J. Pharm. Pharmacol. 1994, 46: 797–800
Received October 27, 1993
Accepted April 8, 1994

Location of Probucol in Lipoproteins Inferred from Compositional Analysis of Lipoprotein Particles. An In-vitro Study

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Abstract—The location of labelled probucol in lipoprotein particles was investigated in-vitro. Human serum was incubated for 4 h at 37°C with [^{14}C]probucol to incorporate probucol into lipoproteins. Serum lipoprotein particles were then isolated according to their apolipoprotein markers by sequential immunoaffinity chromatography at 4°C, and probucol concentration was determined in each lipoprotein fraction. Analysis of probucol distribution vs lipoprotein components revealed that probucol in particles strongly correlated with phospholipid concentration. Analysis of probucol distribution vs lipoprotein physical characteristics showed that probucol strongly correlated with the surface area of the monolayer surrounding the lipidic core of particles constituting phospholipids and free cholesterol. These data support the hypothesis that probucol is preferentially located in the phospholipid/free cholesterol monolayer surrounding the lipid core, in the vicinity of cholesteryl ester at the core surface or in the vicinity of hydrophobic areas of apolipoprotein that faces the monolayer.

Probucol, 4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol), is an antioxidant that also reduces plasma cholesterol concentration in patients with hypercholesterolaemia. It has also been shown to be effective in preventing the progression of atherosclerosis in Watanabe heritable hyperlipidaemic rabbits and reducing tendon xanthoma in man; for a review on these effects, see Zimetbaum et al (1990). The mechanisms of action of the drug have not been completely elucidated at the molecular level; however, those related to the antioxidant properties can reasonably be ascribed to probucol's close interactions with plasma lipoproteins, by which probucol is mainly transported in the blood (Dachet et al 1985).

Lipoproteins were first isolated according to their density. However, the immunological characterization of lipoprotein density classes has shown that each consists of a mixture of lipoprotein particles with similar density or electrical charges but differing in apolipoprotein composition (Alaupovic 1982). Apolipoproteins determine lipoprotein lipid composition, lipoprotein interactions with specific plasma membrane receptors, or may act as enzymatic effectors. Thus, protein particles isolated on the basis of apolipoprotein composition may be ascribed particular physiopathological properties. For these reasons, the distribution of probucol, whose antioxidant action is thought to take place in the lipoprotein itself, was investigated in-vitro among eight apolipoprotein-isolated lipoprotein particles and the determinants of probucol distribution were analysed.

Materials and Methods

Chemicals

[^{14}C]Probucol (11 mCi mmol^{-1}) was kindly provided by Merrell Dow Research Institute (Indianapolis, IN). The radiochemical purity of the drug was 99·5% as assessed by the manufacturer.

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Incorporation of probucol into serum

Blood was withdrawn in glass tubes from two healthy donors by antecubital venipuncture. Samples were allowed to clot at room temperature (21°C) for 2 h and then centrifuged to yield serum. Labelled probucol in ethanol solution (30 μL, 200 μg mL^{-1}) was added to serum while the solution was vortexed to attain a final concentration of approximately 10 μM in 70 mL. Samples were capped under N_2 atmosphere and incubated with gentle orbital agitation (speed N°6) in a Brunswick water bath for 4 h at 37°C. Following incubation, the serum was filtered through a Minisart filter (0·45 μm). This last procedure allowed the separation of the probucol incorporated into serum lipoproteins (filtered) from the probucol free in serum water (unfiltered). The final probucol concentration in the filtered serum was 9 μM.

Isolation of lipoprotein particles from probucol-loaded serum

Serum was subjected to sequential immunoaffinity chromatography at 4°C using antibodies against apolipoproteins (apo), apoA-I, apoA-II, apoA-IV, apoB, apoC-III and apoE, and monitored at 280 nm as previously described in detail (Agnani et al 1991; Bard et al 1991). For simplicity, the lipoprotein particles were named according to their composition in the main apolipoproteins; the following lipoprotein particles were finally obtained, LpA-I, LpA-II : A-I, LpB, LpB : E, LpB : C-III, LpB : C-III : E, Lp(a) and Lp-B : A-I : A-II : A-IV (see Fig. 1). The lipid and apolipoprotein compositions were measured for each fraction as previously described. The volume and radioactivity of each fraction were also measured to determine probucol-associated concentration and probucol distribution among the particles.

