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

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Non-standard abbreviations:

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
CAT = chloramphenicol acetyltransferase
BAEC = Bovine aortic endothelial cells
HAEC = human aortic endothelial cells
HMEC = human microvascular endothelial cells
ICAM-1 = Intercullar adhesion molecule-1
LMB = leukomethylene blue
MCP-1 = monocyte chemoattractant protein-1
ROS = reactive oxygen species
TNF-α = tumor necrosis factor-α
VCAM-1 = vascular cell adhesion molecule-1

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Abstract

Atherosclerosis is a disease of oxidative stress and inflammation. AGI-1067 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 anti-atherosclerotic properties in multiple animal models and in humans. To elucidate its anti-atherosclerotic mechanisms we have evaluated several cellular and molecular properties of AGI-1067 in vitro. AGI-1067 exhibited potent lipid peroxide antioxidant activity comparable to 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, VCAM-1 and MCP-1, in endothelial cells as well as TNF-α, 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 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 anti-atherosclerotic 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 anti-atherosclerotic 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; Iiyama 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 (Cybulsky 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-1β (IL-1β), interleukin-6 (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 (Gosling et al., 1999; Yla-Herttuala et al., 1991; Nelken et al., 1991). 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 (Ali et al., 1999; Hsu and Wen, 2002; Marui et al., 1993; Satriano et al., 1993). 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 include TNF-α and IL-1β, endotoxin, angiotensin II, growth factors, oxidized LDL and hemodynamic forces are known to induce ROS in the vasculature and in leukocytes (Heinecke, 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 (Neish et al., 2001; Ueda et al., 1994), there have been several examples of pharmacologic agents that inhibit the expression of these genes, yet have no effect on NF-κB.

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) is a novel, orally-active, metabolically-stable derivative of probucol that exhibits anti-atherosclerotic activity in several animal models (Meng et al., 2002; Sundell et al., 2003). In addition, the Canadian Antioxidant Restenosis Trial-1 (CART-1) demonstrated a dose-dependent inhibition of post-angioplasty restenosis and improved lumen dimensions of non-intervened coronary artery reference segments, thereby suggesting a direct positive effect of AGI-1067 on atherosclerosis (Tardif et al., 2003; Tardif, 2003). To gain insight into the anti-inflammatory and anti-atherosclerotic 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 anti-oxidant and a selective inhibitor of inflammatory response genes. These biological activities may account for the anti-atherosclerotic and anti-restenotic activity of this new therapeutic agent.

Methods

Cell Culture, Plasmids and Transfections

Human pulmonary artery (HPAEC) and aortic (HAEC) endothelial cells, bovine aortic endothelial cells (BAECs) and human peripheral blood mononuclear cells (hPBMCs) were obtained from Cambrex, Inc (Walkersville, MD). U937 cells were obtained from ATCC. All cells were maintained at 37°C under 5% CO_2 atmosphere. For transient transfection of BAECs, cells were seeded onto a 60mm^2 plate coated with 1% gelatin one day prior to transfection. 2μg of reporter plasmid and 0.5μg of pSV2β-gal (used for normalization of transfection efficiency) were transfected per well using the lipid transfection reagent Lipofectamine Plus, according to the manufacturer's protocol (Life Technologies, Rockville, MD). On the following day, media was removed and fresh media containing compound with or without TNF-α (100U/ml) was added. 48 hr 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, protein content was determined and cellular lysates were 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 hPBMCs

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 hPBMCs. Fresh cryopreserved hPBMCs were pretreated with test compound for 1 hr followed by stimulation with LPS (1μg/ml) for an additional 2 hr 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

HAECs were cultured as above and seeded onto 24- or 96-well plates such that they would reach 90-95% confluency on the following day. Cells were stimulated with TNF-α (1ng/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, Inc., Madison, WI). For determination of VCAM-1 and MCP-1 protein expression, cells were pretreated with test compound for 1 hr and subsequently stimulated with TNF-α in the presence of compound for an additional 4 hr. 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 hr and exposed to TNF-α for 4 hr. 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, Carlsbad, CA) 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 (BioRad, 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.[11] Briefly, two complementary oligonucleotides containing the two NF-κB sites (underlined below) of the human VCAM-1 promoter were annealed in 50mM Tris, pH8.0, 100mM NaCl, and 10mM MgCl. The oligonucleotide sequence is: 5'-GCTGCCCTGGGTTTCCCCTTGAAGGGATTTCCCTCCGCCTCTGCAACAA-3'. Double-stranded, complementary oligonucleotides were labeled at their 5' ends with γ-^{32}P dCTP and T4 polynucleotide kinase. Protein:DNA-binding reactions were performed at room temperature for 30 min in a total reaction volume of 25µl which contained: 3µg nuclear extract, 225 µg/ml BSA, 3 x 10^6 cpm ^{32}P-labeled probe, 0.1 mg/ml polyinosinic:polycytidylic(polydI:dC) acid, and 15µl of binding buffer (12mM HEPES, pH7.4, 4mM Tris, 60mM KCl, 1mM EDTA, 1mM PMSF, 1mM DTT, 12% glycerol). Following incubation, the entire reaction was electrophoresed through a 5% acrylamide gel, dried and exposed to x-ray film.

cDNA Microarray Analysis

HAECs were grown to approximately 90% confluency in EGM-2 media containing 10% FBS. Cells were treated with either 0.1% DMSO or 15µM AGI-1067 in 0.1% DMSO for 15 hr followed by the addition of TNF-α (1ng/ml) for an additional 4 hr. Total cellular

RNA was collected as described above and hybridized to the Atlas™ Human cDNA Expression Array (Clonetech, Inc., Palo Alto, CA) according to the manufacturers protocol. Image analysis and quantitation was performed using a Storm phosphoimager (Molecular Dynamics).

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 (DCF) of the fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCF-DA, Molecular Probes, Eugene, OR). In the intracellular compartment, esterases cleave off the acetate group on H_2DCF-DA, trapping H_2DCF in the intracellular compartment. Conversion of H_2DCF to the fluorescent form, DCF, by endogenous oxidants, was monitored on a microplate fluorimeter, excitation 485nm; emission 530nm. HPAECs or U937 cells were treated with either AGI-1067 or probucol for 3 hr followed by co-treatment with 10μM H_2DCF-DA for an additional 30 min. To determine the effect on H_2O_2-stimulated ROS production, cells were exposed to 200 μM H_2O_2 for 15 min just prior to the end of the experiment. Cells were then washed with PBS and lysed in Tris-buffered

saline containing 0.05% Tween-20 and 0.01% Triton-X100 and absolute fluorescence measured using a fluorimeter (Victor^2, Perkin-Elmer, Boston, MA).

