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SHORT COMMUNICATION

Potent Inhibitory Activities of Hydrophobic *aci*-Reductones (2-Hydroxytetronic Acid Analogs) against Membrane and Human Low-Density Lipoprotein Oxidation

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ABSTRACT. The effects of selected *aci*-reductones, which are hydrophobic ascorbate-related analogs including 4-chlorophenyl-2-hydroxytetronic acid (Cpd A), 4-(1,1′-biphenyl)-2-hydroxytetronic acid (Cpd B), and 4-(4′-chloro-1,1′-biphenyl)-2-hydroxytetronic acid (Cpd C), on membrane and low density lipoprotein (LDL) oxidation were assessed. Hepatic microsomal lipid peroxidation was induced by the ascorbate + Fe(II) chemical system. All three agents inhibited membrane lipid peroxidation in a concentration-dependent manner with the order of potency: Trolox (vitamin E) $\leq$ Cpd A $\ll$ Cpd B $<$ Cpd C; based on the EC_{50} values, Cpd B and Cpd C were 11- and 19-fold, respectively, more potent than Trolox. In contrast to ascorbic acid, all three agents did not display any membrane prooxidative effect in the presence of iron. When human LDL was incubated with 10 μM of Cu(II), LDL oxidation, determined by the formation of thiobarbituric acid reactive substances, followed a typical sigmoidal curve with an initial lag phase. Preincubation of the LDL samples with low micromolar concentrations (1 and 3 μM) of each agents for 30 min before the addition of copper resulted in significant delays of the lag time of LDL oxidation, and the effectiveness of Cpd B and Cpd C was more prominent than that mediated by either Trolox or probucol. Since clinical evidence strongly supports the hypothesis that atherogenesis is initiated by LDL oxidation, the results suggest that these aryl tetronic acid analogs may serve as promising candidates for future therapeutic use as anti-atherogenic agents. BIOCHEM PHARMACOL **55**;11:1921–1926, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ascorbic acid-derived hydrophobic *aci*-reductones; antioxidant activity; copper-induced LDL peroxidation; anti-atherogenic effect

Free radical reactions are believed to participate in the pathogenesis of a wide variety of diseases including cardiovascular diseases such as atherosclerosis, coronary heart disease, and restenosis [1, 2]. Thus, identification of synthetic agents with enhanced antioxidant therapeutic potentials as compared to natural agents remains an area of significant interest and clinical relevance. Previous work from Witiak's laboratory identified several *aci*-reductones that are structurally related to the tetronic acid function in ascorbic acid, some of which exhibited antilipidemic and anti-atherosclerotic properties [3, 4]. Of the agents studied, the 4-aryl-2-hydroxytetronic acids were particularly promising. Because these agents retain the redox activity of ascorbic acid but gain hydrophobicity to varying degrees, it is suggested that these agents might function as lipophilic antioxidants capable of interrupting free radical chain reactions. Because lipid peroxidation of LDL‖ is currently thought to play a critical role in atherogenesis [5, 6], the present study was designed to assess the membrane antioxidant activity of a selected series of newly synthesized 2-hydroxytetronic acid analogs and their relative ability to inhibit LDL oxidation *in vitro*.

MATERIALS AND METHODS

Chemicals and Materials

The selected hydrophobic tetronic acids often referred to as *aci*-reductones (Fig. 1), i.e. 4-(4-chlorophenyl)-2-hydroxytetronic acid (Cpd A or CHTA), 4-(1,1′-biphenyl)-2-hydroxytetronic acid (Cpd B or TX-153), and 4-(4′-chloro-1,1′-biphenyl)-2-hydroxytetronic acid (Cpd C or

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‖ *Abbreviations*: Cpd, compound; LDL, low density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; and WHHL, Watanabe heritable hyperlipidemic rabbits.

