

# AGI-1067: A Multifunctional Phenolic Antioxidant, Lipid Modulator, Anti-Inflammatory and Antiatherosclerotic Agent

CYNTHIA L. SUNDELL, PATRICIA K. SOMERS, CHARLES Q. MENG, LEE K. HOONG, KI-LING SUEN, RUSSELL R. HILL, LAURA K. LANDERS, ANGELA CHAPMAN, DUSTIE BUTTEIGER, MOIRA JONES, DAVID EDWARDS, ALAN DAUGHERTY, MARTIN A. WASSERMAN, R. WAYNE ALEXANDER, RUSSELL M. MEDFORD, and UDAY SAXENA<sup>1</sup>

*AtheroGenics, Inc., Alpharetta, Georgia (C.L.S., P.K.S., C.Q.M., L.K.H., K.-L.S., R.R.H., L.K.L., A.C., D.E., M.A.W., R.M.M., U.S.); University of Kentucky, Lexington, Kentucky (D.B., A.D.); Quintiles, Ltd, Edinburgh, Scotland, United Kingdom (M.J.); and Emory University School of Medicine, Atlanta, Georgia (R.W.A.)*

Received December 13, 2002; accepted February 24, 2003

## ABSTRACT

To explore the therapeutic efficacy and potential mechanisms of action of a new class of antiatherosclerotic drugs, AGI-1067 [mono[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester] (butanedioic acid) was tested in several animal models of atherosclerosis. AGI-1067, a novel phenolic antioxidant, was well tolerated in a 1-year study in hypercholesterolemic cynomolgus monkeys. It lowered low-density lipoprotein cholesterol (LDLc) by 41 and 90% at oral doses of 50 and 150 mg/kg, respectively and increased high-density lipoprotein cholesterol (HDLc) by 107% at the higher dose. In contrast, another phenolic antioxidant, probucol, had a modest LDLc-lowering effect (15% at 250 mg/kg) while decreasing HDLc (37% at 150 mg/kg). Histopathology of the aortas and coronary arteries revealed no atherosclerosis in the AGI-1067 (150 mg/kg) group and minimal-to-moderate atherosclerosis in the vehicle and probucol (150 mg/kg) groups. AGI-1067 also inhibited

atherosclerosis in LDL receptor-deficient (LDLr  $-/-$ ) mice and apolipoprotein E-deficient (ApoE  $-/-$ ) mice even in the absence of a lipid-lowering effect. In LDLr  $-/-$  mice, AGI-1067 reduced aortic atherosclerosis by 49%. In ApoE  $-/-$  mice, AGI-1067 reduced atherosclerosis by 25, 41, and 49% in the arch, thoracic, and abdominal regions of the aorta. AGI-1067 also reduced vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) mRNA levels in lungs of lipopolysaccharide-stimulated mice. At the cellular level, AGI-1067 inhibited tumor necrosis factor- $\alpha$ -inducible expression of VCAM-1, MCP-1, and E-selectin in human aortic endothelial cells (IC<sub>50</sub> values = 6, 10, and 25  $\mu$ M, respectively). These data show that AGI-1067 can inhibit atherosclerosis not only via its lipid-lowering effects but also by having direct anti-inflammatory effects on the vessel wall and suggest that it may be a novel therapeutic agent for coronary artery disease.

Oxidative stress plays a significant role in the pathogenesis of atherosclerosis. Vascular cells produce reactive oxygen species (ROS) that serve as secondary messengers to regulate signal transduction pathways that control gene expression. Abnormal production of ROS in the vasculature can lead to endothelial dysfunction and induction of inflammatory gene expression that contribute to the development of atherosclerosis (Kunsch and Medford, 1999; Ross, 1999).

One of the earliest detectable events in the development of the atherosclerotic plaque is accumulation of leukocytes

within discrete regions of the vasculature. Consistent with this observation, several genes involved in the recruitment and adhesion of leukocytes to the vessel wall, such as vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1), are up-regulated in the early atherosclerotic plaque (Iiyama et al., 1999; Kowala et al., 2000). Furthermore, both of these genes are regulated by ROS-mediated mechanisms that involve redox-regulated transcription factors (Marui et al., 1993; Ping et al., 1999). Results from studies using atherosclerosis-prone mice deficient in VCAM-1 and deficient in or overexpressing MCP-1 support a role for these genes in atherogenesis and suggest that they may be attractive therapeutic targets to treat coronary artery disease (CAD) (Aiello et al., 1999; Gosling et al., 1999; Cybulsky et al., 2001; Dansky et al., 2001).

This work was supported by AtheroGenics, Inc. (Alpharetta, GA).

<sup>1</sup> Current address: Reddy US Therapeutics, Inc., Norcross, GA 30071.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.102.048132.

**ABBREVIATIONS:** ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; CAD, coronary artery disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AGI-1067, mono[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester; HDL, HDL cholesterol; LDLr  $-/-$  mice, LDL receptor-deficient mice; ApoE  $-/-$  mice, apolipoprotein E-deficient mice; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; ICAM-1, intercellular adhesion molecule-1; HAECs, human aortic endothelial cells; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; HBSS, Hanks balanced salts solution; PBS, phosphate-buffered saline; LDLc, LDL cholesterol; HDLc, HDL cholesterol.

Due to the central role of oxidative stress in the pathogenesis of atherosclerosis, antioxidants may be of therapeutic benefit for CAD. Antioxidants inhibit cytokine-activated VCAM-1 and MCP-1 expression in cultured endothelial cells (Weber et al., 1994; Medford, 1995). They also inhibit progression of atherosclerosis in animal models but have not yet shown clinical benefit (Christen and Hennekens, 2000; Keaney, 2000). Probucol is a known lipophilic antioxidant with modest lipid-lowering properties. It is predominantly associated with lipoproteins in the plasma and has been shown to inhibit oxidative modification of LDL (Parthasarathy et al., 1986). Probucol inhibits atherosclerosis in some animals (Daugherty and Roselaar, 1995) and has been evaluated in two clinical trials for its ability to decrease the complications of cardiovascular disease in patients with hypercholesterolemia. In the Probucol Quantitative Regression Swedish Trial (PQRST), it lowered cholesterol but did not induce atherosclerotic regression in the femoral artery (Johansson et al., 1995). In the Fukuoka Atherosclerosis Trial (FAST), probucol lowered cholesterol and stopped progression of atherosclerotic plaques in carotid arteries. Probucol also reduced the incidence of cardiac events when compared with the control group despite lowering HDLc (Sawayama et al., 2002). Nevertheless, chronic clinical use of probucol has been limited since it lowers HDLc and causes QTc interval prolongation (Klein, 1981).

In this article, AGI-1067, a novel antioxidant, anti-inflammatory, and lipid-lowering agent that has antiatherosclerotic properties is described. It is a metabolically stable derivative of probucol that was designed to retain favorable characteristics of probucol (antioxidant and modest lipid-lowering activity) while improving on its liabilities (HDLc lowering, variable and limited oral bioavailability, poor cell permeability, and potential for QTc prolongation) (Meng et al., 2002). In the Canadian Antioxidant Restenosis Trial (CART-1), AGI-1067 dose dependently inhibited restenosis after angioplasty and demonstrated an antiatherosclerotic effect on reference vessel segments that did not undergo angioplasty. Furthermore, unlike probucol, AGI-1067 did not cause prolongation of the QTc interval (Tardif et al., 2003).