Compound uptake studies

HAECs were seeded in 10 cm dishes and were used below passage 9. At approximately 90% confluency, cells were treated with either 5μM AGI-1067 or 5μM 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 hr. At each time point cells were washed twice with 5 ml phosphate-buffered saline (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-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenoxy]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 μg/mL. 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 non-cellular and in cell-based systems. As shown in Figure 1, when evaluated for its ability to inhibit lipid peroxide-mediated oxidation, AGI-1067 demonstrated potent antioxidant activity comparable to 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 intracellular signaling, we examined the ability of AGI-1067 to inhibit intracellular levels of ROS in HPAECs and the promonocytic cell line U937 by fluorescence spectroscopy. As shown in Figure 2a, AGI-1067, but not probucol, inhibited basal levels of ROS in HPAECs 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 10μM. 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 (Figure 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 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, HAECs were incubated with equimolar concentrations of either compound and cell-associated levels of each compound were quantitated at various time points by HPLC analysis. As shown in Figure 3, there was a time-dependent increase in cell-associated AGI-1067 levels; however, the levels of probucol remained below the LLOQ (1μg/ml) for the assay method during the same time period. The concentration of AGI-1067 and probucol in the culture media, as determined by HPLC/UV, were approximately equivalent (4.9μg/ml and 3.5μg/ml respectively). Similar results were observed from a repeat experiment. These data suggest that there is a temporal increase in intracellular or cell-associated AGI-1067 that is not observed with probucol under similar experimental conditions. The increased accumulation of AGI-1067 relative to probucol may be attributable to increased hydrophilicity allowing enhanced uptake across the plasma membrane due to either active or passive transport, or decreased cellular efflux. Regardless of the mechanism, we believe AGI-1067 can be more efficiently taken up into, and/or retained within, the cellular compartment and partition to relevant intracellular sites of ROS production and redox-sensitive signal transduction. This likely accounts for the observed increase in intracellular antioxidant capacity of AGI-1067

relative to probucol (Fig. 2), despite the fact that both compounds exhibit equipotent extracellular antioxidant capacity (Fig. 1).

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 pro-inflammatory 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 hPBMCs *in vitro*. As shown in Figure 4, AGI-1067 resulted in a concentration-dependent reduction in the release of all three cytokines from LPS-activated hPBMCs with apparent IC_{50} values of approximately 1.0μM and 0.5μM, and 0.3μM 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 if AGI-1067 inhibited the inducible expression of endothelial cell redox-sensitive inflammatory response genes, HAECs were treated with AGI-1067 or probucol and, then, stimulated with TNF-α. As shown in Figure 5a, AGI-1067 inhibited the TNF-α-inducible expression of VCAM-1 and MCP-1 in a concentration-dependent manner at 4 hr post-TNF-α with IC_{50} values of ~6μM and 6.4μM, respectively. Probucol, on the other hand, had no effect on either of these genes up to a maximum concentration of 100μM. 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 HAECs 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 HAECs (data not shown).

To determine if 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. HAECs were pretreated with AGI-1067 (6μM or 8μM) for 1hr followed by stimulation with TNF-α for 4 hr. Relative levels of mRNA were determined by real-time quantitative RT-PCR analysis. As shown in Figure 5b, treatment of HAECs 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 Figure 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 EC are difficult to transfect, we used bovine aortic endothelial cells (BAECs) since they exhibit a much higher transfection efficiency. To determine if 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 933bp or 288bp of the human

VCAM-1 promoter fused to the CAT reporter gene. Both of these constructs have previously been shown to respond in BAECs to several pro-inflammatory 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 VCAM-1 constructs with a resulting increase in CAT activity (Figure 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 (Figure 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 588 known genes were analyzed using a cDNA array and samples from TNF-α- and AGI-1067-treated HAECs. A representative quadrant containing 98 genes is shown in Figure 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-fold and 8.5-fold inhibition of hybridization signal for VCAM-1 and E-selectin, respectively. Other

genes whose expression was reduced by a magnitude greater than 2.5-fold, but are not contained within the quadrant shown, included: MCP-1 (2.5-fold), JNK-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 (Figure 7), IL-8, and IL-2r, were not inhibited by AGI-1067 in TNF-α-stimulated HAECs under these conditions. The modulation of expression of these genes by AGI-1067 as determined by expression array hybridization was confirmed by either semi-quantitive or quantitative RT-PCR analysis (figure 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 Figure 6 contain two copies of the NF-κB binding site, and since the results in Figure 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 HAECs pretreated for 1 hour with either the thiol antioxidant PDTC (a known inhibitor of NF-κB activation) or

AGI-1067 and stimulated for 1 hour 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 unlabelled 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 Figure 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 4 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 HAECs 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 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 (Figure 7, C. 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. Pro-inflammatory 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 share 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 (Alexander, 1998; Kunsch and Medford, 1999; Marui et al., 1993).

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, anti-atherosclerotic and anti-restenotic activity; however, because probucol has been shown to cause ventricular arrythmias (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 doesn't 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 to probucol. Interestingly, two research groups have shown that α-tocopherol succinate exhibits enhanced uptake, intracellular antioxidant activity, inhibition of monocyte 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 co-transport mechanisms exist for monocarboxylic acid forms of drugs (Tamai and Tsuji, 1996). Therefore, although the exact mechanism is not know, the monoscuccinic 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 resistant to ex-vivo copper-induced oxidation than vehicle-treated animals. Furthermore, the concentrations at which we observe cellular antioxidant activity are comparable to 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 if 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 which 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 IC_{50} values correlate with plasma levels in our animal models and clinical trials that demonstrate reduced progression of atherosclerosis (Sundell et al., 2003, Tardif et al., 2003). These observations are consistent with numerous reports that demonstrate that antioxidants can inhibit VCAM-1 expression and support the notion that VCAM-1 and MCP-1 are regulated 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 HAECs 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-mediated 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 (HAECs) whereas the study by Zapolska-Downar et al used human umbilical vein endothelial cells. It is well known that endothelial cells derived from different vascular beds, display distinct biological responses. Secondly, 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 hrs) prior to stimulation with TNF- α. When probucol was added 30 min prior to TNF-α, no inhibition of VCAM-1 expression by probucol was observed, consistent with our studies in which probucol was added 1hr prior to TNF- α addition.

The inhibition of inducible VCAM-1 by AGI-1067 is reflected at the level of steady-state mRNA and occurs by inhibition of transcriptional activation of the VCAM-1 promoter. 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 expression, only reflect a modest inhibition of mRNA expression. Therefore, it is possible that AGI-1067 may exert additional 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 expression of VCAM-1 and MCP-1 are not known. One key regulator that is activated by both ROS and cytokine pathways 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 pathophysiologically relevant signals during atherogenesis (Manning, 2001). In the case of oxidant stress, it has been well-documented that ROS activate, whereas a variety of antioxidants inhibit, NF-κB activity. These studies have shown that anti-oxidants 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 nucleus. 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. Similary, another flavonoid, PD098063, inhibited cytokine-induced VCAM-1 with no effect on NF-κB nuclear 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 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 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 to 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 hPBMCs. 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 SMC 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 pro-inflammatory cytokines by AGI-1067 may be another mechanism whereby this compound exerts anti-atherosclerotic 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 anti-atherosclerotic 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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Footnotes

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Figure Legends

Figure 1. Lipid peroxide antioxidant activity of AGI-1067. The colorless agent LMB was incubated together with 13-HpODE and varying concentrations of either AGI-1067 or probucol. The ability of the compounds to inhibit the oxidation of LMB by 13-HpODE was monitored spectrophotometrically and plotted as percent inhibition relative to the vehicle (DMSO) control.

