A Comparison of the Antiatherogenic Effects of Probucol and of a Structural Analogue of Probucol in Low Density Lipoprotein Receptor–deficient Rabbits

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Abstract

The efficacies of probucol and a close structural analogue as antioxidants in the prevention of atherogenesis in LDL receptor-deficient rabbits were compared. The antioxidant potency of the analogue in vitro was equal to that of probucol. Its biological availability was much greater: almost comparable concentrations in total plasma were achieved by feeding 1% probucol (wt/wt) and 0.05% analogue (wt/wt). Total plasma concentrations were comparable, but the concentration of probucol within the LDL fraction was about twice that of the analogue. Probucol slowed lesion progression by almost 50%, confirming earlier reports; the analogue, however, showed no detectable inhibitory effect on atherogenesis. Resistance of LDL to oxidation was measured at the end of the study by incubating it with Cu²⁺ and measuring the rate of diene conjugation. Probucol prolonged the lag time from the control value of 130 min to values >1,000 min. The analogue approximately tripled the lag time (mean, 410 min) and yet failed to slow the atherogenic process. The results suggest that LDL resistance to oxidation must reach a threshold level before there is significant protection against atherogenesis. However, probucol has additional biological effects, possibly not shared by the analogue, that could contribute to its antiatherogenic potential. (J. Clin. Invest. 1994. 94:392–398.) Key words: atherosclerosis • macrophages • oxidation • antioxidant • drug therapy

Introduction

Many lines of evidence, based on studies in cell culture and in experimental animals, suggest that the conversion of native LDL to oxidatively modified LDL is a critical step in the atherogenic process (1, 2). Probably the most compelling evidence in favor of the oxidative modification hypothesis of atherosclerosis is that antioxidants have been shown in six of nine published trials (3–11) to slow the rate of progression of experimental atherosclerosis by more than 50% in LDL receptor-deficient rabbits (3–5) and in cholesterol-fed rabbits (7–9). One study of vitamin E in cholesterol-fed monkeys gave marginal results (11) and one rabbit study was negative (10). A majority of these studies made use of probucol as the antioxidant (3–6, 9, 10). Unfortunately probucol has a cholesterol-lowering effect and several additional biological effects that could very well contribute to or even be primarily responsible for the antiatherogenic effects observed. In some studies the cholesterol-lowering effect of probucol has been taken into account by treating the control group in such a way as to match cholesterol levels (3); in other studies this has not been done (4–6). The additional biological effects that may come into play include the ability of probucol to inhibit release of IL-1 (12), to increase the expression of cholesterol ester transfer protein (13, 14), and to act at an intracellular level to modify oxidative metabolism (15). The possibility that probucol’s antiatherogenic effects are mainly related to its action as an antioxidant is strengthened by the fact that two other antioxidants have been shown to be effective against experimental atherosclerosis. Butylated hydroxytoluene (BHT) was shown by Björkhem and co-workers to inhibit atherosclerosis in cholesterol-fed rabbits (7); N,N’-diphenylphenylenediamine (DPPD) was shown to be effective, again in cholesterol-fed rabbits, by Sparrow and co-workers (8). BHT is structurally closely related to probucol but it does not share the cholesterol-lowering effects of probucol. Thus, the Björkhem study is not confounded by the issue of cholesterol-lowering. On the other hand, BHT has not been tested to see whether it shares some of the other biological properties of probucol. Because of its structural similarity it may very well do so. DPPD is structurally quite different from BHT or probucol and again does not have any cholesterol-lowering effect. The fact that Sparrow and co-workers obtained a result rather similar to that obtained using probucol is consistent with the possibility that probucol works primarily as an antioxidant. However, it does not prove it beyond doubt. Thus there is a need to carry out studies with additional antioxidant compounds and to show unambiguously that the protective effects are due to the antioxidant effect. The present study explores the effects of a new antioxidant, a close structural analogue of probucol (Fig. 1).