## Materials and Methods

**Cynomolgus Monkey Study.** AGI-1067 was evaluated in cynomolgus monkeys in a year-long study by Quintiles (Edinburgh, Scotland). Twenty male monkeys weighing approximately 2 to 3 kg (Shamrock Farms, Small Dole, Henfield, Suffolk, UK) were housed in a group caging system and given free access to water at all times. After a 4-week acclimatization period, they were fed 200 g/day of a high-fat diet (1.7% cholesterol, 11% coconut oil, 11% butter oil, 62.4% monkey chow, 13.0% orange juice, and 0.83% vitamin mix; supplied by SDS, Witham, Essex, UK) for a minimum of 4 weeks before dosing. Upon confirmation of hypercholesterolemia, the animals were randomly allocated into five groups of four animals each and administered vehicle [cremophor/PEG300 (4:1)], 50 mg/kg/day AGI-1067, 150 mg/kg/day AGI-1067, 150 mg/kg/day probucol, or 250 mg/kg/day probucol once daily for 1 year. The vehicle and AGI-1067 were administered by oral gavage at a constant dose weight of 3 g/kg. Probucol was administered in loose-filled gelatin capsules. After 81 days of dosing, one animal in the 150 mg/kg/day AGI-1067 group had to be removed from the study due to a recurring rectal prolapse that was determined not to be drug-related. Animals were monitored daily for clinical signs of toxicity or changes in behavior or appearance. Body weights were recorded shortly after arrival and at weekly

intervals thereafter. Electrocardiograms were performed three times during the acclimatization period and daily thereafter, 1 h after dosing. Blood samples were taken for blood chemistry twice after introduction of the high-fat diet to determine whether or not the cholesterol levels had increased to abnormal levels and weekly thereafter. A blood sample was taken during week 52 for blood chemistry and hematology. Blood was fractionated and cholesterol in the LDL and HDL fraction determined from week 8 until termination of the study. Blood samples were taken by venipuncture; EDTA was used as an anticoagulant for the hematology samples, and lithium heparin was used for the clinical chemistry samples. At the end of the study, the animals were euthanized by intravenous injection of sodium pentobarbitone. All animals were examined externally and then exsanguinated. Organs were weighed and then fixed in 10% formalin and evaluated for histopathology by a veterinary pathologist. Specifically, the left coronary was removed and fixed in formal calcium; the right coronary artery was fixed in situ. Samples of aortic arch and abdominal aorta were also fixed in formal calcium. Two transverse sections each of aortic arch and abdominal aorta were examined, as were two transverse sections of left and right coronary arteries. All tissues were wax-embedded, cut at a nominal thickness of 5  $\mu$ m, and stained with hematoxylin and eosin. Aortas and livers from some animals were also stained with Oil-Red-O to confirm the presence of fat. The histopathology was subject to peer review that was in good agreement with the original histopathologist's findings.

A scoring system was devised that took into account the severity and incidence of atherosclerosis in the coronary arteries and aortas. The atherosclerotic lesions were given a severity rating from 0 to 4 corresponding to none, minimal, slight, moderate, and severe. The incidence of atherosclerosis was determined by the number of animals per group that had lesions that fell into a particular severity category. The total atherosclerosis score was derived by taking the sum of the incidence  $\times$  severity for all the animals in a group. For example, the probucol (150 mg/kg/day) group had one animal with minimal ( $1 \times 1 = 1$ ), one with slight ( $2 \times 1 = 2$ ), one with moderate ( $1 \times 3 = 3$ ), and one with severe ( $1 \times 4 = 4$ ) atherosclerotic lesions in the left coronary arteries for a total score of "10" ( $1 + 2 + 3 + 4 = 10$ ) for the group.

**LDL-receptor (LDLr)  $-/-$  and ApoE  $-/-$  Mouse Atherosclerosis Studies.** Six-week-old LDLr  $-/-$  mice and ApoE  $-/-$  mice (Jackson Laboratories, Bar Harbor, ME), backcrossed for six generations to the C57BL/6 background, were used for these studies. For the LDLr  $-/-$  study, three groups of 20 mice each received a high-fat diet (Harlan Teklad, Indianapolis, IN; TD 88051, containing 1.25% cholesterol and 0.5% sodium cholate) alone or with AGI-1067 or probucol added for 12 weeks. Blood was sampled after 2 and 12 weeks. For the ApoE  $-/-$  mouse atherosclerosis study, three groups of 15 animals each received high-fat chow (Harlan Teklad; TD 97073 containing 1.25% cholesterol) for 12 weeks with or without AGI-1067 or probucol added to the chow at a final concentration of 0.09% w/w. In both studies, AGI-1067 or probucol was added to the chow to deliver approximately 150 mg/kg/day. The mice were then euthanized by halothane overdose, and their circulatory systems were perfused with 4% paraformaldehyde. The aortas were excised, fixed overnight in 4% paraformaldehyde, and adventitial fat removed. Computer-assisted morphometry of en face lesions was used to assess the extent of atherosclerosis. Aortas from LDLr  $-/-$  mice were further evaluated for cholesterol ester content by gas chromatography with flame ionization detection, as described previously (Daugherty et al., 1997).

**Plasma Lipoprotein Profiling and Cholesterol Determination.** Plasma was fractionated by fast-phase liquid chromatography and cholesterol levels in the different lipoprotein fractions determined by an enzymatic assay, as described (Innis-Whitehouse et al., 1998).

**Determination of the Extent of LDL Oxidation Induced by Copper.** LDL in rabbit and monkey plasma was captured by MB47 (an anti-ApoB antibody, a generous gift from J. Witztum; University

of California, San Diego, CA) in 96-well white flat-bottomed MicroFluor (Dynex Technologies, Chantilly, VA) plates at 4°C overnight. The captured LDL was then subjected to *ex vivo* oxidation by 5  $\mu\text{M}$   $\text{Cu}_2\text{SO}_4$  for 20 h at room temperature. Duplicate samples were prepared; one set was used to quantitate the amount of LDL captured by using biotinylated-detecting anti-ApoB antibodies (YE-1 for rabbit samples and MB24 for monkey samples; a generous gift from J. Witztum). The second set of samples was used to quantitate the amount of LDL being oxidized by using a biotinylated antibody to oxidatively-modified LDL (EO6; a generous gift from J. Witztum). EO6 is a monoclonal autoantibody isolated from ApoE  $-/-$  mice (Palinski et al., 1996). EO6 was shown to recognize oxidatively-modified LDL *in vitro* and in atherosclerotic lesions in humans and animals (Palinski et al., 1996). EO6 recognizes oxidized phospholipid, 1-palmitoyl-2-(5-oxovaleroyl)-phosphatidyl-choline (Horkko et al., 1999). A luminescence signal (relative light units) was generated from streptavidin, which was conjugated to Aqualite bound to biotinylated antibodies (Sealite Science, Norcross, GA) and recorded with a Dynex Luminometer (Dynex Technologies). The data are reported as an oxidation score, which is the ratio of relative light units of EO6 signal (20 h of oxidation/no oxidation) and the YE-1 or MB 24 signal (20 h of oxidation/no oxidation).

**In Vivo Lipopolysaccharide (LPS) Challenge Study.** Eleven 6-week-old C57BL/6 mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, *i.p.*). Four 100-mg, 90-day time-release pellets (Innovative Research Associates, Sarasota, FL) containing AGI-1067, probucol, or placebo were implanted subcutaneously for an average daily dose of 133 mg/kg/day. Four mice received AGI-1067, four received probucol, and three received the placebo in this manner. After 1 week, the mice were challenged with an intraperitoneal injection of 1 mg/kg LPS (Sigma-Aldrich, St. Louis, MO) and sacrificed 2 h later. Lungs were removed and immediately frozen in liquid nitrogen for RNA extraction.