Figure 2. Cellular antioxidant activity of AGI-1067. A) HPAECs or the promonocytic cell line U937 were incubated with varying concentrations of either AGI-1067 or probucol and ROS-mediated oxidation of the redox-sensitive dye, H_2DCF was monitored by fluorescence spectroscopy. B) U937 cells were treated with 200μM H_2O_2 for 15 min in the presence or absence of either AGI-1067 or probucol. Values are mean ±SEM, n=3.

Figure 3. Endothelial cell uptake of AGI-1067 and probucol. HAECs were exposed to either 5μM AGI-1067 or probucol. At 0, 0.5, 1.0, 1.5 and 2.0 hr, cells were harvested and levels of cell-associated compound were determined by HPLC/UV analysis. For the probucol-treated samples, the levels were below the LLOQ (1μg/ml) of the assay method at all time points tested. Data is expressed as μg of compound per mg of cellular protein. Data is from a single experiment and is representative of two experiments that each demonstrated similar results.

Figure 4. Effect of AGI-1067 on LPS-induced inflammatory cytokine production from human PBMCs. Human PBMCs were pretreated with either AGI-1067 or probucol for 1 hr followed by stimulation with LPS for 2 hr. Levels of TNF-α (A), IL-1β (B) and IL-6 (C) released into the culture media were determined by ELISA. Data are mean ± SEM of triplicate determinations expressed as pg/ml. Similar results were obtained from two separate experiments.

Figure 5. Effect of AGI-1067 on VCAM-1 and MCP-1 protein and mRNA expression in endothelial cells. A, HAECs were exposed to either AGI-1067 or probucol for 1hr followed by the addition of TNF-α for 4 hr. VCAM-1 and MCP-1 protein levels were determined by ELISA. **B,** HAECs were exposed to AGI-1067 for 1 hr followed by the addition of TNF-α for 3 hr. Relative levels of VCAM-1 and MCP-1-specific mRNA were assessed by RT- PCR following normalization to the housekeeping gene GAPDH. The data shown is representative from several different experiments each yielding similar results. Values represent mean ±SEM, n=3.

Figure 6. Effect of AGI-1067 on transcriptional activation of the VCAM-1 promoter. BAECs were transiently transfected with a minimal VCAM-1/CAT promoter-reporter gene containing either 288 base-pairs (left panel) or 933 base-pairs (right panel) of the VCAM-1 regulatory region. Following transfection, cells were treated with either vehicle alone or increasing concentrations of AGI-1067 for 1 hr followed by addition of TNF-α for 16 hr. Cellular extracts were prepared and CAT activity was determined. Values were normalized for total cellular protein content. The

data shown is representative from several independent experiments with similar results. Values represent mean CAT activity, n=2.

Figure 7. cDNA microarray analysis of AGI-1067-treated TNF-α-activated HAECs. HAECs were exposed to AGI-1067 and stimulated with TNF-α for 4 hrs. Following collection of total RNA, radiolabeled cDNA was synthesized and hybridized to the Atlas™ cDNA Expression Array (Clonetech). Following autoradiography, relative hybridization intensities were determined by phosphoimaging analysis. 1=VCAM-1, 2= E-selectin, 3=ICAM-1.

Figure 8. Effect of AGI-1067 on NF-κB activation. HAECs were exposed to either PDTC (25μM) or AGI-1067 (25μM) for 1hr followed by addition of TNF-α for 1 hr. Nuclear extracts were prepared and evaluated for NF-κB-specific DNA binding activity as described in Materials and Methods. Data shown is representative from several independent experiments with similar results.

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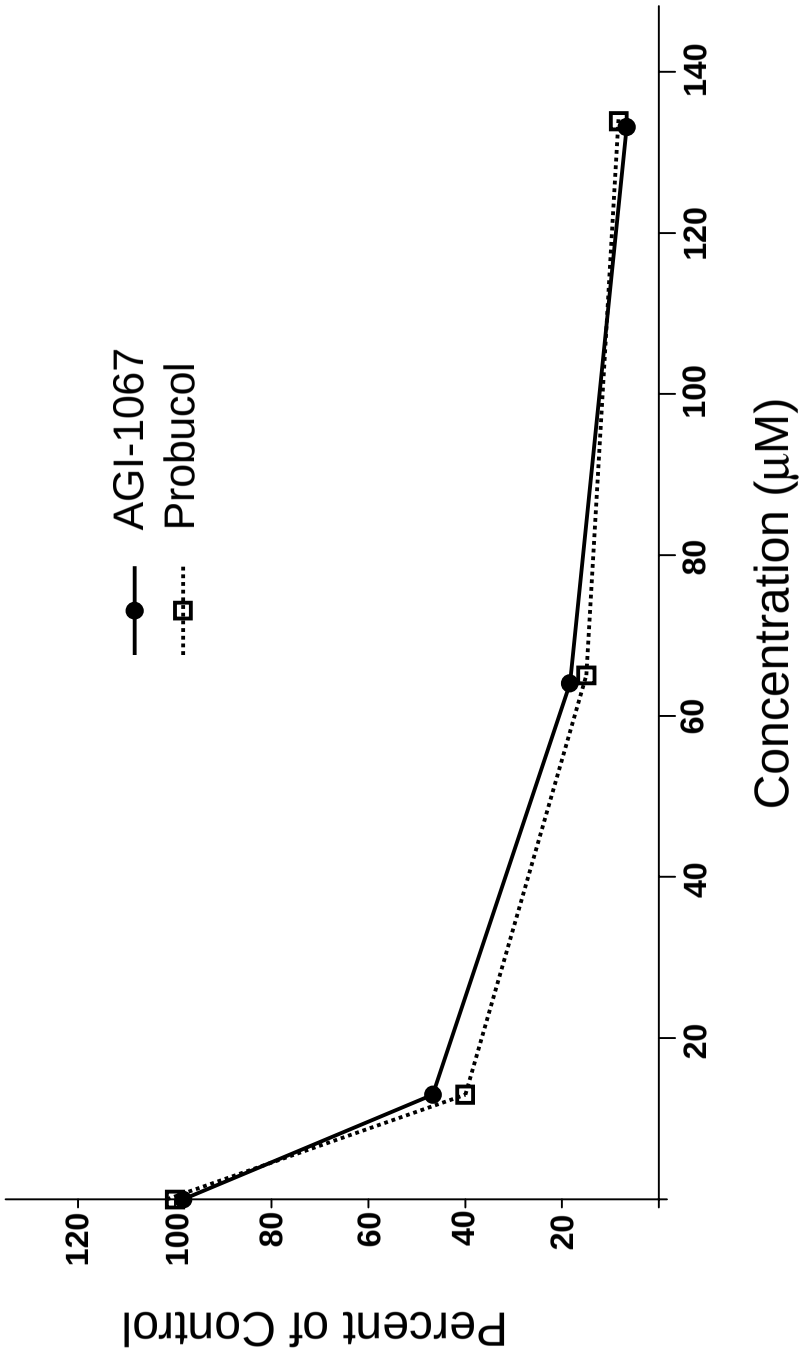
Figure 1



