producing organisms in quantities and purities commensurate with in depth biological and biophysical analysis is not practically possible^{35,37,42} and this represents a major barrier toward studying their antilipoperoxidant potential. Moreover, there are no known biosynthetic methods for site-specifically ¹³C labeling these compounds for solidstate NMR studies. Chemical synthesis thus currently represents the only feasible means of accessing such molecules and their derivatives. However, these large and complex polyene scaffolds present a substantial synthetic challenge due to difficulties in controlling the stereochemistry at each double bond and their sensitivities to light, oxygen, and many reagents, such as protic and Lewis acids.

With the goal of eliminating such synthesis bottlenecks, we recently developed a frontier lego-like synthesis strategy, analogous to peptide coupling, in which a single reaction is employed iteratively to assemble bifunctional haloboronic acid building blocks protected as the corresponding *N*-methyl-iminodiacetic acid (MIDA) boronates.^{43–45} A key feature of this platform is that all of the required functional groups, oxidation states, and stereogenic centers are preinstalled into the building blocks, and these elements are faithfully translated into the growing products due to the mild and stereospecific nature of the Suzuki–Miyaura (SM) cross-coupling-based assembly. This strategy has been applied to access an array of structurally diverse small molecules, including natural products, materials, and pharmaceuticals.^{44–61} We have recently employed this approach to complete the first stereocontrolled total syntheses of peridinin and synechoxanthin on small scale.^{62,63}

Here we describe the application of modular synthesis to access the aforementioned structurally atypical carotenoid natural products produced by organisms that thrive in environments of extreme oxidative stress: peridinin, synechoxanthin, and chlamydaxanthin. Analysis of the antilipoperoxidant activity of these compounds and the ketocarotenoid astaxanthin in model membranes and human primary endothelial cells revealed that peridinin is a potent inhibitor of bilayer lipid peroxidation. Extensive biophysical studies, including state-of-the-art magic-angle spinning SSNMR experiments enabled by a MIDA boronate building block-based synthesis of a site-specifically ¹³C-labeled isotopolog, demonstrated that the potent activity of peridinin relative to astaxanthin is attributable to the unique propensity for peridinin to fully embed within and span the hydrophobic core of lipid bilayers, thus maximizing its effective molarity at the site of the targeted lipid peroxidation chemistry.

RESULTS AND DISCUSSION

Building Block-Based Syntheses of Candidate Natural Products.

The efficiency, flexibility, and stereospecificity of the MIDA boronate-based synthetic platform was initially leveraged to gain on-demand access to the quantities of peridinin necessary for extensive biophysical and biological evaluation (Scheme 1).⁶²

Several enabling features of the MIDA boronate functional group, including compatibility with many different reaction conditions and column chromatography,⁵⁷ facilitated efficient access to the four required building blocks, **BB**₁–**BB**₄. Synthesis of these building blocks is described in detail below in the context of accessing a site-specifically labeled peridinin isotopolog. We observed that the protection of vinyl and polyenyl boron species as the corresponding MIDA boronates imparted significant stability that is typically not observed with such complex boronic acids or boronate esters.

These four building blocks were readily assembled in a fully stereocontrolled fashion using only stereospecific Suzuki–Miyaura couplings in an iterative manner (Scheme 1). Specifically, NaOH-mediated deprotection of MIDA boronate **BB**₁ to the corresponding boronic acid and subsequent cross-coupling to butenolide **BB**₂ yielded tetraenyl MIDA boronate intermediate **6**. Attempts to hydrolyze **6** revealed the instability of the corresponding tetraenyl boronic acid, which translated to minimal or no formation of subsequently targeted cross-coupling product **7**.

We therefore devised a new strategy to reveal the reactivity of the boron terminus of **6** toward coupling yet maintain the integrity of the intermediate. Specifically, the MIDA boronate was directly transesterified to the corresponding pinacol boronic ester, which could be isolated before undergoing efficient boron-selective cross-coupling with all-*trans*-iodotrienyl MIDA boronate **BB**₃ to yield heptaene **7**. The utility and generality of this B-activation strategy for iterative cross-coupling (ICC) has since been further demonstrated by its application in the synthesis of numerous complex polyenyl intermediates, such as those prepared en route to C35-deoxy amphotericin B and the polyene motifs found in greater than 75% of all polyene natural products.^{47,64}

Applying a new method we developed for the stereoretentive SM cross-coupling of haloallenes,⁶² the final coupling of heptaenyl MIDA boronate **7** to **BB**₄ was successfully executed. Specifically, the *in situ* release⁶⁵ of the unstable but highly reactive heptaenyl boronic acid directly from the MIDA boronate was found to best promote coupling to **BB**₄ in good yield and with complete stereoretention to yield the protected carotenoid. Subsequent HF-pyridine-mediated desilylation afforded peridinin (**3**).