Methods

Rabbits and diets. We studied 27 LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits (12 females and 15 males)

1 Abbreviations used in this paper: BHT, butylated hydroxytoluene; DPPD, N, N’-diphenylphenylenediamine; TC, tyramine cellobiose; WHHL rabbit, Watanabe heritage hyperlipidemic rabbit.
from four litters. The animals were divided into three groups, a control group \((n = 10)\), a BM15.0639 group \((n = 11)\), and a probucol group \((n = 6)\). The groups were matched for litter, gender, and plasma cholesterol. Probucol (a gift from Merrell Dow Pharmaceuticals, Inc.) was added to the chow at a concentration of 1% (wt/wt). BM15.0639 [bis(3,5-di-t-tert-butyl-4-hydroxyphenylethyl) propane], a gift from Boehringer Mannheim, Mannheim, Germany, is much more bioavailable than probucol, yielding similar plasma levels at 1/20th the dose of probucol. Therefore it was fed at a concentration of 0.05% (wt/wt). The drugs were added to the chow in diethyl ether and the control chow was similarly treated with plain solvent. The chow was dried for several days before use. The rabbits were fed the diets for 30 wk starting at 13 wk of age. The daily chow ration was gradually increased from 80 to 120 g as the rabbits grew. Extensive studies on the pharmacology and toxicology of BM15.0639 in rabbits were conducted by Boehringer Mannheim. No adverse effects at the doses used were found (H. A. Dresel, unpublished data), and none were found in the present studies.

Plasma lipids and determination of drug levels. Plasma samples were obtained every 2–4 wk after an overnight fast, and cholesterol levels were determined using an automated enzymatic technique (Boehringer Mannheim Diagnostics, Indianapolis, IN). The samples were used to determine drug levels in plasma. The concentrations of probucol and of BM15.0639 were determined using the same HPLC assay. In brief, plasma samples were extracted with methanol/acetonitrile, 3:2 (vol/vol), with addition of 2-panone-bis(3,5-di-t-butyl-4-hydroxyphenyl)methylate as internal standard, and partitioned into heptane. The samples were analyzed by HPLC on a C18-reversed phase column eluted with acetonitrile/heptane/0.1 M ammonium acetate, 92:6:2 (vol/vol). Absorption at 254 nm for probucol and at 234 nm for BM15.0639 was measured. The determination of drug levels in LDL was done using the same method. The concentration of probucol and BM15.0639 in tissue was determined by HPLC after enzymatically digesting, homogenizing, and extracting the tissue (16). In brief, 100 mg of tissue was incubated with 1 ml bacterial collagenase (type I, 5 mg/ml) and 0.5 ml porcine lipase (2 mg/ml) for 3 h at 37°C. Enzymes were from Worthington Biochemicals (Freehold, NJ). To each sample was added 50 μl of ascorbic acid (10 mg/ml). After this digestion the samples were homogenized using a ground-glass pestle-tube system. Internal standard was added and the sample was extracted twice with hexane. The combined hexane phases were dried under a stream of nitrogen. The extracted material was resolubilized in HPLC mobile phase and analyzed as described for plasma drug levels.

Isolation of LDL and LDL modification. LDL \((d = 1.021–1.060 \text{ g/ml})\) was isolated by sequential ultracentrifugation from plasma collected into EDTA after an overnight fast (17). Protein was determined by the method of Lowry et al. (18) with BSA as a standard. At the end of the isolation LDL was extensively dialyzed against PBS containing 2 mM EDTA. LDL was subjected to prooxidative conditions to study its resistance to oxidation as follows: preceding the use of LDL, EDTA was removed by dialysis against PBS. Unlabeled or 125I-labeled LDL was diluted in Ham’s F-10 medium (100 μg LDL protein/ml) and then was incubated in 60-mm plastic dishes with confluent rabbit aortic endothelial cells for 18 h or incubated in PBS in the presence of 5–10 μM CuSO4. The formation of conjugated dienes was measured as the increase in absorption at 234 nm. Lag times were determined graphically as the time point at which the tangent to the curve during the maximum slope of the propagation phase intercepted the time axis. Absorption at the beginning of the reaction was set to zero. Thiobarbituric acid–reactive substances were measured as an index of the degree of lipid peroxidation (19). The extent of LDL oxidation was also assessed in terms of the increase in its rate of degradation by macrophages: 10 μg of 125I-LDL (native or modified) in 0.5 ml DME was added to mouse peritoneal macrophages in 24-well dishes and incubated at 37°C for 5 days. Trichloroacetic acid–soluble radioactivity in the medium and cell-associated radioactivity were then determined.

