Probucol Inhibits ABCA1-Mediated Cellular Lipid Efflux

Elda Favari, Ilaria Zanotti, Francesca Zimetti, Nicoletta Ronda, Franco Bernini, George H. Rothblat

Objective—ATP-binding cassette transporter A1 (ABCA1) mediates the efflux of lipids from cells to lipid-poor apolipoproteins. In this article, we characterize the effect of probucol on cellular ABCA1-mediated lipid efflux.

Methods and Results—Probucol inhibited cholesterol efflux up to 80% in J774 macrophages expressing ABCA1. In Fu5AH hepatoma cells that contain scavenger receptor class B, type I, but not functional ABCA1, we observed no effect of probucol on cholesterol efflux. Probucol inhibited cholesterol efflux from normal human skin fibroblasts but not from fibroblasts from a Tangier patient. Fluorescent confocal microscopy and biotinylation assay demonstrated that in J774 cells probucol impaired the translocation of ABCA1 from intracellular compartments to the plasma membrane. Probucol also inhibited the formation of an ABCA1-linked cholesterol oxidase sensitive plasma membrane domain. Consistent with the inhibitory effect on ABCA1 translocation to the plasma membrane, probucol reduced cell surface–specific [3H]-labeled apolipoprotein-AI binding.

Conclusions—We conclude that probucol is an effective inhibitor of ABCA1-mediated cholesterol efflux without influencing scavenger receptor class B type I–mediated efflux. The inhibition of ABCA1 translocation to the plasma membrane may in part explain the reported in vivo high-density lipoprotein–lowering action of probucol. (Arterioscler Thromb Vasc Biol. 2004;24:2345-2350.)

Key Words: probucol ■ lipid efflux ■ ABCA1 ■ macrophages ■ fibroblasts

Probucol is a lipid-lowering drug that has been extensively investigated since its introduction in the early 1970s. Among its most dramatic effects is the ability to promote the regression of cutaneous and tendinous xanthoma, and this effect appears to be independent of its cholesterol-lowering effect. However, probucol also significantly reduces plasma high-density lipoproteins (HDLs). These facts have made this drug very controversial. It has been reported that probucol treatment induced a more rapid progression of atherosclerotic lesions in apolipoprotein E (apoE) knockout mice. In contrast, a recent study demonstrated that probucol treatment of scavenger receptor class B type I (SR-BI)/apoE double knockout mice prevents the dramatic early coronary heart disease and death that is a characteristic of these animals.

Just as the data on probucol effects in vivo are contradictory, so too are the results from a number of experiments that have been conducted to study the action of probucol on cholesterol metabolism in vitro. Yamamoto et al measured the effect of probucol on the change in cholesterol mass on incubation of THP-1 macrophages with HDLs and observed a significant reduction consistent with a probucol-mediated increase in the net efflux of cholesterol. A similar result was obtained by Goldberg and Mendez using human skin fibroblasts. In contrast, a subsequent investigation failed to observe any probucol stimulation in the efflux of cholesterol from a number of cell types. It has been demonstrated that specific cell surface proteins play an important role in mediating the flux of cholesterol between cells and extracellular acceptors (for a review see Yancey et al). SR-BI facilitates the selective uptake of HDL cholesteryl ester (CE) and enhances the bidirectional flux of free cholesterol (FC) between cells and HDLs. ATP-binding cassette A1 (ABCA1) has been shown to bind lipid-free or lipid-poor apoproteins and to transfer both cholesterol and phospholipid from the cell membrane to the apoprotein.

Even before the identification of ABCA1 as a mediator of cell lipid efflux to apoproteins, it was demonstrated that probucol treatment of cells produced a marked inhibition of apo-AI–mediated lipid efflux from macrophages. It has now been shown that this inhibition is accompanied by complete or partial inhibition of binding of the apoprotein to the probucol-treated cells.

Thus, there is no question that treatment of cells with probucol can impact cellular lipid efflux. However, the effect of the drug and the mechanisms by which it produces changes in efflux remain obscure and contradictory. Because of a renewed interest in probucol, and because of our growing knowledge of the roles of SR-BI and ABCA1 on cell cholesterol flux, we have now conducted studies to obtain additional information on the effect of probucol on lipid flux.
For this investigation, we have focused on the impact of probucol treatment on the expression, intracellular distribution, and cholesterol efflux–mediating ability of ABCA1.

