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Letter

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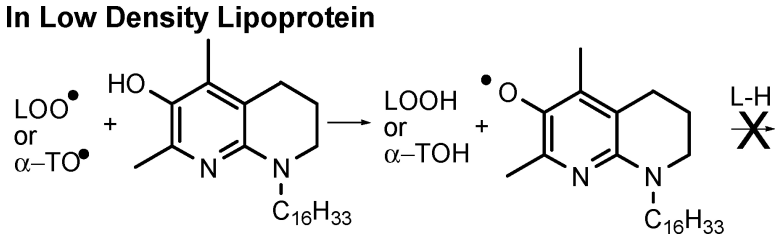

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Lipid-Soluble 3-Pyridinol Antioxidants Spare α-Tocopherol and Do Not Efficiently Mediate Peroxidation of Cholesterol Esters in Human Low-Density Lipoprotein

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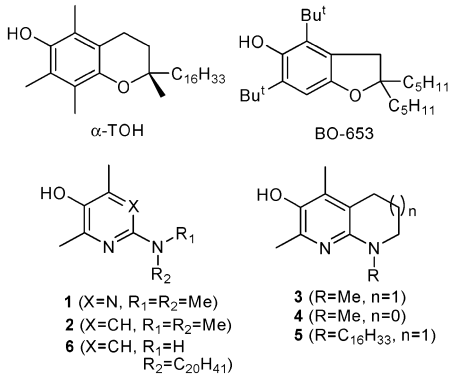
Abstract: Because α-tocopherol (α-TOH) mediates the peroxidation of cholesterol-esterified lipids in human low-density lipoprotein (LDL) in vitro and has displayed disappointing results against the onset and development of atherosclerosis, it may not be appropriate for use as a therapeutic in the prevention and/or treatment of the disease. Herein are described the experimental results demonstrating that 3-pyridinols spare α-TOH, do not efficiently mediate lipid peroxidation, and protect lipoprotein tryptophan residues in human LDL.

The widely accepted oxidation theory of atherosclerosis is based on the observation that the oxidative modification of low-density lipoprotein (LDL), believed to be initiated by the radical-mediated peroxidation of cholesterol-esterified lipids, can lead to an increased and uncontrolled uptake of cholesterol by macrophages.[1] This event triggers a cascade of cellular processes that lead to the formation of fatty streaks and eventually atherosclerotic lesions in the artery wall. Vitamin E, of which α-tocopherol (α-TOH) is the most potent form, is the major lipid-soluble antioxidant in blood plasma and LDL. Given that it is one of the most effective peroxyl-radical-trapping antioxidants known,[2] the potential of α-TOH supplementation as a preventive or therapeutic strategy to combat atherosclerosis has been the subject of many epidemiological and biochemical studies. The results of these clinical trials have been disappointing, prompting the question of whether α-TOH is an appropriate antioxidant for use as a therapeutic in the prevention or treatment of the disease.[3] Indeed, Ingold, Stocker, and co-workers have shown that in the absence of coantioxidants such as ascorbate (vitamin C) or reduced coenzyme-Q_{10}, α-TOH mediates the peroxidation of LDL lipids, rendering it a prooxidant under these conditions.[4]

Recently, we reported that substituted phenolic compounds containing one or two nitrogen atoms in the aromatic ring (3-pyridinols[5] and 5-pyrimidinols[6]) are extremely effective peroxyl-radical-trapping chain-breaking antioxidants in homogeneous organic solutions. For example, **1**−**4** react with peroxyl radicals with rate constants 2- to 90-fold greater than that measured for their reaction with α-TOH. These results prompted



us to explore whether the greater reactivity of these compounds would enable them to protect LDL lipids from peroxidation in the absence of coantioxidants by not mediating the chain reaction as does α-TOH. Herein we describe the results of experiments in which lipid-soluble variants of two of these compounds (**5** and **6**) spare α-TOH, do not mediate the peroxidation of cholesterol esters, and inhibit the oxidation of tryptophan residues in human LDL.[7]

