9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.96, 148.40, 134.27, 119.42, 113.27, 112.11, 78.87, 67.33, 56.25, 48.38, 48.27, 40.66, 37.26, 37.17, 36.81, 34.34, 29.49, 20.77; IR (KBr) ν_{max} 2944, 2924, 2864, 1510, 1452, 1426, 1376, 1260, 1150, 1134, 1028 cm^{-1}; mass spectrum (DCl-NH_3), exact mass calcd for $C_{16}H_{22}NO_2$ ($M^+ = P - CH_2NH_2$) 260.1651, found 260.1650.

1-Methyl-5-[3-(*endo*-bicyclo[2.2.1]hept-2-yloxy)-4-methoxyphenyl]-2-imidazolidinone (24). Preparation of imidazolidinone **24** was accomplished from **23** in the fashion described above for the synthesis of imidazolidinone **4**. The desired product was obtained in 38% yield as a white solid: mp 148.5–149.5 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85–6.65 (m, 3 H), 5.85 (bs, 1 H), 4.60 (m, 1 H), 4.45 (dd, 1 H, J = 5.5, 7.1 Hz), 3.83 (s, 3 H), 3.68 (dd, 1 H, J = 6.6, 7.1 Hz), 3.21 (dd, 1 H, J = 5.5, 6.6 Hz), 2.61 (bs, 3 H), 2.55 (m, 1 H), 2.23 (m, 1 H), 2.01 (m, 2 H), 1.59–1.45 (m, 1 H), 1.43–1.30 (m, 4 H), 1.22–1.05 (m, 1 H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 163.37, 149.86, 148.85, 131.89, 119.44, 112.45/113.30, 112.10, 78.97, 62.93, 56.25, 56.21, 47.67, 40.66, 37.29, 37.17, 36.82, 29.49, 28.80, 20.79; IR (KBr) ν_{max} 1134, 1229, 1259, 1497, 1683, 2958, 3227 cm^{-1}; mass spectrum (DCl-NH_3), m/z 222 (base); exact mass calcd for $C_{18}H_{24}N_2O_3$ 316.1787, found 316.1781. Anal. ($C_{18}H_{24}N_2O_3$) C, H, N.

1-Methyl-5-[3-((1*SR*,2*RS*,6*RS*,7*SR*,8*SR*)-*endo*-tricyclo-[5.2.1.0^{2,6}]dec-8-yloxy)-4-methoxyphenyl]-2-imidazolidinone (25). Imidazolidinone **25** was prepared in the fashion described above for imidazolidinone **24** with catechol and (1*RS*,2*RS*,6*RS*,7*SR*)-tricyclo[5.2.1.0^{2,6}]-8-decanone[34] as starting material: mp 149–151 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.9–6.8 (m, 1 H), 5.25 (bs, 1 H), 4.65 (m, 1 H), 4.41 (ddd, 1 H, J = 2.0, 6.0, 6.1 Hz), 3.82 (s, 3 H), 3.68 (dd, 1 H, J = 6.0, 6.1 Hz), 3.21 (dd, 1 H, J = 6.0, 6.1 Hz), 2.62 (m, 1 H), 2.60 (s, 3 H), 2.38 (m, 1 H), 2.11–1.89 (m, 5 H), 1.86 (m, 1 H), 1.65 (m, 1 H), 1.30–0.85 (m, 5 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.19/163.72, 149.76/149.71, 148.73, 131.68, 119.36, 112.26/112.13, 111.80, 78.28, 62.89/62.83, 56.17, 47.74, 47.62/47.57, 45.12, 41.01, 38.18/38.14, 36.96/36.91, 32.62, 31.82/31.77, 31.23/31.17, 28.77, 27.08; IR (KBr) ν_{max} 3228, 2942, 2886, 2857, 1694, 1678, 1512, 1450, 1259, 1230, 1023 cm^{-1}; mass spectrum (DCl-NH_3), m/z 222 (base); exact mass calcd for $C_{21}H_{28}N_2O_3$ 356.2099, found 356.2120. Anal. ($C_{21}H_{28}N_2O_3$) C, H, N.