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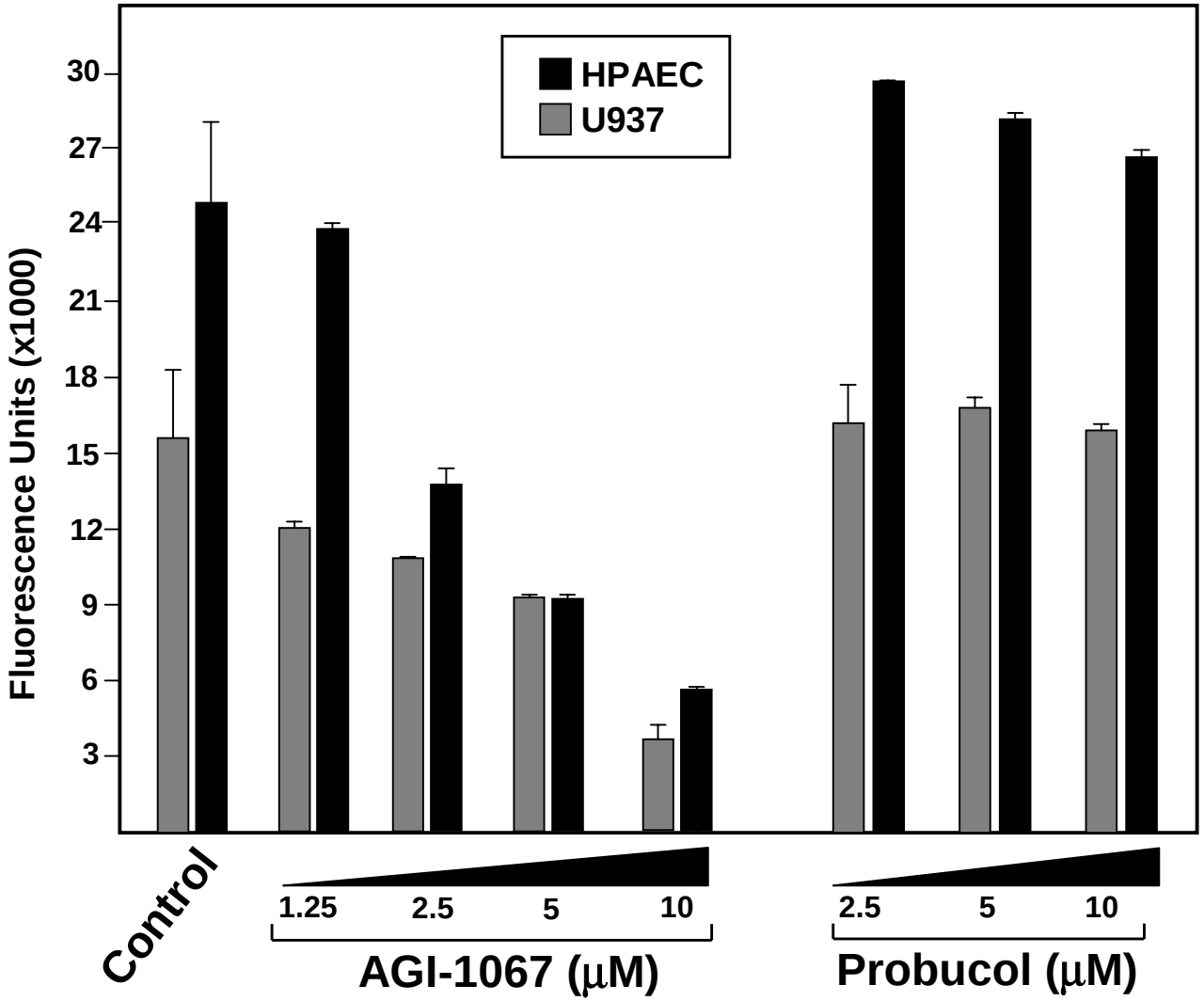

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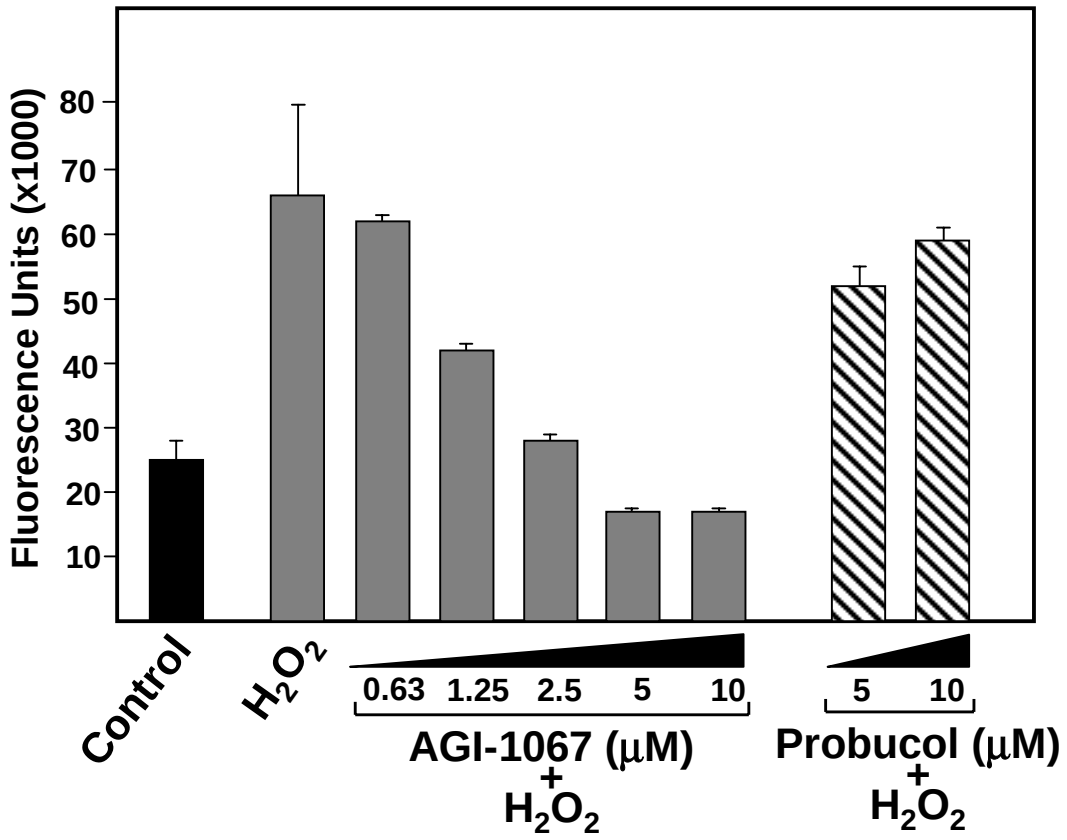