Methods

Materials
Acetylated low-density lipoproteins (LDLs) were prepared as previously described. Apo-AI was purified from human blood plasma, as previously described, and it was provided by L. Calabresi at University of Milan (Italy). [125I]-labeled apo-AI ([125I]-apo-AI) was provided by M.A. Connelly at State University of New York (SUNY, Stony Brook, NY). For details, please see the online Methods, available at http://atvb.ahajournals.org.

Cells
J774 mouse macrophages were cultured in RPMI medium 1640 supplemented with 10% FCS. Fu5AH rat hepatoma cells were grown in DMEM with 5% FCS. Human fibroblasts were grown in DMEM supplemented with glutamine, nonessential amino acids, sodium pyruvate, and FCS. All the culture media were supplemented with 50 µg/mL gentamicin.

Assay of Cellular Cholesterol and Phospholipid Efflux
Cells were seeded in 24-well plates until 80% confluent. Cells were then labeled with 2 µCi/mL [1,2-3H] cholesterol for 24 hours and subsequently incubated overnight in medium containing 0.2% BSA with or without either 5 µg/mL 22-hydroxycholesterol (22-OH)/10 µmol/L 9-cis retinoic acid (9cRA) or 0.3 mmol/L of 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (cpt-cAMP). Efflux was promoted to various acceptors after the incubation of cells with or without 10 µmol/L probucol. Time zero values were obtained from cell walls harvested before lipid acceptor addition. The percent release of lipid was calculated as: (cpm in medium per cpm time zero) × 100. For phospholipid efflux assay, cells were treated as previously described and phospholipids were extracted by the Bligh and Dyer method.

Assay of Cholesterol Oxidase
Cholesterol oxidase treatment was essentially as previously described. Briefly, cells were labeled with 3 µCi/mL [1,2-3H] cholesterol. Cholesterol oxidase (1 U/mL) was added, and cells were incubated for 4 hours. Lipid was extracted with isopropanol, and radioactive cholesterol and cholestene were separated using thin-layer chromatography and quantified.

Western Blots
Cell monolayers were lysed with 1% Triton X-100, 0.5% Nonidet P-40, 10 mmol/L tris buffer and homogenized through a 27-gauge needle. Equal amounts of protein (24 µg) were separated on 3% to 8% tris-acetate gel and transferred to polyvinylidene fluoride membrane. Blot was performed with an anti-ABCA1 rabbit polyclonal antibody. Chemiluminescence was used to visualize the proteins according to the manufacturer’s instructions.

Confocal Microscopy
J774 macrophages were grown on 8-well multichamber slides and incubated with 0.3 mmol/L cpt-cAMP for 18 hours to induce the expression of ABCA1. In each slide, half of the wells were then treated with 10 µmol/L probucol in serum-free medium for 2 hours. Cells were fixed with methanol and incubated with an anti-ABCA1 rabbit polyclonal antibody for 40 minutes at room temperature, then incubated with an anti-rabbit Ig coupled with fluorescein isothiocyanate. Slides were mounted with a cover slide and observed by confocal microscopy (Molecular Dynamics Multiprobe 2001). For further details on the experimental conditions please see online Methods.

J774 Cell Surface Binding of 125I-Lipid–Free Apo-AI
J774 were treated according to the following protocol: during the efflux period, cells were treated with [125I] apo-AI in presence or absence of a 20-fold excess of unlabeled apo-AI for 4 hours. After the incubation, cells were washed several times with PBS and lysed with 0.1 mol/L NaOH. Radioactivity in cell lysate was measured by γ-counting, after which aliquots were taken for protein determination. Specific binding was calculated by subtraction of nonspecific from total binding.

Cell Surface Biotinylation Assay
Cell monolayers were grown in 60-mm Petri dishes and incubated with 0.3 mmol/L cpt-cAMP for 18 hours to induce the expression of ABCA1. Half of the dishes were then treated with 10 µmol/L probucol in serum-free medium for 2 hours. Cells were washed with PBS and treated with Sulfo-NHS-LC-LC-Biotin (Pierce) in PBS/calcium-magnesium buffer for 30 minutes at room temperature. Monolayers were washed with PBS supplemented by 100 mmol/L glycine and were lysed with a 1% Triton X-100, 0.5% Nonidet P-40, 10 mmol/L tris buffer. Biotinylated proteins were separated from nonbiotinylated proteins by using Monomeric Avidin Column (ImmunoPure Immobilized Monomeric Avidin Kit, Pierce). Biotinylated proteins (14.2 µg) were separated on 3% to 8% tris-acetate gels and transferred to polyvinylidene fluoride membrane. Western blot analysis was performed as described.