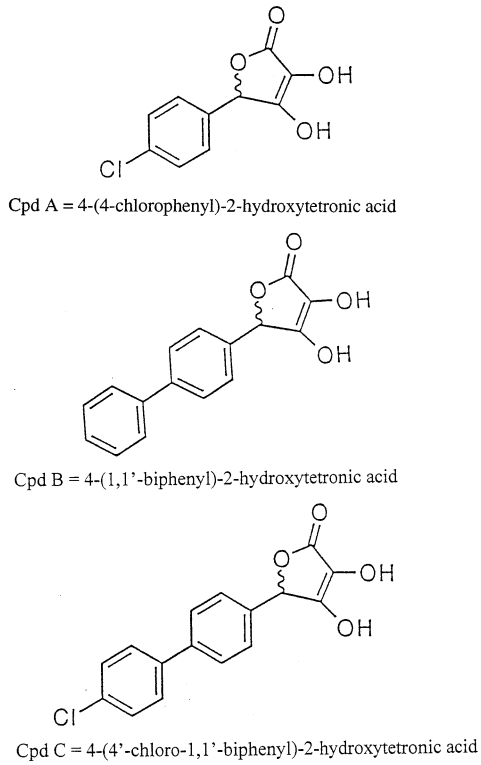

FIG. 1. Chemical structures of the 2-hydroxytetronic acid analogs studied.

TX-117) were provided by OXIS International Inc. Aliquots of LDL (L-2139) from human plasma, as well as most other chemicals, were obtained from Sigma. Sephadex G-25 M (PD-10) columns were purchased from Pharmacia Biotech.

Membrane Lipid Peroxidation Induced by Ascorbic Acid + Fe(II)

Hepatic microsomal membranes from male Sprague–Dawley rats (250 g) were isolated by differential centrifugation as described previously [7, 8]. The membranes (0.2 mg protein/mL) in PBS (150 mM of NaCl, 10 mM of potassium phosphate, pH 7.4) were preincubated with the 2-hydroxytetronic acid analogs (0.067 to 6.7 μM) for 20 min at 30° before the final addition of ferrous sulfate (10 μM) and ascorbic acid (100 μM). After 20 min of incubation, membrane lipid peroxidation and the extent of drug-mediated inhibition were determined by the colorimetric TBARS method [9]. To avoid nonspecific color formation, 0.01% butylated hydroxytoluene was included during the heating step at 80° [10]. Concentrations of all three analogs up to 100 μM were found not to interfere with the TBARS assay. To determine the potential prooxidant effects of these analogs, the agents at different concentrations (10–500 μM) were incubated with the microsomes in the presence of 10 μM of $FeSO_4$.

LDL Oxidation and Lag-Time Determination

The EDTA content of the LDL samples was removed by gel filtration with PD-10 Sephadex G25-M filtration columns; PBS (nitrogen purged) was used as the eluent. The LDL samples (50 μg of protein/mL of PBS) were preincubated with or without (controls) each agent (1–20 μM) for 30 min at 37°. Oxidation of LDL was induced by the final addition of 10 μM of $CuSO_4$. The time course of LDL oxidation, measured by increases of TBARS formation, was followed up to 24 hr. The lag time before the onset of upward oxidation was estimated according to the method of Esterbauer *et al.* [11].

Protein content was determined by the procedure of Bradford [12] using the Pierce Coomassie Protein Plus assay kit (Pierce Chemical). Statistical analyses were performed by the unpaired Student's *t*-test.

RESULTS

Potent Membrane Antioxidant Activities

As an initial system to assess the antioxidant activity of the selected 2-hydroxytetronic acid analogs, a microsomal membrane system was employed, and lipid peroxidation was induced by the well-established chemical free radical system consisting of ascorbic acid + Fe(II). Because all three analogs are relatively more hydrophobic than ascorbic acid, the analogs were preincubated for at least 15 min before the addition of the free radical components. The antioxidant effects of the analogs were measured as percent inhibition of TBARS formation, and compared with the free radical controls, which were measured to be 57 ± 6 nmol of MDA equivalents/mg of protein at 20 min of incubation (mean ± SD, $N = 4$). As represented by Fig. 2, all three compounds provided concentration-dependent inhibition of the induced lipid peroxidation. The EC_{50} values were calculated to be 3.02, 0.28, and 0.16 μM, respectively, for Cpd A, Cpd B, and Cpd C. For comparison, the EC_{50} for Trolox, a vitamin E analog, was 3.05 μM. Trolox was used instead of α-tocopherol because of its much faster membrane uptake (which is similar to that for the three *aci*-reductones). Thus, while the antioxidant potency of Cpd A was comparable to that of Trolox, Cpd B and Cpd C were 10.9- and 19.1-fold, respectively, more potent. Additional control experiments were performed, which showed that none of these agents (at concentrations up to 100 μM) had any effect on the TBARS assay. The rank order of potency of the analogs correlated well with their relative hydrophobicity indexes (partitioning coefficients), which were measured to be 0.71, 1.96, and 2.67, respectively, for Cpd A, Cpd B, and Cpd C in an octanol/water system.