Extent of aortic lesions. Each rabbit was given 1,000 IU heparin and then was deeply anesthetized with sodium pentobarbital (50 mg/kg). The systemic circulation was perfused with 2 liters of isotonic PBS containing 2 mM EDTA through a large-bore cannula introduced into the apex of the left ventricle while collecting the effluent from the severed right ventricle. The aorta was then fixed in situ with half-strength Karnovsky’s solution for 20–25 min. A perfusion pressure of 80 mm Hg was maintained during the entire procedure, using large elevated reservoirs. The entire aorta was removed and cleaned of loose adventitial tissue. The thoracic and abdominal aortas were divided 5 mm proximal to the celiac artery. Each segment was opened longitudinally and fixed in half-strength Karnovsky’s for an additional 24 h. The aortas were stained with Sudan IV, pinned flat on wax beds, and covered with PBS. They were then photographed and digitally recorded using a Cohu solid-state camera connected to a personal computer via a data-technology 2851 frame grabber board. The captured image was processed using image pro II software (Media Cybernetics, Inc., Silver Spring, MD). The areas of sudanophilic lesions and the total area of each aortic segment were determined (20). The extent of lesions was expressed as percent of total aortic surface area involved.

Metabolic studies. In subsets of four rabbits from each of the three different treatment groups, arterial LDL degradation rates were determined at killing. LDL was isolated from pools of plasma of each group as described above. The LDL was first iodinated conventionally with 125I using 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodogen; Pierce Chemical Co. Rockford, IL). It was then covalently linked to 125I-tyramine cellobiose (125I-T-C), a lysosomally trapped tracer, with cysteic chloride as described (21). After dialysis against PBS containing 2 mM EDTA, <1% of the radioactivity of each isotope was soluble in 10% (wt/vol) trichloroacetic acid. Radioactivity extractable into chloroform/methanol, 1:1 (vol/vol), was 2.4±0.34% for 125I and 1.47±0.33% for 123I. The specific activities of 125I and 123I-T-C ranged from 281 to 462 and 65.4 to 126 cpm/ng protein, respectively. The LDL was used 2–3 d after labeling, which was 3–4 d after initial isolation. The doubly labeled LDL (6.97±1.29 × 10⁴ cpm of 123I and 1.80±0.42 × 10⁴ cpm of 125I) was injected intravenously after the rabbits had been injected with 3 mg of NaI to prevent uptake of radioiodide by the thyroid. The animals received homologous LDL. The plasma decay of labeled LDL was followed over 72 h by obtaining 12 serial samples of blood beginning at 10 min after injection. At killing the systemic circulation was perfused with PBS; the aortas were fixed in situ, dissected, stained, and photographed; and the sudanophilic lesion area was determined as described above. The aortic arch was separated from the descending thoracic aorta 1–2 mm below the ductus scar. Sudan positive atherosclerotic lesions and negative nonlesioned areas were cut out of each aortic segment and weighed. The 125I and 123I contents of tissue and plasma samples were measured in a well-type γ-scintillation-counter (Compu Gamma; LKB Instruments Inc., Gaithersburg, MD) with cor-
Table 1. Inhibition of Cu²⁺- and Endothelial Cell–Promoted LDL Oxidation In Vitro