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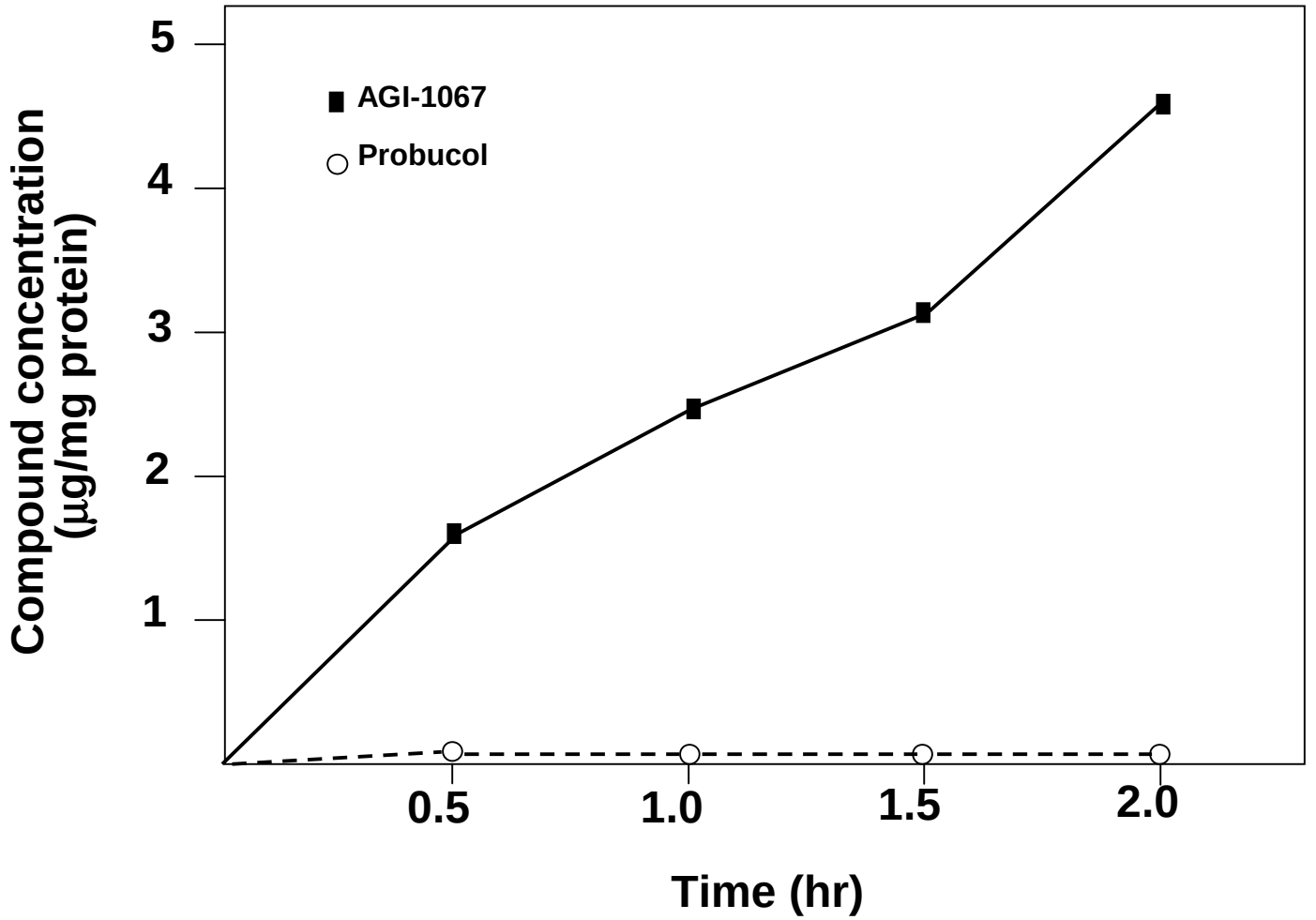

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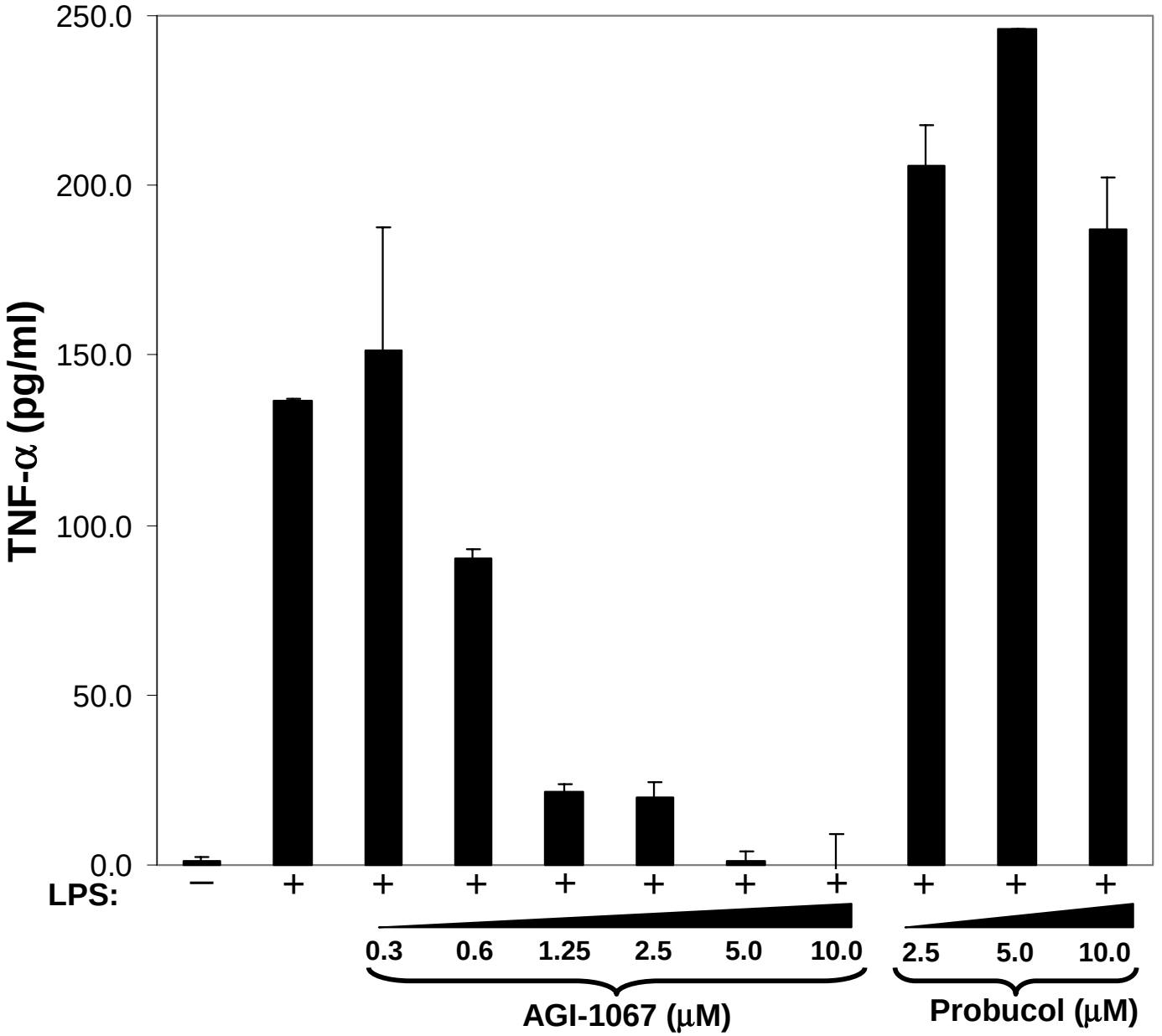