The lipid-soluble 3-pyridinols **5** and **6** were prepared by a modification of our published route to **3** (see Supporting Information for details).[5] The compound was incorporated into human LDL by addition of a small volume of **5** as a concentrated solution in DMSO to plasma freshly separated from whole blood (final plasma concentration of **5** of 75 and 120 μM and <3% v/v DMSO). The supplemented LDL was subsequently isolated from the plasma using sequential density ultracentrifugation as described elsewhere.[8] To remove low molecular mass compounds such as KBr, aqueous antioxidants, and unincorporated **5**, the LDL was passed through two consecutive PD-10 gel filtration columns, eluting with cold phosphate-buffered saline (PBS). Upon disruption of the LDL and analysis of its constituents by RP-HPLC with electrochemical (EC) detection (0.5 V), an average of 7 and 20 molecules of **5** were found per LDL particle in each of the supplemented mixtures along with an average of 5 molecules of endogenous α-TOH. Throughout, we will refer to these two supplemented mixtures as sLDL-7 and sLDL-20, respectively, and to the native, unsupplemented LDL as nLDL. All three samples were oxidized simultaneously under exactly the same conditions, and the data are shown in Figure 1 (see figure caption for experimental details).[9]

The oxidation of nLDL (panel A) proceeded as we have reported previously[8b] and as others[4] have found when using similar water-soluble azo-initiator-derived peroxyl radicals. The endogenous α-TOH is steadily consumed over the first 4 h and does not provide any protection against oxidation of the cholesterol-esterified linoleate, with the rate of peroxidation at a maximum while α-TOH is present (R_p = 48 μM h^{-1} from 0 to 3 h). The oxidations of sLDL-7 and sLDL-20 (panels B and C) display dramatically different profiles. In both samples,

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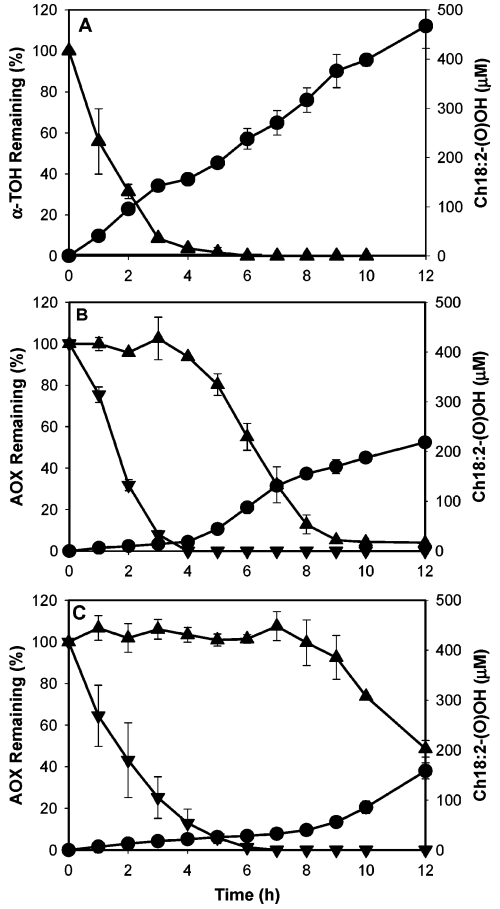

Figure 1. Oxidation of human LDL (0.75 mg/mL protein in PBS) initiated by decomposition of the azo initator C-0 (0.5 mM final concentration) at 37 °C. Total cholesterol linoleate hydroperoxides (●) were monitored at 234 nm by HPLC following their reduction to the corresponding alcohols with PPh_3, and antioxidant (AOX) consumption was monitored by RP-HPLC with EC and UV detection, α-TOH (▲) and **5** (▼) at 295 and 330 nm, respectively: (A) nLDL; (B) sLDL-7; (C) sLDL-20.

the endogenous α-TOH is spared in favor of consumption of the more reactive pyridinol. α-TOH is consumed only following the disappearance of the pyridinol (at about 4 and 7 h for sLDL-7 and sLDL-20, respectively). Furthermore, R_p is reduced by 1 order of magnitude in the presence of the pyridinol (4.6 and 5.1 μM h^{-1} in sLDL-7 (0−3 h) and sLDL-20 (0−6 h), respectively) relative to that determined in the presence of α-TOH in nLDL. In direct contrast to the foregoing, supplementing LDL with additional α-TOH increases the rate of lipid peroxidation.[10]