Statistical Analysis
Results were presented as means and standard deviations of triplicate determinations. Significant differences were established by t test using Graph Pad Prism (GraphPad Software Inc).

Results
Efflux of cholesterol to apo-AI from J774 macrophages was stimulated up to 4-fold by the exposure to cpt-cAMP. Probucol treatment for 2 hours inhibited this efflux by 80%±0.06 (Figure 1). Stimulation with cpt-cAMP upregulated ABCA1 expression, and probucol interfered with the ABCA1 efflux to lipid-free apolipoproteins without affecting the protein expression (Figure 1, inset). We tested the probucol effect on Fu5AH cells, a cell line that expresses high levels of SR-BI protein, but not functional ABCA1. Probucol did not influence cholesterol efflux from Fu5AH to HDL (% efflux to HDL was 9.98±1.28 and 11.24±0.69 after probucol pretreatment), showing that the probucol effect was specific for ABCA1-mediated cholesterol efflux. In J774 cells upregulated for ABCA1 using the liver X receptor and retinoic X receptor (LXR-RXR) agonists 22-OH and 9cRA and exposed to probucol for 2 hours, 1 µmol/L of probucol inhibited efflux by 50% and reached the maximum inhibition at 5 µmol/L (Figure 1A, available online at http://atvb.ahajournals.org). We performed a time course experiment using 10 µmol/L of probucol. The results show a 60% inhibition of apo-AI–mediated cholesterol efflux from J774 cells treated with LXR-RXR agonists after only 15 minutes exposure to probucol, and by 2 hours cholesterol efflux was maximally inhibited (Figure 1B). We next evaluated phospholipid efflux to further test the probucol effect on ABCA1 activity. The 3-fold increase of phospholipid efflux to apo-AI stimulated by LXR-RXR agonists was abolished in the presence of probucol (% efflux to apo-AI was 4.42±0.001 and 1.20±0.12 after probucol pretreatment).
As shown in Figure II (available online at http://atvb.ahajournals.org), using normal and Tangier fibroblasts up-regulated for ABCA1 by incubation with 9-cRA and 22-OH, apo-AI–mediated efflux was inhibited by probucol in normal fibroblasts but probucol treatment had no effect on Tangier cells that are unable to express ABCA1.

The turnover of ABCA1 protein can be rapid,11,23 thus probucol inhibition of lipid efflux could be linked to accelerated ABCA1 turnover and degradation. However, this does not appear to be the case, because the probucol inhibition is exceptionally rapid (15 minutes; Figure IB) and the level of ABCA1 protein is not significantly reduced after a 2-hour exposure to probucol (Figure 1, inset). Because ABCA1 localization is in the plasma membrane and late endosome compartments,24 we used fluorescent confocal microscopy to determine whether probucol could interfere with the protein distribution in cells. Consistent with previous studies,24 we observed ABCA1 protein both at the cell periphery and intracellular locations in cells stimulated by cpt-cAMP (Figure 2A). After a 2-hour exposure to probucol, ABCA1 protein was almost exclusively present in an intracellular location (Figure 2B). Similar results were obtained using J774-loaded cells by 50/μg/mL of acetylated LDLs in the same experimental conditions (Figure III, available online at http://atvb.ahajournals.org).

Vaughan and Oram25 demonstrated that the expression of ABCA1 in cells produces an increase in the size of membrane cholesterol pool. Thus, we tested whether probucol interfered with the movement of cholesterol to plasma membrane domains. To this end, we evaluated cholesteneone formation after exposure of cells to cholesterol oxidase under conditions leading to oxidation of FC present in the plasma membrane.21 As expected,25 in J774 macrophages the upregulation of ABCA1 by cpt-cAMP increased by almost 3-fold the cholesterol in the plasma membrane sensitive to oxidation by cholesterol oxidase. This process was absent in cells exposed to probucol (Figure 3A). The experiment was also performed using only 2-hour incubation with the enzyme, and we obtained similar results (% cholesteneone moves from 4.50±0.17 in control cells to 9.90±0.16 in cAMP treated cells, and 2-hour incubation with probucol reduces this value back to 5.90±0.60).