The demonstrated utility of the building block-based ICC strategy for the efficient synthesis of polyenes suggested that this approach could be further applied to readily access the similarly challenging-to-isolate aromatic carotenoid synechoxanthin (**4**).⁶³ The C₂-symmetric synechoxanthin structure indicated the opportunity to design a highly convergent route. We noted that in contrast to the standard iterative cross-coupling approach in which a boronic acid is united with the halide terminus of a bifunctional halo-MIDA boronate block,

the distribution of functional groups in synechoxanthin suggested that inverting the polarity of the bifunctional building blocks would be advantageous. Specifically, electron poor boranes generally couple inefficiently due to competing protodeboronation and homocoupling pathways,^{66,67} whereas electron deficient halides are often excellent coupling partners. Guided by the goal of positioning the electron withdrawing carboxymethyl groups on the halide coupling partners, synechoxanthin was retrosynthesized into three building blocks, **BB**₅–**BB**₇ as shown in Scheme 2.

Building block assembly commenced with the coupling of electronically activated aryl iodide **BB**₅ to the reactive pinacol boronic ester terminus of the novel bisborylated **BB**₆ to deliver the dienyl MIDA boronate **8** (Scheme 2). Maintenance of the optimal building block polarity required transformation of the MIDA boronate to the corresponding halide **9**, which was achieved with complete stereoretention via treatment with NaOMe and I₂. Subsequent coupling of the activated dienyl iodide **9** with a second equivalent of the **BB**₆ yielded the key tetraenyl MIDA boronate **10**. Analogous to the final coupling in the synthesis of peridinin, we found that in situ release and coupling of the unstable polyenyl boronic acid intermediate was optimal. In this case, the hydrolysis of two equivalents of **10** and bidirectional double cross-coupling to the activated halide *trans*-1-iodo-2-bromoethylene **BB**₇ afforded synechoxanthin bismethylester, which underwent carboxylic acid deprotection to give the natural product **4**.

We next sought to access the glycolipidated carotenoid chlamydxanthin (**5**). A previously reported preparation of this small molecule proceeded via the initial bis-glycosidation of astaxanthin followed by lipidation of the primary glucose hydroxyls to form the corresponding oleoyl esters.³⁷ Our attempts to utilize this route to access multimilligram quantities of chlamydxanthin were fruitless. To more efficiently access assayable quantities of this complex natural product, we thus designed an alternative modular semisynthetic route in which astaxanthin was bis-functionalized via stereocontrolled glycosidation with a sugar donor **BB**₈ that is prefunctionalized with the requisite oleic acid (Scheme 3). This approach is highly convergent, thereby minimizing the number of transformations through which the sensitive polyene core would be carried. Implementation of this strategy, however, necessitated a sugar protecting group which would facilitate both the stereoselective formation of the required β -glycosidic linkages via neighboring group participation and global deprotection under mild conditions that avoid hydrolysis of the sensitive primary lipid esters.

Pivaloate esters have been applied as C2–OH protective groups in glycosylation reactions to achieve near perfect β -selectivity via anchimeric assistance while sterically blocking orthoester formation. Removing such sterically bulky esters, however, typically requires harsh deprotection conditions. Toward addressing this challenge, a series of pivaloate groups containing a tethered masked nucleophile poised for intramolecular cyclization and release of the free alcohol have been developed.^{68–70} The redox sensitive nature of the chlamydxanthin polyene and hydrolytic sensitivity of the lipid esters necessitated particularly mild nucleophile unmasking conditions. Although not previously employed for glycosylations, we identified 4-(*tert*-butyl-dimethyl-silyl)oxy-2,2-dimethyl-butyric acid (TDMB, **13**), which was reported by Trost for the mild and selective protection and

deprotection of a secondary alcohol in the total synthesis of (+)-cyclophellitol,⁷⁰ as a promising candidate. We hypothesized that removal of the *tert*-butyl-dimethyl silyl (TBS) ether with buffered HF and subsequent mild base-catalyzed intramolecular cyclization would allow for selective TDMB removal.

Preparation of the desired sugar donor **BB**₈ began with selective acylation of the primary alcohol of **11** utilizing oleoyl chloride to afford **12** (Scheme 3a). Peracylation of **12** with TDMB **13** mediated by *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide (EDC) and dimethylaminopyridine (DMAP) was achieved to yield the desired product **14**. Initial attempts to oxidize the C1 thioether of **14** to the corresponding sulfoxide were plagued by limited conversion or decomposition, therefore we applied mercury trifluoroacetate-mediated hydrolysis conditions to access the hemiacetal **15** as a 1:1.4 α : β mixture of anomeric alcohols. Formation of the anomeric 2,2,2-trichloroacetimidate proceeded to provide **BB**₈ as the final sugar donor, anticipating a double Schmidt glycosidation reaction with astaxanthin.

