Attenuation of Atherosclerosis in a Modified Strain of Hypercholesterolemic Watanabe Rabbits With Use of a Probucol Analogue (MDL 29,311) That Does Not Lower Serum Cholesterol

Simon J.T. Mao, Mark T. Yates, Roger A. Parker, Eric M. Chi, and Richard L. Jackson

Probucol is a drug that lowers plasma cholesterol in both humans and animals. In low density lipoprotein (LDL) receptor–deficient Watanabe rabbits, probucol reduces the progression of atherosclerosis. This effect may be attributed to the antioxidant and/or the cholesterol-lowering properties of the drug. In the present report we studied the antiatherogenic effect of a probucol analogue (MDL 29,311) that possesses antioxidant activity but that does not lower cholesterol. Modified Watanabe rabbits (11–12 weeks of age) produced by crossing British Brown and Japanese Watanabe rabbits were fed normal chow (n=8), chow containing 1% probucol (n=9), or chow containing 0.1% (n=9), 0.5% (n=8), or 1% (n=6) probucol analogue. After 70 days serum cholesterol levels and the percent area of sudanophilic lesions in the thoracic region of aortas were determined. Total serum cholesterol was significantly lowered (p<0.05) in the probucol group (560±54 mg/dl) compared with that of controls receiving no drug (731 ±67 mg/dl) but was not lowered in the analogue groups (722-802 mg/dl). The lesioned area (mean%±SEM) in the probucol group (16±3) was significantly lower (p<0.01) than in the controls (52±8). There were 43±7%, 33±8%, and 35±5% of lesions for the 0.1%, 0.5%, and 1% analogue groups, respectively. After combining the data for the 0.5% and 1% analogue groups, the value (34%) was lower than that of the controls and almost reached significance (p=0.066). The mean serum drug concentration in the 1% probucol group was 58±4 μg/ml compared with 13±2, 44±8, and 74±8 μg/ml for the 0.1%, 0.5%, and 1% analogue groups, respectively. Thus, the decreased effectiveness of the probucol analogue in preventing atherosclerosis could not be explained by a lack of bioavailability. LDLs isolated from rabbits treated with the drug were resistant to Cu²⁺-induced lipid peroxidation, as determined by thiobarbituric acid–reactive substances. The resistance within the analogue groups was dependent on the number of antioxidant molecules per LDL particle. However, there was no significant difference in atherosclerotic lesions between these two groups, suggesting, although not definitively, that the maximal antiatherogenic effect had been reached. Our data suggest that the antioxidant activity of this class of compounds may play an important role in reducing atherosclerosis, but not in reducing cholesterol levels, and that hypocholesterolemic and possibly other activities of probucol might further enhance its antiatherogenic activity. (Arteriosclerosis and Thrombosis 1991;11:1266–1275)

The formation of foam cells derived from monocyte–macrophages in the arterial wall is thought to be an important event in the pathogenesis of atherosclerosis. 1–7 In vitro, macrophages accumulate cholesteryl esters when incubated with acetylated low density lipoproteins (LDLs)8,9; malondialdehyde (MDA)-modified LDL,10 endothelial cell–, smooth muscle cell–, monocyte–, or macrophage-incubated LDL,11–15; oxidatively modified LDL,15–21; or LDL aggregates.22 Recently, the presence of MDA epitopes, MDA modified LDL, or both in the atheroma of Watanabe heritable hyperlipidemic (WHHL) rabbits and humans has been reported.23–25
Probucol, a potent antioxidant, is a drug that lowers plasma total cholesterol in both humans and animals. It also causes a rapid and marked regression of cutaneous and tendon xanthomas in humans. In vitro, probucol inhibits oxidative modification of LDL resulting from its antioxidant property. Carew et al have shown that probucol inhibits the rate of progression of atherosclerotic lesions in WHHL rabbits. In this study, control WHHL rabbits were treated with lovastatin, a cholesterol-lowering agent, at doses that maintained plasma cholesterol in the two groups at approximately the same levels. Kita et al have reported similar results on the antiatherogenic effects of probucol in a comparable study of WHHL rabbits. The evidence favors the conclusion that the antioxidant activity of probucol may attribute to its antiatherogenic action. Because both the cholesterol-lowering and the antioxidant activity of probucol could contribute to the reduction of atherosclerosis, the present study was designed to use a probucol analogue (bis(3,5-di-tert-butyl-4-hydroxyphenylthio)methane) (MDL 29,311) that possesses antioxidant but not cholesterol-lowering activity to determine its effect on atherogenesis in the WHHL rabbit.