We performed a similar experiment using normal and Tangier human skin fibroblasts incubated with the LXR-RXR
agonists to upregulate ABCA1. Only in normal cells LXR-RXR stimulation induced an increase in the cholesterol oxidase sensitive pool. Probucol completely inhibited this effect. In Tangier cells, the cholestenone formation was unaffected by either LXR-RXR stimulation or probucol treatment (Figure 3B).

To test the effect of probucol on apo-AI cell surface binding, J774 macrophages, cpt-cAMP–stimulated, were treated with probucol for 2 hours and [125I]apo-AI binding was measured at 4°C. As shown in Figure 4, binding was increased by cAMP treatment, and exposure to probucol reduced the specific binding of [125I]apo-AI. Probucol had no effect on the nonspecific binding. To further provide biochemical support for the concept that probucol impaired the ABCA1 trafficking from the intracellular compartments to the plasma membrane, we measured the ABCA1 localization on the cell surface by biotinylation assay. The results showed that the expression of ABCA1 protein on the cell surface is fully inhibited by probucol (Figure 4, inset).

**Discussion**

Even before the identification of ABCA1 as a mediator of lipid efflux, it was demonstrated that probucol treatment of cells would produce a marked inhibition of apolipoprotein-mediated lipid efflux from macrophages.12,13 This inhibition of lipid efflux to apoproteins was accompanied by an inhibition of binding of the apoprotein to the probucol-treated cells.12 In contrast to the apoprotein-mediated efflux, probucol was shown not to have an effect on FC efflux to LDL,12 a process that results largely by unmediated or SR-BI–mediated cholesterol exchange.8 Using macrophages pretreated with cAMP, a procedure we now know up regulates ABCA1, Sakr et al13 demonstrated that exposure to probucol produced a rapid and marked inhibition of apo-AI–mediated efflux.

One of the most dramatic effects of probucol treatment of J774 macrophages is the rapidity (15 minutes) of the inhibition of lipid efflux to apo-AI in J774-expressing ABCA1. Because of the pronounced hydrophobicity of probucol, it can be anticipated that the compound rapidly partitions into the plasma membrane, we measured the ABCA1 localization on the cell surface by biotinylation assay. The results showed that the expression of ABCA1 protein on the cell surface is fully inhibited by probucol (Figure 4, inset).

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There are a number of mechanisms by which probucol could exert its inhibitory effect. Rapidity of probucol effect and Western blot analysis (Figure 1B and Figure 1, inset) exclude an effect on ABCA1 expression or degradation. Studies by Vaughan and Oram25 demonstrated that the
presence of ABCA1 in the plasma membrane of baby hamster kidney cells resulted in an increase in the pool of cholesterol in the plasma membrane that became susceptible to oxidation by cholesterol oxidase. Here we demonstrated that upregulation of ABCA1 in J774 results in an increase in cholesterol oxidase-sensitive cholesterol pool and that probucol completely inhibited the formation of this lipid domain (Figure 3A). That this increase in sensitivity to cholesterol oxidase is an ABCA1-linked response is further demonstrated by the observation that treatment of normal human fibroblasts with LXR-RXR ligands resulted in an increase in the size of the oxidase-sensitive pool and elevated cholesterol efflux which was inhibited by probucol. A similar treatment of Tangier fibroblasts produced none of these responses (Figure 3B). It has been reported that cAMP-treated cells exposed to probucol exhibit reduced specific binding of apo-AI compared with control cells.12 In this study, probucol largely inhibited cAMP-induced upregulation of binding, whereas in a previous study there was a somewhat less dramatic inhibition.16 Differences in the experimental conditions can account for the observed differences in binding. The previous study used cholesterol-loaded cells,16 whereas in the present protocol the cells were cholesterol-normal. Cell cholesterol enrichment may increase the binding of an extracellular acceptor independently of cellular metabolism,26 an effect that might oppose the inhibitory activity of probucol. Neufeld et al24 demonstrated that ABCA1 protein resides both on the cell surface and on intracellular vesicles and traffics between these 2 compartments. In this study, the reduced lipid efflux, cholesterol membrane pool, and apo-AI binding produced by probucol correlate well with the shift in the distribution of ABCA1 from the plasma membrane compartment to an internal location, as evidenced by confocal analysis and biontinylated assay (Figure 2, Figure III, and Figure 4, inset). Recently, an inhibitory effect of probucol on apo-AI binding was also reported in WI-38 human fibroblasts.27 However, these authors did not observe any effect of probucol on ABCA1 protein or cholesterol cellular distribution. We do not have a clear explanation for this apparent discrepancy with our present data. The difference in cell lines and conditions of probucol presentation to cells may account for some of the different conclusions reached in this article.