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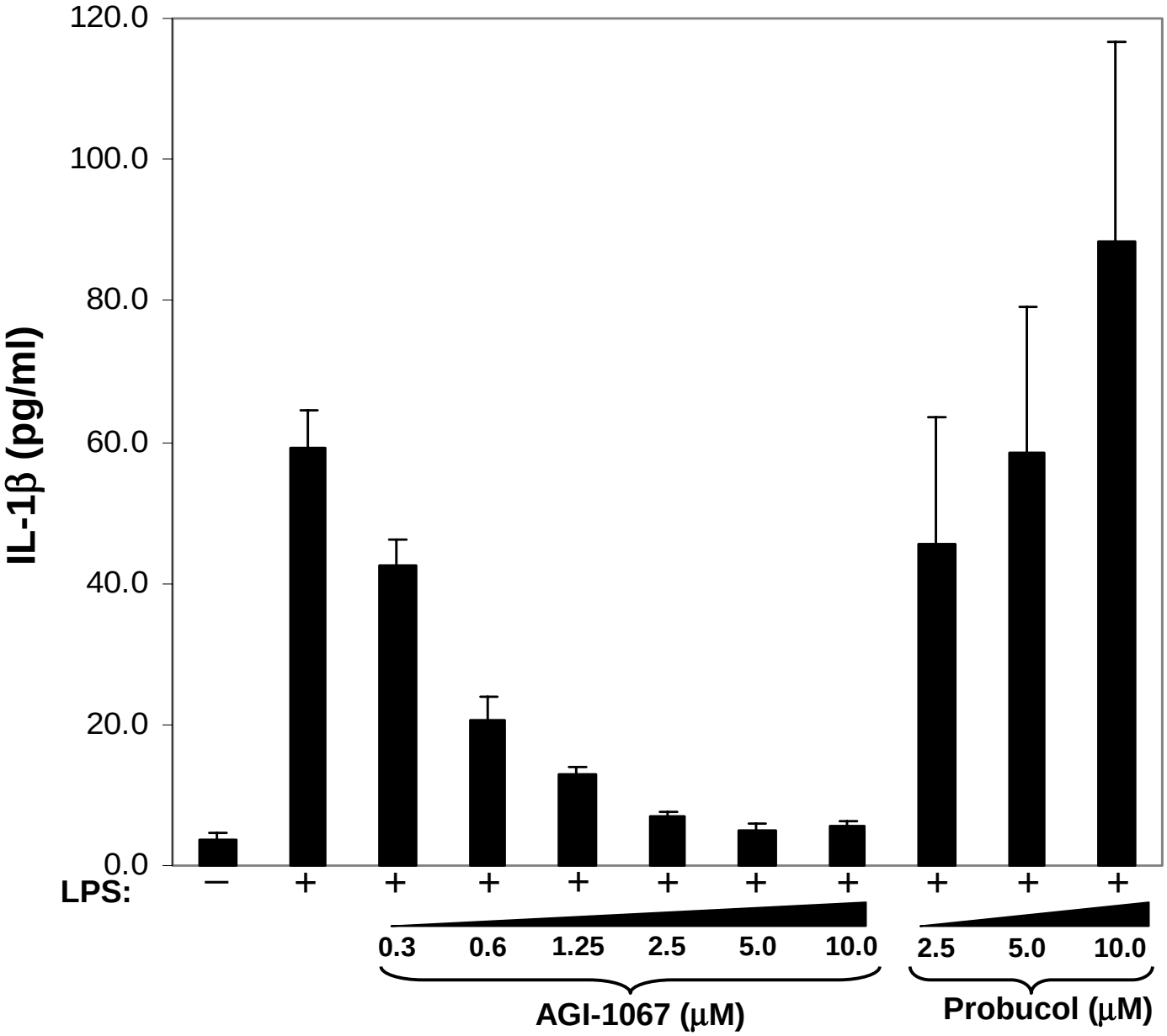

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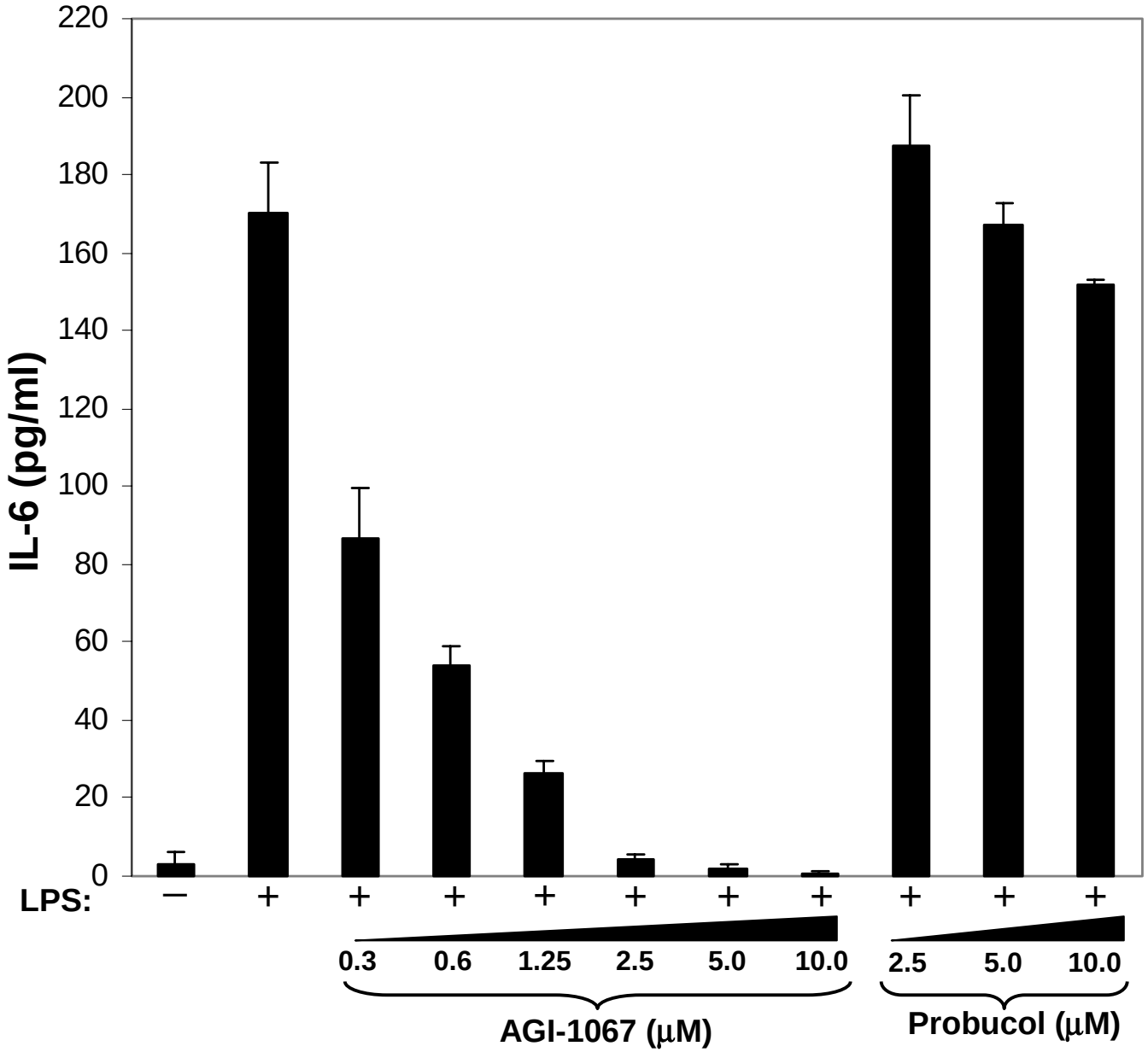

