AGI-1067: A multifunctional phenolic antioxidant, lipid modulator, anti-inflammatory and anti-atherosclerotic agent

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AtheroGenics, Inc.
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Abbreviations:
LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; LDLr-/- mice, LDL receptor deficient mice; ApoE -/- mice, Apolipoprotein E deficient mice.

Section Assignment: Cardiovascular
ABSTRACT

To explore the therapeutic efficacy and potential mechanisms of action of a new class of anti-atherosclerotic drugs, AGI-1067 was tested in several animal models of atherosclerosis. AGI-1067, a novel phenolic antioxidant, was well tolerated in a one-year study in hypercholesterolemic cynomolgus monkeys. It lowered LDLc by 41% and 90% at oral doses of 50 and 150 mg/kg, respectively, and increased HDLc by 107% at the higher dose. In contrast, another phenolic antioxidant, probucol, had a modest LDL-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 LDLr -/- mice and 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 VCAM-1 and MCP-1 mRNA levels in lungs of LPS-stimulated mice. At the cellular level, AGI-1067 inhibited TNF-α-inducible expression of VCAM-1, MCP-1, and E-selectin in human aortic endothelial cells (IC50 values = 6 µM, 10 µM, and 25 µM, 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 upregulated 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) (Cybulsky et al., 2001; Dansky et al., 2001; Aiello et al., 1999; Gosling et al., 1999).

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 (Keaney, 2000; Christen and Hennekens, 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 to the control group despite lowering HDL cholesterol (Sawayama et al., 2002). However, chronic clinical use of probucol has been limited since it lowers HDL cholesterol and causes QTc interval prolongation (Klein, 1981).

In this paper, AGI-1067, a novel antioxidant, anti-inflammatory and lipid-lowering agent that has anti-atherosclerotic 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 anti-atherosclerotic 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, in press).
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 2-3 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 four-week acclimatization period they were fed 200 g per day of a high fat diet (1.7% cholesterol, 11% coconut oil, 11% butter oil, 62.4% monkey chow, 13.0% orange juice, 0.83% vitamin mix; supplied by SDS, Witham, Essex, UK) for a minimum of 4 weeks prior to dosing. Upon confirmation of hypercholesterolemia, the animals were randomly allocated into five groups of four animals each and administered vehicle (cremophor:PEG300 (4:1)), AGI-1067 50 mg/kg/day, AGI-1067 150 mg/kg/day, probucol 150 mg/kg/day or probucol 250 mg/kg/day once daily for one 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 AGI-1067 150 mg/kg/day 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 hr 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 study. Blood samples were taken by venepuncture; 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, 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 µm, 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 0-4 corresponding to none, minimal, slight, moderate and severe. The incidence of atherosclerosis was determined as 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 X severity for all the animals in a group. For example, the probucol (150 mg/kg/day) group had one animal with minimal (1 X 1=1), one with slight (2 X 1=2), one with moderate (1 X 3=3) and one with severe (1 X
4=4) atherosclerotic lesions in the left coronary arteries for a total score of “10” (1 + 2 + 3 + 4 = 10) for the group.

**LDLr -/- and ApoE -/- Mouse Atherosclerosis Studies**

Six-week old LDL-receptor (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 twenty 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% wt/wt. 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 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 (FPLC) 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-Apo B antibody, a generous gift from J. Witztum) 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 µM Cu₂SO₄ for 20 hr 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, 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 (POPVC) (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, Chantilly, VA). The data is reported as an oxidation score, which is the ratio of relative light units of EO6 signal (20 hr oxidation/no oxidation) and YE-1 or MB 24 signal (20 hr oxidation/no oxidation).

In Vivo Lipopolysaccharide (LPS) Challenge Study

Eleven six-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 one week, the mice were challenged with an intraperitoneal injection of 1 mg/kg LPS (Sigma Chemicals, St. Louis, MO) and sacrificed 2 hr 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 µg total RNA was separated on a 1% denaturing formaldehyde agarose gel, then transferred to a nylon membrane. Membranes were hybridized with $^{32}$P-labelled probes to mouse VCAM-1, MCP-1/JE and β-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, ICAM-1, and E-selectin expressed on the surface of activated human aortic endothelial cells (HAECs) (Clonetics, Inc., Walkersville, MD) 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 - 4.0 X 10$^4$ cells/ml in endothelial growth media + 10% fetal bovine serum (FBS) (Clonetics, Inc.). The media was changed 24 hr later and TNF-α (10 units/well) was added either with AGI-1067 (2.5-10 µM), probucol (≤100 µM) or vehicle (DMSO). Cells were then incubated at 37 °C with 5% CO$_2$ for 16 hr for measurement of VCAM-1 and ICAM-1 expression or 6 hr 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% FBS (250 µl) were added to the wells with or without a primary antibody (0.25 µg/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 µl/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 15-30 min and the reaction stopped by adding 75 µl of 8N sulfuric acid/well. Absorbance was measured at 450 nm. MCP-1 levels in the media after incubation of the cells with TNF-α and test compounds for 16 hr, were determined by ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