Lack of Prooxidant Activity

In the presence of iron, it is well known that ascorbic acid can display both antioxidant or prooxidant activities, de-

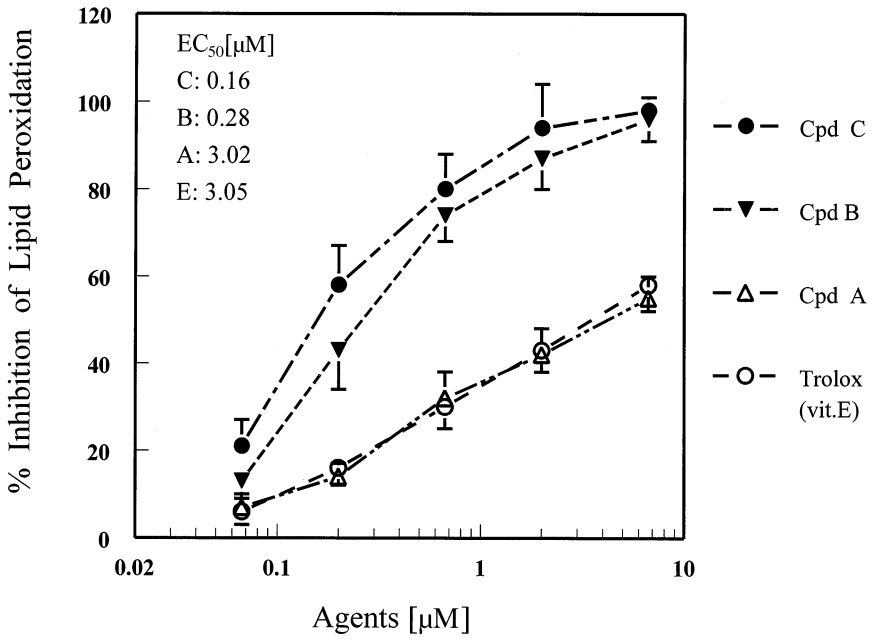

FIG. 2. Potent inhibitory effects of the 2-hydroxytetronic acid analogs against membrane lipid peroxidation induced by ascorbic acid + Fe(II). Hepatic microsomes (0.20 mg protein/mL) were incubated with each analog (0.067 to 6.7 μM) for 20 min at 30° before the addition of freshly prepared Fe(II) (10 μM) and ascorbic acid (100 μM). After 20 min of incubation, samples were assayed for TBARS formation and expressed as percent inhibition relative to the free radical controls. Values are means ± SD of 4–6 different determinations. The EC_{50} values are calculated from the linear slopes of the graphs.

pending on the concentration used [13–15]. The following experiments were performed to assess whether the 2-hydroxytetronic acid analogs would display similar properties. We previously reported that ascorbic acid < 250 μM was prooxidative in the presence of iron, whereas ascorbic acid > 500 μM was antiperoxidative [15]. In the present study, ascorbic acid at 100 μM greatly promoted membrane lipid peroxidation (Fig. 3). However, at the same concentration, none of the three analogs (Cpds A, Cpd B, and Cpd C) induced any prooxidant activity as did ascorbic acid. In data not shown, all three analogs at other concentrations (10–500 μM) did not display any prooxidant activity.

Potent Inhibitory Effects against LDL Oxidation

Because LDL oxidation has been implicated as an important contributing factor in the development of atherosclerosis, the following experiments were designed to assess the antiperoxidative potency of these 2-hydroxytetronic acid