The glycosidation of astaxanthin (($\pm$)-**2**) with **BB**₈ under 2-chloro-6-methylpyridinium triflate buffered conditions in hexanes or methylene chloride resulted in no conversion or undesired silyl transfer from TDMB to astaxanthin, respectively. After extensive optimization of solvent mixtures, time, and temperature, we found that a 2:1 mixture of hexanes and methylene chloride at 23 °C for 3 h sufficiently suppressed silyl transfer and provided the bis-glycosidated protected chlamydxanthin **16** (Scheme 3b). Conditions previously used to remove the TDMB directing group relied on the use of a protic acid to promote both alcohol desilylation and cyclization to the lactone.⁷⁰ Anticipating acid-mediated decomposition of the polyene, we investigated basic deprotection conditions. Buffered HF-pyridine readily removed all six TBS groups, but induced only minimal cyclization and TDMB removal. Alternatively switching to a two-step protocol in which the TBS groups were removed using HF-pyridine followed by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) base-mediated cyclization provided efficient access to chlamydxanthin (**5**).

Analysis of Antilipoperoxidant Activity.

With robust, modular access to peridinin, synechoxanthin, and chlamydxanthin in hand, we analyzed their antilipoperoxidant activities in direct comparison to astaxanthin. To specifically probe the inhibition of nonenzymatic bilayer lipid peroxidation and avoid the variability associated with use of lipid mixtures isolated from biologic sources,^{71,72} a fully synthetic chemically defined liposome system was optimized. Specifically, we prepared large unilamellar vesicles (LUVs) composed of 75% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 25% of the polyunsaturated phospholipid 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. These polyunsaturated fatty acyl chains contain a low concentration of lipid hydroperoxides (LOOH) derived from autoxidation,⁷³ exposure of which to CuCl₂ initiates lipid peroxidation.^{71,73} The extent of lipid peroxidation was quantified by measuring formation of the lipid decomposition product malondialdehyde (MDA) via HPLC analysis of the corresponding thiobarbituric acid adduct.⁷⁴

Consistent with prior reports, astaxanthin inhibited lipid peroxidation in this liposome-based assay, albeit with low potency.^{13,26} Specifically, >5 mol % astaxanthin relative to total lipid

was required to reach 90% inhibition of lipid peroxidation (Figure 2a). Head-to-head analysis of the antilipoperoxidant activity of the three atypical carotenoids in the same liposome-based system revealed that chlomydaxanthin demonstrated no improvement relative to astaxanthin. Synechoxanthin showed a moderate increase in activity. Remarkably, peridinin proved to be a potent antilipoperoxidant (Figure 2a). A concentration of only 0.5 mol % peridinin relative to lipid was sufficient to achieve nearly complete protection against nonenzymatic bilayer lipid peroxidation. This is more than an order of magnitude lower than the concentration of astaxanthin required to achieve similar activity in this same system. These results diverge from previous lipid peroxidation inhibition studies performed in chemically undefined liposomes prepared from egg yolk PC which concluded that peridinin was less or similarly effective relative to astaxanthin.⁷² Monitoring the time course of lipid peroxidation by tracking both MDA formation and consumption of the polyunsaturated arachidonoyl (20:4) fatty acyl phospholipid chain further demonstrated the superior activity of peridinin relative to astaxanthin (Figure 2b,c) in this liposome-based assay. While 0.5 mol % astaxanthin afforded modest inhibition of lipid peroxidation over the course of 10 h, 0.5 mol % peridinin provided almost complete protection.

We developed a complementary *in vivo* assay to quantify lipid peroxidation in primary human umbilical vein endothelial cells (HUVECs), a model system with physiologic relevance for atherosclerosis.⁷⁵ We found that incubating HUVECs in serum-free basal media leads to the reproducible onset of lipid peroxidation as judged by MDA quantification. Cell counts were measured at the beginning and end of the assay to confirm differences in MDA were not the result of differences in cell number (Figure S4).

Peridinin proved to be an effective inhibitor of lipid peroxidation in HUVECs, whereas astaxanthin was only minimally effective. Specifically, a concentration of 140 nM peridinin was sufficient to reach 50% inhibition of lipid peroxidation compared to >40 μ M astaxanthin required to reach the same threshold (Figure 2d, S5). Thus, in this primary endothelial cell-based assay, peridinin is >200 times more effective than astaxanthin.

