Selective Inhibition of Endothelial and Monocyte Redox-Sensitive Genes by AGI-1067: A Novel Antioxidant and Anti-Inflammatory Agent


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ABSTRACT

Atherosclerosis is a disease of oxidative stress and inflammation. AGI-1067 [butanediolic acid, mono[4-[[1-[[3,5-bis[1,1-dimethylethyl]-4-hydroxyphenyl][thio]-1-methylethyl][thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester] is a metabolically stable derivative of, yet pharmacologically distinct from, the antioxidant drug probucol. It is a member of a novel class of orally active, antioxidant, anti-inflammatory compounds termed vascular protectants and exhibits antiatherosclerotic properties in multiple animal models and in humans. To elucidate its antiatherosclerotic mechanisms, we have evaluated several cellular and molecular properties of AGI-1067 in vitro. AGI-1067 exhibited potent lipid peroxide antioxidant activity comparable with probucol yet demonstrated significantly enhanced cellular uptake over that observed with probucol. AGI-1067, but not probucol, inhibited basal levels of reactive oxygen species (ROS) in cultured primary human endothelial cells and both basal and hydrogen peroxide-induced levels of ROS in the promonocytic cell line, U937. Furthermore, AGI-1067 inhibited the inducible expression of the redox-sensitive genes, vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1, in endothelial cells as well as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 production in peripheral blood mononuclear cells, whereas probucol had no effect. cDNA array hybridization experiments demonstrated that AGI-1067 selectively inhibited the expression of only a subset of TNF-α-responsive and nuclear factor-κB (NF-κB)-inducible genes in endothelial cells. The inhibitory effect of AGI-1067 on inducible VCAM-1 gene expression occurred at the transcriptional level, yet AGI-1067 had no effect on the activation of the redox-sensitive transcription factor NF-κB. These studies suggest that the anti-inflammatory and antiatherosclerotic properties of AGI-1067 may be due to selective inhibition of redox-sensitive endothelial and monocyte inflammatory gene expression. These studies provide a molecular basis for understanding the mechanism of action of this new class of therapeutic antiatherosclerotic compounds.

It is now well accepted that atherosclerosis can be viewed as a disease of chronic inflammation (Libby, 2002). Adhesion of leukocytes to vascular endothelium and their subsequent recruitment into the arterial wall are important in the early pathogenesis of the disease. Vascular cell adhesion molecule-1 (VCAM-1), a protein whose expression is induced on the endothelial cell surface by a variety of pathophysiological conditions, is largely responsible for the attachment of leukocytes to the endothelium (Cybulsky and Gimbrone, 1991). In addition to VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and E-selectin are other adhesion molecules that participate in leukocyte adhesion and transmigration. The expression of VCAM-1 is up-regulated at early time points in experimental models of atherosclerosis and correlates with monocyte adhesion and lesion development (Li et al., 1993; Dopp et al., 1995; Haynesworth et al., 1995).

ABBREVIATIONS: VCAM-1, vascular cell adhesion molecule-1; AGI-1067, butanediolic acid, mono[4-[[1-[[3,5-bis[1,1-dimethylethyl]-4-hydroxyphenyl][thio]-1-methylethyl][thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester; CAT, chloramphenicol acetyltransferase; BAEC, bovine aortic endothelial cells; HMEC, human microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; LMB, leukomethylene blue; MCP-1, monocyte chemoattractant protein-1; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IL, interleukin; HPAEC, human pulmonary artery endothelial cells; hPBMC, human peripheral blood mononuclear cells; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; RT-PCR, real-time polymerase chain reaction; H$_2$DCF, 2′,7′-dichlorofluorescein; DA, diacetate; PBS, phosphate-buffered saline; ISTD, internal standard; HPLC, high-performance liquid chromatography; LLOQ, lower limit of quantitation; EC, endothelial cell; PDTC, pyrrolidinedithiocarbamate; PD98063, 2-(3-amino-phenyl)-8-methoxy-chromene-4-one.
Iyama et al., 1999). VCAM-1 expression has also been demonstrated in human coronary atherosclerotic plaques, which is consistent with a potential role for this adhesion molecule in the disease (O’Brien et al., 1993). Perhaps the strongest evidence for a role of VCAM-1 in atherosclerosis is the observation that disruption of the fourth immunoglobulin domain in VCAM-1, which is required for interaction with its counter receptor on leukocytes (VLA-4), results in a marked, gene dosage-dependent reduction in monocyte staining and atherosclerotic lesion formation in a murine model of atherosclerosis (Cybulska et al., 2001; Dansky et al., 2001). This same effect was not observed in animals with a deficiency in ICAM-1; cholesterol levels, lipoprotein profiles, and number of circulating leukocytes were comparable between the VCAM-1-null and wild-type animals. Cumulatively, these observations suggest a prominent role for VCAM-1 in the pathogenesis of atherosclerosis.