1-Methyl-5-[3-(*endo*-benzobicyclo[2.2.1]hept-2-yloxy)-4-methoxyphenyl]-2-imidazolidinone (26). Preparation of imidazolidinone **26** was accomplished in the same fashion as described above for the synthesis of imidazolidinone **24** with catechol and benzobicyclo[2.2.1]-2-heptanone[38] as starting materials: mp 167–168 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22–7.01 (m, 4 H), 6.78–6.67 (m, 3 H), 5.79 (bs, 1 H), 5.12 (m, 1 H), 4.38 (m, 1 H), 3.73 (m, 1 H), 3.68 (m, 1 H), 3.59 (s, 3 H), 3.40 (bs, 1 H), 3.25 (m, 1 H), 2.66 (m, 1 H), 2.64 (s, 3 H), 2.45 (m, 1 H), 1.91 (m, 1 H), 1.78 (m, 1 H), 1.25 (m, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.34, 150.10/150.05, 148.70, 148.24, 142.63/142.59, 132.21/132.16, 126.13, 125.48, 124.07, 120.22, 120.08, 113.85, 113.70, 78.29, 62.75, 56.65, 48.16, 48.03, 47.61, 43.60, 36.26, 38.79; IR (KBr) ν_{max} 3208, 2967, 1514, 1490, 1444, 1259, 1232, 1134 cm^{-1}; mass spectrum (DCl-NH3), m/z (base); exact mass calcd for $C_{22}H_{24}N_2O_3$ 364.1787, found 364.1769. Anal. ($C_{22}H_{24}N_2O_3 \cdot {}^1/_3H_2O$) C, H, N.

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Supplementary Material Available: Tables of NMR data for intermediates not referred to specifically in the text (8 pages). Ordering information is given on any current masthead page.

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Antioxidant Activity of Probucol and Its Analogues in Hypercholesterolemic Watanabe Rabbits

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Probucol (1) and probucol analogues with the substitutions at the disulfide-linked carbon (**2**, **3**) and an additional substitution at a *tert*-butyl of each phenolic ring (**4**) were tested for their ability to lower total serum cholesterol and prevent aortic atherosclerosis in modified Watanabe heritable hyperlipidemic (WHHL) rabbits and to inhibit Cu^{2+}-induced lipid peroxidation of isolated plasma low-density lipoproteins (LDL). After 84 days of feeding 1% of each compound in rabbit chow, probucol was effective in lowering serum cholesterol, whereas **2**–**4** were not. The concentration of drug in serum and LDL was **2** > **1** > **3** > **4**. Probucol and analogues prevented Cu^{2+}-induced oxidation of LDL in vitro to an extent that directly related to their concentrations in LDL. The decrease in lipid oxidation was directly correlated with the inhibition of both oxidized-LDL-induced cholesteryl ester synthesis in cultured macrophages and to the inhibition of aortic atherosclerosis in vivo. These results show that the antioxidant activity of probucol and analogues is directly related to their concentration in LDL, which may explain their pharmacological activity in reducing atherosclerosis.

Early atherosclerosis in men and in animals is characterized by the presence of lipid-laden foam cells in the arterial intima.[1-6] Circulating monocytes or macrophages which enter the arterial wall and accumulate lipid are one of the sources of foam cells.[7,8] It has been proposed by several investigators that atherosclerosis may be initiated, or at least enhanced, by lipid peroxides and other compounds formed by the peroxidation of polyunsaturated lipids and oxidized proteins in the arterial wall.[8-13] Recent studies[14-16] have demonstrated that oxidatively modified low-density lipoproteins (LDL) are taken up by cultured macrophages, causing massive cholesterol accumulation. If LDL were to be oxidatively modified in vivo, one could expect that the prevention of LDL lipid peroxidation would attenuate foam-cell formation. In support of this