<table>
<thead>
<tr>
<th>Additions</th>
<th>TBARS nmol MDA/ml</th>
<th>Degradation by macrophages ( \mu g/5 h + mg protein )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>None</td>
<td>1.4; 0.8</td>
</tr>
<tr>
<td>LDL incubated with endothelial</td>
<td>1 µM probucol</td>
<td>2.5; 9.15</td>
</tr>
<tr>
<td>cells</td>
<td>2.5 µM probucol</td>
<td>3.7; 7.30</td>
</tr>
<tr>
<td></td>
<td>5 µM probucol</td>
<td>6; 1.20</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>39; 9.00</td>
</tr>
<tr>
<td></td>
<td>1 µM BM15.0639</td>
<td>Nondetectable; 1.05</td>
</tr>
<tr>
<td></td>
<td>2.5 µM BM15.0639</td>
<td>Nondetectable; 1.45</td>
</tr>
<tr>
<td></td>
<td>5 µM BM15.0639</td>
<td>Nondetectable; 1.13</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>48; 6.75</td>
</tr>
<tr>
<td>LDL incubated with ( \text{Cu}^{2+} )</td>
<td>1 µM probucol</td>
<td>52; 6.05</td>
</tr>
<tr>
<td></td>
<td>2.5 µM probucol</td>
<td>52; 4.65</td>
</tr>
<tr>
<td></td>
<td>5 µM probucol</td>
<td>23; 0.86</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>50; 6.45</td>
</tr>
<tr>
<td></td>
<td>1 µM BM15.0639</td>
<td>55; 3.95</td>
</tr>
<tr>
<td></td>
<td>2.5 µM BM15.0639</td>
<td>41; 0.95</td>
</tr>
<tr>
<td></td>
<td>5 µM BM15.0639</td>
<td>6; 1.00</td>
</tr>
</tbody>
</table>

\( ^{125} \text{I-LDL} (100 \mu g/ml) \) was incubated for 18 h with either 5 µM \( \text{Cu}^{2+} \) or endothelial cells in F-10 medium. The extent of LDL oxidation in the presence of probucol or BM15.0639 was then assessed by measuring thiobarbituric acid–reactive substances (TBARS) and the rate of degradation by macrophages.

Analogue was even more potent in this in vitro test system, inhibiting oxidation of LDL almost completely even at 1 µM.

Oxidation induced by incubation with copper ions was also inhibited effectively by both compounds. Probucol again showed inhibition at 2.5 µM and almost completely blocked at 5 µM; the analogue showed a definite effect at 1 µM and was completely inhibitory at 2.5 µM. It should be noted that the concentrations of drug actually reached within the LDL particles under these conditions is not known; the drugs were simply added in ethanolic solution but no measurements of the fraction entering the LDL particles were made.

Both drugs were also shown to inhibit LDL oxidation assessed in terms of increased electrophoretic mobility and the ability of the oxidatively modified LDL to induce accumulation of cholesterol esters in macrophages. These results were consonant with those described above, i.e., the analogue had a somewhat greater potency than that of probucol itself (data not shown).

**Results**

Inhibition of LDL oxidation in vitro. The potencies of BM15.0639 and probucol in the inhibition of LDL oxidation in vitro were compared. As shown in Table I, probucol showed a partial inhibition of LDL oxidation by endothelial cells at 2.5 µM and almost completely blocked oxidation at 5 µM. The analogue was even more potent in this in vitro test system, inhibiting oxidation of LDL almost completely even at 1 µM.

Oxidation induced by incubation with copper ions was also inhibited effectively by both compounds. Probucol again showed inhibition at 2.5 µM and almost completely blocked at 5 µM; the analogue showed a definite effect at 1 µM and was completely inhibitory at 2.5 µM. It should be noted that the concentrations of drug actually reached within the LDL particles under these conditions is not known; the drugs were simply added in ethanolic solution but no measurements of the fraction entering the LDL particles were made.