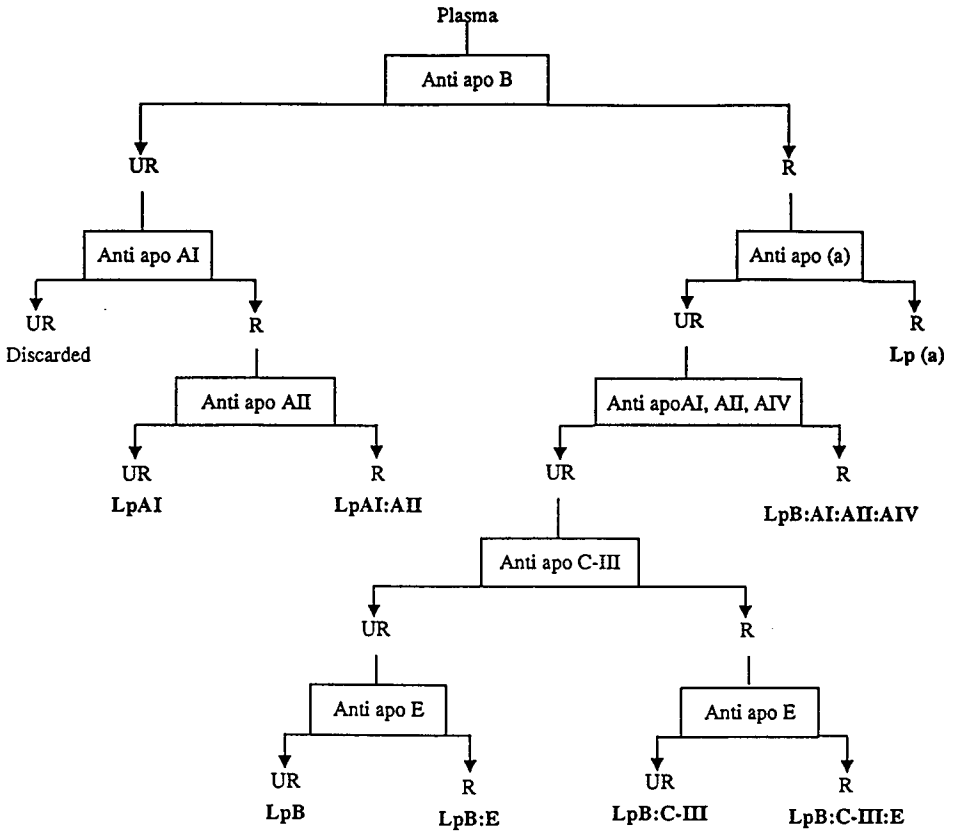

FIG. 1. Flow diagram for immunoaffinity column chromatography isolation of lipoprotein particles. R, Retained fraction, UR, unretained fraction. The boxes represent affinity columns containing monospecific antibody directed against the apolipoprotein indicated.

Data analysis

Probucol concentration in lipoprotein fractions was subjected to regression analysis, independent variables being the various lipid or apolipoprotein concentrations. The distribution of probucol was also analysed in terms of physical characteristics of the particles (volumes, areas). These were determined according to Shen et al (1977). In brief, free cholesterol, cholesteryl ester, phospholipid, triglyceride and protein, composing the particle were ascribed specific contributions to the total volume or area of the lipoprotein. The diverse lipoprotein particles were assumed to have the same structural geometry, the cholesteryl ester and triglyceride components constitute a hydrophobic spherical core surrounded by an amphiphilic monolayer composed of phospholipid, cholesterol and proteins. Cholesterol occupies the inner half of the surface monolayer of phospholipid, in contact with acyl chains, and proteins the outer half, in the head group region. The molecular volumes of cholesteryl ester and triglyceride are 1068 and 1556 $Å^3$ and occupy a sphere of radius (R) ~ 20·5 Å, R being the particle radius. At the core interface, cholesterol and phospholipid have molecular areas of 39·1 and 68·5 $Å^2$. At the particle-water interface, the protein surface area is 15·6 $Å^2$ per amino acid and the phospholipid molecular area is 62·7 $Å^2$; an average residue weight of 100 per amino acid was assumed. The following apolipoprotein mol. wts were assumed, A-I 28 013, A-II 17 014, A-IV 44 000, B 555 555, C-III 8000, E 33 793.