Figure 5a

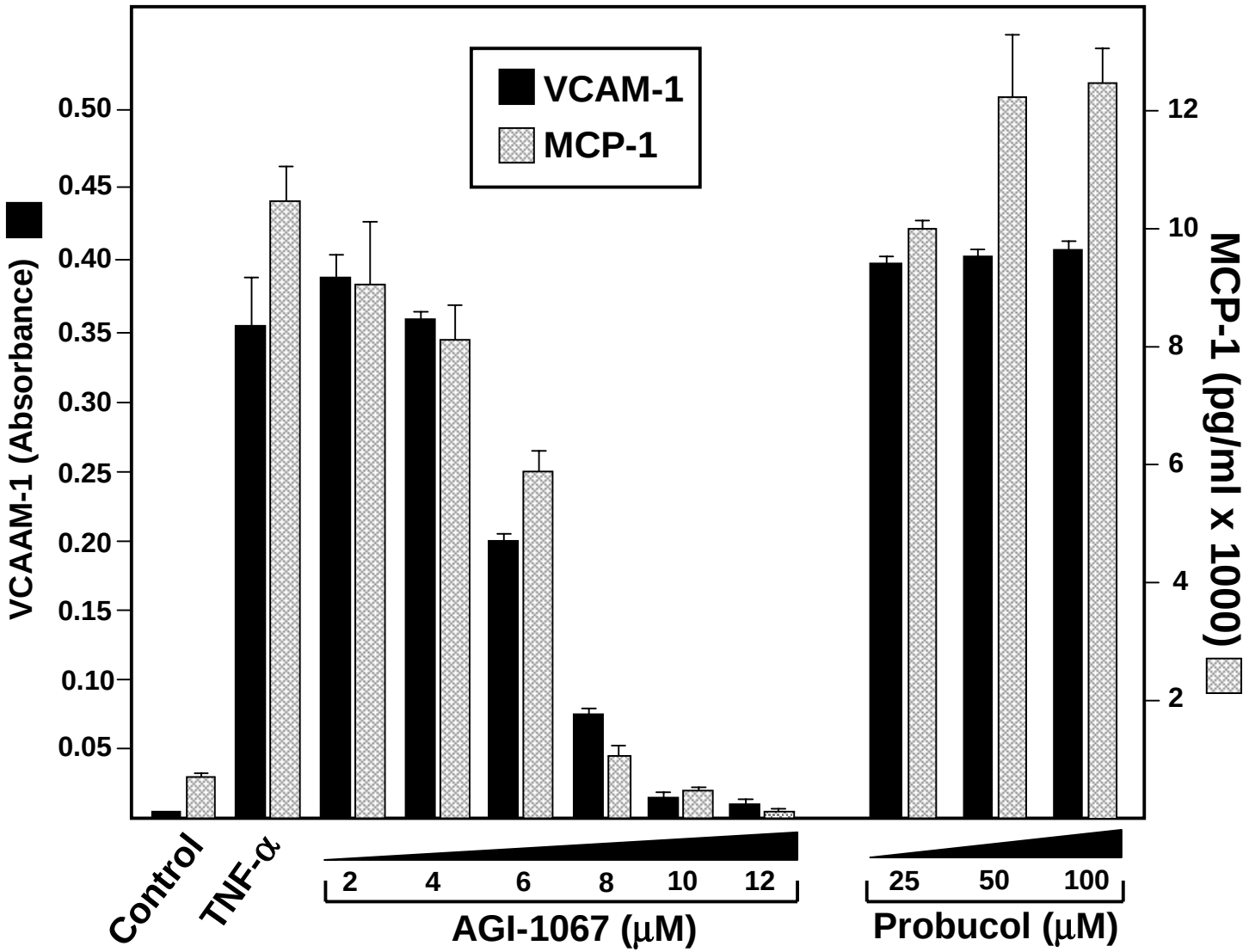

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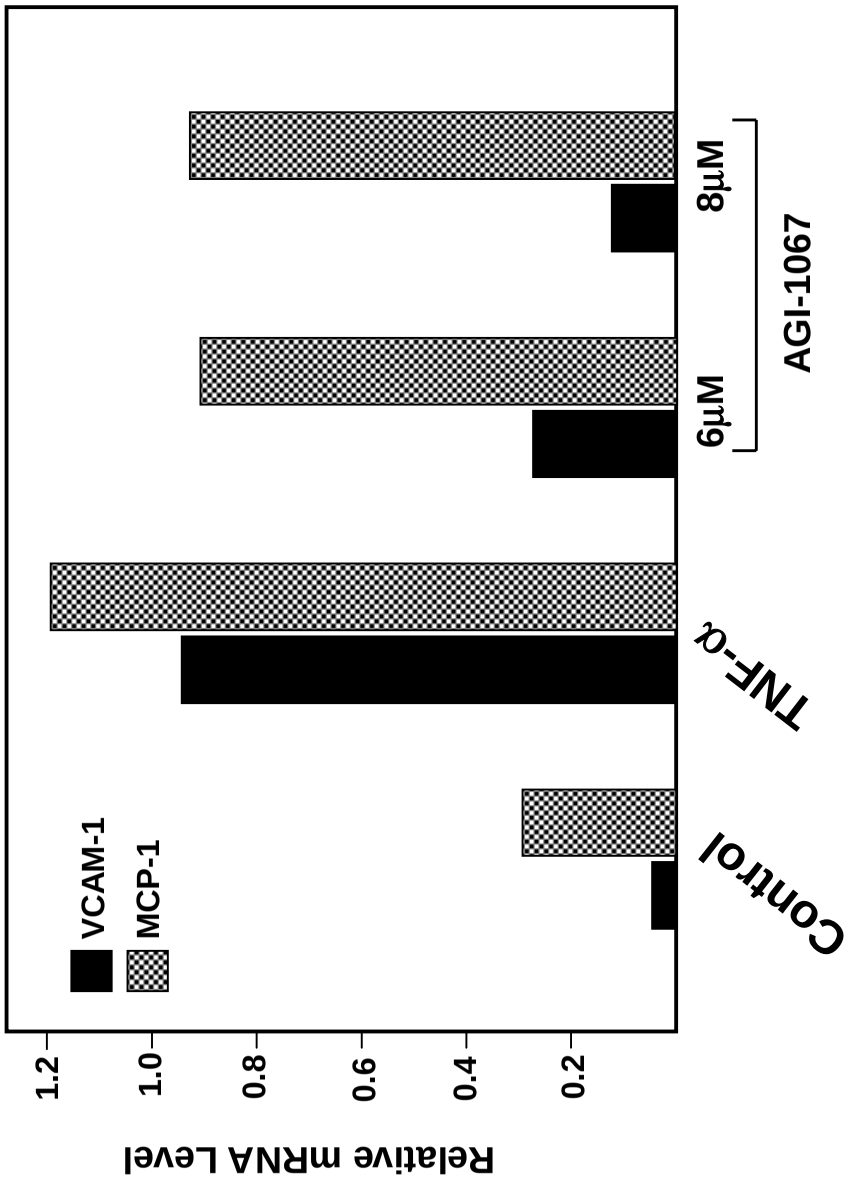
Figure 5b