Statistical significance was determined by ANOVA with comparisons made using Fishers Protected LSD post hoc test. Where appropriate, a Students 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 (Figure 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 to probucol (150 and 250 mg/kg/day) in a yearlong 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 (Figure 2). Total cholesterol levels at the end of the study expressed as the percent change from pre-treatment levels (-1 week) were 112 ± 61% for the control group; 14 ± 15% and -39 ± 13% for the 50 and 150 mg/kg/day AGI-1067 groups, respectively; and 84 ± 30% and 130 ± 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 (Figure 3a). HDLc levels were no different than the vehicle group at the 50 mg/kg/day dose and were increased 107% (p<0.05) at the 150 mg/kg/day when compared with vehicle controls (Figure 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 to 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 (Figure 4). The probucol (150 mg/kg/day) group actually appeared to have a greater degree of atherosclerosis of the coronary arteries compared to the AGI-1067 150 mg/kg/day group or any of the other groups (Figure 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.

The fact 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 \textit{ex vivo} oxidation by Cu$_2$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 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 ~ 150 mg/kg/day for 12 weeks decreased 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 atherosclerotic lesion area by 21% (p=NS, N=20) (Figure 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 LDLr -/- mice was decreased by 32% when compared to vehicle controls (p<0.05, N=20), whereas probucol did not have a significant effect (Figure 6b). Similar results were seen in the ApoE -/- mouse model. AGI-1067 (150 mg/kg/day) inhibited atherosclerosis by 25%, 41% and 49% compared to untreated controls in the arch, thoracic and abdominal aortic regions,
respectively (p<0.05; N=15) (Figure 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 upregulation of pro-inflammatory 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 hr after LPS-challenge of placebo-treated mice (Figure 8a and b). Prior treatment with AGI-1067 at ~133 mg/kg/day for one 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 pro-inflammatory genes VCAM-1 and MCP-1 at plasma levels that are well tolerated.

Effects on Redox-Mediated Processes in Human Aortic Endothelial Cells (HAECs)

In order 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 µM) added to HAECs concomitantly with TNF-α for 16 hr inhibited VCAM-1 cell surface expression (IC₅₀ = 6 µM, average of 3 experiments). AGI-1067 had no effect on the cell surface expression of
intercellular adhesion molecule-1 (ICAM-1) at the same concentrations (Figure 9). In contrast, probucol failed to inhibit the TNF-α-inducible cell surface expression of VCAM-1 or ICAM-1 at concentrations as high as 100 μM (Figure 9). AGI-1067 also inhibited TNF-α induction of two other redox-sensitive inflammatory proteins, MCP-1, and the adhesion molecule, E-selectin, with IC_{50} values of 10 μM and 25 μM, 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 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 Vesselinovitch, 1983; Sasahara et al, 1994; Daugherty et al, 1995) and seemed to actually worsen atherosclerosis at the 150 mg/kg/day dose.

The anti-atherosclerotic effects of AGI-1067 were also demonstrated in two commonly used models of hypercholesterolemia-induced atherosclerosis, the LDLr -/- mouse and the ApoE -/- mouse (Ishibashi et al, 1994; Zhang et al, 1992). 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 anti-atherosclerotic properties independent of its lipid-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 anti-atherosclerotic
effect of probucol in LDLr−/- and ApoE−/- mice, in apparent contradiction to several
reported studies that showed a pro-atherosclerotic 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 5-11X lower dose of probucol used in our study, thereby
suggesting that part of the pro-atherosclerotic effect seen in these other studies may be
caused by toxicity due to high plasma levels.

These data led us to surmise that the anti-atherosclerotic 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 upregulation of
VCAM-1 and MCP-1. The model involved challenging mice with LPS and evaluating
the effect of pretreatment 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 upregulation 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-α-activated
endothelial cells to a greater extent than ICAM-1. Subsequently AGI-1067 was also
found to inhibit the TNF-α-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-α-induced VCAM-1, MCP-1 and E-selectin expression in cultured endothelial cells when compared to 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 method of activation, cell density, cell type used, drug concentration and length of exposure. The greater activity of AGI-1067 when compared to 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 to 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 media (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 (Kleinveld et al, 1994). AGI-1067 was shown to be equipotent to probucol in this 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 anti-atherosclerotic 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 anti-atherosclerotic properties and its ability to inhibit redox-sensitive processes in vascular wall cells. These data support the central role of abnormal redox-signalling 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.
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References


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Figure Legends

Figure 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).