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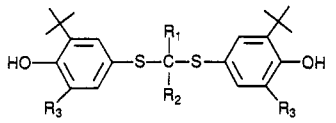
Table I. Chemical Structure and Some Physicochemical Properties of Probucol and Its Analogues



compd	R_1	R_2	R_3	formula	mw	anal.	mp, °C	% purity (HPLC)
probucol	CH_3	CH_3	*t*-Bu	$C_{31}H_{48}O_2S_2$	516.8	C, H, S	117–118	>99
2	H	H	*t*-Bu	$C_{29}H_{44}O_2S_2$	488.8	C, H, S	94–95	>98
3	CH_3	$CH_3(CH_2)_2$	*t*-Bu	$C_{33}H_{52}O_2S_2$	544.9	C, H, S	130–132	>98
4	CH_3	$CH_3(CH_2)_3$	Me	$C_{28}H_{42}O_2S_2$	474.8	C, H, S	126–128	>98

Table II. Serum Cholesterol Levels before and after Treatment with Probucol and Analogues

compd	no. of animals	cholesterol concn, mg/dL ± SEM			
		day 0	day 28	day 56	day 84
control	10	773 ± 77	953 ± 57	905 ± 53	875 ± 48
probucol	4	658 ± 128	728 ± 61	731 ± 38	698 ± 66[a]
2	5	890 ± 73	905 ± 39	766 ± 59	734 ± 46
3	4	685 ± 80	885 ± 75	796 ± 39	782 ± 60
4	5	1005 ± 66	1042 ± 71	1079 ± 92	1095 ± 120

[a] Significantly different from control on day 84, $p < 0.05$.

hypothesis, Carew et al.[17] and Kita et al.[18] have recently shown that the treatment of Watanabe heritable hyperlipidemic (WHHL) rabbits with probucol, a potent lipid-soluble antioxidant,[19-21] prevents the progression of atherosclerosis. In humans, low serum levels of α-tocopherol, an important antioxidant in vivo,[22,23] seem to correlate with an increased incidence of myocardial infarction.[24] Such data suggest that the presence of serum antioxidants, particularly in LDL, may attenuate atherosclerosis by preventing LDL lipid peroxidation and foam-cell formation.

In the present report, we have prepared several probucol analogues with different alkyl group substitutions at the central disulfide carbon and an additional substitution at a *tert*-butyl of each phenolic ring (Table I). We examined the effectiveness of these compounds in preventing LDL lipid peroxidation and foam-cell formation in vitro and in the attenuation of atherosclerosis in WHHL rabbits.