In addition to adhesion molecules, chemokines such as monocyte chemoattractant protein-1 (MCP-1) and cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) play a role in the recruitment of circulating leukocytes to the site of damaged or injured endothelium and perpetuation of the inflammatory process. Several studies have demonstrated a correlation between MCP-1 or its receptor and atherosclerosis in human and animal models of the disease (Nelken et al., 1991; Yla-Herttuala et al., 1991; Gosling et al., 1999). Both MCP-1 and VCAM-1 expression are inducible in the vascular endothelium by a variety of stimuli, including cytokines and oxidant stress. Many investigators have shown that redox signals modulate the expression of several inflammatory genes, including VCAM-1, MCP-1, TNF-α, IL-1β, and IL-6, and antioxidants reduce the expression of these genes (Marui et al., 1993; Satriano et al., 1993; Ali et al., 1999; Hsu and Wen, 2002). These observations have provided further support to the oxidation hypothesis of atherosclerosis (Witztum, 1994) by demonstrating that key inflammatory genes are regulated by redox signaling.

The precise oxidant signals that confer inducible expression to redox-sensitive genes are unknown. Multiple activating signals to the endothelium, including TNF-α and IL-1β, endotoxin, angiotensin II, growth factors, oxidized LDL, and hemodynamic forces, are known to induce ROS in the vascularity and in leukocytes (Heinrecke, 1999; Harrison et al., 2003). Generation of intracellular ROS in response to these stimuli has been proposed to serve as a signaling event in the activation of the transcription factor NF-κB and the enhanced expression of vascular redox-sensitive genes (Kunsch and Medford, 1999). Despite the fact that NF-κB plays a major role in the inducible expression of VCAM-1 and MCP-1 (Ueda et al., 1994; Neish et al., 2001), there have been several examples of pharmacologic agents that inhibit the expression of these genes yet have no effect on NF-κB.

AGI-1067 is a novel, orally active, metabolically stable derivative of probucol that exhibits antiatherosclerotic activity in several animal models (Meng et al., 2002; Sundell et al., 2003). In addition, the Canadian Antioxidant Restenosis Trial-1 demonstrated a dose-dependent inhibition of postangioplasty restenosis and improved lumen dimensions of nonintervened coronary artery reference segments, thereby suggesting a direct positive effect of AGI-1067 on atherosclerosis (Tardif, 2003; Tardif et al., 2003). To gain insight into the anti-inflammatory and antiatherosclerotic mechanisms of AGI-1067 and to differentiate its biological properties from probucol, we examined its effects on redox-sensitive expression of inflammatory genes in endothelial and peripheral blood mononuclear cells. Our results demonstrate that AGI-1067 is a potent intracellular antioxidant and a selective inhibitor of inflammatory response genes. These biological activities may account for the antiatherosclerotic and antirestenotic activity of this new therapeutic agent.

Materials and Methods

Cell Culture, Plasmids, and Transfections. Human pulmonary artery (HPAEC) and aortic (HAEC) endothelial cells, bovine aortic endothelial cells (BAEC), and human peripheral blood mononuclear cells (hPBMC) were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). hPBMC cells were obtained from American Type Culture Collection (Manassas, VA). All cells were maintained at 37°C under 5% CO₂ atmosphere. For transient transfection of BAEC, cells were seeded onto a 60-mm² plate coated with 1% gelatin 1 day prior to transfection. Two micrograms of reporter plasmid and 0.5 μg of pSV2β-galactosidase (used for normalization of transfection efficiency) were transfected per well using the lipid transfection reagent LipofectAMINE Plus, according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). On the following day, media was removed, and fresh media containing compound with or without TNF-α (100 U/ml) was added. Forty-eight hours later, cells were harvested by scraping, and cell lysates were prepared by three cycles of freeze-thaw in a dry ice/methanol bath followed by 2 min of sonication. Cell lysates were collected, clarified by centrifugation, and protein content was determined and cellular lysates stored at −70°C. Chloramphenicol acetyltransferase (CAT) activity was determined as previously described (Marui et al., 1993). Plasmids 288VCAM-CAT and 933VCAM-CAT were a generous gift of Douglas C. Dean, Washington University School of Medicine (Iademarco et al., 1992).

Determination of IL-1β, IL-6, and TNF-α Levels in hPBMC. The effect of AGI-1067 and probucol on lipopolysaccharide (LPS)-stimulated cytokine secretion was determined by measuring TNF-α, IL-6, and IL-1β secreted into culture medium of hPBMC. Fresh cryopreserved hPBMC were pretreated with test compound for 1 h followed by stimulation with LPS (1 μg/ml) for an additional 2 h in the presence of AGI-1067 or probucol. Cell supernatants were collected and assayed for cytokine levels by ELISA (R&D Systems, Minneapolis, MN).