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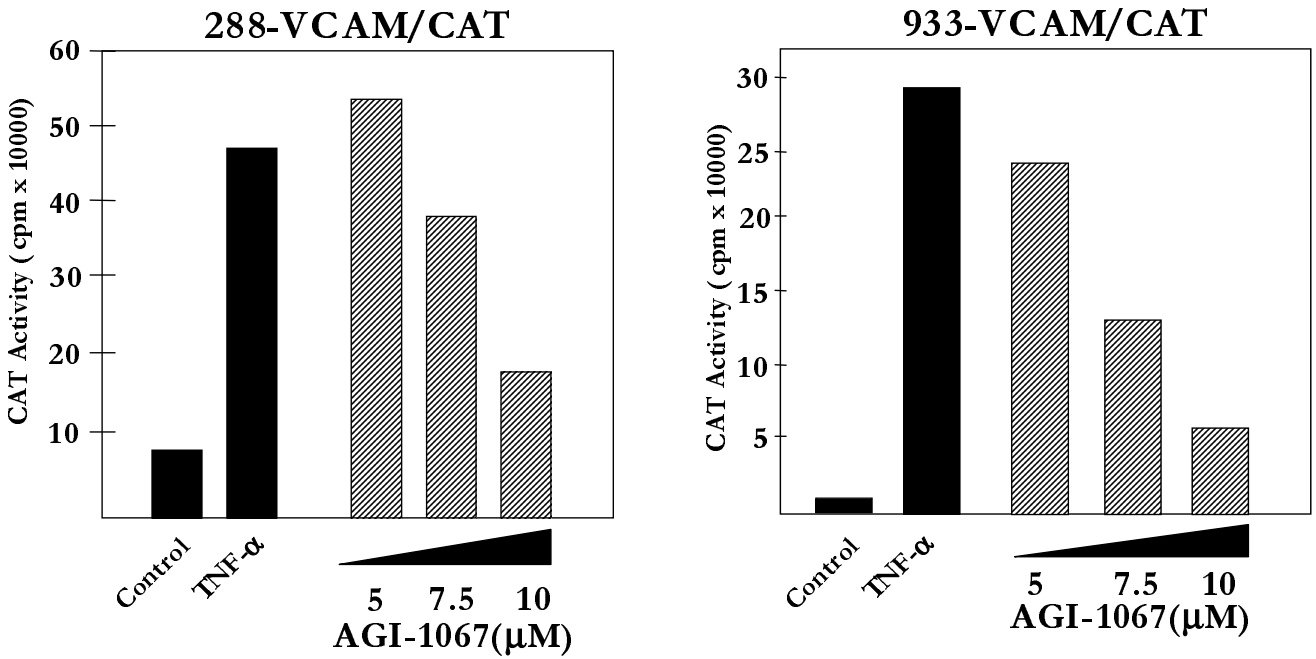

Figure 7

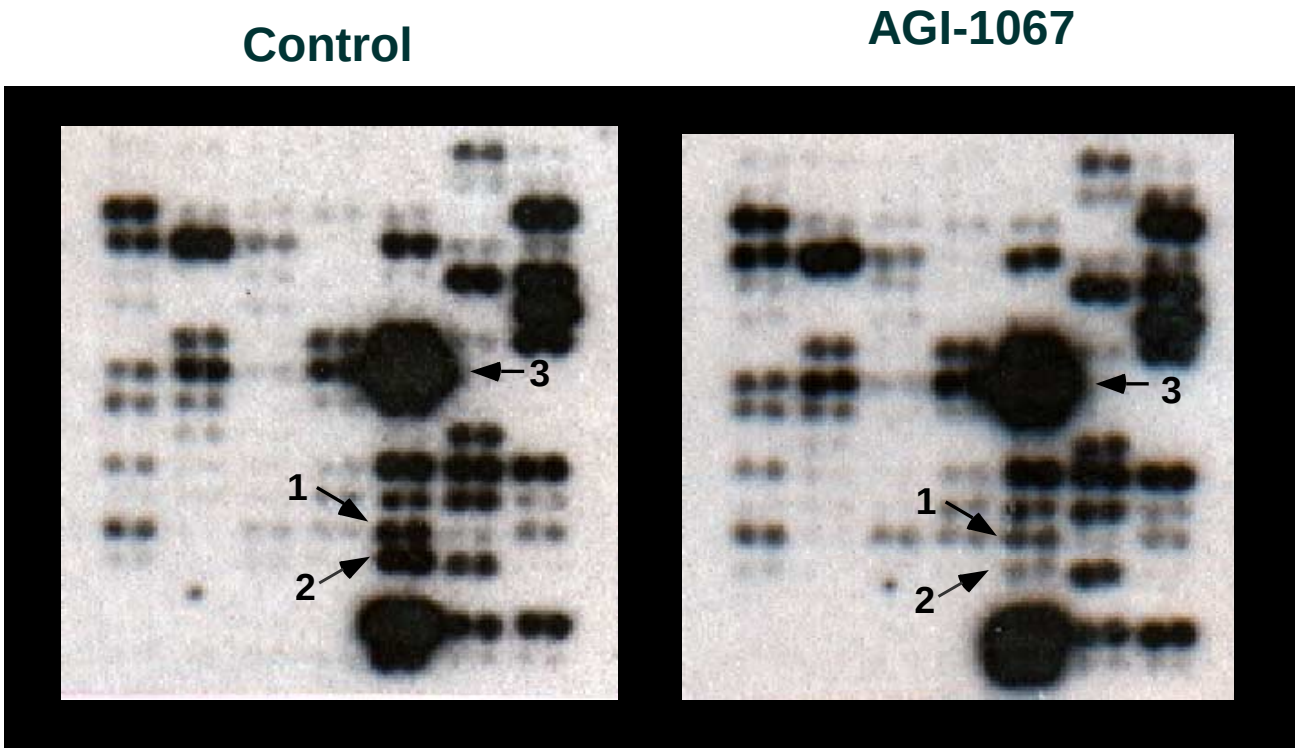

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Figure 8