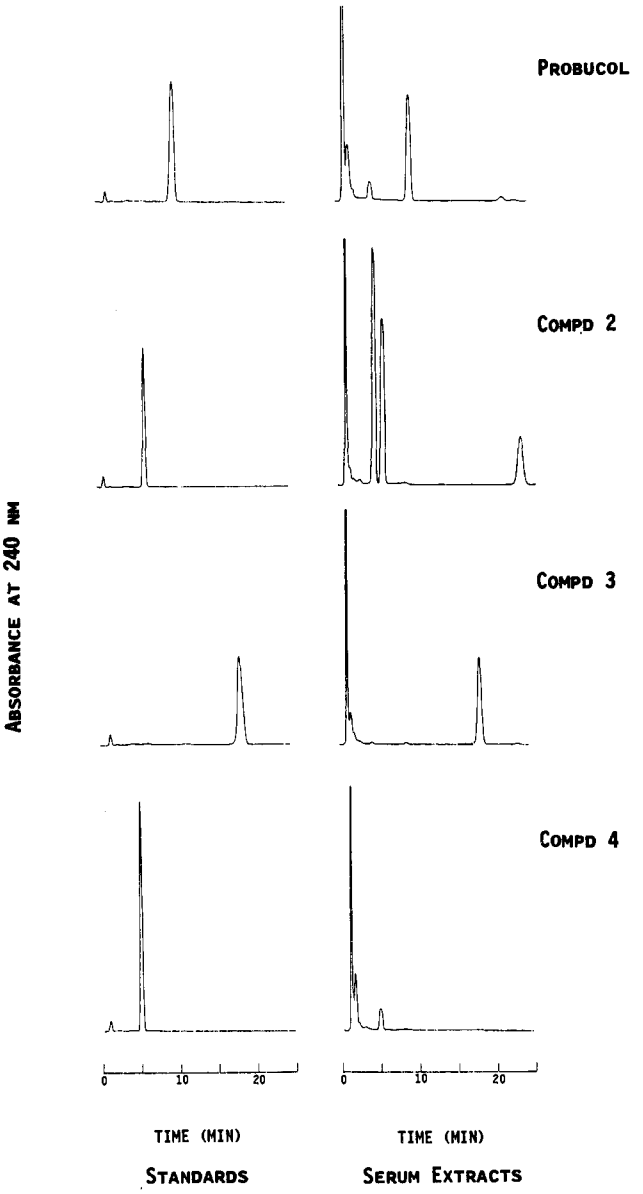


Figure 1. HPLC profiles of probucol and its analogues. One hundred microliters of serum of WHHL rabbits fed with probucol or its analogues (at day 84) was used for ether/ethanol extraction. Identification of each compound found in serum was further confirmed by GC–mass spectral analyses. Additional peaks shown in the compound **2** panel were found to be the metabolites of compound **2** (see ref 21).

Results

Table I shows the chemical structures and some physicochemical properties of probucol (**1**) and the three analogues (**2–4**) used in the present study. Serum cholesterol

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Table III. Drug Concentrations in Serum and in the LDL Fraction

compd	drug concn, μg/mL serum	drug concn, μg/mL LDL[a]	protein concn in LDL, mg/mL	molar content[b] of compd in LDL, molecules/ LDL particle
control			3.79 ± 0.13	
probucol	56 ± 13	41 ± 6	3.57 ± 0.21	11.3 ± 1.2
2	135 ± 17	65 ± 9	3.26 ± 0.26	20.3 ± 3.3
3	42 ± 4	23 ± 2	3.78 ± 0.24	6.2 ± 0.8
4	6.4 ± 0.5	3 ± 0.2	4.68 ± 0.55	0.7 ± 0.05

[a] To determine the drug concentrations in the LDL fraction, LDL were isolated at d = 1.019–1.063 g/mL. The isolated LDL were then brought up to the initial serum volume and the drug concentrations were determined. [b] All the values represent the mean ± SEM of each group. For calculations, molecular weight (mw) of the LDL protein used was 500 000, since each LDL particle contains one copy of apoB (mw ≈ 500 000), which is the major protein moiety of LDL (greater than 95%). The following formula was used for the calculation:

$$\frac{\text{compound concn } (\mu g/mL) \text{ in LDL}/\text{mw of compound}}{\text{LDL protein concn } (\mu g/mL)/500\,000} = \frac{\text{molecules of compound}}{\text{LDL particle}}$$

levels before, during, and after treatment of each group are given in Table II. As compared to the control group at day 84, only probucol significantly lowered serum cholesterol levels ($p < 0.05$). Probucol did not lower cholesterol at day 84 as compared to the value at day 0. This is because the plasma cholesterol levels continue to increase throughout the study period. Body weight (kg ± SEM) at day 84 for control and compound (1–4) treated groups was 3.04 ± 0.06 and 2.97 ± 0.08, 2.76 ± 0.12, 2.96 ± 0.08, and 2.94 ± 0.08, respectively. There were no significant differences in body weight among the groups ($p > 0.1$). Serum drug concentrations at the time of sacrifice (day 84) were determined by a high-performance liquid chromatography (HPLC) technique. Typical HPLC profiles of ether/ethanol-soluble material from the serum of treated animals are shown in Figure 1. Drug concentrations in serum and LDL fraction are given in Table III. The retention times of the compounds (Figure 1) with a C-18 reverse-phase column increased with the number of alkyl groups on the disulfide-linked carbon of probucol, with the exception of compound 4. Although 4 has one more alkyl group than 3 at the central carbon, the substitution of a methyl for a *tert*-butyl in each phenolic ring of 4 may account for less hydrophobicity and shorter retention time as compared to those of 1–3. Furthermore, drug concentrations in serum appeared to be negatively correlated with the chain length of alkyl groups (Table III). Compound 2 yielded the highest concentration in serum as well as in LDL with 20 molecules of 2 in each LDL particle as compared to 11 molecules for probucol. Regardless of the final serum concentration, greater than 50% of each compound was present in LDL (Table III).