Administration of probucol to both humans and animals has been shown to lower HDL levels.28 This effect involves a probucol-related increase of plasma CE transfer protein and a production of HDL with an enhanced ability to promote cholesterol efflux.29 We propose that in addition to this mechanism the drug may reduce hepatic HDL production by inhibiting the apo-AI interaction with ABCA1. Despite the reduction in HDL, probucol is still very effective in promoting the regression of xanthomas.5,5 Also, a study by Braun et al9 demonstrated that administration of probucol to SR-BI/apoE double knockout mice reversed red blood cell abnormal morphology, restored normal FC/CE ratios, and blocked the development of coronary heart disease. A number of different mechanisms could be responsible for probucol-mediated regression of xanthomas and its antiatherogenic effects. A decrease in foam cell lipid content, and hence regression of xanthomas, could reflect either a reduction in cholesterol influx or stimulation of macrophage cholesterol efflux. Probu- col is a potent antioxidant, and the presence of the drug in plasma has been shown to increase the resistance of LDL to oxidative modification and the subsequent uptake of modified LDL by scavenger receptors.2 An alternative hypothesis is that enhanced efflux of cholesterol plays a role in xanthoma regression. The results of this study demonstrate that a probucol stimulation of macrophage cholesterol efflux would not occur through an ABCA1 pathway. In the absence of ABCA1-mediated efflux, passive diffusion or SR-BI appears to be a good candidate. However, it is unlikely that probucol is acting directly on cells to increase the level or activity of SR-BI, because drug treatment of SR-BI–rich Fu5AH cells failed to enhance cholesterol efflux to HDL, and feeding probucol to mice does not change the SR-BI mRNA14 or protein20 in liver. Rather, it is possible that probucol may generate lipoprotein particles more active in promoting net cholesterol efflux from foam cells. In support of this model is the observation by Rinninger et al30 that HDL from probucol-treated mice have increased CE selective uptake into Chinese hamster ovary cells compared with control mouse HDL. These modifications in HDL composition could also increase SR-BI bidirectional flux of FC and the net flux of cholesterol out of cholesterol-enriched foam cells.31

Acknowledgments

This work was supported by grant No. QLGI-1999-01007 from the European Union; by grants from Compagnia di San Paolo, the Istituto Nazionale Ricerche Cardiovascolari, and the USA-Italy Agreement (to F.B.); and Heart, Lung, and Blood Institute (NHLBI) grants HL22633 and HL63768 (to G.H.R.). The authors would like to thank Prof. S. Calandra of University of Modena, Italy, for providing the Tangier fibroblast. We thank Prof. G. Orlandini, University of Parma, Italy, for his assistance on using the confocal microscopy.

References


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Arterioscler Thromb Vasc Biol. 2004;24:2345-2350; originally published online October 28, 2004;
doi: 10.1161/01.ATV.0000148706.15947.8a
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure 1

A. 

% Efflux from J774 / 4h

[µM]

B. 

% Inhibition of Efflux from J774

hours of pretreatment
Figure I

% Efflux from Normal Human Fibroblasts / 4h

9cRA+22-OH+apo-Al

Ctrl  apo-Al  Probufol

% Efflux from Tanger Human Fibroblasts / 4h

9cRA+22-OH+apo-Al

Ctrl  apo-Al  Probufol
**Figure Legends**

**Fig. I:** Concentration-dependent (panel A) and time-dependent (panel B) effect of probucol on apo-AI-mediated cholesterol efflux in J774 cells. Monolayers were labeled as in Fig 1. Cells were then equilibrated with 0.2% BSA and incubated with 10μM 9cRA and 5μg/ml 22-OH for 18h. Monolayers were then incubated in the presence of increasing concentrations of probucol for 2h (panel A) or in the presence of 10μM probucol for the indicated time periods (panel B). After probucol treatment, cells were washed and than incubated with RPMI containing 25μg/ml lipid-free apo-AI for 4h. Data are from a representative experiment with triplicate wells (n = 3). Values are expressed as means ± S.D.