Measurement of VCAM-1, ICAM-1, and MCP-1 Protein Expression. HAEC were cultured as above and seeded onto 24- or 96-well plates such that they would reach 90 to 95% confluence on the following day. Cells were stimulated with TNF-α (1 ng/ml) in the presence or absence of either AGI-1067 or probucol dissolved in 0.1% DMSO. Following treatment, cells were examined for signs of cellular toxicity either visually or by measurement of lactate dehydrogenase (Promega, Madison, WI). For determination of VCAM-1 and MCP-1 protein expression, cells were pretreated with test compound for 1 h and subsequently stimulated with TNF-α in the presence of compound for an additional 4 h. VCAM-1 expression was measured by ELISA as described previously (Sundell et al., 2003). For determination of secreted MCP-1, cells were treated with test compound for 1 h and exposed to TNF-α for 4 h. The level of MCP-1 secreted into the culture supernatant was measured by ELISA (R&D Systems).

RNA Analysis. RNA was collected by total lysis in Trizol (Invitrogen) and precipitated using isopropanol. RNA integrity was monitored by visual observation of the ratio of the 28S to 18S rRNA by ethidium bromide gel electrophoresis. To measure RNA levels for specific genes, quantitative real-time polymerase chain reaction (RT-PCR) was used with gene-specific oligonucleotide primers and amplification with an iCycler thermocycler (Bio-Rad, Hercules, CA).
Quantitative assessment of VCAM-1 and MCP-1 RNA levels were calculated relative to the level of the housekeeping gene GAPDH.

**Electrophoretic Mobility Shift Assay.** Nuclear extracts were prepared, and the assay was performed, as described previously (Marui et al., 1993). Briefly, two complementary oligonucleotides containing the two NF-κB sites (shown below) of the human VCAM-1 promoter were annealed in 50 mM Tris (pH 8.0), 100 mM NaCl, and 10 mM MgCl. The oligonucleotide sequence is: 5'-GCTGCCCT-GGTTTTCCCCCTGAAAGATTCCCTCGCTCTGCAACAA-3'. Double-stranded, complementary oligonucleotides were labeled at their 5’ ends with [γ-32P]dCTP and T4 polynucleotide kinase. Protein-DNA binding reactions were performed at room temperature for 30 min in a total reaction volume of 25 μl, which contained 3 μg of nuclear extract, 225 μg/ml bovine serum albumin, 3 × 104 cpm [32P]-labeled probe, 0.1 mg/ml polyinosinic-polycytidylic acid, and 15 μl of binding buffer (12 mM HEPES (pH 7.4), 4 mM Tris, 60 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, and 12% glycerol). Following incubation, the entire reaction was electrophoresed through a 5% acrylamide gel, dried, and exposed to X-ray film.

cDNA Microarray Analysis. HAEC were grown to approximately 90% confluence in Endothelial Cell Medium-2 (Cambrex Bio Science Walkersville, Inc.) containing 10% fetal bovine serum. Cells were treated with either 0.1% DMSO or 15 μM AGI-1067 in 0.1% DMSO for 15 h followed by the addition of TNF-α (1 ng/ml) for an additional 4 h. Total cellular RNA was collected as described above and hybridized to the Atlas Human cDNA Expression Array (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer’s protocol. Image analysis and quantitation was performed using a Storm phosphorimager (Amersham Biosciences Inc., Piscataway, NJ).

**Leukomethylene Blue (LMB) Assay.** The LMB assay uses hemoglobin-catalyzed oxidation of colorless N-benzoylleukomethylene blue to detect the presence of lipid hydroperoxides. Reduction of lipid hydroperoxides by antioxidants to the alcohol form results in a concomitant reduction in the oxidation of LMB, which can be monitored spectrophotometrically. LMB activity was measured as described previously (Somers et al., 2000).

**Determination of Cellular Reactive Oxygen Species.** Cellular ROS levels were measured by detecting the fluorescence intensity of the oxidized product (dichlorofluorescein) of the fluorescent probe 2′,7′-dichlorofluorescein (H2DCF)-diacetate (DA; Molecular Probes, Eugene, OR). In the intracellular compartment, esterases cleave off the acetate group on H2DCF-DA, trapping H2DCF in the intracellular compartment. Conversion of H2DCF to the fluorescent form, dichlorofluorescein, by endogenous oxidants, was monitored on a microplate fluorimeter, excitation 485 nm, emission 530 nm. HAEC or U937 cells were treated with either AGI-1067 or probucol for 3 h followed by cotreatment with 10 μM H2DCF-DA for an additional 30 min. To determine the effect on H2O2-stimulated ROS production, cells were exposed to 200 μM H2O2 for 15 min just prior to the end of the experiment. Cells were then washed with phosphate-buffered saline (PBS) and lysed in Tris-buffered saline containing 0.05% Tween 20 and 0.01% Triton X-100, and absolute fluorescence was measured using a fluorimeter (Victor2; PerkinElmer Wallac, Boston, MA).