In addition to its cholesterol-lowering effect, studies have shown that probucol prevents atherosclerosis in WHHL rabbits.[17,18] The mechanism of this effect is thought to involve prevention of lipid peroxidation of LDL. For this reason, we next tested whether the presence of these compounds in LDL protects against LDL lipid peroxidation. LDL isolated at sacrifice (day 84) from each WHHL rabbit were incubated with Cu^{2+} ($CuSO_4$, 5 μM) to initiate peroxidation, and the content of lipid peroxides was determined by the thiobarbituric acid (TBA) assay using malondialdehyde as a standard. When the content of lipid peroxides in LDL was plotted against the drug

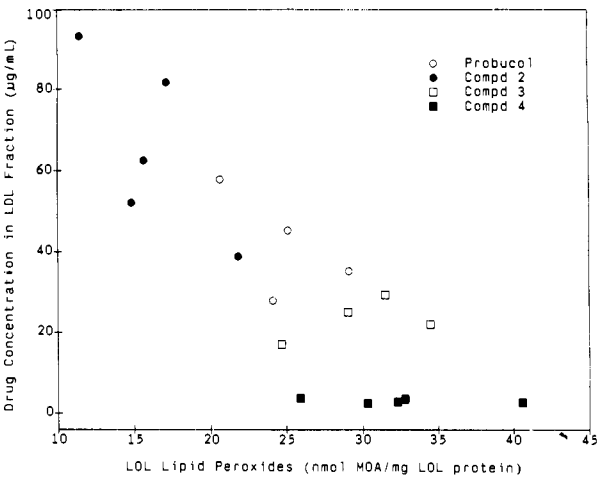

Figure 2. Effect of probucol and its analogue concentrations in LDL on lipid peroxidation. LDL (100 μg) isolated from each treated WHHL rabbit (at day 84) were used for Cu^{2+}-induced lipid peroxidation.

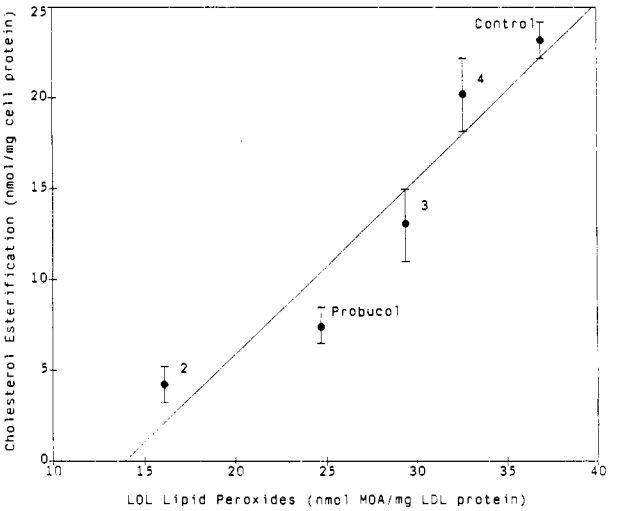

Figure 3. Effect of Cu^{2+}-oxidized LDL on the cholesterol esterification in mouse peritoneal macrophages. LDL isolated from each treated WHHL rabbit (at day 84) were oxidatively modified by Cu^{2+} prior to the cholesterol esterification experiment. The error bar represents the mean ± SEM for each treated group.

concentrations in LDL, it was found that the extent of Cu^{2+}-induced peroxidation increased with decreasing drug concentration (Figure 2).