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**Plasma drug levels and degree of protection of plasma LDL against oxidative modification ex vivo.** Previous studies of pharmacodynamics at Boehringer Mannheim laboratories had shown that BM15.0639 was much more readily absorbed than probucol (H. Dresel, personal communication). In fact, comparable blood levels were reached at 1/20th the dose of probucol. As shown in Fig. 2, total plasma concentrations of the analogue and of probucol were almost exactly the same when probucol was included in the diet at 1% (w/w) and the analogue at 0.05% (w/w). However, even though the mean total plasma concentrations of the two drugs were similar, the concentration of probucol in the LDL particles was approximately twice as high as that of the analogue: 27.8 nmol probucol/mg LDL protein versus 12 nmol of analogue/mg LDL at 30 wk. Both drugs were transported almost exclusively in lipoproteins, i.e., almost none was found in the 1.21 bottom fraction. The higher concentrations of probucol per LDL particle reflected in part the decrease in total plasma lipoproteins in the probucol-treated group (i.e., the drug was distributed among a smaller total number of lipoprotein particles) and the fact that a higher percentage of the total plasma lipids was found in the LDL fraction in the probucol-treated groups (i.e., the VLDL dropped to a greater extent than did the LDL so that the fraction of total plasma lipids represented by the LDL increased as did its share of the lipophilic drug).

LDL was isolated from the plasma at the end of the feeding period and tested for its resistance to oxidation ex vivo. The
samples were incubated in the presence of 5 or 10 μM CuSO₄ and the rate of conjugated diene formation was followed by measuring absorption at 234 nm. As shown in Fig. 3, LDL from animals treated with probucol was almost completely protected against oxidative modification for over 1,000 min under these conditions. In contrast, LDL from the animals treated with the analogue, although showing a definite extension of lag time compared with control LDL, was much less well protected. The diene conjugation lag time for the analogue-treated animals was 313 min in the presence of 10 μM copper and 410 min in the presence of 5 μM copper. The control samples showed lag times of only a little over 100 min.

To determine whether the results obtained at the termination of the study were representative or not, we studied a small number of animals fed the drugs for 14 d or for 9 wk. As shown in Table II, the results were very similar to those obtained at the end of the study, i.e., the lag time for diene conjugation was prolonged to a much greater extent in the LDL from probucol-treated animals than in the LDL from the analogue-treated animals and both were considerably longer than the value in the controls.

Effects of treatment. None of the animals showed any signs of toxicity and the weight gain in all groups was similar. Total plasma cholesterol in the untreated controls was 788±113 mg/dl during the studies. The analogue did not decrease plasma cholesterol at all (745±102 mg/dl) but probucol, as expected, did (581±115 mg/dl during the study). Lipoprotein profiles, carried out at time zero and after 18 wk of feeding showed that HDL accounted for only <2% of the total plasma cholesterol and there were no significant differences in HDL levels between the probucol-treated and the analogue-treated groups; both showed a drift downward during the course of the study. The LDL fraction accounted for 27% of the total plasma cholesterol at the beginning of the study. However, because there was a large decrease in VLDL cholesterol in the probucol group (~50%), LDL accounted for a larger fraction of total plasma cholesterol at the end of the study. As discussed below, these shifts are relevant to the observed changes in drug concentration within lipoprotein particles.

The extent of sudanophilic lesions in the aortas of the three groups is shown in Table III. The quantification of surface lesion areas by digital imaging showed significantly less atherosclerosis in the probucol-treated animals compared with the control animals (P < 0.01). The total arterial surface area involved in lesions was reduced by almost 50%. The reduction of arteriosclerosis was highly significant (P < 0.01) in all three segments of the aorta. The slowing of the atherosclerotic process was most pronounced in the descending thoracic aorta (−71%). On the contrary, in rabbits treated with BMJ15.0639 the extent of aortic lesions was unchanged compared with the control group. The small differences in individual segments of the aorta were statistically not significant.

In an attempt to assess the extent to which the decrease in plasma cholesterol caused by probucol might have contributed to its observed antiatherogenic effect, a statistical analysis of variance with cholesterol as covariant was performed. Because there was no difference in lesion area between the control group and the analogue-treated group, the results in those two groups were pooled (32.8±6.9% of aortic surface covered by lesions).