**Compound Uptake Studies.** HAEC were seeded in 10-cm dishes and were used below passage 9. At approximately 90% confluence, cells were treated with either 5 μM AGI-1067 or 5 μM probucol dissolved in 0.1% DMSO, and cell and culture supernatant samples were collected at 0, 0.5, 1.0, 1.5, and 2.0 h. At each time point, cells were washed twice with 5 ml of PBS, scraped and collected with 5 ml of PBS, and transferred to a 15-mL conical tube. The cell suspension was centrifuged at 11,000 rpm for 3 min. The supernatant was discarded, the pellet was recovered and resuspended in 100 μl of PBS, and protein concentration was determined (Bio-Rad). Compound concentrations were determined using an internal standard (ISTD) spiking technique with a structurally related compound [2-[4-[[1-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methyl-ethyl][thiol]-2,6-bis(1,1-dimethylethyl)phenoxyl]butanoic acid] as the ISTD. The method employs protein precipitation (acetonitrile) to isolate the compound and ISTD from the sample matrix followed by HPLC/UV detection. The lower limit of quantitation (LLOQ) for the assay was set at 1 μM/l. Two independent studies were performed, with each demonstrating similar results.

**Results**

**AGI-1067 Demonstrates Antioxidant Activity.** We evaluated the ability of AGI-1067 to function as an antioxidant both in noncellular and in cell-based systems. As shown in Fig. 1, when evaluated for its ability to inhibit lipid peroxide-mediated oxidation, AGI-1067 demonstrated potent antioxidant activity comparable with that observed with probucol, a well studied antioxidant drug. These observations are consistent with previously published data on the ability of probucol to inhibit lipid peroxide-mediated oxidation (Steinberg, 1986) and demonstrate that the addition of a succinate ester moiety to probucol to form AGI-1067 (Meng et al., 2002) does not compromise its antioxidant activity. Because ROS play an important role in the pathogenesis of atherosclerosis and other inflammatory diseases, in part via modulation of intracellular signaling, we examined the ability of AGI-1067 to inhibit intracellular levels of ROS in HAEC and the promonocytic cell line U937 by fluorescence spectroscopy. As shown in Fig. 2a, AGI-1067, but not probucol, inhibited basal levels of ROS in HAEC in a concentration-dependent manner. Similarly, AGI-1067 inhibited basal levels of ROS in U937 cells, whereas probucol had no effect. Maximum inhibition of ROS levels was observed in both cell types at 10 μM. At the concentrations tested, there were no visible signs of cell stress or cytotoxicity. AGI-1067 also inhibited hydrogen peroxide-induced production of ROS in U937 cells (Fig. 2b), whereas probucol had no effect. Taken together, these data demonstrate that AGI-1067 has lipid peroxide antioxidant activity and can function as a potent intracellular antioxidant in multiple cell types.

**AGI-1067 Demonstrates Enhanced Endothelial Cell Uptake over Probucol.** Because AGI-1067 is the monosuccinic acid ester of probucol, and other succinate esters exhibit enhanced cellular uptake (Fariss et al., 2001), we reasoned

![Fig. 1. Lipid peroxide antioxidant activity of AGI-1067. The colorless agent LMB was incubated together with 13-[S-(Z,E)]-9,11-hydro-per-oxoyctadecadienoic acid and varying concentrations of either AGI-1067 or probucol. The ability of the compounds to inhibit the oxidation of LMB by 13-[S-(Z,E)]-9,11-hydro-peroxoyctadecadienoic acid was monitored spectrophotometrically and plotted as percent inhibition relative to the vehicle (DMSO) control.](image-url)
that the observed differences in the ability of AGI-1067 and probucol to inhibit intracellular ROS may be due to differences in cellular uptake and/or distribution within intracellular compartments. To address differences in cellular uptake between AGI-1067 and probucol, HAEC were incubated with equimolar concentrations of either AGI-1067 or probucol, and ROS-mediated oxidation of the redox-sensitive dye, H$_2$DCF, was monitored by fluorescence spectroscopy (A). U937 cells were treated with 200 μM H$_2$O$_2$ for 15 min in the presence or absence of either AGI-1067 or probucol (B). Values are mean ± S.E.M., n = 3.

AGI-1067 Inhibits the Release of Inflammatory Cytokines from Activated Human Peripheral Blood Mononuclear Cells. Activated macrophages have been implicated as key pathogenic regulators of the atherogenic process. The proinflammatory cytokines, TNF-α, IL-1β, and IL-6 are released by activated macrophages and are present in the atherosclerotic lesion. We evaluated the effect of AGI-1067 on the release of these important cytokines in hPBMC in vitro. As shown in Fig. 4, AGI-1067 resulted in a concentration-dependent reduction in the release of all three cytokines from LPS-activated hPBMC with apparent IC$_{50}$ values of approximately 1.0 and 0.5 μM and 0.3 μM for TNF-α, IL-1β, and IL-6, respectively. Probucol, on the other hand, had no effect at similar concentrations.