Peridinin is also an order of magnitude more potent in HUVECs than the recently reported antilipoperoxidant OH-pen⁷⁶ (Figure S6). We further note that, as mentioned above and consistent with prior reports, we observed prolipoperoxidant activity of β -carotene in liposomes as evidenced by increased MDA formation relative to vehicle (Figure S1).^{13,14} In the same liposome-based assay, α -tocopherol showed activity similar to that of astaxanthin. In contrast, in HUVECs α -tocopherol was more potent than astaxanthin (Figure S2). The antilipoperoxidant activity of α -tocopherol in HUVECs may be primarily due to mechanisms other than direct inhibition of nonenzymatic bilayer lipid peroxidation. Notably, previous studies have demonstrated a diverse range of activities for α -tocopherol *in vivo*, including inhibition of lipoxygenase-mediated enzymatic lipid peroxidation,¹⁵ and modulation of inflammatory pathways via inhibition of protein kinase C and/ or transcription factors.¹⁶

Peridinin Concomitantly Inhibits Lipid Peroxidation and Monocyte Binding in Human Endothelial Cells.

With this highly potent inhibitor of lipid peroxidation in hand, we next sought to apply peridinin to probe the capacity of this small molecule to concomitantly inhibit lipid peroxidation and monocyte-endothelial cell adhesion in HUVECs.¹⁰ Switching the endothelial cells from complete to basal media resulted in an increase in both lipid peroxidation (Figure 2e) and monocyte binding (Figure 2f,g, S7). Upon HUVEC treatment with 2 μ M astaxanthin, we observed no reduction in lipid peroxidation and no change in monocyte binding. In contrast, treatment with 2 μ M peridinin caused a significant reduction in both lipid peroxidation and the number of bound monocytes (Figure 2e–g). These results are consistent with a model in which the bilayer lipid peroxidation-promoted binding of monocytes to human endothelial cells can be mitigated by a small molecule antilipoperoxidant, thus attenuating this hallmark atherosclerotic phenotype.¹¹ Enzymatically formed lipid oxidation products can also stimulate monocyte-endothelial cell adhesion, and may further contribute to this process.^{77,78}

Probing the Increased Potency of Peridinin Relative to Astaxanthin.

With the superior antilipoperoxidant potency of peridinin relative to astaxanthin established, we next sought to understand the underpinnings of this differential activity. Relative to astaxanthin, peridinin does not show an increased capacity to modify lipid lateral diffusion in POPC bilayers (Figure S8a–c)^{13,79–83} nor does it more rapidly react with various radical species (Figure S8d,e).^{19,73,84,85}

Alternatively, light microscopy analysis of the HUVECs employed in the aforementioned monocyte binding assay revealed an intriguing difference. Specifically, large dark extracellular aggregates were observed in the samples incubated with astaxanthin, but no such aggregates were observed in the peridinin-treated cells (Figure 2g, S7c,d). This suggested that the two carotenoids may possess different capacities to localize in cellular or lipid membrane environments. Specifically, we questioned whether peridinin may more readily insert into lipid bilayers, thereby increasing its effective molarity at the site necessary to promote antilipoperoxidant activity, while astaxanthin may self-aggregate extramembranously.

Peridinin has a Higher Effective Molarity in Lipid Bilayers.

Carotenoid localization and/or orientation with respect to the lipid bilayer has previously been proposed to have an important effect on antilipoperoxidant activity,^{17,86–88} but experimentally demonstrating this relationship has proven to be challenging. A wide range of biophysical studies applying UV–visible spectroscopy,⁸⁹ fluorescence spectroscopy,⁸⁶ X-ray diffraction,¹³ differential scanning calorimetry,⁹⁰ linear dichroism,⁹¹ and computational modeling^{17,86} have all been interpreted as supportive of a model in which polar carotenoids, such as astaxanthin, are primarily membrane-embedded and align approximately parallel to the bilayer normal. Definitively assigning the relative membrane localization and alignment requires Ångstrom-level resolution, however, and this is challenging or impossible to achieve with most of these biophysical techniques.

We thus applied a paramagnetic relaxation enhancement (PRE)-based magic-angle spinning (MAS) SSNMR experiment, which is based upon the pioneering theoretical work of Solomon⁹² and has been extended to quantitative analysis of protein structure by the Jaroniec group⁹³ and for studies of protein immersion depth by Lorigan and co-workers.^{94,95} In a recent study, we advanced this approach specifically for the purpose of characterizing small molecule-membrane interactions with high spatial resolution.⁹⁶ In this experiment, the PRE for a ¹³C nucleus can be quantified by measuring the change in longitudinal relaxation rate ($R_1 = 1/T_1$) upon addition of a lipid-appended stable radical spin label, such as 4,4-dimethyloxazolidine-*N*-oxyl (DOXYL). The PRE magnitude depends on $1/r^6$ (where r is the distance from the radical to the ¹³C nucleus), resulting in especially large ($>1 \text{ s}^{-1}$) effects for distances up to $\sim 10 \text{ \AA}$ and still significant ($>0.1 \text{ s}^{-1}$) values for distances up to $20 \text{ \AA}$; this capability enables differentiation between intramembranous vs extramembranous carotenoid populations (Figure 3a).