**Fig. II:** Probucol activity on FC efflux from normal and Tangier human fibroblasts. Monolayers were labeled with 4μCi/ml [³H]cholesterol for 24h in medium with 1% FCS in the presence of 2μg/ml ACAT inhibitor. Cells were then incubated over night in the presence (solid bars) or absence (hatched bars) of 10μM 9-cRA and 5μg/ml 22-OH in 0.2% BSA followed by incubation with or without probucol for 2h. After this period of time cells were incubated with RPMI containing 25μg/ml lipid-free apo-AI as cholesterol acceptors for 4h. Data are from a representative experiment with triplicate wells (n = 3). Values are expressed as mean ± S.D.

**Fig. III:** Fluorescent confocal microscopy analysis of probucol effect on ABCA1 localization in J774 mouse macrophages. Cells were plated very diluted in RPMI medium with 10% FCS in a 8 wells chamber slides. After 24 h cells were cholesterol loaded in medium with 1% FCS in the presence of 50 μg/ml of AcLDL and 2μg/ml ACAT inhibitor. Cells were than equilibrated with 0.2% BSA and incubated with 0.3 mM cpt-cAMP for 18 h prior the probucol exposure. After the cpt-cAMP treatment, cells were incubated without (A-B) or with (C-D) 10 μM probucol as described in Experimental Procedures. After 2 h cells were chilled on ice then fixed by exposure to
methanol for 2 min. After fixation, cells were extensively washed and then incubated 1 h with the primary rabbit polyclonal antibody to ABC1 diluted 1:50 in DPBS. Cells were then washed very well and incubated with a fluorescent secondary antibody for 1 h. Panel A and C or B and D represent two different experiments conducted in the same conditions (see “Methods”).
Methods

Materials

Cell culture media and phosphate buffer solution for washing were purchased from BioWhitaker (Walkersville, MD); Fetal calf serum (FCS), bovine serum albumin (BSA), 22-hydroxycholesterol (22-OH), cis-9-retinoic acid (9cRA), 8-(4-Chlorophenylthio)adenosine 3', 5'-cyclic monophosphate (cpt-cAMP), glutamine, gentamicin, sodium pyruvate, 4-cholesten-3-one, Nonidet-P40 (NP-40), Triton X-100 and probucol were purchased from Sigma (St. Louis, MO, USA). Tissue culture flasks and plates were from Corning (Corning, NY, USA) and Falcon (Lincoln, NY, USA). Cholesterol oxidase (streptomyces enzyme) was purchased from Roche (Basel, Switzerland). Organic solvents were purchased from Merck (Darmstadt, Germany); [1,2-^3^H]cholesterol and [methyl-^3^H]choline chloride were from Amersham Biosciences (Uppsala, Sweden). The acyl-CoA cholesterol acyltransferase (ACAT) inhibitor, Sandoz 58-035, was a gift from Novartis (Basel, Switzerland). Multichamber slides (Lab-Tek(R) II) for confocal analysis were purchased from Nalge Nunc International (NY, USA). Anti-ABCA1 rabbit polyclonal antibody was purchased from Novus Biologicals (Cambridge, UK) and anti-rabbit Ig coupled with FITC from Southern Biotechnology (Birmingham AL, USA).

Cpt-cAMP, 22-hydroxycholesterol, cis-9-retinoic acid and probucol solutions

Cpt-cAMP was dissolved in distilled water at 30 mM and stored at -20°C. 22-OH and 9cRA were dissolved in DMSO at 2 mg/ml and 4mM respectively and stored at -20°C. A stock solution of probucol dissolved at 10 mM in ethanol was prepared and stored at -20°C. For experiments, an initial solution containing 200 μM probucol, 2% ethanol (v/v) and 4% BSA in FCS-free culture medium was prepared and then diluted to final concentration of 10 μM probucol, 0.1% ethanol (v/v) and 0.2% BSA. Control medium was prepared without probucol.
**Confocal microscopy**

Control wells cells were treated as described but in the absence of the primary antibody to exclude second antibody non-specific binding. In addition, control experiments were performed as described above, but incubating the cells with probucol after fixation, in order to exclude the theoretical possibility that membrane bound probucol could react with the revealing system and give false positive results.