Methods

Probucol and its analogue MDL 29,311 were synthesized as described previously. The purity of each compound (>98%) was determined by mass spectral analysis and reverse-phase high-performance liquid chromatography (HPLC).

Source of Modified Watanabe Heritable Hypertensive Rabbits and Administration of Probucol and Its Analogue

Probucol or its analogue was mixed and repelleted with Purina rabbit chow by Purina (Richmond, Ind.). The final content of the compound was 1% (wt/wt) for probucol and 0.1%, 0.5%, or 1% for the probucol analogue. The drug content was confirmed by extraction of the compound from the chow and quantification by HPLC. British half-lap WHHL rabbits were provided by Thomas Parker and Thomas Donnelly (Rogosin Institute, New York, N.Y.). This modified strain of WHHL rabbit was obtained by cross-breeding British half-lap rabbits with Japanese WHHL rabbits. All modified WHHL rabbits were homozygous for the LDL receptor deficiency. The biochemical and pathological features of this modified WHHL rabbit have been described by Gallagher et al. The protocol for the administration of probucol and its analogue and the number of animals used in this study are summarized in Figure 2. Briefly, expansion of modified WHHL litters was obtained by superovulating four female WHHL rabbits with follicle-stimulating hormone and human chorionic gonadotropin; the female rabbits were then artificially inseminated with sperm from seven WHHL male donors. The embryos were transferred to 14 pseudopregnant normal foster New Zealand White rabbits for gestation. Forty WHHL rabbits (22 females and 18 males, aged 11–12 weeks) from seven litters were equally divided into control (containing no drug), probucol, and probucol analogue groups. Each rabbit was fed 100 g/day of the diet for 70 days. After this time animals were killed by intravenous injection of pentobarbital (1–1.5 ml/kg body wt).
Plasma was collected for the isolation of lipoproteins, and serum was used for the determination of lipid levels and drug concentrations.

**Determination of Drug Concentrations**

Rabbit serum (100 μl) or LDL sample was added dropwise to 2 ml of an ether/ethanol mixture (3:1, vol/vol) while it was being vortexed. The mixture was vortexed for an additional 2 minutes and then centrifuged for 15 minutes at 2,000 rpm (90g). The supernatant fractions were transferred to borosilicate glass tubes (12×75 mm) and dried under N2 gas. The dried extracts were then dissolved in 200 μl acetonitrile/hexane/0.1 M ammonium acetate (90:6.5:3.5, vol/vol/vol) and subjected to HPLC. The separations were performed by use of 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 A; Waters) was used with the mobile phase acetonitrile/water (85:15, vol/vol) at a flow rate of 1.5 ml/min as previously described. Standards were prepared by dissolving the compound in acetonitrile. Analytical recovery was determined by addition of the drug into WHHL rabbit serum containing no drugs. LDLs (d=1.019-1.063 g/ml) were isolated from the plasma of each WHHL rabbit by the method of Havel et al., and stored in PBS containing 0.01% NaN3 without EDTA, were used for lipid peroxidation. LDLs (250 μg) in a volume less than 100 μl were diluted with distilled water (double deionized) to give a final volume of 1.45 ml. CuSO4 (50 μl) was then immediately added to a final concentration of 5 μM, followed by an incubation at 37°C for 0–6 hours. The reaction was stopped by adding 100 μl 50 mM Na3EDTA. Then, 50 μg Cu2+-treated LDL was added to 1.5 ml 20% trichloroacetic acid and vortexed. Finally, 1.5 ml 0.67% thiobarbituric acid in 0.05N NaOH was added and incubated at 90°C for 30 minutes. Samples were centrifuged at 1,500 rpm for 10 minutes, and the absorbance of the supernatant was read at 532 nm (Ultraspex Plus, 4054 ultraviolet/visible spectrophotometer; LKB) to determine the content of lipid peroxides (thiobarbituric acid–reactive substances) according to the procedure of Yagi. A standard curve (0–5 nmol) of MDA was generated by using MDA bis(di-methylacetal) as a reference to determine the lipid peroxidation content of Cu2+-treated LDL.