Macrophages are known to possess a limited number of receptors for normal LDL but have a separate receptor, termed scavenger receptor, which recognizes LDL that have undergone lipid peroxidation.[25,26] Oxidatively modified LDL (oxidized LDL) are taken up by the cells with enhanced efficiency, so that cholesteryl esters rapidly accumulate within the macrophage, resulting in their conversion to foam cells. In Figure 3, we demonstrate that Cu^{2+}-oxidized LDL from control animals substantially increased the synthesis of cholesteryl ester in mouse peritoneal macrophages. However, LDL isolated from drug-treated groups were resistant to lipid peroxidation (Figure 2) and resulted in less cholesteryl esterification (Figure 3). Compound 2 in LDL was more effective than probucol in

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Table IV. Prevention of Cu^{2+}-Induced Lipid Peroxidation of LDL Isolated from Drug-Treated Groups vs Percent Atherosclerotic Lesions in WHHL Rabbits[a]

compd	lipid peroxidation,[b] nmol MDA/mg LDL protein	% lesion
control	37 ± 2	67 ± 6
probucol	25 ± 2	47 ± 17
2	16 ± 2	39 ± 15
3	30 ± 2	53 ± 17
4	33 ± 2	81 ± 6

[a] Animal numbers used in each group are listed in Table I. [b] Lipid peroxidation is determined by thiobarbituric acid using MDA as a standard. Each value represents the mean ± SEM of each group.

the inhibition of cholesteryl esterification, whereas compounds 3 and 4 were less effective and this is consistent with the fact that their concentration in LDL was less than that of probucol or compound 2.

Finally, the extent of fatty streak lesions in the aortas of WHHL rabbits, treated with and without drug, were examined. Lesions in the thoracic region, determined by a digital-imaging system based on the density of sudanophilic staining, are given in Table IV. Mean percent lesions in the compound 2 group (39%) was less than that for probucol (47%) and untreated groups (67%). There was a close relationship between the antioxidant activity of isolated LDL and the development of aortic lesions ($r = 0.87$; $p < 0.01$). In the 4-treated group, the drug concentration in plasma or LDL was low, the protection against lipid peroxidation was minimal, and there was no effect on the prevention of atherosclerosis.

Discussion

The major pharmacological action of probucol is cholesterol lowering. The mechanism of this action, however, is still not well detailed. It appears to be independent of LDL receptor mediated clearance since the drug is effective in LDL receptor deficient patients with hypercholesterolemia.[27] In addition, Naruszewicz et al.[28] demonstrated that LDL isolated from probucol-treated WHHL rabbits had an increased fractional catabolic rate in probucol-treated or untreated group. Therefore, one suggested mechanism by which probucol lowers LDL levels relates to the intrinsic changes in the structure of plasma LDL containing probucol. Theoretically the intrinsic changes of LDL structure should be greater with more drug molecules in each LDL particle. The present study shows that compound 2 resulted in 2 times more molecules per LDL particle than probucol (20.3 vs 11.3). The cholesterol lowering effect of 2, however, was not as great as that of probucol. Therefore, the incorporation of drug into LDL cannot fully explain the cholesterol lowering. The mechanism by which compound 2 and probucol reached high concentrations in serum remains unknown. The hydrophobicity of each compound (1–3) around the disulfide-linked carbon may play an important role in determining their intestinal uptake.

Recent clinical and experimental studies have established that elevated plasma cholesterol and LDL are associated with accelerated atherogenesis. For this reason we have chosen WHHL rabbits for the present study. The earliest recognized gross lesions in atherogenesis are the fatty streaks, characterized by an accumulation of foam cells loaded with cholesterol esters. Oxidatively modified LDL enhance foam-cell formation.[14-18] This oxidative modification, however, can be prevented in vitro by probucol.[19-21] In Table IV, we show that 2 in isolated LDL from WHHL rabbits was the most effective in protection against lipid peroxidation. In in vitro experiments, we directly added probucol, compound 2, 3, or 4 into native LDL at the same final concentrations (50 μg/5 mg of LDL protein); the antioxidant activity of each compound as assessed by Cu^{2+} oxidation was equally effective in protecting LDL from lipid peroxidation (data not shown). In addition, the amount recovered in LDL was the same for each compound. Therefore, the superiority of the antioxidant effect of 2 found in vivo in WHHL rabbits' LDL may be related to its concentration in LDL. We speculate that the high concentration of 2 found in LDL in vivo might be due to its bioavailability rather than its ability to incorporate into LDL.