The PRE experiments required access to highly ¹³C-enriched astaxanthin and peridinin. Uniformly ¹³C-enriched astaxanthin (U-¹³C-astaxanthin, **17**) was produced via biosynthesis utilizing the red yeast *Xanthophyllomyces dendrorhous* strain UBV-AX2 with fully ¹³C-labeled glucose as the primary carbon source.⁹⁷ This growth protocol yielded 65% ¹³C incorporation (Figure 3b).

Because of the challenges associated with the production and isolation of peridinin from its producing organism, this compound was not amenable to a similar biosynthetic strategy for isotopolog generation. However, leveraging the inherent efficiency and flexibility of our MIDA boronate building block-based total synthesis, we were able to rapidly access site-specifically ¹³C-labeled derivatives simply by incorporating the corresponding site-selectively labeled building blocks into the same synthetic route.⁶² Because they represent sites on peridinin that span the polyene core and have resolved carbon signals in the ¹³C spectrum, we selected the C7 and C7' polyene carbons near the terminal rings contained in BB₁ and BB₄, and C15 and C15' carbons at the center of the polyene contained in BB₃ for ¹³C enrichment (Figure 3c, **18**).

Applying the route developed by de Lera and co-workers,⁹⁸ installation of the ¹³C-labeled C7 and C7' carbons in BB₁ and BB₄ commenced with lithiation and trapping of vinyl iodide **19** with ¹³C paraformaldehyde to access allylic alcohol **20** (Scheme 4a). Subsequent Sharpless epoxidation, Swern oxidation, and Colvin rearrangement furnished alkyne **23**. This intermediate was elaborated to either ¹³C BB₁ via hydroboration and transesterification to the corresponding MIDA boronate,⁶² or to ¹³C BB₄ through TBS deprotection, iodoallene and acetate formation, and TMS protection (Scheme 4c).⁹⁸

The synthesis of ¹³C₂-labeled BB₃ began with bis-¹³C labeled trimethylsilylacetylene **25** (Scheme 4b). Alkyne germylation, silyl deprotection,⁹⁹ and hydrostannylation¹⁰⁰ yielded the ¹³C₂ bismetalated olefin **27**. Selective Stille coupling at the vinyl stannane to dienyl iodide **28** followed by iododegermylation afforded ¹³C₂ BB₃.⁶² The three ¹³C-labeled blocks and natural abundance BB₂ were iteratively cross-coupled with complete stereospecificity and subjected to global desilylation to yield C7, C7', C15, C15' ¹³C-labeled peridinin (¹³C₄-peridinin, **18**) (Figure S9).

For solid-state NMR studies, we first prepared a sample of U- ^{13}C -astaxanthin (**17**) in POPC bilayers as MLVs and used a series of ^{13}C chemical shift correlation experiments (including 2D ^{13}C - ^{13}C with supercycled POST-C7 (SPC7) mixing^{101,102} and 3D ^{13}C - ^{13}C - ^{13}C with SPC7 and DARR mixing¹⁰³ in the first and second indirect dimensions) to *de novo* assign the ^{13}C resonances of astaxanthin (Figure S10a,b). Interestingly, the U- ^{13}C -astaxanthin spectra exhibited multiple cross peaks that were uniquely assignable to at least two states, specifically a major and at least one minor state (Figure 4a).

A second sample was similarly prepared with $^{13}\text{C}_4$ -peridinin (**18**) in POPC bilayers, and we assigned ^{13}C resonances from the corresponding well resolved solution phase ^{13}C shifts (Figure S10c,d). In contrast to the multiple states observed for astaxanthin, the $^{13}\text{C}_4$ -peridinin samples showed only a single set of sharp peaks consistent with a single peridinin population (Figure 4b).

PRE measurements were next performed for a POPC-only control, the U- ^{13}C -astaxanthin-POPC sample, and the $^{13}\text{C}_4$ -peridinin-POPC sample in the presence of 5 mol % 16-DOXYL-PC. These experiments were performed in accordance with published procedures⁹³⁻⁹⁶ utilizing a standard ^{13}C inversion recovery pulse sequence with 20 recovery time values. Specifically, under identical experimental conditions, samples without and with the DOXYL reagent were measured and T_1 values determined by integrating the signal intensity for each signal at each time point, and then explicitly fitting the data to single or double-exponential expressions (eqs 1 and 2 in the Supporting Information). Pulse delays were sufficiently long to ensure near-equilibrium magnetization. The populations as extracted from these fits were in excellent agreement with the ratio obtained by direct integration of isolable signals, and consistent with the conclusion that astaxanthin exists in at least two states. Additional details of the fitting programs and specific methods utilized are included in the Supporting Information.