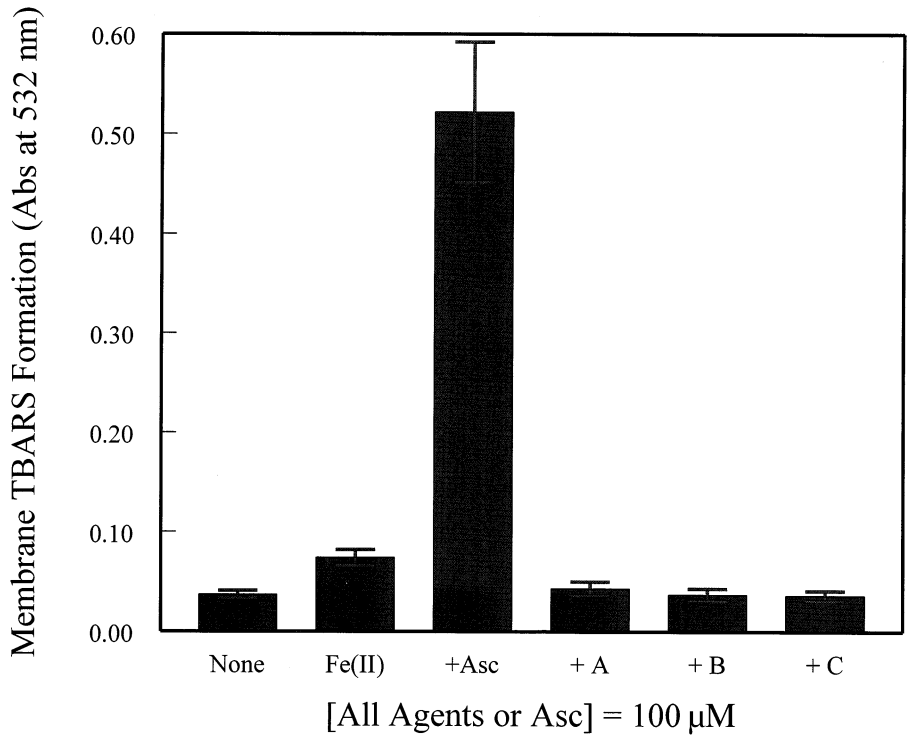

FIG. 3. Lack of a prooxidative effect of the analogs on microsomal membrane lipid peroxidation in the presence of 10 μM of Fe(II). Hepatic microsomes (0.20 mg of protein/mL) were preincubated with each of the analogs (100 μM) for 10 min before the addition of Fe(II) (10 μM). After 20 min of incubation, lipid peroxidation was assessed by the TBARS absorbance at 532 nm. Values are means ± SD of 4–6 different determinations. Other conditions were as described in Fig. 2.