AGI-1067 Inhibits Redox-Sensitive Inflammatory Gene Expression in Human Aortic Endothelial Cells. To determine whether AGI-1067 inhibited the inducible expression of endothelial cell redox-sensitive inflammatory response genes, HAEC were treated with AGI-1067 or probucol and then stimulated with TNF-α. As shown in Fig. 5a, AGI-1067 inhibited the TNF-α-inducible expression of VCAM-1 and MCP-1 in a concentration-dependent manner at 4 h post-TNF-α with IC$_{50}$ values of ~6 and 6.4 μM, respectively. Probucol, on the other hand, had no effect on either of these genes up to a maximum concentration of 100 μM. At the concentrations used, there were no visible effects on either cell stress or cytotoxicity as measured by release of the mitochondrial enzyme, lactate dehydrogenase. Similar results have been obtained for the inhibition of TNF-α-inducible VCAM-1 expression by AGI-1067 in various lots of primary HAEC and in human microvascular endothelial cells (data not shown). In addition to TNF-α, AGI-1067 also inhibited IL-1β-mediated induction of VCAM-1 and MCP-1 expression in HAEC (data not shown).

To determine whether the inhibition of VCAM-1 and MCP-1 protein by AGI-1067 was at the level of gene expression, we examined its effect on TNF-α-inducible levels of steady-state mRNA for these genes. HAEC were pretreated with AGI-1067 (6 or 8 μM) for 1 h followed by stimulation with TNF-α for 4 h. Relative levels of mRNA were determined by real-time quantitative RT-PCR analysis. As shown in Fig. 5b, treatment of HAEC with TNF-α resulted in a large increase in the expression of VCAM-1 and MCP-1 mRNA. AGI-1067 inhibited the levels of mRNA for VCAM-1 in a concentration-dependent fashion and, to a lesser extent, those for MCP-1. These observations suggest that inhibition of TNF-α-inducible VCAM-1 and MCP-1 protein by AGI-1067 as shown in Fig. 5a is reflected at the level of steady-state mRNA.
AGI-1067 Inhibits VCAM-1 Transcriptional Activation. To further define the mechanism of inhibition of these redox-sensitive genes by AGI-1067, we used the VCAM-1 gene as a model system. Because human ECs are difficult to transfect, we used BAEC, since they exhibit much higher transfection efficiency. To determine whether the effect of AGI-1067 was due to decreased transcriptional activation of the VCAM-1 gene, we studied its effects using a minimal VCAM-1 promoter fragment containing either 933 or 288 base pairs of the human VCAM-1 promoter fused to the CAT reporter gene. Both of these constructs have previously been shown to respond in BAEC to several proinflammatory agents including TNF-α and IL-1β (Marui et al., 1993; Wolle et al., 1996). Treatment with TNF-α resulted in activation of both the 933- and 288-base pair VCAM-1 constructs, with a resulting increase in CAT activity (Fig. 6). Pretreatment of cells with AGI-1067 demonstrated a concentration-dependent inhibition of TNF-α-inducible VCAM-1-mediated promoter activity from both promoter constructs. These data are consistent with the observed inhibition by AGI-1067 of TNF-α-induced steady-state mRNA for VCAM-1 (Fig. 5b) and demonstrate that AGI-1067 inhibits TNF-α-inducible VCAM-1 gene expression through transcriptional inhibition at the level of the VCAM-1 promoter. The inhibition of the VCAM-1 promoter constructs is not due to general transcriptional inhibition, as we observed no effect of AGI-1067 on transcriptional activation of a control reporter gene containing a minimal promoter driving the expression of β-galactosidase.

AGI-1067 Inhibits a Small Subset of TNF-α-Activated Endothelial Cell Genes. To further define the effect of AGI-1067 on TNF-α-inducible endothelial cell gene expression, the relative expression levels of 585 known genes were analyzed using a cDNA array and samples from TNF-α- and AGI-1067-treated HAEC. A representative quadrant containing 98 genes is shown in Fig. 7. Treatment with AGI-1067 resulted in a reduction in the hybridization signal to both the VCAM-1 and E-selectin probes. Densitometric scanning of the autoradiograph revealed approximately 5.0- and 8.5-fold inhibition of hybridization signal for VCAM-1 and E-selectin, respectively. Other genes, the expression of which was reduced by a magnitude greater than 2.5-fold but are not contained within the quadrant shown, included MCP-1 (2.5-fold), c-Jun NH₂-terminal kinase-3 kinase (3.6-fold), and bone morphogenic protein-4 (5.8-fold). Interestingly, several other TNF-α-inducible and NF-κB-regulated genes did not exhibit inhibition by AGI-1067. For example, the mRNA levels of IL-6, a TNF-α- and NF-κB-regulated gene, were slightly increased in endothelial cells treated with AGI-1067. Similarly, the expression of ICAM-1 (Fig. 7), IL-8, and IL-2r were not inhibited by AGI-1067 in TNF-α-stimulated HAEC under these conditions. The modulation of expression of these genes by AGI-1067 as determined by expression array hybridization was confirmed by either semiquantitative or quantitative RT-PCR analysis (Fig. 5b; data not shown). These results demonstrate that AGI-1067 is a selective inhibitor of only a subset of TNF-α-inducible genes. Furthermore, the fact that several known NF-κB-regulated genes were not inhibited suggests that AGI-1067 does not globally inhibit a common signaling pathway that converges on NF-κB (see also results below).