**Northern Blot Analysis.** Total RNA was extracted from mouse lungs using TriPure isolation reagent (Roche Applied Science, Indianapolis, IN). For Northern blot analysis, 30  $\mu\text{g}$  of total RNA was separated on a 1% denaturing formaldehyde agarose gel and then transferred to a nylon membrane. Membranes were hybridized with  $^{32}\text{P}$ -labeled probes to mouse VCAM-1, MCP-1/JE, and  $\beta$ -actin, as described previously (Marui et al., 1993). Laser densitometry and digital analysis of scanned images were used for quantitation of autoradiograms.

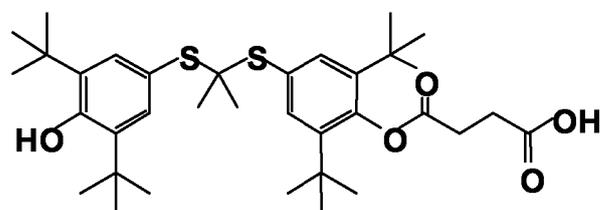
**Cell Culture and Enzyme-Linked Immunosorbent Assay (ELISA).** VCAM-1, intercellular adhesion molecule-1 (ICAM-1), and E-selectin expressed on the surface of activated human aortic endothelial cells (HAECs) (Cambrex Bio Science Walkersville, San Diego, CA) and MCP-1 secreted into the cell media were assessed by ELISA. HAECs passaged less than eight times were plated onto 24-well tissue culture dishes at a density of 3.6 to 4.0  $\times 10^4$  cells/ml in endothelial growth medium + 10% fetal bovine serum (Cambrex Bio Science Walkersville). The medium was changed 24 h later and TNF- $\alpha$  (10 units/well) was added either with AGI-1067 (2.5–10  $\mu\text{M}$ ), probucol ( $\leq 100$   $\mu\text{M}$ ), or vehicle (dimethyl sulfoxide). Cells were then incubated at 37°C with 5%  $\text{CO}_2$  for 16 h for measurement of VCAM-1 and ICAM-1 expression or 6 h for E-selectin. After visually assessing the cells for overt signs of toxicity, cells were washed with 1 ml/well of a 1:1 mixture of Hanks balanced salts solution (HBSS) and phosphate-buffered saline (PBS). HBSS/PBS + 5% fetal bovine serum (250  $\mu\text{l}$ ) were added to the wells with or without a primary antibody (0.25  $\mu\text{g}/\text{ml}$ ) and incubated for 30 min at 37°C. The primary antibodies used were mouse anti-human VCAM-1, ICAM-1, and E-selectin antibodies [Southern Biotechnology Associates (SBA), Birmingham, AL]. The cells were then washed twice with 0.5 ml/well of HBSS/PBS, and a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (SBA; diluted 1:500) was added. After a 30-min incubation at 37°C, the cells were washed four times with 0.5 ml of HBSS/PBS, and 250  $\mu\text{l}$ /well of substrate solution added (3% hydrogen peroxide, 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine in water). The cells were incubated at room temperature in the dark for 15 to 30 min, and the reaction was stopped by adding 75  $\mu\text{l}$  of 8 N sulfuric

acid/well. Absorbance was measured at 450 nm. MCP-1 levels in the medium after incubation of the cells with TNF- $\alpha$  and test compounds for 16 h were determined by ELISA (R&D Systems, Minneapolis, MN).

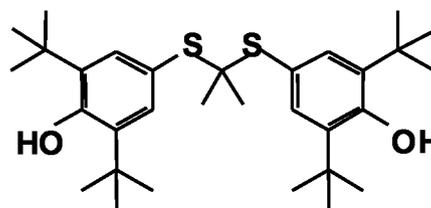
**Statistical Analysis.** Statistical significance was determined by analysis of variance with comparisons made using Fishers' protected LSD post hoc test. Where appropriate, a Student's *t* test was used. An experimental group was considered statistically significantly different from control when  $p < 0.05$ .

## Results

**Effects of AGI-1067 in Hypercholesterolemic Monkeys.** AGI-1067 is a novel phenolic antioxidant (Fig. 1) that lowers LDLc in multiple species (Meng et al., 2002). Given these properties, we hypothesized that AGI-1067 may have utility in CAD. To address this assumption, AGI-1067 was evaluated in hypercholesterolemic cynomolgus monkeys at oral doses of 50 and 150 mg/kg/day, and results were compared with probucol (150 and 250 mg/kg/day) in a year-long study. AGI-1067 dose dependently reduced plasma cholesterol levels. In contrast, probucol had no effect at 150 mg/kg/day and a modest cholesterol-lowering effect at 250 mg/kg/day (Fig. 2). Total cholesterol levels at the end of the study, expressed as the percent change from pretreatment levels ( $-1$  week) were  $112 \pm 61\%$  for the control group,  $14 \pm 15$  and  $-39 \pm 13\%$  for the 50 and 150 mg/kg/day AGI-1067 groups, respectively, and  $84 \pm 30$  and  $130 \pm 24\%$  for the 150 and 250 mg/kg/day probucol groups, respectively. Triglyceride levels were not significantly affected by either compound in this study. Evaluation of plasma cholesterol levels in the LDL and HDL fractions after 1 year of therapy revealed that LDLc levels in the AGI-1067-treated groups were reduced by 41 and 90% ( $p < 0.05$ ) at the 50 and 150 mg/kg/day doses, respectively, when compared with vehicle controls (Fig. 3a). HDLc levels were not different from the vehicle group at 50

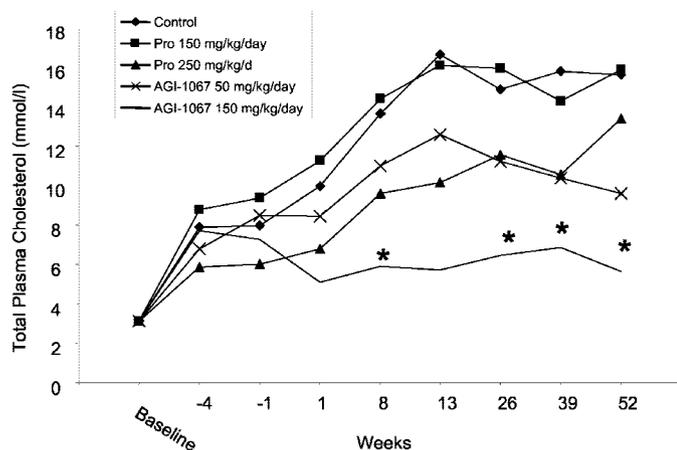


**AGI-1067**



**Probucol**

**Fig. 1.** The chemical structure of AGI-1067 (butanedioic acid, mono [4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester).