Results

Table 1 summarizes the composition of particles and their probucol content. This first shows that probucol distributes preferentially in apoB-containing particles, and this may be related to the apolipoprotein composition (other than apoB) or to the specific lipid environment related to this apolipoprotein. Regression analyses showed a good correlation ($r = 0·983$, $P < 0·01$) between particle-associated probucol and particle phospholipid concentration (Fig. 2); coefficients of correlation were lower for cholesteryl ester ($r = 0·802$, $P < 0·05$), cholesterol ($r = 0·701$, $P > 0·05$ NS) and apoA-II ($r = 0·633$, $P > 0·05$), and even lower for the other particle components. Physical characteristics of particles, particularly volume and surface, are dependent on these components, and therefore, they were examined as possible probucol-binding determinants.

The analyses were performed with normalized data, assuming one apoB per apoB-containing particle (including LpB : A-I : A-II : A-IV) and four apoA-I for apoA-I-containing particles, to obtain the number of apolipoprotein, lipid and drug molecules per particle (data of Table 1). Fig. 3 shows the relationship between the volume of the hydrophobic core and the number of probucol molecules associated with the particles. The correlation was good when data from Lp(a) was excluded from the analysis ($r = 0·983$, $P < 0·01$); inclusion of Lp(a) led to non-significant correlation

Table 1. Molecular composition and probucol content of particles.

Particle	ApoA-I[a]	ApoA-II	ApoC-III	ApoE	ApoB[b]	CE	Chol	TG	PL	Probucol
LpB	0·7	0·3	0·2	0·1	1	1964	837	216	702	10·8
LpB : E	0·5	0·2	1·3	0·9	1	1244	837	500	649	8·1
LpB : C-III : E	0·1	0·3	14·9	1·8	1	2182	2181	3090	2030	48
LpB : C-III	0·02	0·04	1·2	0·04	1	794	454	368	417	8·6
Lp(a)	0·01	0·15	4·89	0·0	1	932	716	3611	537	2·15
LpB : A-I : A-II : A-IV	15·4	16·4	9·2	3·3	1	1392	632	452	839	13
LpA-I	4	0·005	0·03	0·0	0·003	64	24	14	66	1·3
LpA-II : A-I	4	3·15	0·23	0·004	0·001	124	33	15	109	2·15

[a] Four apoA-I per apoA-I-containing particle. [b] One apoB per apoB-containing particle (including LpB : A-I : A-II : A-IV). CE = cholesteryl ester, Chol = cholesterol, TG = triglyceride, PL = phospholipid.

($r = 0{\cdot}629$, $P > 0.05$). The analyses with other physical parameters gave poorer results, with always a deviation caused by Lp(a) data.

Absolute experimental data (non-normalized) were used in further analyses, since normalization could induce a constraint on the data (one apoB or four apoA-I per particle). A very good relationship ($r = 0{\cdot}919$, $P < 0{\cdot}01$) with a satisfactory distribution of points around the regression line was observed between particle-associated probucol and the surface of the phospholipid/cholesterol monolayer surrounding the hydrophobic core (Fig. 4). The representation was poorer when the surface at the particle/water interface was used as the independent variable, although the correlation was good (Fig. 5, $r = 0{\cdot}901$, $P < 0{\cdot}05$). Regression analysis for all particles between the hydrophobic core volume and particle-associated probucol was not statistically significant ($r = 0{\cdot}758$, $P > 0{\cdot}05$).