Lack of Effect of AGI-1067 on NF-κB Nuclear Translocation. Cytokine-inducible VCAM-1 gene expression is regulated, in part, via the redox-sensitive transcription factor, NF-κB (Neish et al., 2001). Since both VCAM-1 promoter elements used in the experiments depicted in Fig. 6 contain two copies of the NF-κB binding site, and since the results in Fig. 7 suggest that not all NF-κB-regulated genes are affected by AGI-1067, we directly examined the ability of AGI-1067 to inhibit TNF-α-inducible NF-κB activation. Nuclear extracts were prepared from HAEC pretreated for 1 h with either the thiol antioxidant PDTC (a known inhibitor of NF-κB activation) or AGI-1067 and stimulated for 1 h with TNF-α. NF-κB DNA binding activity was assessed by electrophoretic mobility shift assay using a double-stranded oligonucleotide probe containing the two NF-κB binding sites from the VCAM-1 promoter. NF-κB-specific binding was assessed by 1) competition of binding activity by excess unlabeled oligonucleotide.
beled probe, 2) lack of effect of competition with a probe containing a mutant copy of the NF-κB binding element, and 3) absence of binding of nuclear extracts to the VCAM-1 mutant probe (data not shown). As shown in Fig. 8, treatment with TNF-α resulted in the induction of two bands representing NF-κB-specific binding activity. Treatment with 25 μM PDTC significantly reduced NF-κB binding activity; however, treatment with 25 μM AGI-1067, a concentration more than four times the IC_{50} for VCAM-1 inhibition, failed to inhibit NF-κB. In addition, we have observed that PDTC, but not AGI-1067, inhibited the TNF-α-induced nuclear translocation of the p65 subunit of NF-κB in endothelial cells as assessed by immunofluorescence microscopy (data not shown). Also, immunoblot analysis demonstrated no change in the nuclear levels of p65 in HAEC following stimulation with TNF-α (Piper and Kunsch, unpublished observations). These data demonstrate that although AGI-1067 inhibits redox-sensitive gene expression in endothelial cells, this effect is independent of NF-κB nuclear translocation and

Fig. 4. Effect of AGI-1067 on LPS-induced inflammatory cytokine production from hPBMC. hPBMC were pretreated with either AGI-1067 or probucol for 1 h followed by stimulation with LPS for 2 h. Levels of TNF-α (A), IL-1β (B), and IL-6 (C) released into the culture media were determined by ELISA. Data are mean ± S.E.M. of triplicate determinations expressed as picogram(s) per milliliter. Similar results were obtained from two separate experiments.
DNA binding. This conclusion is supported by the fact that the inducible expression of several NF-κB-regulated genes was not affected by treatment with AGI-1067 (Fig. 7C; Kunsch, unpublished observations).

**Discussion**

The expression of inflammatory genes in the vessel wall is a central event in the early pathogenesis leading to the development of atherosclerosis. Among them are the cellular adhesion molecules VCAM-1, ICAM-1, and E-selectin and the chemoattractant MCP-1. Proinflammatory signals, including cytokines and oxidant stress, along with several of the well-established risk factors, such as hyperglycemia, hyperlipidemia, hypertension, and smoking, play prominent roles in the pathogenesis of coronary artery disease. Although representing unique physiological stresses and having distinct signaling mechanisms, each shares a common feature of inducing changes in the vascular redox state. These changes in redox homeostasis modulate second messenger signaling pathways that result in changes in gene expression, cellular proliferation, and viability. Although the exact nature of the oxidant signals that couple changes in redox state to alterations in signal transduction is poorly understood, antioxidants that target these events result in inhibition of key redox-sensitive inflammatory genes in the vessel wall (Marui et al., 1993; Alexander, 1998; Kunsch and Medford, 1999).

AGI-1067, an anti-inflammatory and antioxidant compound with preclinical and clinical efficacy in atherosclerosis and restenosis, was designed to improve upon some of the chemical and pharmacological properties of probucol. Probucol is a drug with well characterized lipid peroxide antioxidant...
tant, antiatherosclerotic, and antirestenotic activity; how-
ever, because probucol has been shown to cause ventricular arrhythmias (QTc prolongation) and a reduction in levels of HDL cholesterol, the clinical utility of probucol has been limited. The introduction of a succinate moiety onto one of the phenol groups in AGI-1067 renders it slightly more hydrophilic than probucol. Although this modification does not change the antioxidant potential of AGI-1067, it does result in enhanced cellular uptake and/or cellular retention and, as a result, exhibits more potent intracellular antioxidant activity and improved inhibition of inflammatory genes when compared with probucol. Interestingly, two research groups have shown that α-tocopherol succinate exhibits enhanced uptake, intracellular antioxidant activity, inhibition of mono-
cyte adhesion, and inhibition of cytokine-induced VCAM-1 relative to α-tocopherol (Erl et al., 1997; Fariss et al., 2001). It has also been suggested that specific proton cotransport mechanisms exist for monocarboxylic acid forms of drugs (Tamai and Tsuji, 1996). Therefore, although the exact mech-
nism is not known, the monosuccinic acid moiety of AGI-
1067 likely accounts for the improved cellular uptake relative to probucol.