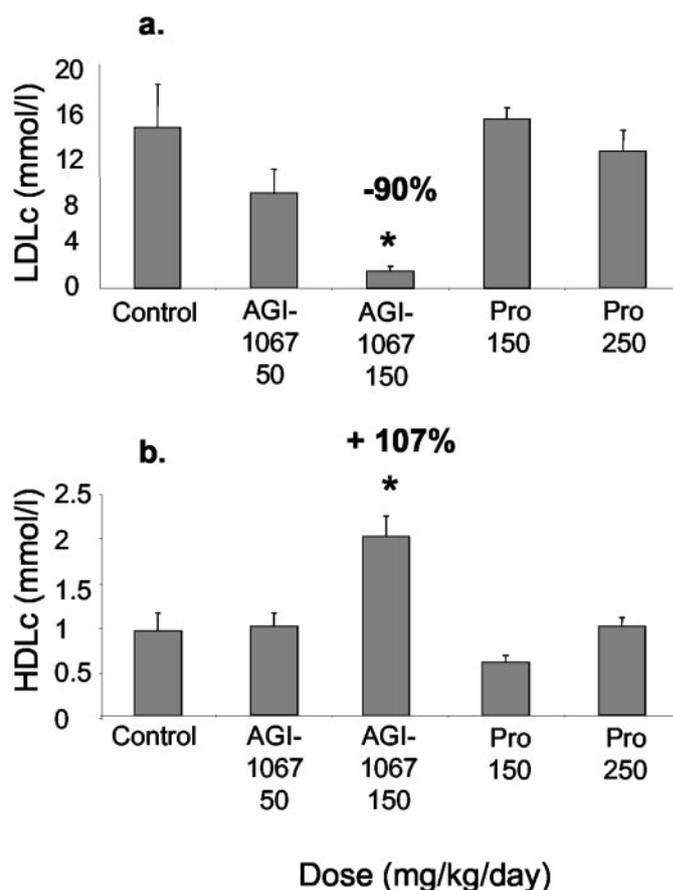
**Fig. 2.** Total plasma cholesterol versus time curves from hypercholesterolemic cynomolgus monkeys treated with vehicle, AGI-1067 (50 and 150 mg/kg/day), or probucol (Pro) (150 and 250 mg/kg/day) for 1 year. AGI-1067 dose dependently lowered total plasma cholesterol levels. The effect was seen as early as 1 week after onset of dosing and was sustained throughout the course of the study. Probucol decreased total cholesterol at 250 mg/kg/day and had no effect at 150 mg/kg/day. \*,  $p < 0.05$  compared with control group.

mg/kg/day and were increased 107% ( $p < 0.05$ ) at the 150 mg/kg/day dose when compared with vehicle controls (Fig. 3b). In contrast, probucol did not have a statistically significant effect on LDLc levels and decreased HDLc by 37% at 150 mg/kg/day and had no effect at 250 mg/kg/day when compared with vehicle controls.

Semiquantitative histopathological analyses of cross sections of the aortas and coronary arteries revealed the presence of atherosclerotic lesions in the aortas of all groups except those treated with AGI-1067 at 150 mg/kg/day and in the coronary arteries of all groups except the AGI-1067 (150 mg/kg/day) and probucol (250 mg/kg/day) groups. The degree and incidence of atherosclerosis in the other groups were variable and ranged from minimal-to-moderate in terms of severity (Fig. 4). The probucol (150 mg/kg/day) group actually appeared to have a greater degree of atherosclerosis of the coronary arteries compared with the 150 mg/kg/day AGI-1067 group or any of the other groups (Fig. 5). These results demonstrate that AGI-1067 decreased LDLc and increased HDLc and demonstrated a qualitative inhibitory effect on atherosclerosis in primates.

AGI-1067 was well tolerated in this study with all animals gaining weight. CBC and clinical chemistry parameters were not significantly different from the vehicle controls in any of the treated groups. No QTc prolongation was noted in any of the animals in this study.

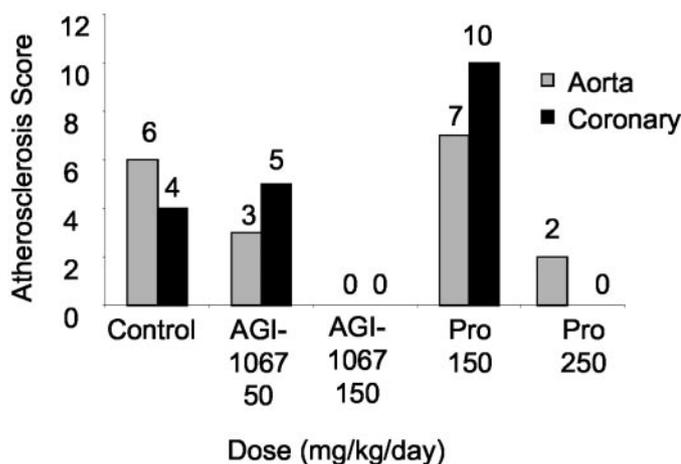
That AGI-1067 is a lipophilic antioxidant associated primarily with lipoproteins in the plasma (data not shown) suggested to us that it would also function to protect LDL from oxidation. In a separate experiment, LDL from hypercholesterolemic monkeys treated with several different doses of AGI-1067 (1, 5, and 25 mg/kg/day for 4 weeks) was found to be dose dependently resistant to ex vivo oxidation by  $\text{Cu}_2\text{SO}_4$ . The oxidation state of LDL was assessed with a monoclonal antibody (EO6) to oxidatively modified LDL. LDL from untreated, hypercholesterolemic monkeys had an oxidation score (EO6/anti-monkey apoB antibody reactivity) of 3.2 compared with 1.5, 0.8 ( $p < 0.05$ ), and 0.3 ( $p < 0.05$ ) for animals treated with AGI-1067 at doses of 1, 5, and 25 mg/kg/day, respectively. In a similar study in rabbits, LDL



**Fig. 3.** LDLc and HDLc levels in cynomolgus monkeys treated with vehicle, AGI-1067, or probucol (Pro) for 1 year. a, LDLc was decreased at the 50 and 150 mg/kg/day AGI-1067 doses, whereas probucol did not have a significant effect at 150 or 250 mg/kg/day. b, AGI-1067 significantly increased HDLc levels at the 150 mg/kg/day dose. In contrast, probucol decreased HDLc at the 150 mg/kg/day dose. \*,  $p < 0.05$  compared with control group.

from hypercholesterolemic rabbits treated with AGI-1067 or probucol at a dose of 150 mg/kg/day demonstrated similar ability to protect LDL from ex vivo oxidation. These data suggest that additional properties of AGI-1067 beyond its ability to protect LDL from oxidation are likely to contribute to the differential inhibitory effects of the two compounds on atherosclerosis progression.

**Inhibition of Atherosclerosis in the Absence of a Lipid-lowering Effect.** To separate the lipid-lowering effects of AGI-1067 from any direct vascular anti-inflammatory effects, the compound was further evaluated using a more quantitative approach in two transgenic mice models, the LDLr  $-/-$  and the ApoE  $-/-$  mice. AGI-1067 did not lower lipids in the LDLr  $-/-$  mice and caused a transient lipid lowering effect in ApoE  $-/-$  mice; probucol caused transient lipid lowering in both models (Table 1). Triglyceride levels were unaffected by AGI-1067 treatment. In the LDLr  $-/-$  mice, AGI-1067 at  $\sim 150$  mg/kg/day for 12 weeks decreased the atherosclerotic lesion area in the aorta by 49% ( $p < 0.05$ ,  $n = 20$ ) when compared with vehicle controls ( $n = 16$ ) as assessed by digital morphometry. Probucol, at the same dose, decreased the atherosclerotic lesion area by 21% ( $p =$  not significant,  $n = 20$ ) (Fig. 6a). The extent of atherosclerosis was also assessed by the more quantitative method of measuring cholesterol ester content of the aorta. The cholesterol ester content of the aortic arch in AGI-1067-treated



**Fig. 4.** A semiquantitative assessment of atherosclerosis (see *Materials and Methods*) in the aorta and coronary arteries of hypercholesterolemic monkeys treated with vehicle, AGI-1067, or probucol (Pro). There was no atherosclerosis detected in the aortas and coronary arteries of the AGI-1067 (150 mg/kg/day) group nor in the coronary arteries of the probucol (250 mg/kg/day) group. Probucol appeared to increase atherosclerosis in the coronary arteries of the (150 mg/kg/day) group.