Although we have demonstrated that these compounds are mostly associated with LDL, the physiological usefulness in regard to their direct protection against LDL peroxidation in the circulation may not be crucial. For example, Haberland et al.[29] have shown that malondialdehyde (MDA), the byproduct of lipid peroxides, is only associated with LDL inside the arterial wall but not in the circulation. Other studies have also provided strong evidence that lipid peroxidation is taking place within the atherosclerotic lesion (discussed by Halliwell[30]). Thus, it may be important to design a compound that can be effectively incorporated into LDL in vivo to prevent lipid peroxidation inside arterial wall.

In summary, this study shows that compound 2, when incorporated into LDL, protects against LDL lipid peroxidation, inhibits cholesterol reesterification in macrophages, and prevents progression of atherosclerosis in WHHL rabbits. This compound may provide further insight in establishing the relationship between the antiatherogenesis and antioxidants.

Experimental Section

Probucol [bis[(3,5-di-*tert*-butyl-4-hydroxyphenyl)thio]propane] and its analogues (Figure 1) 2 (MDL 29,311), 3 (MDL 27,272), and 4 (MDL 29,097) were synthesized as described previously.[31] The purity of each compound was determined by mass spectral analysis and reverse-phase high-performance liquid chromatography (HPLC).

Administration of Probucol and Analogues. Probucol and analogues were mixed and repelleted with Purina rabbit chow by Purina (Richmond, IN). The final drug content was 1% (wt/wt); the drug concentrations were confirmed by extracting the chow and measuring the compound by HPLC. British half-lop Watanabe heritable hyperlipidemic (WHHL) rabbits were obtained from Drs. Thomas Parker and Thomas Donnelly (Rogosin Institute, New York, NY). This modified strain of WHHL was obtained after cross-breeding British half-lop rabbits (BHL) and WHHL rabbits. All rabbits were homozygous for LDL receptor deficiency. The biochemical and pathological features of this modified WHHL are described by Gallagher et al.[32] Twenty-eight WHHL rabbits (age 14 ± 1 weeks) were divided into a control group containing no compound ($n = 10$) and probucol ($n = 4$) and analogue 2 ($n = 4$), 3 ($n = 5$), and 4 ($n = 5$) groups. Each rabbit was fed 100 g/day of the respective diet. Twelve weeks later plasma was collected for the isolation of LDL and the animals were sacrificed by intravenous injection of pentobarbital. Serum was used for the determination of total cholesterol by an enzymatic method.[33]

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Determination of Serum Drug Concentrations. Rabbit serum (100 μL) was added dropwise to 2 mL of an ether/ethanol mixture (3/1, v/v) while vortexing. The samples were vortexed for an additional 2 min and then centrifuged for 15 min at 2000 rpm (500g). The supernatant fractions were transferred to borosilicate glass tubes (12 × 75 mm) and dried under N_2 gas. The dried extracts were then dissolved in 200 μL of acetonitrile/hexane/0.1 M ammonium acetate (90/6.5/3.5, v/v/v) and subjected to reverse-phase HPLC. The separations were carried out with a Waters 600E System equipped with a 990 photodiode-array detector; fractions were monitored at 240 nm. A Deltapak C18 reverse-phase column (15 cm × 3.9 mm, 300 Å, Waters) was used with a mobile phase of acetonitrile/water (85/15, v/v) at a flow rate of 1.5 mL/min as previously described.[34]

Preparation of Macrophage Monolayers. Peritoneal cells were harvested from CD-1 mice in phosphate-buffered saline (PBS, 0.01 M sodium phosphate, 0.12 M NaCl, pH 7.4) as described previously.[35] The fluid from 50 mice (5 × 10^6 cells per mouse) was pooled, and the cells were collected by centrifugation (1400 rpm, 10 min, 4 °C) and washed twice with PBS. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% (v/v) fetal calf serum (FCS) and 50 μg/mL gentamycin to give 3 × 10^6 cells/mL. Aliquots (1.0 mL) of this cell suspension were dispensed onto petri dishes (35 × 10 mm) and then incubated in a 5% CO_2 incubator. After incubation for 2 h at 37 °C, the monolayers were washed twice with 2 mL of DMEM and then incubated for 18 h with DMEM containing 20% FCS. Cells were then washed twice with PBS, refed DMEM, and used for the experiment.