**Determination of Low Density Lipoprotein Lipid Peroxidation Induced by Cu2+**

Freshly prepared LDLs, stored in PBS containing 0.01% NaN3 without EDTA, were used for lipid peroxidation. LDLs (250 μg) in a volume less than 100 μl were diluted with distilled water (double deionized) to give a final volume of 1.45 ml. CuSO4 (50 μl) was then immediately added to a final concentration of 5 μM, followed by an incubation at 37°C for 0–6 hours. The reaction was stopped by adding 100 μl 50 mM Na3EDTA. Then, 50 μg Cu2+-treated LDL was added to 1.5 ml 20% trichloroacetic acid and vortexed. Finally, 1.5 ml 0.67% thiobarbituric acid in 0.05N NaOH was added and incubated at 90°C for 30 minutes. Samples were centrifuged at 1,500 rpm for 10 minutes, and the absorbance of the supernatant was read at 532 nm (Ultraspex Plus, 4054 ultraviolet/visible spectrophotometer; LKB) to determine the content of lipid peroxides (thiobarbituric acid–reactive substances) according to the procedure of Yagi. A standard curve (0–5 nmol) of MDA was generated by using MDA bis(di-methylacetal) as a reference to determine the lipid peroxidation content of Cu2+-treated LDL.

**Determination of Atherosclerotic Lesions**

Immediately after the animals were killed, each aorta was dissected from the ascending arch to the iliac bifurcation. Extraneous adipose tissue was removed, and the aorta was opened longitudinally and rinsed several times with saline. Some of the aortic arch and abdominal regions of aortas in control and probucol groups were dissected for drug determination and other studies not reported in this investigation. The aortas were then stained with Sudan IV by a procedure described previously and photographed. Only the

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**TABLE 1. Body Weight and Serum Total Cholesterol Levels of Modified Watanabe Heritable Hyperlipidemic Rabbits Before and After Treatment**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day 0</th>
<th>Day 70</th>
<th>Day 0</th>
<th>Day 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>1.81±0.09</td>
<td>2.67±0.09</td>
<td>608±33</td>
<td>731±67</td>
</tr>
<tr>
<td>Probucol (1%)</td>
<td>9</td>
<td>1.72±0.12</td>
<td>2.63±0.08</td>
<td>625±31</td>
<td>560±54</td>
</tr>
<tr>
<td>Analogue (0.1%)</td>
<td>9</td>
<td>1.72±0.09</td>
<td>2.60±0.06</td>
<td>696±52</td>
<td>722±82</td>
</tr>
<tr>
<td>Analogue (0.5%)</td>
<td>8</td>
<td>1.75±0.06</td>
<td>2.66±0.06</td>
<td>609±61</td>
<td>738±74</td>
</tr>
<tr>
<td>Analogue (1%)</td>
<td>6</td>
<td>1.76±0.09</td>
<td>2.75±0.12</td>
<td>645±34</td>
<td>802±54</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*p<0.05 compared with control group at day 70.

†Not significant when compared with control and other analogue groups at day 70.
areas of sudanophilic lesions in the thoracic region of the aorta were digitized by use of a Magiscan image analysis system (Joyce-Loebl Ltd.). Stained versus unstained areas were distinguished by the imaging system based on the density of Sudan IV red staining. The corresponding lesioned and nonlesioned areas were quantified accordingly.

Other Methods

For the preparation of plasma, blood was collected in EDTA (0.1% final concentration) and centrifuged at 4°C to remove cells. Cholesterol was determined by an enzymatic method. Identification of probucol and analogue metabolites was confirmed by mass spectrum and nuclear magnetic resonance.