LDLr  $-/-$  mice was decreased by 32% when compared with vehicle controls ( $p < 0.05$ ,  $n = 20$ ), whereas probucol did not have a significant effect (Fig. 6b). Similar results were seen in the ApoE  $-/-$  mouse model. AGI-1067 (150 mg/kg/day) inhibited atherosclerosis by 25, 41, and 49% compared with untreated controls in the arch, thoracic, and abdominal aortic regions, respectively ( $p < 0.05$ ;  $n = 15$ ) (Fig. 7). In contrast, probucol (150 mg/kg/day) inhibited atherosclerosis by 46% ( $p < 0.05$ ;  $n = 15$ ) in the thoracic aorta but had no statistically significant effect in the arch and abdominal regions. The ability to inhibit atherosclerosis even in the absence of a lipid-lowering effect suggests that other functionalities of the compound may account for this activity.

**In Vivo Anti-Inflammatory Properties.** The potential in vivo anti-inflammatory effects of AGI-1067 were investigated in a murine model of LPS-induced acute inflammation that results in up-regulation of proinflammatory genes. When lungs from non-LPS, placebo-treated mice were evaluated, there was no expression of VCAM-1 or MCP-1 (as assessed by Northern analysis), whereas mRNAs from both these genes were increased 2 h after LPS-challenge of placebo-treated mice (Fig. 8, a and b). Prior treatment with AGI-1067 at  $\sim 133$  mg/kg/day for 1 week significantly inhibited the LPS-induced increase in lung VCAM-1 and MCP-1 mRNA levels. Probucol, at the same dose, had a modest and more variable inhibitory effect. These data corroborate and extend our in vitro studies by demonstrating that AGI-1067 acts as an anti-inflammatory agent in vivo by inhibiting the induction of the proinflammatory genes VCAM-1 and MCP-1 at plasma levels that are well tolerated.

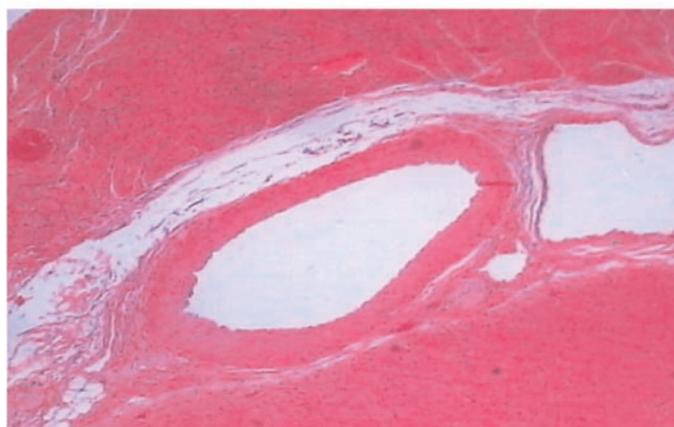
**Effects on Redox-Mediated Processes in HAECs.** To determine whether AGI-1067 directly affects the cells of the blood vessel wall, the compound was evaluated in cultured HAECs for its effects on redox-sensitive inflammatory gene expression. AGI-1067 (2.5–10  $\mu$ M) added to HAECs concomitantly with TNF- $\alpha$  for 16 h inhibited VCAM-1 cell surface expression ( $IC_{50} = 6$   $\mu$ M, average of three experiments). AGI-1067 had no effect on the cell surface expression of ICAM-1 at the same concentrations (Fig. 9). In contrast, probucol failed to

a.



**Probucol (150 mg/kg/day)**

b.



**AGI-1067(150 mg/kg/day)**

**Fig. 5.** Photomicrographs of hematoxylin and eosin-stained cross sections of the left coronary arteries of a cynomolgus monkey treated with probucol (150 mg/kg/day) (a) or AGI-1067 (150 mg/kg/day) (b).

inhibit the TNF- $\alpha$ -inducible cell surface expression of VCAM-1 or ICAM-1 at concentrations as high as 100  $\mu$ M (Fig. 9). AGI-1067 also inhibited TNF- $\alpha$  induction of two other redox-sensitive inflammatory proteins, MCP-1 and the adhesion molecule E-selectin, with  $IC_{50}$  values of 10 and 25  $\mu$ M, respectively; probucol also had no effect on the inducible expression of these genes (data not shown). These data demonstrate that AGI-1067 can act directly on endothelial cells to inhibit redox-sensitive processes such as the induction of inflammatory genes.

## Discussion

The novel, orally deliverable phenolic antioxidant and anti-inflammatory compound, AGI-1067, was well tolerated in hypercholesterolemic monkeys when administered for 1 year. AGI-1067 demonstrated dose-dependent lowering of LDL cholesterol and marked elevation of HDL cholesterol. Probucol, by contrast, modestly lowered LDL and HDL cholesterol. Qualitative histopathological assessment of this long-term

TABLE 1

Total plasma cholesterol levels from AGI-1067- and probucol-treated LDLr  $-/-$  and ApoE  $-/-$  mice. Mice were administered AGI-1067, probucol, or vehicle (untreated controls) as an admixture in chow for 12 weeks at an approximate daily dose of 150 mg/kg/day. Total plasma cholesterol (milligrams per deciliter) was determined after 2 and 12 weeks of treatment and is expressed as the mean  $\pm$  S.E.M.

Treatment	LDLr $-/-$ Mice		ApoE $-/-$ Mice	
	2 Week	12 Week	2 Week	12 Week
Untreated controls	1060 $\pm$ 12	1360 $\pm$ 20	1053 $\pm$ 71	726 $\pm$ 31
AGI-1067	1172 $\pm$ 16	1365 $\pm$ 19	646 $\pm$ 140*	987 $\pm$ 48*
Probucol	877 $\pm$ 23	1203 $\pm$ 14	736 $\pm$ 19	811 $\pm$ 75

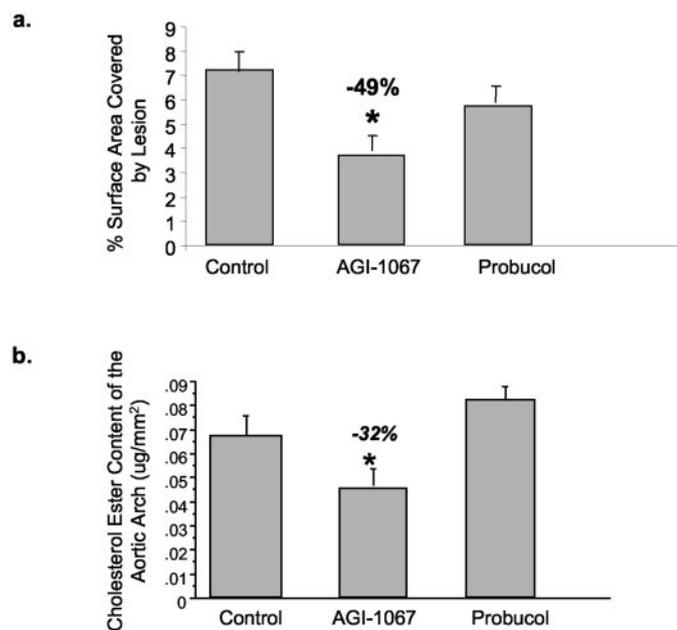
\*  $P < 0.05$  compared to untreated controls.

study revealed the absence of atherosclerosis in the aortas and coronaries of the AGI-1067 high-dose group. Probucol inhibited atherosclerosis only at the 250 mg/kg/day dose, consistent with previously reported studies (Wissler and Veselinovitch, 1983; Sasahara et al., 1994; Daugherty and Rose-laar, 1995) and seemed to actually worsen atherosclerosis at the 150 mg/kg/day dose.