Discussion

The present in-vitro results strongly support the hypothesis that probucol distribution among lipoprotein particles is mainly determined by their lipid composition, as previously reported (Urien et al 1984). Accordingly, the high number of probucol molecules in apoB-containing particles, as compared with apoA-containing particles, can be reasonably ascribed to their large size, mainly determined by a high lipid content. The relationship observed between probucol distribution in particles and phospholipid, cholesteryl ester and cholesterol suggested that probucol binding could take place where these lipids are located in the lipoprotein particles. Because the lipid concentrations in particles are not independent variables, the multivariate analysis technique could not be used to determine the exact relationship between probucol binding and these lipid concentrations. However, the structural organization of lipoprotein particles is related to their molecular components, and probucol binding could then be analysed in terms of core volume (triglyceride/cholesteryl ester combination) or areas (phospholipid/cholesterol or phospholipid/proteins). The close correlation observed with phospholipid indicated that probucol should be located in the monolayer surrounding the particle core, whereas the good correlation with cholesteryl ester suggested a role for the hydrophobic core (although no correlation was found with triglyceride). The results of the regression analyses with volume and surface terms showed that probucol binding was closely related to the monolayer composed of cholesterol and phospholipid, the relationship to the water-particle external monolayer being less obvious. Consequently, the major part of probucol in lipoprotein particles can be reasonably located in the phospholipid/cholesterol monolayer lining the surface of the core. Probu-

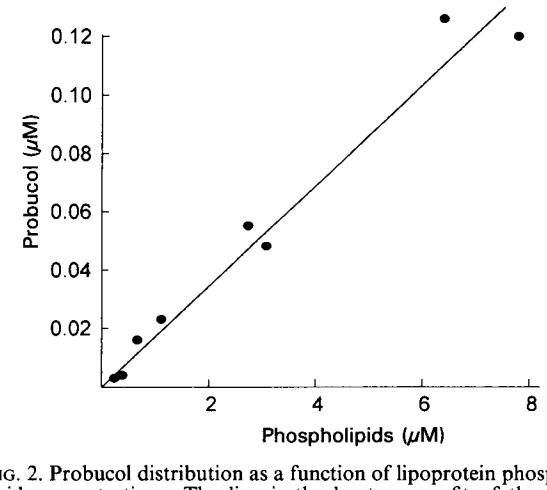

FIG. 2. Probucol distribution as a function of lipoprotein phospholipid concentrations. The line is the least-square fit of the data points, $r = 0{\cdot}983$, $P < 0{\cdot}01$.

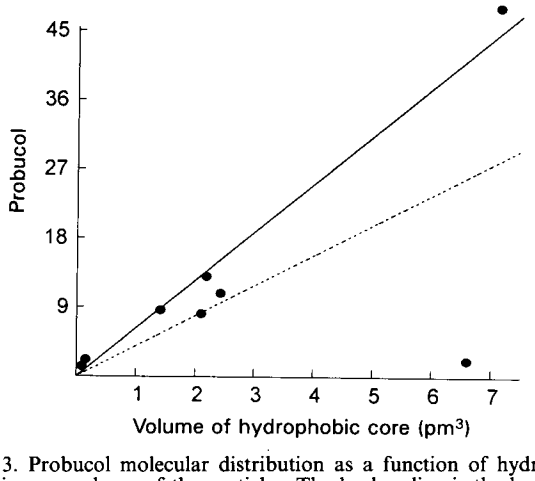

FIG. 3. Probucol molecular distribution as a function of hydrophobic core volume of the particles. The broken line is the least-square fit of all the data points, $r = 0{\cdot}629$, NS. The solid line is the least-square fit of the data points when Lp(a) data (*) is excluded, $r = 0{\cdot}983$, $P < 0{\cdot}01$.