Table II. Effects of Probucol and of BMJ15.0639 on Cu²⁺-catalyzed Oxidation of Plasma LDL after 2, 9, and 30 wk of Treatment

<table>
<thead>
<tr>
<th>Length of treatment</th>
<th>Control</th>
<th>BMJ15.0639</th>
<th>Probucol</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 d</td>
<td>137.1 (2)</td>
<td>403.7 (3)</td>
<td>935.8 (3)</td>
</tr>
<tr>
<td>9 wk</td>
<td>135.0 (2)</td>
<td>496.7 (3)</td>
<td>928.3 (3)</td>
</tr>
<tr>
<td>30 wk</td>
<td>123.0 (10)</td>
<td>410.0 (11)</td>
<td>&gt;1080 (6)</td>
</tr>
</tbody>
</table>

LDL (100 μg/ml) was incubated in PBS and oxidation was initiated by adding 5 μM Cu²⁺. Lag times were determined as described in Methods. Values in parentheses are number of animals.

Table III. Extent of Aortic Lesions in WHHL Rabbits after Treatment with BMJ15.0639 or Probucol

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total aorta</th>
<th>Arch thoracic</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of surface area involved</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 10)</td>
<td>32.8±5.8</td>
<td>66.0±11.6</td>
<td>29.1±9.0</td>
</tr>
<tr>
<td>BMJ15.0639 (n = 11)</td>
<td>32.8±4.9</td>
<td>71.5±13.8</td>
<td>27.0±3.8</td>
</tr>
<tr>
<td>Probucol (n = 6)</td>
<td>17.8±3.8</td>
<td>47.6±10.4</td>
<td>8.4±9.4</td>
</tr>
</tbody>
</table>

Surface areas of sudanophilic lesions were determined by digital imaging and expressed as percentage of total arterial surface. Probucol treatment decreased the extent of arteriosclerotic lesions significantly (P < 0.01) compared with untreated and BMJ15.0639-treated animals.
plasma, rabbits, treated BM15.0639-treated examine 4. found were in the wall lesions, in the aorta by assessed. Comparing BM15.0639 (n = 19) animals with 0.01% Probucol (n = 10) animals was significantly decreased in animals treated with probucol (Table IV). Animals treated with BM15.0639 showed no significant difference in fractional cata-
boric rates within lesions.

**Drug levels in aortic tissue and histology.** The concentrations of probucol and BM15.0639 in aortic tissue were determined in a separate set of animals after 2 and 18 wk of treatment. At both time intervals, the concentrations of the compounds were almost identical, both in normal aortic tissue and in lesioned areas. After 18 wk of treatment both compounds were found at much higher concentrations within lesions than in normal aorta. The drug levels measured after 18 wk are shown in Fig. 4.

The cellular composition of lesioned arterial tissue was examined by immunohistology. We used the monoclonal antibodies RAM-11, HIF-35, and MDA2, specific for macrophages, smooth muscle cells, and malondialdehyde-modified LDL, respectively. Comparing lesions at similar stages of development, no obvious treatment effect on cellular composition was seen. To examine the distribution of lipids within the arterial wall, frozen sections were prepared and stained with Sudan black B. A representative number of sections from all three segments of the aorta were analyzed. No obvious change in lipid distribution was found among the three treatment groups.

**Discussion**

These results confirm the effectiveness of probucol as an anti-atherogenic drug in LDL receptor-deficient rabbits. As in previous studies (3–5), the probucol-treated animals showed about a 50% inhibition in the extent of lesions, and the LDL from these animals strongly resisted oxidative modification in vitro. As in the studies of Carew et al. (3), we were able to show that the rate of uptake of injected native LDL into atherosclerotic lesions was sharply reduced in the probucol-treated ani-
mals. This is very likely because probucol inhibits oxidation of LDL, for the following reasons. First, earlier studies have shown that most of the uptake of injected native LDL into lesions in these rabbits is attributable to uptake into macrophages (30). Second, Watanabe rabbits express almost no functional native LDL receptors and, in any case, these would be expected to be downregulated in the face of the very high plasma LDL concentrations found. In fact, in situ hybridization studies (31) have shown that the LDL receptor is markedly downregulated in atherosclerotic lesions but that the acetyl LDL receptor is strongly expressed. Thus it seems reasonable to conclude that most of the uptake of injected native LDL into lesions in this and related studies is attributable to macrophage uptake via the acetyl LDL receptor, which means the LDL must have been first converted from the native form to the oxidatively modified form. Probucol would be expected to inhibit this conversion and the decreased incorporation into lesions in the probucol-treated animals is consistent with this interpretation. Alternative interpretations, however, are possible as discussed below.