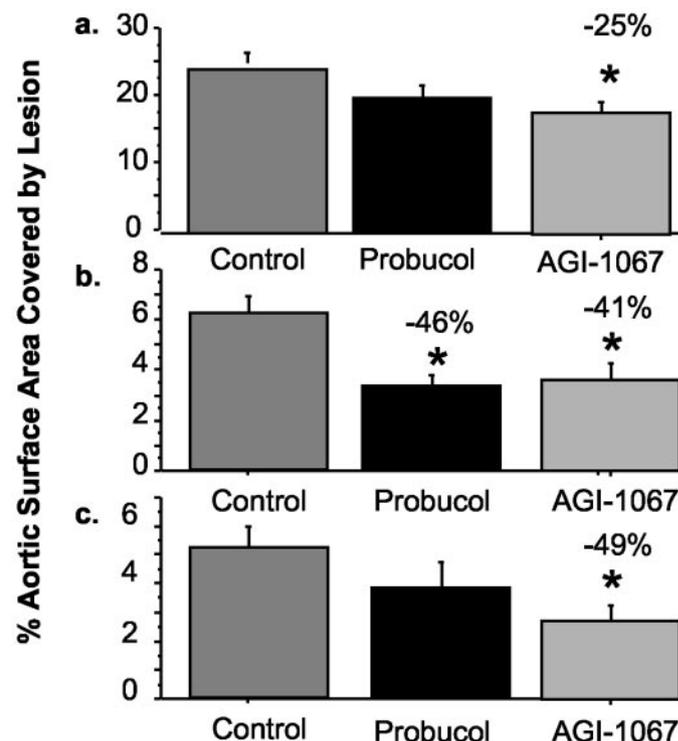
The antiatherosclerotic effects of AGI-1067 were also demonstrated in two commonly used models of hypercholesterolemia-induced atherosclerosis, the LDLr  $-/-$  mouse and the ApoE  $-/-$  mouse (Zhang et al., 1992; Ishibashi et al., 1994). AGI-1067 had no effect on cholesterol levels in the LDLr  $-/-$  mice and transient effects in the ApoE  $-/-$  mice. The lack of a sustained lipid-lowering effect in these mice may be due to the lack of the LDL receptor in the case of LDLr  $-/-$  mice and its down-regulation in ApoE  $-/-$  mice that occurs after prolonged exposure to high cholesterol levels (Brown and Goldstein, 1975). Irrespective of the mechanism, the inability of AGI-1067 to lower plasma cholesterol levels in these two mouse strains allowed for a direct assessment of the compounds antiatherosclerotic properties independent of its lip-

id-lowering effects. In spite of having either no or transient lipid-lowering effects in these mice, AGI-1067 still significantly inhibited atherosclerosis to a greater extent than probucol, which had modest lipid-lowering effects in these models. We report a modest antiatherosclerotic effect of probucol in LDLr  $-/-$  and ApoE  $-/-$  mice in apparent contradiction to several reported studies that showed a proatherosclerotic effect of the drug in these same mouse strains (Zhang et al., 1997; Moghadasian et al., 1999). One possible explanation for these disparate results is the 5 to 11 $\times$  lower dose of probucol used in our study, thereby suggesting that part of the proatherosclerotic effect seen in these other studies may be caused by toxicity due to high plasma levels.

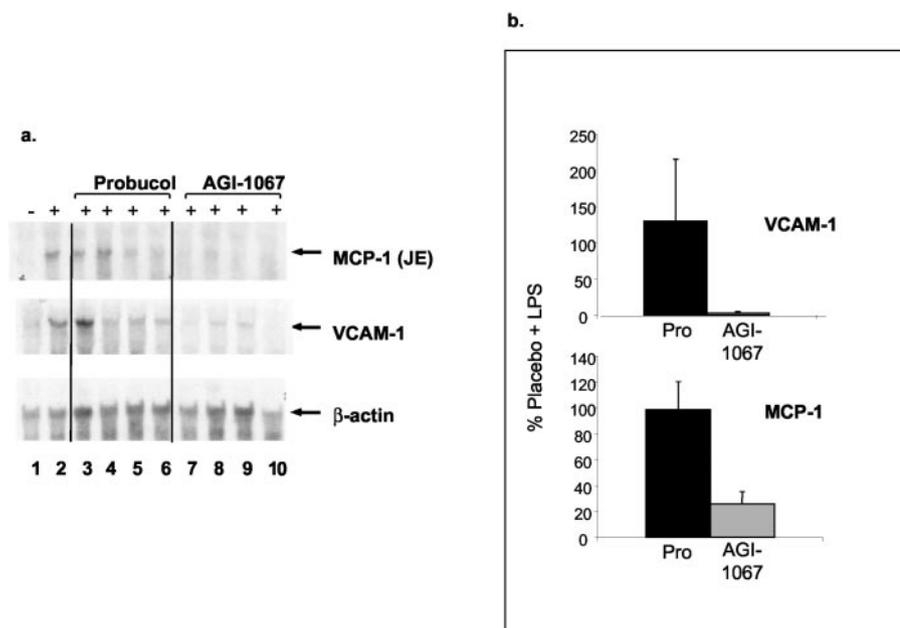
These data led us to surmise that the antiatherosclerotic properties observed in these studies may be due to AGI-1067's anti-inflammatory properties. This was directly assessed in a murine model of acute inflammation that resulted in up-regulation of VCAM-1 and MCP-1. The model involved challenging mice with LPS and evaluating the effect of pre-



**Fig. 6.** Effect of AGI-1067 and probucol on atherosclerosis in LDLr  $-/-$  mice. a, AGI-1067 inhibited atherosclerosis as determined by morphometric analysis of en face lesions. The data are expressed as the percentage of the total aortic surface area covered by lesion. b, the extent of atherosclerosis was also assessed by quantitation of the cholesterol ester content of the aorta. AGI-1067 reduced the cholesterol ester content of the aortas, whereas probucol had no effect. AGI-1067 and probucol were administered to LDLr  $-/-$  mice at an approximate dose of 150 mg/kg/day for 12 weeks. \*,  $p < 0.05$  compared with untreated controls.



**Fig. 7.** Effect of AGI-1067 and probucol on atherosclerosis lesion area in ApoE  $-/-$  mice. The data are expressed as the percentage of the aortic surface area covered by lesion in the aortic arch (a), thoracic (b), and abdominal regions (c). AGI-1067 and probucol were administered to ApoE  $-/-$  mice at an approximate dose of 150 mg/kg/day for 12 weeks. \*,  $p < 0.05$  compared with untreated controls.