For a POPC-only control, measurement of the PRE for the POPC carbons demonstrated a maximum value on the $\omega-1$ and $\omega-2$ carbons and a steady decrease in PRE value for carbons approaching the phosphatidylcholine headgroup (Figure S11). These results were observed in all three samples and are consistent with a well-formed membrane in which the 16-DOXYL spin label assumes a position near the center of the bilayer.

Analysis of the T_1 relaxation curves for astaxanthin carbons 1-4, 6, and multiple polyene carbons in the U- ^{13}C -astaxanthin sample revealed unambiguously that a single-exponential fit was not sufficient, but a two-exponential component fit yielded excellent agreement (Figure S12, S13). Specifically, fitting the entirety of the data with a single-exponential decay model resulted in chi-squared values of 35.6 and 90.0 for samples without and with 16-DOXYL-PC respectively, whereas the double-exponential model reduces the chi-squared values to 14.6 and 20.7 for samples without and with 16-DOXYL-PC. The smaller chi-squared values in the latter case are consistent with a major astaxanthin population that exhibits negligible PRE ($<0.1\text{ s}^{-1}$) and one or more minor astaxanthin populations with PRE values of at least 1 s^{-1} (and for several sites, $>4\text{ s}^{-1}$) (Figure 4c). In addition, for carbon 6 in particular, we observed two resolved peaks in the ^{13}C 1D spectrum, consistent with a major and a minor population, which demonstrate negligible PRE for the major population signal

and a strong PRE for the minor population signal (Figure 4c, S14). These results are consistent with a model in which astaxanthin exists in at least two states. In this model, the majority of astaxanthin resides >20 Å from the spin label, i.e., outside the hydrophobic core of the lipid bilayer, and one or more minor populations of astaxanthin (estimated to account for roughly 10–20%) reside close to the spin label within the lipid bilayer.

When we alternatively analyzed the $^{13}\text{C}_4$ -peridinin POPC sample, we observed a large (>3 s $^{-1}$) PRE for all four labeled carbons (Figure 4d, S17). Spin-diffusion experiments, in which the mobile water and lipid acyl chain signals are selected by a T_2 filter and correlated to the ^{13}C signals of the small molecule,¹⁰⁴ provided a second line of evidence firmly in support of peridinin being primarily membrane-inserted (Figure 4e, S19). Specifically, strong lipid-peridinin but no water-peridinin correlations were observed for carbons 15 and 15' of peridinin. Thus, in contrast to the results with astaxanthin, the single peridinin population is fully embedded in the hydrophobic core of POPC bilayers.

Intriguingly, we also noted that for the $^{13}\text{C}_4$ -peridinin sample containing the 16-DOXYL spin label, carbons 15 and 15' of peridinin showed a larger PRE (8–10 s $^{-1}$) than the 7 and 7' carbons (3–4 s $^{-1}$) (Figure 4d). This suggests that peridinin is oriented nearly parallel to the bilayer normal and thus spans the hydrophobic core, maximizing the effective molarity of its polyene motif to the site of lipid peroxidation. Further evidence supporting this model came from the spin-diffusion experiments for the 7 and 7' carbons. Strong lipid-peridinin spin-diffusion was again observed, and interestingly, weaker but clearly observable water-peridinin spin diffusion was now observed (Figure 4f, S19).

To further probe this putative orientation of peridinin in the bilayer, we performed a systematic series of PRE experiments, each employing different PC lipids with DOXYL spin labels on carbons 5, 7, 10, 12, 14, or 16 (Figure 5a). The relative magnitude of the PRE values observed for carbons 7/7' and 15/15' across these experiments is consistent with a peridinin orientation parallel to the bilayer normal (Figure 5b, S20). Specifically, for the 5-DOXYL-PC and 7-DOXYL-PC probes, a larger PRE was observed for C7/7' relative to C15/15'. The opposite was observed for the 14-DOXYL-PC and 16-DOXYL-PC lipids, and a transition between these two extremes was observed with the intervening probes.