The probucol analogue studied here (BM15.0639) was just as effective as an antioxidant in vitro as probucol. Because of the much greater bioavailability of the analogue and because we wanted to try to achieve comparable plasma levels of the two compounds, the analogue was fed at only 0.05% in the diet whereas probucol was fed at 1% (wt/wt). The total plasma concentrations of the two drugs at the end of the studies were in fact about the same. Lesion development, however, was not affected by the treatment with BM15.0639. The rates of LDL degradation within arterial lesion sites of BM15.0639-treated rabbits was also not significantly different from control animals, suggesting that oxidation of LDL was not being inhibited. LDL isolated from plasma of the analogue-treated animals at the end of the study was protected against oxidation, but not nearly as well as LDL from probucol-treated rabbits. Whereas probucol prolonged the diene conjugation lag time to values of ≥1,000 min, the analogue only extended the lag time to ~ 400 min (compared with the normal value of 130 min). Although the total plasma concentrations were similar, the number of molecules of probucol per LDL particle was twice the number of analogue molecules per particle. This was accounted for by the

<table>
<thead>
<tr>
<th></th>
<th>Arch</th>
<th>Descending thoracic</th>
<th>Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (n = 4)</td>
<td>24.8±3.6</td>
<td>21.0±11.9</td>
<td>10.9±1.9</td>
</tr>
<tr>
<td>BM15.0639 (n = 4)</td>
<td>18.4±5.4</td>
<td>21.5±12.6</td>
<td>8.0±4.7</td>
</tr>
<tr>
<td>Probucol (n = 4)</td>
<td>13.1±4.5</td>
<td>10.0±8.9</td>
<td>5.9±2.3</td>
</tr>
</tbody>
</table>

Analysis of variance with repeated measures was used to compare data between groups. Differences between probucol-treated and untreated animals were significant (P = 0.001). BM15.0639-treated animals were not different from untreated at P = 0.372.
factors: (a) the total plasma cholesterol of the probucol-treated animals fell by 25% so that the drug was distributed into a smaller total number of lipoprotein particles than in the case of the analogue-treated group and (b) the fraction of the total plasma cholesterol present in the LDL fraction increased by almost 50%. The latter was mainly due to a large drop in VLDL cholesterol. Thus, the larger number of probucol molecules in the LDL may be enough to explain the difference in degree of protection against oxidation. It is also possible that metabolites of probucol carried in LDL (not measured in these studies) may make a contribution to the protection against oxidation seen in the probucol-treated group. The analogue probably is not metabolized in the same way as probucol. Lacking the sulfur atoms in the bridge (see Fig. 1), its metabolism may be limited to the aromatic rings. Whereas metabolites of probucol were detected by HPLC, no metabolites of BM15.0639 were noted. Whatever the mechanism involved, the key question is whether the difference in degree of protection against oxidative modification ex vivo is sufficient to explain the ineffectiveness of the analogue in slowing the progression of atherosclerotic lesions. Compatible with this conclusion is the fact that the incorporation of injected native LDL into arterial lesions was not inhibited in the analogue-treated rabbits.