Results

Table 1 lists the body weights and serum cholesterol levels before and after drug treatment. Each rabbit was given 100 g of the chow per day; there was no significant difference in body weights among the groups at the end of the study (day 70). Both control-
**FIGURE 4.** Biotransformation pathway for probucol reported for the monkey in liver and adipose tissues. Detection of the bisphenol (peak 1 in Figure 3), spiroquinone (peak 2 in Figure 3), and diphenoquinone (peak 3 in Figure 3) metabolites of probucol in rabbit serum suggests that biotransformation of probucol in the rabbit is the same as in the monkey.

**TABLE 3.** Cu²⁺-Induced Lipid Peroxidation of Low Density Lipoprotein Isolated From Modified Watanabe Heritable Hyperlipidemic Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>0 hour</th>
<th>3 hours</th>
<th>6 hours</th>
<th>Drug molecules per LDL particle†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>1.8±0.4</td>
<td>39.1±0.9</td>
<td>39.6±1.0</td>
<td>...</td>
</tr>
<tr>
<td>Probuco 1.0%</td>
<td>9</td>
<td>1.4±0.3</td>
<td>0.8±0.3</td>
<td>2.7±0.3</td>
<td>10.0±0.5</td>
</tr>
<tr>
<td>Analogue 0.1%</td>
<td>9</td>
<td>1.3±0.2</td>
<td>27.1±1.3</td>
<td>42.7±1.2</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Analogue 0.5%</td>
<td>8</td>
<td>1.5±0.5</td>
<td>5.2±0.5</td>
<td>31.1±2.6</td>
<td>8.4±0.9</td>
</tr>
<tr>
<td>Analogue 1.0%</td>
<td>6</td>
<td>1.5±0.6</td>
<td>2.4±0.5</td>
<td>7.6±4.3</td>
<td>12.9±1.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
LDL, low density lipoprotein; conc, concentration.

*Thiobarbituric acid-reactive substances (TBARS) were determined with the use of malondialdehyde (MDA) as a reference. Briefly, 250 μg of each LDL sample was subjected to 0–6 hours' oxidation at 37°C by addition of 5 μM Cu²⁺ in a final volume of 1.5 ml. Reaction was stopped by addition of 100 μl 50 mM Na₂EDTA. Fifty micrograms of each treated sample was then assayed for TBARS.

†For calculations, molecular weight (MW) of LDL protein used was 550,000, as each LDL particle contains one copy of apolipoprotein B (MW —550,000), the major protein moiety of LDL (>95%). The following formula was used for the calculation:

\[
\text{Compound conc (μg/ml) in LDL/MW of compound} = \frac{\text{Drug molecules per LDL particle}}{\text{LDL protein conc (μg/ml)/550,000}}
\]

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and probucol analogue (MDL 29,311)-treated groups showed an increase in serum cholesterol (ranging from 12% to 24%) from day 0 to day 70. In probucol-treated groups, however, cholesterol was decreased by 10% (625±31 versus 560±54 mg/dl). Compared with the control group at day 70 (560±54 versus 731±67 mg/dl), probucol significantly lowered cholesterol by 23% (p<0.05), which is consistent with previous reports. In contrast, serum cholesterol levels in analogue-treated groups were not decreased compared with the control animals.

To investigate whether the lack of a cholesterol-lowering effect in the analogue groups was due to differences in bioavailability of the drug, drug concentrations in rabbit serum were determined by an HPLC technique. Typical HPLC profiles for probucol and its analogue extracted from serum are illustrated in Figure 3. As shown in Table 2, the serum analogue concentrations were correlated with the increased doses given in the rabbit chow. The concentration in the 1% analogue group (73.5±7.9 µg/dl) was significantly greater than that of the 1% probucol group (57.5±3.8 µg/ml). Thus, the lack of a cholesterol-lowering effect by the analogue could not be explained by decreased bioavailability.