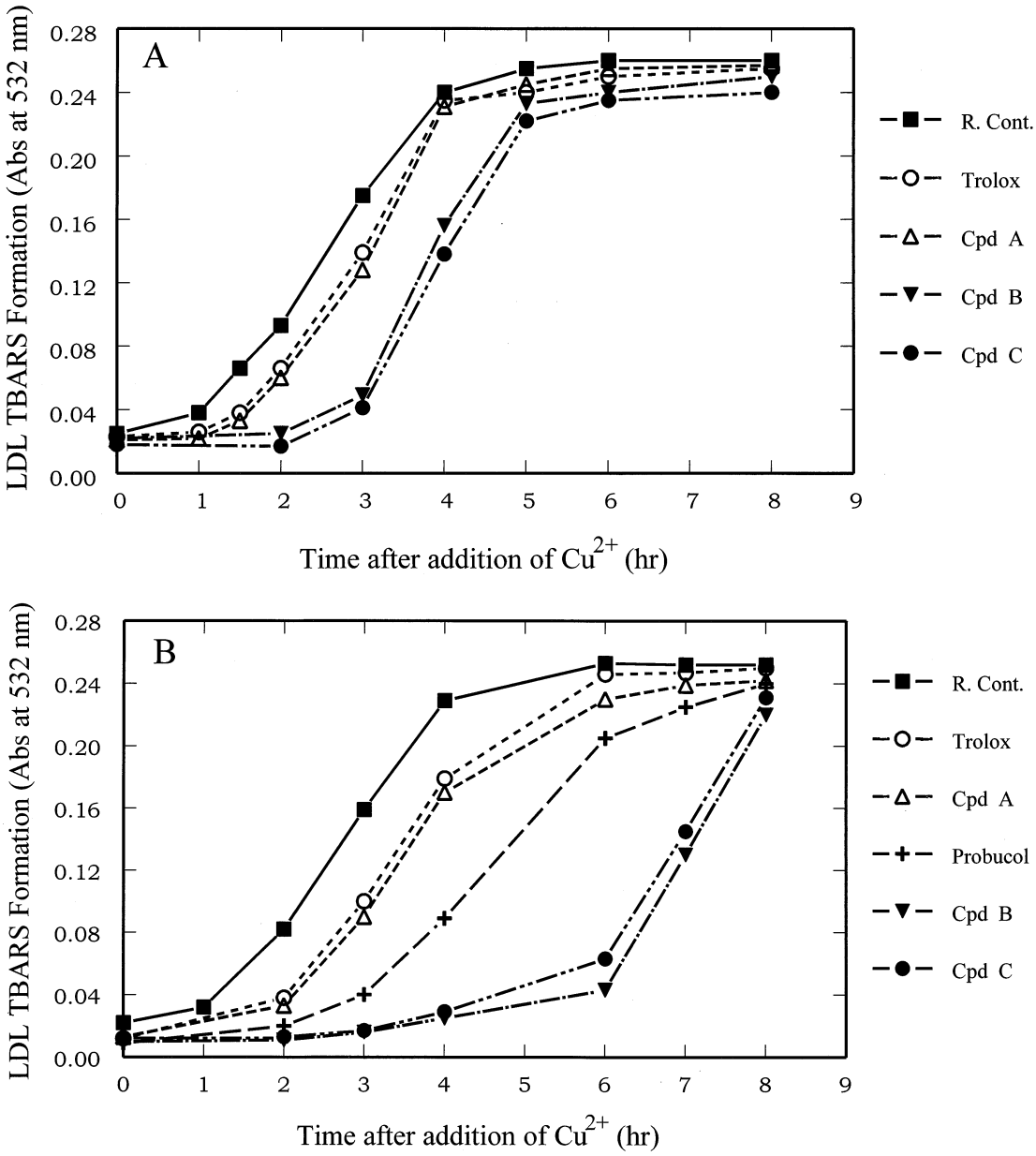

FIG. 4. Relative efficacy of (A) 1-μM and (B) 3-μM concentrations of the 2-hydroxytetronic acid analogs, vitamin E, and probucol to increase the resistance (lag time) of LDL against Cu(II)-induced oxidation. Human LDL (50 μg of protein/mL) in PBS was preincubated with each agent or analog (1 and 3 μM) for 30 min (37°) before the addition of freshly prepared $CuSO_4$ (10 μM). The time course of LDL lipid peroxidation was measured by increases in TBARS formation (abs at 532 nm). The lag time before the onset of upward oxidation was determined according to the method of Esterbauer *et al.* [11]. Data are representative effects of the three analogs and other agents from 3 to 5 separate determinations.

analogs against LDL oxidation *in vitro*. Under our system, upon addition of copper (10 μM), LDL oxidation, determined by the TBARS method, followed typical sigmoidal curve kinetics described by Esterbauer *et al.* [11] with an initial lag phase followed by a sharp propagation and final plateau phases (Fig. 4). The extent of lag time was interpreted as the antioxidant resistant capacity of the LDL. Results in Fig. 4A and 4B represent typical effects of the three analogs (1- and 3-μM concentrations) on the prolonging of lag time. While Cpd A, like vitamin E, appeared to have only moderate effects on the lag-time delay, both Cpd B and Cpd C delayed the lag time substantially longer: on an average, 3 μM of the two analogs delayed the lag time by ~530%, and 1 μM of the analogs, by about 225% of controls (Table 1). In comparison, a 3-μM concentration of vitamin E (or Cpd A) only had a modest effect (~169% of controls). However, probucol (3 μM) did provide an intermediate effect and prolonged the lag time to about 257% of controls (Table 1). Thus, Cpd B and Cpd C both delayed the LDL oxidation lag times to a much greater extent than did either vitamin E or probucol.

TABLE 1. Effects of the 2-hydroxytetronic acid analogs on prolonging the lag time of LDL oxidation induced by Cu(II)

	Lag time (min)	
Agents added	(1 μM)	(3 μM)
Control [Cu(II) alone]	61 ± 12	
α-Tocopherol	79 ± 12	104 ± 16
Probucol		157 ± 26
Cpd A	81 ± 8	105 ± 12
Cpd B	135 ± 18*	330 ± 50†
Cpd C	140 ± 22*	320 ± 38†

Values (means ± SD, $N = 3–5$) are derived from the experimental data of Fig. 4.
*$P < 0.05$ vs α-tocopherol.
†$P < 0.01$ vs α-tocopherol or probucol.

DISCUSSION

Ascorbic acid is a well-known water-soluble antioxidant in biological systems. However, because of its water solubility, the antioxidant ability of this compound is limited to the aqueous compartment [16]. In this communication, we present evidence that the 2-hydroxytetronic acid analogs, which are structurally related to ascorbic acid but are relatively much more hydrophobic due to the presence of aryl aromatic and chloride substitutions, possess potent membrane and LDL antioxidant activities. Using the membrane oxidation model, it appears that the relative antioxidant potency for the three analogs is directly proportional to their hydrophobicities. Furthermore, the membrane antioxidant activities for both Cpd B and Cpd C were determined to be an order of magnitude more potent than that for vitamin E (Trolox). These data support the argument that hydrophobicity (and, hence, lipophilicity) is an important parameter for the membrane antioxidant efficacy of the analogs. This notion has been supported by our previous observations [17, 18] demonstrating that the antioxidant activities of the β-adrenergic receptor blockers and calcium channel blockers correlated well with their lipid-partition coefficients. Interestingly, the three 2-hydroxytetronic acids, although retaining redox potentials similar to that of ascorbic acid [4], did not display the prooxidant properties of the parent compound often exhibited in the presence of transition metals [13–15].