Little or no information is available that relates effectiveness of antioxidants in vitro to their antiatherogenic potential. We do not know whether there is a graded relationship or a threshold relationship. The latter possibility is a real one. Consider the transport of LDL into and out of the arterial wall. LDL particles will on the average reside within the artery wall for some defined time interval (mean residence time). Schwenke and Carew (32) have estimated the mean residence time of LDL particles in the normal rabbit aorta and in the aorta of animals with experimental atherosclerosis. Under normal conditions, with a short residence time within the artery wall, LDL might undergo very little oxidative damage during transit. Once it reenters the plasma compartment, the chances that it will reenter the artery are extremely small, since only a very small fraction of LDL turnover is attributable to arterial uptake. If the degree of oxidation of a particular LDL particle goes beyond a certain point during its passage through the artery wall, that LDL may become a target for uptake via scavenger receptors or it may undergo complexing with connective tissue matrix, or it may aggregate with other LDL particles. In that case, its fate may be to stay indefinitely within the artery wall and wind up being taken up by macrophage scavenger receptors. The central point here is that in order to be effective, an antioxidant residing in the LDL particle may need to protect it for a length of time near to or greater than the residence time of the LDL within the artery wall. That time may not be the same as the diene conjugation lag time in absolute terms, because the latter is measured under artificial in vitro conditions that may or may not reflect the pro-oxidant “stress” within the artery wall, but there might be some proportionality. It is conceivable that there is a threshold level of protection that would represent the minimum necessary to exert an antiatherogenic effect. In this connection, a recently completed study of the effectiveness of probucol in Macaca nemestrina may be pertinent. Sasahara and co-workers (33) showed that probucol (1% wt/wt in the diet) inhibited lesion formation by ~50% in the thoracic aorta (P < 0.001). No effects were seen in the abdominal aorta nor in the iliac arteries, possibly because lesions were more advanced in these arterial segments. The investigators measured diene conjugation lag time in the LDL isolated from these animals at the end of the study. What they found was that there was a negative correlation between the extent of lesion area and the prolongation of the diene conjugation lag time. They suggested that diene conjugation lag times needed to be = 400 min in order to significantly inhibit lesion formation. This value is near the diene conjugation lag time achieved with the analogue in the present studies, an effect that was not associated with inhibition of lesion formation. This should not be overinterpreted because of the species difference but the coincidental finding of a similar “threshold” in prolongation and lag time is worth noting.

Although the proposed antioxidant mechanism is appealing, it is still unclear whether this is the major underlying mechanism of probucol’s action. This is especially true, because BM15.0639 failed to slow the progression of atherosclerosis despite protecting LDL against oxidation ex vivo. The degree to which LDL was protected was remarkably high compared with maximally achievable protection with natural antioxidants, such as vitamin E. Probucol has a number of additional effects which therefore need to be considered. First, probucol does have a cholesterol-lowering effect and cholesterol levels were somewhat lower in the probucol-treated group. However, the difference was small and, as discussed above, correction for the effect of the degree of hypercholesterolemia on the extent of lesion formation accounts for only a very small part of the probucol effect in lesion formation. In the studies of Carew et al. (3) the control rabbits were treated with a small dose of lovastatin, just enough to match the plasma cholesterol levels in those treated with probucol. Thus, a difference in cholesterol levels did not contribute to the antiatherogenic effect of probucol in those studies. In the studies of Sparrow et al. (8), using DPPD, and the studies by Björkhem et al. (7), testing BHT, there were no differences in cholesterol level and the effectiveness of those two antioxidants in cholesterol-fed rabbits is clearly not due to a cholesterol-lowering effect. For all of these reasons it does not seem likely that the different results in the present studies are due to the small decrease in cholesterol levels induced by probucol.

A second category of explanation for the difference in effectiveness of the two compounds against atherogenesis is that probucol has additional biological properties that may not shared by the analogue. As pointed out in the introduction, several of these properties could be relevant to its antiatherogenic effect (12–15). The analogue has not yet been tested to determine whether it shares these properties with probucol.

Whatever the explanation of the unexpected ineffectiveness of BM15.0639 against atherogenesis, these negative results are instructive. Many investigators, including ourselves, have tended to assume that the relative effectiveness of antioxidants as antiatherogenic agents would parallel their effectiveness in protecting LDL in ex vivo measurements of oxidation under controlled conditions. Clearly things are more complex. Much more work is needed to sort out what the true relationship is and to search for other relevant variables.

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References