Cholesterol Esterification. To assess the effect of LDL or oxidized LDL on the cholesterol esterification of macrophages, incorporation of [^{14}C]oleate into cellular cholesterol [^{14}C]oleate (cholesterol esterification) was measured by using a method described previously.[36] In brief, 1.3 × 10^6 dpm of [^{14}C]oleate complexed to bovine serum albumin (BSA) was added into each dish. LDL or oxidized LDL (50 μg) were added to the culture media and incubated at 37 °C for 20 h. Cellular cholesteryl [^{14}C]oleate was extracted into hexane/2-propanol (3/2, v/v) and separated from other lipids by a thin-layer chromatography.[37]

Preparation of Oxidized LDL. WHHL rabbit plasma samples were collected in Na_2 EDTA (0.1% final concentration) for the isolation of LDL after feeding the compounds for 84 days. LDL were isolated from each rabbit plasma using a sequential ultracentrifugation technique at d = 1.019–1.063 g/mL.[38] LDL were then dialyzed against PBS at 4 °C for 24 h. Oxidized LDL were prepared in a manner similar to that described previously.[18] In brief, 5 mg of LDL protein in 1 mL of PBS, pH 7.4, were incubated with 5 μM $CuSO_4$ at 37 °C for 16 h. Na_2 EDTA was added to a final concentration of 0.1% to stop the lipid peroxidation. The oxidized LDL were exhaustively dialyzed against PBS, pH 7.4, prior to the cholesterol-esterification experiment.

Determination of LDL Lipid Peroxidation Induced by Cu^{2+}. One hundred micrograms of each LDL sample was brought to a volume of 1.5 mL with distilled water. Lipid peroxidation was initiated by the addition of $CuSO_4$ to a final concentration of 5 μM, followed by an incubation at 37 °C for 3 h. The reaction was stopped by adding 100 μL of 50 mM Na_2 EDTA. Fifty micrograms of LDL from the reaction mixture was added to 1.5 mL of 20% trichloroacetic acid and vortexed. Finally, 1.5 mL of 0.67% thiobarbituric acid (TBA) in 0.05 N NaOH was added and the mixture was incubated at 90 °C for 30 min. Samples were centrifuged at 1500 rpm for 10 min. The absorbance of the supernatant fractions was determined at 532 nm (Ultrospec K, 4053 UV/visible spectrophotometer, LKB) to determine the content of lipid peroxides (TBA-reactive substances) according to the procedure of Yagi.[39] A standard curve (0–5 nmol) of malondialdehyde (MDA) was generated by using malonaldehyde bis(dimethyl acetal) (Aldrich) as a reference to determine the lipid peroxidation content in Cu^{2+}-treated LDL.

Determination of Atherosclerotic Lesions. Immediately following sacrifice by intravenous injection of sodium pentobarbital, the aortas were dissected from the ascending arch to the ileal bifurcation. Extraneous adipose tissue was removed, and the aortas were opened longitudinally and rinsed several times with saline. The aortas were stained with Sudan IV by using a procedure described previously[40] and photographed. The areas of sudanophilic lesions in the thoracic region of the aorta were digitized with a Magiscan image analysis system (Joyce-Loebl Ltd.). Stained versus unstained areas were distinguished by the imaging system on the basis of the density of Sudan Red staining. The corresponding lesioned and nonlesioned areas were quantitated accordingly.

Registry No. 1, 23288-49-5; 2, 129895-82-5; 3, 26067-78-7; 4, 27428-28-0.

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