Figure 2. Total plasma cholesterol versus time curves from hypercholesterolemic
cynomolgus monkeys treated with vehicle, AGI-1067 (50 and 150 mg/kg/day) or
probucol (150 and 250 mg/kg/day) for one year. AGI-1067 dose-dependently lowered
total plasma cholesterol levels. The effect was seen as early as one 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 to
control group.

Figure 3. LDLc and HDLc levels in cynomolgus monkeys treated with vehicle, AGI-
1067 or probucol for one 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 HDL cholesterol levels at the 150
mg/kg/day dose. In contrast, probucol decreased HDL at the 150 mg/kg/day dose.
* p<0.05 compared with control group.

Figure 4. A semiquantitative assessment of atherosclerosis (see methods) in the aorta
and coronary arteries of hypercholesterolemic monkeys treated with vehicle, AGI-1067
or probucol. 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.

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

**Figure 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 percent 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 to untreated controls.

**Figure 7.** Effect of AGI-1067 and probucol on atherosclerosis lesion area in ApoE-/- mice. The data are expressed as the percent of the aortic surface area covered by lesion in the (a) aortic arch, (b) thoracic and (c) abdominal regions. 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 to untreated controls.

**Figure 8.** Analysis of mRNA levels for VCAM-1, MCP-1/JE and β-actin in lungs from LPS-challenged mice. (a) Northern blot of mRNA from control (-) and LPS (+) challenged mice that were pre-treated for 7 days prior to LPS challenge with subcutaneous time release pellets containing placebo, (lanes 1-2), probucol (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 hrs 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 β-actin. The data are shown as the percent of the placebo and LPS control. Error bars represent sem.

**Figure 9.** Effect of AGI-1067 on TNF-α inducible endothelial cell surface expression of VCAM-1 and ICAM-1. (a) Data from a representative experiment demonstrating concentration-dependent inhibition of TNF-α-induced VCAM-1 cell surface expression by AGI-1067 on HAECs and lack of effect on ICAM-1 expression as detected by ELISA. The data are presented as the percent of the vehicle (DMSO) + TNF-α control. (b) Lack of effect of probucol on TNF-α induced VCAM-1 and ICAM-1 expression.
Figure 2

Total Plasma Cholesterol (mmol/l)

Weeks

Baseline

Control
Pro 150 mg/kg/day
Pro 250 mg/kg/day
AGI-1067 50 mg/kg/day
AGI-1067 150 mg/kg/day
Figure 3

(a) LDLc (mmol/l)

Control | AGI-1067 50 | AGI-1067 150 | Probucol 150 | Probucol 250
--- | --- | --- | --- | ---
| | | | |

-90%

(b) HDLc (mmol/l)

Control | AGI-1067 50 | AGI-1067 150 | Probucol 150 | Probucol 250
--- | --- | --- | --- | ---
| | | | |

+107%

Dose (mg/kg/day)
Figure 4

The graph shows the atherosclerosis score at different doses of AGI-1067 and Pro in control and coronary vessels.

- Control
- AGI-1067 50
- AGI-1067 150
- Pro 150
- Pro 250

Aorta
Coronary

Dose (mg/kg/day)
Atherosclerosis Score

6 4
3 5
0 0
7 10
2 0

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Figure 6

a.

- Control
- AGI-1067
- Probucol

% Surface Area Covered by Lesion

-49%

b.

- Control
- AGI-1067
- Probucol

Cholesterol Ester Content of the Aortic Arch (ug/mm²)

-32%
Figure 7

% Aortic Surface Area Covered by Lesion

- Control
- Probulcol
- AGI-1067

a. -25%
b. -46%
c. -41%
d. -49%
Figure 8

(a) Immunoblot analysis of MCP-1 (JE), VCAM-1, and β-actin expression in cells treated with Pro, AGI-1067, and their combinations.

(b) Quantitative analysis of VCAM-1 and MCP-1 expression in the presence of Placebo + LPS and treatment with Pro or AGI-1067.
Figure 9

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Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDLr -/- Mice</th>
<th>12 wk</th>
<th>ApoE -/- Mice</th>
<th>12 wk</th>
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<tr>
<td>Untreated Controls</td>
<td>1060 +/- 12</td>
<td>1360 +/- 20</td>
<td>1053 +/- 71</td>
<td>726 +/- 31</td>
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<tr>
<td>AGI-1067</td>
<td>1172 +/- 16</td>
<td>1365 +/- 19</td>
<td>646 +/- 140 *</td>
<td>987 +/- 48 *</td>
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<tr>
<td>Probucol</td>
<td>877 +/- 23</td>
<td>1203 +/- 14</td>
<td>736 +/- 19</td>
<td>811 +/- 75</td>
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</tbody>
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Total plasma cholesterol levels from AGI-1067 and probucol-treated LDLr -/- and ApoE-/- mice. LDLr -/- mice and ApoE-/- 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 (mg/dL) was determined after 2 and 12 weeks of treatment and is expressed as the mean +/- SEM. * P<0.05 compared to untreated controls.