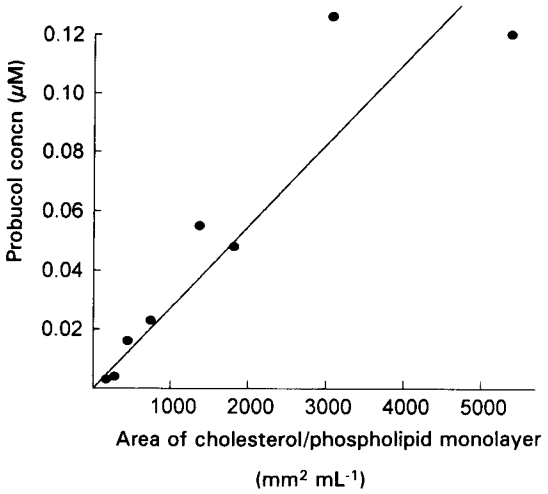

FIG. 4. Probucol distribution as a function of lipoprotein cholesterol/phospholipid monolayer area ($mm^2 mL^{-1}$). The line is the least-square fit of the data points, $r = 0.919$, $P < 0.01$.

col molecules are likely to locate also in packed cholesteryl ester molecules of the core or in the vicinity of hydrophobic amino acid residues facing the phospholipid cholesterol monolayer. Finally, the observation that Lp(a) had very little probucol in spite of its large core volume is an additional data for surface location.

Recent studies have demonstrated that probucol incorporation into lipoproteins is the basis of its antioxidant properties, preventing low density lipid oxidative modification. Probucol did not completely prevent peroxidation of low density lipoproteins, although this resulted in a complete protection of low density lipoprotein structure (McLean & Hagaman 1989). A possible explanation is that probucol could prevent the transfer of peroxidized lipids to the surface of low density lipoprotein, which confines the reaction of peroxidized lipids to internal lysines (instead of reacting with water interface lysines). This is compatible with the physical location of probucol in the cholesterol/phospholipid monolayer in the vicinity of hydrophobic areas of apolipoproteins.

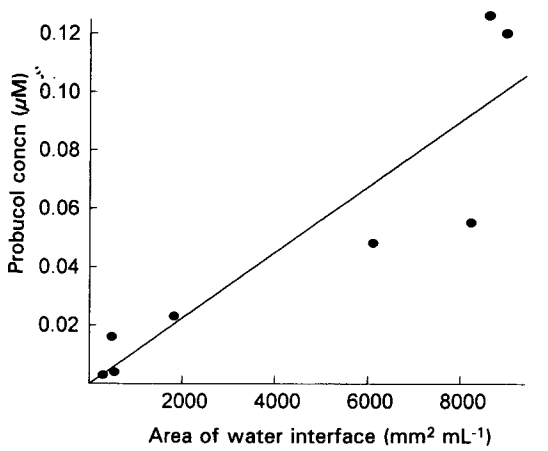

FIG. 5. Probucol distribution as a function of lipoprotein water interface area ($mm^2 mL^{-1}$). The line is the least-square fit of the data points, $r = 0.901$, $P < 0.05$.

In addition, probucol could be easily included in phosphatidylcholine liposomes at concentrations much higher than its antioxidative concentration, and probucol molecules were located in the C1-C8 methylene region in the glycerol backbone region of the bilayer, whereas tocopherol was located in the fatty acyl chain region of the bilayer (McLean & Hagaman 1990). It has also been demonstrated that probucol, like tocopherol, is a potent free-radical terminator inside lipoproteins, and that probucol antioxidant function is concentration-dependent (Barnhart et al 1989). In that study, oxidation products of probucol were suggested to be located close to or inside the lipoprotein core. This again favours the location of probucol in the cholesterol/phospholipid monolayer in contact with cholesteryl ester of the particle core.

In conclusion, the analysis of probucol distribution vs lipoprotein particle chemical composition and physical characteristics strongly suggests that probucol molecules are preferentially located in the cholesterol/phospholipid monolayer, at the boundary with the particle core or with hydrophobic regions of apolipoproteins. This location appears compatible with the antioxidant function and mode of action ascribed to this drug.

Acknowledgements

The authors thank Merrell Dow (Indianapolis, IN) for providing radiolabelled probucol, Dr Pascal Danglas (Merrell Dow France, Paris) for his co-operation and Mr Bruno Derudas for his skilful technical assistance. We also thank le Ministère de la Santé, France and Le Réseau de Pharmacologie Clinique for financial support.

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