In support of the ability of AGI-1067 to function as an antioxidant, Sundell et al. (2003) have previously shown that isolated LDL from AGI-1067-treated animals is more resis-
tant to ex vivo copper-induced oxidation than vehicle-treated animals. Furthermore, the concentrations at which we ob-
servem cellular antioxidant activity are comparable with the efficacious plasma drug levels observed in animal models and in clinical trials (Sundell et al., 2003; Tardif et al., 2003). The assay used for determination of intracellular ROS in this study did not identify the precise species that is/are inhibited by AGI-1067. Further studies will be needed to determine whether AGI-1067 functions to merely quench intracellular ROS or to modulate the activity and/or levels of key proteins involved in ROS homeostasis. Taken together, these results suggest that AGI-1067 maintains the well characterized in vitro antioxidant properties of probucol but, unlike probucol, demonstrates improved cellular antioxidant activity that is likely attributable to improved cellular uptake.

Because AGI-1067 exhibited potent cellular antioxidant activity, we examined its ability to affect redox-sensitive inflammatory gene expression in both ECs and mononuclear cells in vitro. AGI-1067 exhibited a concentration-dependent inhibition of TNF-α- and IL-1β-inducible expression of VCAM-1 and MCP-1 gene expression, and the IC50 values correlate with plasma levels in our animal models and clinical trials that demonstrate reduced progression of athero-
sclerosis (Sundell et al., 2003; Tardif et al., 2003). These observations are consistent with numerous reports that demon-
strate that antioxidants can inhibit VCAM-1 expression and support the notion that VCAM-1 and MCP-1 are regu-
lated via redox-sensitive pathways in the vasculature.

Interestingly, in our studies, probucol had no effect on TNF-α-induced VCAM-1 or MCP-1 expression in HAEC in vitro. However, studies by Fruebis et al. (1997, 1999) have suggested that probucol inhibits both basal and inducible levels of VCAM-1 in in vivo models of atherogenesis. In these studies, the inhibitory effect of probucol may have merely been due to its potent inhibition of LDL oxidation, thus reducing a major stimulus for VCAM-1 expression, rather than a direct effect on oxidant or other inflammatory-medi-
ated induction of VCAM-1 on the endothelium. Also, Zapolska-Downar et al. (2001) have reported an effect of probucol on cytokine-induced expression of VCAM-1 in endothelial cells in vitro. There are two potential explanations for the apparent discrepancy between their studies and the results reported in our study. The first could be due to the fact that we used endothelial cells derived from the aorta (HAEC), whereas the study by Zapolska-Downar et al. used human umbilical vein endothelial cells. It is well known that endo-
thelial cells derived from different vascular beds display dis-
tinct biological responses. Second, the inhibition of VCAM-1 by probucol observed by Zapolska-Downar et al. was only apparent when cells were pretreated with probucol for long periods of time (24 or 48 h) prior to stimulation with TNF-α. When probucol was added 30 min prior to TNF-α, no inhibi-
tion of VCAM-1 expression by probucol was observed, consis-
tent with our studies in which probucol was added 1 h prior to TNF-α addition.

The inhibition of inducible VCAM-1 by AGI-1067 is re-
flected at the level of steady-state mRNA and occurs by in-
hibition of transcriptional activation of the VCAM-1 pro-

motor. Unlike VCAM-1, where inhibition of steady-state mRNA by AGI-1067 correlates well with inhibition of protein expression, inhibition of MCP-1 steady-state mRNA levels by AGI-1067 is less dramatic. With repeated experiments, we have found that concentrations of AGI-1067 that result in nearly complete inhibition of inducible MCP-1 protein ex-
pression only reflect a modest inhibition of mRNA expres-
sion. Therefore, it is possible that AGI-1067 may exert addi-
tional post-transcriptional inhibition of MCP-1 expression. Further experimentation is needed to address the specific nature of regulation of inducible MCP-1 expression by AGI-
1067.