Previous studies showed that Cpd A displayed antilipidemic activities superior to that produced by clofibrate in the cholesterol-fed rat model [3]. Clofibrate undergoes hydrolysis to clofibric acid *in vivo*, but unlike 2-hydroxytetronic acid, this metabolite does not possess a biologically relevant redox potential. Clofibrate is notorious for its potential serious side-effects in both humans and animals [19, 20]. In contrast, the *aci*-reductone (Cpd A) is well tolerated and has no observable hematological toxicity nor any major organ toxicity, such as hepatomegaly caused by clofibrate [3, 4, 21, 22]. The available evidence suggested that the tetronic acid mediated its antilipidemic effect by a mode(s) of action clearly different from that of clofibrate [3, 4, 21]. These modes of action may include (but are not limited to) inhibition of arachidonic acid metabolism, antiaggregatory effect, and scavenging of superoxide anions [3, 22, 23]. We suggest that the observed antilipidemic activity of Cpd A might be related to its antioxidant activity. According to the "oxidative-modification hypothesis," the initial oxidation of LDL constitutes the critical step of atherogenesis [5, 6]. This hypothesis is strongly supported by the evidence that LDL oxidation occurs *in vivo* and contributes to the clinical manifestations of atherosclerosis (see review in Ref. 6). To support this argument, it has been well-established that probucol can reduce the formation of atherosclerosis in both WHHL rabbits and cholesterol-fed rabbits; this action has been attributed to its anti-LDL-oxidation activity, independent of its cholesterol-lowering effect (see review in Ref. 5). Antioxidants may also prevent oxidative stress related to neointimal formation and vascular remodeling. Indeed, in a recent double-blind, randomized clinical trial, probucol administration was shown to be able to effectively decrease the incidence and severity of restenosis after angioplasty, an effect also tentatively attributed to its antioxidant activity [24].

In this communication, we determined that the antioxidant potency of Cpd A is comparable to that of vitamin E. Upon the introduction of a biphenyl ring, as represented by Cpd B and the biphenyl ring + chloride substitution as represented by Cpd C (Fig. 1), both the hydrophobicity and antioxidant activities were proportionally enhanced. The results from the LDL oxidation model show that both Cpd B and Cpd C at concentrations as low as 1 μM significantly prolonged the lag time > 200% of control; at 3 μM, the lag time was prolonged ≥ 500% of controls by both analogs (Fig. 4, Table 1). Additionally, both Cpd B and Cpd C at concentrations ≥ 20 μM delayed TBARS formation by more than 24 hr (data not shown). Thus, these two 2-hydroxytetronic acid analogs (Cpd B and Cpd C) displayed better efficacy compared with probucol in delaying the LDL oxidation, suggesting their potential therapeutical utility. Preliminary results using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity test¶ indicate that none of these three agents (up to 20 μM) displayed any toxicity to cultured endothelial cells over 24 hr. Also, *in vivo* administration of these agents to rats at daily doses up to 30 μmol/kg for a week was well tolerated. However, additional *in vivo* studies are clearly required to define the relevance of the concentrations used as related to their pharmacokinetics, tissue distribution, and potential toxicity. More specific experiments should include determination of whether or not administration of these *aci*-reductones to animals would result in their incorporation into LDL and a concomitant increase in resistance of the LDL to oxidative modification *ex vivo*. Nevertheless, the

¶ Mak IT, Hopper AT, Witiak DT, Ziemniak J and Weglicki WB, Potent protective effects of hydrophobic *aci*-reductones (ascorbic analogs) against oxidative endothelial cell injury. *Oxygen Society Meeting*, Pasadena, CA, Nov. 1995, (Abstr.) p. 42.

current experimental data indicate that the more potent *aci*-reductions of Cpd B and Cpd C should be studied further to determine if these compounds are potential therapeutic agents for diseases such as atherogenesis and restenosis, where lipid peroxidation may play a major role in pathogenesis.

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