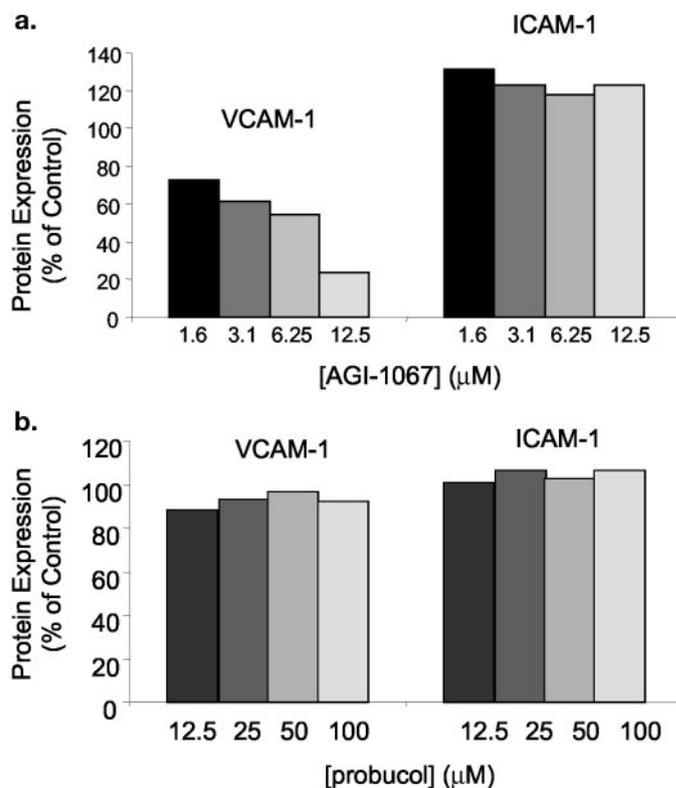
**Fig. 8.** Analysis of mRNA levels for VCAM-1, MCP-1/JE, and  $\beta$ -actin in lungs from LPS-challenged mice. **a.** Northern blot of mRNA from control (-) and LPS (+)-challenged mice that were pretreated for 7 days before LPS challenge with subcutaneous time release pellets containing placebo (lanes 1–2), probucol (Pro) (lanes 3–6), or AGI-1067 (lanes 7–10) at a dose of approximately 133 mg/kg/day. The mice were sacrificed and mRNA collected 2 h after the LPS challenge. Individual lanes in the Northern blot represent different individual mice. **b.** densitometric analysis of mean VCAM-1 and MCP-1 transcript levels from the probucol- and AGI-1067-treated groups that were normalized to  $\beta$ -actin. The data are shown as the percentage of the placebo and LPS control. Error bars represent S.E.M.

treatment with AGI-1067 and probucol on the expression of these genes in the lung. Whereas probucol had a modest and heterogeneous effect on the expression of these genes, AGI-1067 inhibited the LPS-inducible up-regulation of VCAM-1 and MCP-1, thus demonstrating that the compound has *in vivo* anti-inflammatory activity.

To address whether AGI-1067 functions to inhibit redox-sensitive processes in the vasculature, we evaluated the compound for its effects on inflammatory gene expression. AGI-1067 selectively inhibited inducible VCAM-1 expression in TNF- $\alpha$ -activated endothelial cells to a greater extent than ICAM-1. Subsequently, AGI-1067 was also found to inhibit the TNF- $\alpha$ -inducible endothelial expression of MCP-1 and E-selectin, thereby suggesting that these genes may share similar regulatory mechanisms with VCAM-1. The relative selectivity of AGI-1067 for VCAM-1 inhibition versus ICAM-1 infers that AGI-1067 treatment might not decrease the body's ability to fight infection.

Under the conditions used in this study, probucol was less active in inhibiting TNF- $\alpha$ -induced VCAM-1, MCP-1, and E-selectin expression in cultured endothelial cells when compared with AGI-1067. Reported effects of probucol on the expression of these genes vary (Kaneko et al., 1996; Tanaka et al., 1998; Zapolska-Downer et al., 2001). This may be due to differences in protocol such as the method of activation, cell density, cell type used, drug concentration, and length of exposure. The greater activity of AGI-1067 when compared with probucol in inhibiting redox-sensitive inflammatory gene expression may be due to its enhanced cell permeability. The carboxylic acid in the side chain of AGI-1067 would be expected to decrease its lipophilicity compared with probucol; this may increase its cell permeability and access to intracellular sites of redox regulation relative to probucol. This is consistent with the observation that in endothelial cell culture AGI-1067 partitions with the cellular fraction, whereas probucol is predominantly associated with the medium (C. Kunsch, unpublished data). Consistent with its *in vitro* activities, AGI-1067 also inhibited induction of VCAM-1 and MCP-1 expression in an *in vivo* model of LPS-induced endothelial activation to a greater extent than probucol at plasma levels that were well tolerated.

Substantial evidence indicates that oxidatively modified lipoproteins can contribute to atherogenesis (Witztum, 1994). Treatment of hypercholesterolemic animals with lipophilic antioxidants such as vitamin E and probucol can protect their LDL from *ex vivo* oxidation (Kleinvelde et al., 1994). AGI-1067 was shown to be equipotent to probucol in this



**Fig. 9.** Effect of AGI-1067 on TNF- $\alpha$ -inducible endothelial cell surface expression of VCAM-1 and ICAM-1. **a.** data from a representative experiment demonstrating concentration-dependent inhibition of TNF- $\alpha$ -induced VCAM-1 cell surface expression on HAECs by AGI-1067 and lack of effect on ICAM-1 expression as detected by ELISA. The data are presented as the percentage of the vehicle (dimethyl sulfoxide) + TNF- $\alpha$  control. **b.** lack of effect of probucol on TNF- $\alpha$  induced VCAM-1 and ICAM-1 expression.

regard with LDL from animals treated with both compounds similarly resistant to ex vivo oxidation by copper. These data are consistent with the two compounds' similar antioxidant properties (Meng et al., 2002) and propensity to partition with lipoproteins in the plasma. Thus the ability of AGI-1067 to protect LDL from oxidation may contribute to its antiatherosclerotic properties but does not explain the differential pharmacological effects of AGI-1067 and probucol.

The pharmacology data presented here suggest that AGI-1067 works by a novel mechanism that is distinct from statins, which are currently the therapeutic agents of choice for CAD patients. Whereas statins reduce the risk of developing CAD primarily by decreasing LDLc, AGI-1067 is anticipated to inhibit the progression of the disease and possibly reverse it by acting directly on the vessel wall to protect it from oxidative damage. As such, AGI-1067 belongs to a new class of compounds known as vascular protectants that are predicted to be efficacious in patients with CAD that do not have elevated cholesterol levels as well as those that are at risk for CAD due to other risk factors such as diabetes and hypertension.

In summary, AGI-1067 inhibited atherosclerosis in primate and mouse models of hypercholesterolemia-induced atherosclerosis. Furthermore, in mice, it inhibited atherosclerosis by a mechanism unrelated to its lipid-lowering properties. AGI-1067 was distinguished from probucol in these studies by its superior lipid-modulating, anti-inflammatory and antiatherosclerotic properties, and its ability to inhibit redox-sensitive processes in vascular wall cells. These data support the central role of abnormal redox-signaling in the pathogenesis of atherosclerosis and, taken together with promising clinical data, suggest that AGI-1067 may be a novel and safe therapeutic agent for CAD as well as restenosis after percutaneous coronary intervention.

#### Acknowledgments

We acknowledge Drs. Charles Kunsch, James A. Sikorski, and Anne M. Whalen for critically reviewing this manuscript.