Although inflammatory cytokines, such as TNF-α, have been shown to induce the production of ROS in cells of the
vasculature, the precise signals that drive the inducible ex-
pression of VCAM-1 and MCP-1 are not known. One key regula-
tor that is activated by both ROS and cytokine path-
ways in ECs is the pleiotropic transcriptional activator, NF-
κB. NF-κB regulates the transcriptional activity of many
inflammatory, immune response, and proliferative genes in
multiple cell types and is activated in response to patho-
physiologically relevant signals during atherogenesis (Mann-
ing, 2001). In the case of oxidant stress, it has been well
documented that ROS activate, whereas a variety of antioxidant
agents inhibit, NF-κB activity. These studies have shown that
antioxidants such as PDTC or N-acetylcysteine primarily
inhibit the phosphorylation and degradation of IκB and the
resultant release of NF-κB and its translocation to the nu-
cleus. Our results demonstrate that unlike PDTC, AGI-1067,
despite its antioxidant activity, had no effect on the TNF-α-
inducible nuclear translocation of NF-κB. Similar findings
have been reported with other antioxidant pharmacologic
agents. For example, Gerritsen et al. (1995) demonstrated
that the flavonoid apigenin inhibited both VCAM-1 and
ICAM-1 gene expression without affecting NF-κB nuclear
translocation. Similarly, another flavonoid, PD089063, inhib-
ited cytokine-induced VCAM-1 with no effect on NF-κB nu-
clear translocation (Wolle et al., 1996). Also, Umetani et al.
(2000) showed that a novel cell adhesion molecule inhibitor,
K-7174, inhibited endothelial VCAM-1 expression through
regulation of GATA transcriptional factors but not NF-κB.
These observations suggest that not all antioxidants inhibit cytokine-induced NF-κB activation in endothelial cells and that perhaps other redox-sensitive signals downstream of TNF-α and independent of NF-κB may be targeted by AGI-1067 in regulation of VCAM-1 and MCP-1 expression.

We used gene expression profiling to provide a comprehensive evaluation of those EC genes that are modulated by AGI-1067. In this study, we did not see any effect on inhibition of ICAM-1, a well characterized NF-κB-regulated gene, or on other NF-κB-regulated genes, including IL-6, IL-8, and the IL-2 receptor. The lack of effect on ICAM-1 mRNA levels in this study supports our observations that, at similar concentrations, AGI-1067 is a more selective inhibitor of VCAM-1 versus ICAM-1 protein in ECs (Sundell et al., 2003). In addition, other antioxidants such as probucol (Zapolska-Downar et al., 2001), α-tocopherol (Erl et al., 1997), PDTC (Marui et al., 1993), and PD098063 (Wolle et al., 1996) show no effect on inducible ICAM-1 expression in vitro. The lack of effect by AGI-1067 on multiple NF-κB-regulated genes supports our studies demonstrating no effect on NF-κB nuclear localization. In addition, we observed that only a small subset of TNF-α-inducible genes was inhibited by AGI-1067, implying that the mechanism of action of AGI-1067 is highly selective. Although the precise molecular target(s) of AGI-1067 is/are not known, we would propose that there are AGI-1067-sensitive redox signals that are involved in the selective regulation of key atherogenic endothelial inflammatory genes, such as VCAM-1, MCP-1, and E-selectin. These genes, in addition to possibly others, likely share unique oxidant-sensitive transcriptional regulatory pathways that are targeted by AGI-1067. Future studies will be required to help define the precise signaling pathways and molecular interactions that are unique to these genes that are modulated by AGI-1067.

In addition to effects on EC inflammatory response genes, we have demonstrated that AGI-1067 inhibits LPS-induced secretion of TNF-α, IL-1β, and IL-6 from hPBMC. These cytokines are produced primarily by T-cells and monocytes locally at sites of inflammation. Elevated expression of these genes has been observed in atherosclerotic plaques, and their expression activates a multitude of inflammatory events involved in EC and smooth muscle cell activation, matrix deposition, and monocyte recruitment. Furthermore, recent evidence suggests that elevated levels of inflammatory markers, in particular IL-6, are associated with increased cardiovascular risk (Blake and Ridker, 2003). Therefore, inhibition of production of these proinflammatory cytokines by AGI-1067 may be another mechanism whereby this compound exerts antiatherosclerotic activity.

In summary, we have demonstrated that AGI-1067 demonstrates potent cellular and extracellular antioxidant activity. AGI-1067 inhibits the secretion of several redox-sensitive inflammatory cytokines from activated monocytes and demonstrates selective inhibition of a subset of redox-sensitive EC genes, including VCAM-1, MCP-1, and E-selectin. This selective inhibition can be explained, in part, by the lack of effect of AGI-1067 on nuclear translocation of NF-κB. Since NF-κB regulates a multitude of immune response genes, AGI-1067 should not act as a global immunosuppressive compound. In fact, preclinical phase I and phase II clinical trials completed to date show no adverse effects on immune function. Taken together, the properties of this novel antioxidant and anti-inflammatory compound provide a mechanistic framework for understanding its antiatherosclerotic activity in both animal models and in humans and provide further support that vascular protection through maintenance of redox homeostasis and modulation of inflammatory genes offers an attractive therapeutic approach to the treatment of atherosclerosis and coronary artery disease.

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