In addition to the parent compounds, three metabolites of probucol and the analogue were identified in WHHL rabbit serum (Figure 3). These three metabolites eluted at retention times of 5 minutes (bisphenol), 24 minutes (spiroquinone), and 25 minutes (diphenoquinone), or peaks 1, 2, and 3, respectively (Figures 3C and 3D). The chemical structure and proposed metabolic pathways are described in Figure 4. This biotransformation pathway is based on that previously reported for the monkey (Macaca mulatta) in liver and adipose tissues. In this pathway, probucol is first oxidized to the intermediate spiroquinone, and then the spiroquinone disproportionates to the diphenoquinone, which is then reduced to the bisphenol. In the analogue-treated rabbits, spiroquinone was not detected (Figure 3D). The spectral characteristics of these metabolites are shown in Figure 5. Diphenoquinone is a greenish-yellow compound with a maximal absorbance at 420 nm. Thus, the presence of this metabolite explains why plasma or lipoprotein fractions isolated from cholesterol-fed animals treated with probucol are greenish-yellow. The physiological and pharmacological roles of these metabolites are currently unknown.

The extent of Sudan IV-stained fatty streak lesions in the aortas of the five groups of WHHL rabbits are shown in Figure 6. Macroscopically, probucol-treated groups had significantly less atherosclerosis than did the analogue-treated groups. Some of the aortic arch and abdominal regions of control and probucol groups were removed for the determination of drug content and other studies. Only the surface lesions in the thoracic region were further quantified by a digital imaging system. Figure 7 shows that the percent lesion (mean±SEM) in the probucol group (16±3%) was significantly lower than that in the control group (52±8%) (p<0.01). The effect of the analogue in reducing the extent of atherosclerosis was apparent but was not as significant as that of the probucol group. There was no significant difference in lesion percentage (33±8% versus 35±5%) between the 0.5% and the 1% analogue-treated groups (p>0.1). The combined 0.5% and 1% analogue groups (34%) were different from untreated controls (52±8%), and this difference was almost significant (p=0.066). Interestingly, the protective effect of the analogue seems to be maximal at a drug concentration of 0.5% in the diet, or 44 µg/ml in the serum (Table 2).

We have attempted to determine the presence of the drug in the aorta. Except in lesion-involved areas, neither probucol nor the analogue was detectable in the arterial wall (data not shown). This result indicates that the drug may not directly incorporate into the arterial wall. The drug found in lesion areas must
be carried by atherogenic lipoprotein particles. For this reason, we next determined the drug concentrations in isolated plasma LDL from the rabbits. As shown in Table 2, approximately 70% of the drug in serum was present in LDL in all treated groups.

The resistance to oxidative modification of LDL with probucol and its analogue in isolated LDL particles was also studied (Table 3). Lipid peroxidation of LDL was induced by incubation with Cu²⁺ for 0–6-hours, and the content of thiobarbituric acid-reactive substances was measured. Within the analogue groups, a positive correlation between antioxidant activity and molar drug concentration in LDL was observed. LDL isolated from the 0.1% analogue group showed a slight protective effect against lipid peroxidation; presumably, this was due to the presence of a limited number of antioxidant molecules per LDL particle (2.4 molecules/LDL particle). The number of probucol molecules found in each LDL particle (10/LDL) is similar to that found in human subjects with familial hypercholesterolemia who received probucol (500 mg) twice a day for 6 months or longer. Although the LDL isolated from the 1% analogue group contained a greater number of molecules (12.9/LDL) and has higher antioxidant activity than that from the 0.5% group (8.4/LDL) (Table 3), the protection against atherosclerosis was not enhanced further in vivo (Figures 6 and 7).

Discussion

The modified Watanabe rabbits used in the present study are hybrids of the Japanese WHHL and British half-lop species. This species of animal was chosen for its rapid and reproducible development of aortic lesions. For example, it normally takes 10–12 weeks after weaning to develop 50% surface involvement of fatty streak lesions in this modified strain compared with 1 year in Japanese WHHL rabbits. In
addition, the hybrid animal does not develop hypertriglyceridemia, a trait of the WHHL rabbit.