#### References

- Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, and Milos PM (1999) Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **19**:1518–1525.
- Brown MS and Goldstein J (1975) Regulation of the activity of the low-density lipoprotein receptor in human fibroblasts. *Cell* **6**:307–316.
- Christen WG and Hennekens CH (2000) Antioxidant vitamins and cardiovascular disease: evidence from observational epidemiologic studies and randomized trials, in *Antioxidants and Cardiovascular Disease* (Tardiff JC and Bourassa MG ed) pp135–143, Kluwer Academic Publishers, Norwell, MA.
- Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Ramos Gutierrez JC, Connelly PW, and Milstone DS (2001) A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* **107**:1209–1210.
- Dansky HM, Barlow CB, Lominska C, Sikes JL, Kao C, Weinsaft J, Cybulsky MI, and Smith JD (2001) Adhesion of monocytes to arterial endothelium and initiation of atherosclerosis are critically dependent on vascular cell adhesion molecule-1 gene dosage. *Arterioscler Thromb Vasc Biol* **21**:1662–1667.
- Daugherty A, Pure E, Delfel-Butteiger D, Chen S, Leforovich J, Roselaar SE, and Rader DJ (1997) The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E  $-/-$  mice. *J Clin Invest* **100**:1575–1580.
- Daugherty A and Roselaar SE (1995) Lipoprotein oxidation as a mediator of atherogenesis: insights from pharmacological studies. *Cardiovasc Res* **29**:297–311.
- Gosling J, Slaymaker S, Gu L, Tseng S, Zlot CH, Young SG, Rollins BJ, and Charo IF (1999) MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* **103**:773–778.
- Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Freidman P, Dennis EA, Curtiss LK, et al. (1999) Monoclonal antibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest* **103**:117–128.
- Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, and Cybulsky MI (1999) Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res* **85**:199–207.

- Innis-Whitehouse W, Li X, Brown VW, and Le N (1998) An efficient chromatographic system for lipoprotein fractionation using whole plasma. *J Lipid Res* **39**:679–690.
- Ishibashi S, Goldstein JL, Brown MS, Herz J, and Bruns DK (1994) Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest* **93**:1885–1893.
- Johansson J, Olsson AG, Bergstrand L, Elinder LS, Nilsson S, Erickson U, Molgaard J, Holme I, and Walldius G (1995) Lowering of HDL<sub>2b</sub> by probucol partly explains the failure of the drug to affect femoral atherosclerosis in subjects with hypercholesterolemia. A Probuco Quantitative Regression Swedish Trial (PQRST) Report. *Arterioscler Thromb Vasc Biol* **15**:1049–1056.
- Kaneko M, Hayashi J, Saito I, and Miyasaka N (1996) Probuco down-regulates E-selectin expression on cultured human vascular endothelial cells. *Arterioscler Thromb Vasc Biol* **16**:1047–1051.
- Keaney JF (2000) Antioxidants and vascular disease: animal studies, in *Antioxidants and Cardiovascular Disease* (Tardiff JC and Bourassa MG ed) pp 101–116, Kluwer Academic Publishers, Norwell, MA.
- Klein L (1981) QTc-interval prolongation produced by probucol. *Arch Intern Med* **141**:1102–1103.
- Kleinvelde HA, Demacker PN, and Stalenhoef AF (1994) Comparative study on the effect of low-dose vitamin E and probucol on the susceptibility of LDL to oxidation and the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb* **14**:1386–1391.
- Kowala MC, Reece R, Beyer S, Gu C, and Valentine M (2000) Characterization of atherosclerosis in LDL receptor knockout mice: macrophage accumulation correlates with rapid and sustained expression of MCP-1/JE. *Atherosclerosis* **149**:323–330.
- Kunsch C and Medford RM (1999) Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* **85**:753–766.
- Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, and Medford RM (1993) Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant sensitive mechanism in human vascular endothelial cells. *J Clin Invest* **92**:1866–1874.
- Medford RM (1995) Antioxidants and endothelial expression of VCAM-1: A molecular paradigm for atherosclerosis. *Cardiovascular Disease* (Gallo LL) 2nd ed, pp 121–127, Plenum Press, NY.
- Meng CQ, Somers PK, Rachita CL, Holt LA, Hoong LK, Zheng XS, Simpson JE, Hill RR, Olliff LK, Kunsch C, et al. (2002) Novel phenolic antioxidants as multifunctional inhibitors of inducible VCAM-1 expression for use in atherosclerosis. *Bioorg Med Chem Lett* **12**:2545–2548.
- Moghadasian MH, McManus BM, Godin DV, Rodrigues R, and Frohlich JJ (1999) Proatherogenic and antiatherogenic effects of probucol and phytosterols in apolipoprotein E-deficient mice: possible mechanisms of action. *Circulation* **99**:1733–1739.
- Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, and Witztum JL (1996) Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest* **98**:800–814.
- Parthasarathy S, Young SG, Witztum JL, Pittman RC, and Steinberg D (1986) Probuco inhibits oxidative modification of low density lipoprotein. *J Clin Invest* **77**:641–644.
- Ping D, Boekhoudt GH, Rogers EM, and Boss JM (1999) Nuclear factor- $\kappa$ B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant protein-1 gene. *J Immunol* **162**:727–734.
- Ross R (1999) Atherosclerosis—an inflammatory disease. *N Engl J Med* **340**:115–126.
- Sasahara M, Raines EW, Chait A, Carew TE, Steinberg D, Wahl PW, and Ross R (1994) Inhibition of hypercholesterolemia-induced atherosclerosis in the nonhuman primate by probucol. *J Clin Invest* **94**:155–164.
- Sawayama Y, Shimizu C, Maeda N, Tatsukawa M, Kinukawa N, Koyanagi N, Koyanagi S, Kashiwagi S, and Hayashi J (2002) Effects of probucol and pravastatin on common carotid atherosclerosis in patients with asymptomatic hypercholesterolemia. Fukuoka Atherosclerosis Trial (FAST). *J Am Coll Card* **39**:610–616.
- Tanaka K, Hayashi K, Shingu T, Kugu Y, Nomura K, and Kajiyama G (1998) Probuco inhibits neointimal formation in carotid arteries of normocholesterolemic rabbits and the proliferation of cultured rabbit vascular smooth muscle cells. *J Cardiovasc Drugs Ther* **12**:19–28.
- Tardiff JC, Gregoire J, Schwartz L, Title L, Laramée L, Reeves F, Lesperance J, Bourassa MG, L'Allier PL, Glass M, et al. (2003) Effects of AGI-1067 and probucol after percutaneous coronary interventions. *Circulation* **107**:552–558.
- Weber C, Erl W, Pietsch A, Strobel M, Zeigler-Heitbrock HWL, and Weber PC (1994) Antioxidants inhibit monocyte adhesion by suppressing nuclear factor- $\kappa$ B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. *Arterioscler Thromb Vasc Biol* **14**:1665–1673.
- Wissler RW and Vesselinovitch D (1983) Combined effects of cholestyramine and probucol on regression of atherosclerosis in Rhesus monkey aortas. *Appl Pathol* **1**:89–96.
- Witztum JL (1994) The oxidation hypothesis of atherosclerosis. *Lancet* **344**:793–795.
- Zapolska-Downer D, Zapolski-Downer A, Markiewski M, Ciechanowicz A, Kaczmarczyk M, and Naruszewicz M (2001) Selective inhibition by probucol of vascular cell adhesion molecule-1 (VCAM-1) expression in human vascular endothelial cells. *Atherosclerosis* **155**:123–130.
- Zhang SH, Reddick RL, Avdievich E, Surlis KL, Jones RG, and Reynolds JB (1997) Paradoxical enhancement of atherosclerosis by probucol treatment in apolipoprotein E-deficient mice. *J Clin Invest* **99**:2858–2866.
- Zhang SH, Reddick RL, Piedrahita JA, and Maeda N (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science (Wash DC)* **258**:468–471.

**Address correspondence to:** Dr. Cynthia L. Sundell, Discovery Research, AtheroGenics, Inc., 8995 Westside Parkway, Alpharetta, GA 30004. E-mail: csundell@atherogenics.com