The observed results can be ascribed to two important properties of **5**, which can be inferred from model studies of the analogous N-methylated derivative **3**.[5a] First, **5** is ∼30-fold more reactive toward peroxyl radicals than is α-TOH. Second, the aryloxyl radical derived from **5** is 2 kcal/mol more stable than that derived from α-TOH and, as such, is expected to be a poorer mediator of the peroxidation chain reaction:

$$\alpha\text{-TO}^{\bullet} + \text{lipid-H} \rightarrow \alpha\text{-TOH} + \text{lipid}^{\bullet} \qquad (1)$$

Oxidation of LDL surface phospholipids and apoprotein B-100 (apoB-100), the 4536 amino acid protein associated with LDL, is not inhibited by α-TOH.[1,3,11] The

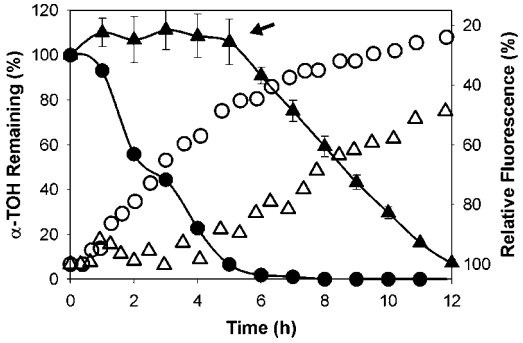

Figure 2. Relative tryptophan fluorescence (open symbols, $\lambda_{ex} = 295$ nm, $\lambda_{em} = 326$ nm) and consumption of α-TOH (closed symbols) monitored for a solution of human LDL with $[\alpha\text{-TOH}]_0 = 8$/particle (○, ●) and a pyridinol-supplemented sample with $[\mathbf{5}]_0 = 5$/particle (△, ▲) over the course of a C-0 initiated oxidation such as that in Figure 1. The arrow indicates the time at which **5** was consumed in the supplemented LDL.

oxidation of these important LDL constituents is thought to be critical in the decreased recognition of the lipoprotein by the LDL receptor and its increased recognition and uptake by macrophages.

We have begun to examine the effect of **5** and **6** on LDL phospholipid and protein oxidation. We have found that (1) phosphatidylcholine-esterified linoleates in the phospholipid-rich surface of the LDL particle are more resistant to oxidation when a pyridinol is present than those in the native LDL (Figure S1 of Supporting Information).[12] (2) The fluorescence of highly oxidizable apoB100 tryptophan residues, ablated as they are oxidized,[13] is preserved in the presence of a pyridinol (Figure 2). (3) Lipogel electrophoresis of pyridinol supplemented LDL is unchanged under conditions of oxidation in which native LDL is substantially altered, as evidenced by its electrophoretic mobility (Figure S2). Taken together, these observations suggest that the pyridinols preserve LDL better than endogenous antioxidants under conditions of oxidative stress.

BO-653,[14] a synthetic phenolic peroxyl-radical-trapping antioxidant, has recently shown promise in preventing LDL oxidation in the absence of coantioxidants and is now in phase II clinical trials following very encouraging in vivo studies that demonstrated reduced neointimal thickening in restenosis (arterial blockage following angioplasty) models and reduced atherosclerotic lesions in normal and transgenic mice.[14c] Given that **5** is much more reactive to peroxyl radicals in homogeneous organic solution than BO-653 (which possesses reactivity similar to that of α-TOH)[14a] but yields an aryloxyl radical that is similarly unreactive toward lipoprotein lipids,[14b] it appears that it, and lipid-soluble analogues of other pyridinol and pyrimidinol antioxidants (including **6**), should be studied further both as antioxidants in LDL and for their potential antiatherogenic properties in animal models.[15]

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Supporting Information Available: Supplemental oxidation data (Figures S1 and S2) and preparation of and spectral data for pyridinols **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Studies of pyridinol oxidation to be published in due course suggest that quinone-imines are among the products formed. The reactivity of these compounds is important with regard to the in vivo efficacy of the compounds.

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