Carew et al. have shown that probucol inhibits the rate of degradation of LDL in fatty streak lesions of WHHL rabbits and slows atherosclerosis development. One possible mechanism for this activity is that the antioxidant property of probucol present in LDL prevents the oxidative modification of LDL. Oxidatively modified LDLs are the most likely atherogenic lipoproteins in vivo, and they are thought to play an important role in the atherogenesis in WHHL rabbits.33

The exact site at which probucol prevents LDL oxidation, however, has not been demonstrated. Haberland et al. have recently shown that a product of lipid peroxides, MDA, is colocalized with apo B of LDL in the aortic lesions of WHHL rabbits, and this finding has been further confirmed by other reports.24,25,47 These results suggest that MDA may modify apo B of LDL and accelerate foam cell formation. MDA-modified LDLs, however, have not been found in plasma,23,25 and most of the MDA epitopes are found associated with the nonlipoprotein fractions.25 It is conceivable that an MDA modification of LDL might occur inside the arterial wall rather than in the circulation, as there are naturally occurring antioxidants in the circulation that may protect LDL from lipid peroxidation. The prevention of LDL oxidation must be directly from the probucol associated with LDL. Nagano et al. and Ku et al. have also shown that the presence of probucol in macrophages does not prevent foam cell transformation. It is worth mentioning that some water-soluble antioxidants in plasma should not be totally ignored, as they might also transport into the arterial wall to have some antioxidative role.

Thus, it further suggests that plasma itself is a good antioxidant. Steinberg et al. suggest that the in vivo LDL oxidation must occur extravascularly and that it requires a favorable microenvironment protected from naturally occurring antioxidants. Except for α-tocopherol, these plasma antioxidants are not associated with LDL, and we speculate that they might not be as effective as probucol in preventing the lipid peroxidation of LDLs that are present in the arterial wall. Several studies33,34,55 have proposed that the direct association of antioxidants in LDL plays a beneficial role for antiatherogenic activity in modified WHHL rabbits.33

Although probucol is a potent antioxidant, it also reduces plasma cholesterol in animals. The mechanism by which probucol reduces total serum cholesterol in WHHL rabbits still remains unclear. The present study, however, suggests that the cholesterol-lowering effect of probucol may be independent of its antioxidant activity because the analogue, which possesses antioxidant activity, did not affect cholesterol levels (Table 1). The lipid-lowering activity may also account for a factor in antiatherogenesis. Using this probucol analogue, we thought it would be one approach to investigate the importance of an antioxidant in preventing atherosclerosis. The present stud-
ies show that the antiatherogenic effect of MDL 29,311 was dependent on the concentration of the drug. The maximal protection from atherosclerosis by the probucol analogue was reached approximately between 40 and 70 μg/ml in serum, or between 30 and 50 μg/ml in LDL (Table 2). Although without offering ultimate proof, Figure 7 predicts that further increases of the antioxidant concentration in plasma may not attenuate atherosclerotic lesions. The reduction of lesions by probucol was greater than that by the analogue (Figure 4), indicating that the cholesterol-lowering effect may partially be responsible for the additional protection from atherosclerosis. Furthermore, probucol may have other actions, such as enhancement of reverse cholesterol transport,68-69 increase of cholesterol ester transfer in the HDL subfraction,60 and inhibition of interleukin-1β secretion,61,62 that may not be possessed by the analogue. LDLs isolated from WHHL rabbits and containing probucol were slightly more resistant against in vitro oxidative modification than were LDLs containing analogue (Table 3). Whether this may also account for some additional protection from atherosclerosis in vivo is not known at the present time. In particular, we do not know that the Cu2+-induced lipid peroxidation in vitro is equivalent to that which occurs inside the arterial wall. For example, LDL from the 1% analogue–treated group showed a much greater antioxidant activity than that of the 0.5% group, yet the higher concentration did not exhibit further protection against atherosclerosis. Thus, the data suggest that 1) the structure of the drug molecule determines its antioxidant activity in LDL, 2) the concentration of antioxidant in LDL is positively correlated with the protection against oxidative modification in vitro, and 3) the antiatherogenic effect resulting from an antioxidant might be saturable in vivo. In other words, the antioxidant activity of LDL may play an important but not complete role in the prevention of atherosclerosis.

Acknowledgments

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**Key Words**: probucol • probucol analogue • antioxidant • cholesterol lowering • atherosclerosis • Watanabe heritable hyperlipidemic rabbits
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