Thus, in contrast to the primarily extramembranous localization of astaxanthin, peridinin is fully embedded within and spans the hydrophobic core of lipid bilayers. We conclude that the corresponding increase in effective molarity at the site of the targeted lipid peroxidation chemistry likely enables peridinin to act as a potent inhibitor of nonenzymatic bilayer lipid peroxidation. Furthermore, these experiments describe the localization of astaxanthin relative to model membranes and demonstrate for the first time that the majority of astaxanthin lies outside the lipid bilayer. Thus, the lack of potency observed for astaxanthin is likely due, at least in part, to its propensity to preferentially localize extramembranously.

CONCLUSION

These findings collectively demonstrate that peridinin is a potent and membrane-embedded small molecule antilipoperoxidant, in contrast to astaxanthin, which is less potent and is

primarily localized outside the lipid bilayer. Furthermore, the peridinin-mediated inhibition of bilayer lipid peroxidation mitigates monocyte-endothelial cell adhesion to HUVECs, supporting a mechanism by which bilayer lipid peroxidation may contribute to atherogenesis. Detailed, direct comparisons of the biological and biophysical properties of astaxanthin and peridinin revealed a potentially generalizable design principle for guiding the development of other small molecule antilipoperoxidants. Specifically, antilipoperoxidant activity can be increased by maximizing the effective molarity of small molecules within the hydrophobic core of lipid bilayers. These findings also provide a mechanistic explanation for the weak antilipoperoxidant activity of astaxanthin observed here and in previous studies.

This study exemplifies the enabling potential of building block-based synthesis to illuminate complex small molecule function.^{44,45} The efficient and flexible nature of our route to peridinin readily enabled access to this natural product as well as rapid generation of a site-selectively ¹³C-labeled derivative. This modular approach to synthesis has widespread potential to enable better understanding and optimization of complex small molecule function. Combining synthesis with frontier SSNMR methods further provided the opportunity to probe membrane localization with resolution that is unmatched by other widely used biophysical techniques. It has been proposed that carotenoid localization and orientation with respect to the lipid bilayer may influence antilipoperoxidant activity,¹³ yet assessment of this connection has remained limited by the lack of high-resolution methods for assessing small molecule-membrane interactions. Application of the SSNMR PRE experiment allowed assessment of this relationship for astaxanthin and peridinin with unprecedented resolution. While all of our data are consistent with this biophysical model, we note that peridinin may also exert biological activity via binding to as-of-yet unidentified protein targets.

This same combination of synthesis and SSNMR has the additional potential to systematically interrogate the chemical features of peridinin which promote membrane insertion, and alternatively, those of astaxanthin which facilitate extramembranous localization. The aggregation behavior of numerous polar and apolar carotenoids has been demonstrated in hydrated organic solution and lipid environments.^{89,105,106} Astaxanthin aggregation in hydrated organic solutions has been attributed to its planarity, symmetry, and capacity for intermolecular hydrogen bonding.¹⁰⁷ These structural features may facilitate the formation of extramembranous aggregates stabilized by intermolecular hydrogen bonding between the α -hydroxy ketone motifs of adjacent molecules and π - π stacking interactions of the polyene.¹⁰⁷ Alternatively, peridinin possesses an atypical nonsymmetric and nonplanar structure, which is the result of five tailoring enzyme modifications of the typical carotenoid scaffold of zeaxanthin.¹⁰⁸ We speculate that these alterations decrease the affinity of peridinin for self-aggregation and bias insertion within the hydrophobic core of lipid bilayers, thus contributing to the observed potent antilipoperoxidant activity. This suggests that similar nonplanar allene-containing carotenoids such as fucoxanthin, mimulaxanthin, paracentrone, dinoxanthin, and neoxanthin may also more readily embed within membranes and therefore function as potent antilipoperoxidants.^{109,110} Synergizing the building block-based synthesis of a series of carotenoids with SSNMR methods would enable testing of these hypotheses.

Peridinin may also have broader potential to act as an effective chemical probe to better understand the still enigmatic role of bilayer lipid peroxidation in the pathogenesis of many other human diseases, including asthma,^{111,112} intracerebral hemorrhage,¹¹³ and Alzheimer's disease.^{114,115} Peridinin may also represent a promising starting point for the development of pharmaceuticals that selectively inhibit nonenzymatic bilayer lipid peroxidation. For example, the oxidation of lipids in an alternative location, namely low density lipoprotein (LDL) particles, has been shown to play a key role in atherogenesis.¹¹ Oxidized LDL (oxLDL) in the subendothelial space of the arterial wall activates signaling pathways resulting in the binding, migration, and differentiation of circulating monocytes.¹¹ The success of small molecule drugs that reduce LDL concentrations in the bloodstream and thereby reduce cardiovascular events in patients has provided clinical evidence supporting the importance of this mechanism.¹¹⁶ A similar strategy predicated upon reducing the quantity of oxidizable substrate is not viable for inhibiting bilayer lipid peroxidation-mediated monocyte binding, as the substrate in this case is endogenous phospholipids within endothelial cell bilayer membranes. Although further studies are required to establish whether free radical lipid peroxidation reactions may be a causative factor in the pathogenesis of atherosclerosis, our findings suggest that highly effective small molecule inhibitors of bilayer lipid peroxidation may minimize monocyte adhesion and thereby provide benefit. Such an antilipoperoxidant might be particularly impactful for patients deficient in antilipoperoxidant proteins such as GSTs known to possess an increased risk for atherosclerosis.^{4,5} Deficiencies in these same protein antilipoperoxidants have also been associated with increased risk for asthma⁶ and Alzheimer's disease.⁷

In summary, peridinin is a potent and membrane-embedded inhibitor of bilayer lipid peroxidation and likely has much more to teach us about how the biology-illuminating and therapeutic potential of small molecule bilayer antilipoperoxidants might be realized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Carotenoid antiliperoxidants. (a) β -Carotene and astaxanthin are commonly applied as small molecule antiliperoxidants. (b) The structurally atypical carotenoids peridinin, synechoxanthin, and chlamydaxanthin.

Figure 2.

Peridinin is a potent antilipoperoxidant. (a) Lipid protection afforded by the commonly applied antilipoperoxidant astaxanthin and the three atypical carotenoids chlamydaxanthin, synechocanthin, and peridinin in liposomes at 10 h as determined by HPLC quantification of the lipid oxidation product MDA. (b) Time course for inhibition of MDA formation and (c) arachidonoyl (20:4) phospholipid chain consumption by astaxanthin and peridinin in liposomes. (d) Small molecule-mediated lipid protection in primary HUVECs at 2 h as determined in panel a. (e) Effect of 2 μM astaxanthin and peridinin on MDA formation in primary HUVECs incubated in basal media. (f) Binding of primary CD14⁺ monocytes to primary HUVECs. (g) Representative images of monocytes bound to HUVECs. Arrow indicates a monocyte. Scale bar equals 100 μm . Data are mean $\pm$ s.d. for panels a–c and mean $\pm$ s.e.m. for panels d–f; $n = 3$ –16 biological replicates; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant; * is comparison to vehicle; # is comparison to astaxanthin.

Figure 3.

SSNMR paramagnetic relaxation enhancement effect (PRE) experiment to determine the relative bilayer localization of peridinin and astaxanthin. (a) No PRE effect between a 16-DOXYL-PC spin label and a ^{13}C carotenoid nuclei is expected for an extramembranously localized species, while a robust PRE effect is expected for a membrane inserted species. (b) U- ^{13}C -astaxanthin was biosynthesized with 65% ^{13}C incorporation utilizing the red yeast *X. dendrorhous*. (c) Building block-based route for site-specifically ^{13}C -labeled peridinin.

Figure 4.

Astaxanthin localizes primarily extramembranously and peridinin inserts within lipid membranes. (a) ^{13}C - ^{13}C cross correlation spectrum of U- ^{13}C -astaxanthin in POPC MLVs by SSNMR. In insets, major population peaks are dark blue, minor population peaks are light blue, and ambiguous peaks are black. (b) ^{13}C - ^{13}C SPC7 correlation spectrum of $^{13}\text{C}_4$ -peridinin in MLVs by SSNMR. Peridinin peaks are red and POPC peaks are black. (c) PRE effect of 16-DOXYL-PC on U- ^{13}C -astaxanthin in MLVs by SSNMR. Data for the major population is on the left and minor population(s) is on the right. (d) PRE effect of 16-

DOXYL PC on $^{13}\text{C}_4$ -peridinin in MLVs by SSNMR. (e,f) Spin diffusion from water and POPC to the peridinin 7 and 15 carbons. Data are mean $\pm$ s.e.m.; $n = 8$ technical replicates for panels c and d.

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Figure 5.

Peridinin inserts within lipid membranes approximately parallel to the bilayer normal. (a) DOXYL-PC spin label series experiment to determine the relative orientation of peridinin within the lipid bilayer. (b) PRE effect from $^{13}\text{C}_4$ -peridinin to the 5-, 7-, 10-, 12-, 14-, and 16-DOXYL-PC spin label series. Data are mean $\pm$ s.e.m.; $n = 8$ technical replicates.

Scheme 1.
Building Block Synthesis of Peridin (3)⁶²

Scheme 2.
Building Block Synthesis of Synechoxanthin (4)⁶³

Scheme 3.
Synthesis of the Sugar Donor BB₈ and Chlamydxanthin (5)

Scheme 4.
Synthesis of ^{13}C -Labeled Peridinin Building Blocks