AGIX-4207 [2-[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenoxy]acetic Acid], a Novel Antioxidant and Anti-Inflammatory Compound: Cellular and Biochemical Characterization of Antioxidant Activity and Inhibition of Redox-Sensitive Inflammatory Gene Expression


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ABSTRACT

The pathogenesis of chronic inflammatory diseases, including rheumatoid arthritis, is regulated, at least in part, by modulation of oxidation-reduction (redox) homeostasis and the expression of redox-sensitive inflammatory genes including adhesion molecules, chemokines, and cytokines. AGIX-4207 [2-[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenoxy]acetic acid] is a novel, orally active, phenolic antioxidant and anti-inflammatory compound with antirheumatic properties. To elucidate its anti-inflammatory mechanisms, we evaluated AGIX-4207 for a variety of cellular, biochemical, and molecular properties. AGIX-4207 exhibited potent antioxidant activity toward lipid peroxides in vitro and displayed enhanced cellular uptake relative to a structurally related drug, probucol. This resulted in potent inhibition of cellular levels of reactive oxygen species in multiple cell types. AGIX-4207 selectively inhibited tumor necrosis factor (TNF)-α-inducible levels of the redox-sensitive genes, vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1, with less inhibition of E-selectin, and no effect on intracellular adhesion molecule-1 expression in endothelial cells. In addition, AGIX-4207 inhibited cytokine-induced levels of ROS, reactive oxygen species in endothelial cells and human fibroblast-like synoviocytes as well as lipopolysaccharide-induced release of TNF-α, IL-1β, and IL-6 from human peripheral blood mononuclear cells. AGIX-4207 did not inhibit TNF-α-induced nuclear translocation of nuclear factor of the κ-enhancer in B cells (NF-κB), suggesting that the mechanism of action is independent of this redox-sensitive transcription factor. Taken together, these results provide a mechanistic framework for understanding the anti-inflammatory and antirheumatic activity of AGIX-4207 and provide further support for the view that inhibition of redox-sensitive inflammatory gene expression is an attractive approach for the treatment of chronic inflammatory diseases.

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ABBREVIATIONS: VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; RA, rheumatoid arthritis; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; TNF-α, tumor necrosis factor-α; ROS, reactive oxygen species; AGIX-4207, 2-[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenoxy]acetic acid; HAEC, human aortic endothelial cell; HPAEC, human pulmonary artery endothelial cell; PBMC, peripheral blood mononuclear cell; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; FLS, fibroblast-like synoviocyte; NF-κB, nuclear factor of the κ-enhancer in B cells; PDTC, pyrrolidinedithiocarbamate; PCR, polymerase chain reaction; LMB, leukomethylene blue; 13-HpODE, 13-[Z,E]-9,11-hydroperoxyoctadecadienoic acid; PBS, phosphate-buffered saline; ISTD, internal standard; EC, endothelial cell; EMSA, electrophoretic mobility shift assay; H$_2$DCF, 2',7-dichlorodihydro fluorescein.
cule-1 (ICAM-1), E-selectin, and P-selectin. During chronic inflammation, this normally regulated process goes awry, and a state of constant immune activation with accompanying inflammation predominates. The importance of adhesion molecules, including VCAM-1 and ICAM-1, in chronic inflammatory diseases such as rheumatoid arthritis (RA), has been reviewed in detail (Postigo et al., 1993; Tak et al., 1995; McMurray, 1996).

In addition to cellular adhesion molecules, cytokines play a central role in the activation and perpetuation of the immune response. Proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, produced by both leukocytes and the endothelium, activate surrounding target tissues and cell types including macrophages, fibroblasts, and T-cells to produce additional inflammatory cytokines and mediators. Furthermore, the expression of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and IL-8 can be induced in the endothelium and mediate the migration of leukocytes to sites of active inflammation. In RA, these cytokines and chemokines are produced in abundance, and a substantial body of experimental evidence points to a central role for these cytokines in the disease pathogenesis (Koch et al., 1992; Gong et al., 1997; Ellingsen et al., 2001).

One of the biological consequences of vascular inflammation is the production of reactive oxygen species (ROS), released both extracellularly from activated leukocytes (i.e., neutrophils) as well as intracellularly in key cell types involved in the mediation of inflammatory responses. ROS can be toxic and not only cause macromolecular damage to biomolecules (DNA, proteins, lipids) but recently have been recognized as important intracellular signaling mediators (Suzuki et al., 1997; Finkel, 2003). The role of ROS as mediators of inflammatory gene expression has been well characterized in cell types of the vasculature (Kunsch and Medford, 1999; Griendling et al., 2000; Harrison et al., 2003), and recently, it has become evident that ROS also play an important role in regulating inflammatory processes in other cell types as well, e.g., monocytes and T-cells (Haddad and Land, 2002; Hsu and Wen, 2002; Finkel, 2003).

There is a large body of evidence demonstrating a role for ROS and oxidant stress in the pathogenesis of RA. Both preclinical and clinical studies have demonstrated a correlation between markers of oxidant stress with progression of disease and the potential beneficial effects of antioxidant supplementation or therapy (Heliovaara et al., 1994; Bauerova and Bezek, 1999; Darlington and Stone, 2001; Bandt et al., 2002; De Leo et al., 2002; Paredes et al., 2002; Cerhan et al., 2003; Jaswal et al., 2003; Kamanli et al., 2004). Although a complete understanding of how oxidative stress participates in the pathogenesis of RA is lacking, there is evidence demonstrating that expression of several inflammatory genes that participate in RA (including endothelial VCAM-1, MCP-1, and IL-6 and TNF-α and IL-β in monocytes) is regulated by redox-sensitive pathways (Marui et al., 1993; Haddad and Land, 2002; Hsu and Wen, 2002).

We have recently described a series of monoether derivatives of the antioxidant drug probucol (Meng et al., 2004) as having the ability to inhibit the inducible expression of VCAM-1. One of these compounds, AGIX-4207 (Fig. 1), is a novel antioxidant and anti-inflammatory compound with pharmacological efficacy in preclinical models of inflammation, including RA (Sundell et al., 2004), and has demonstrated safety upon oral administration to humans in clinical studies. Here, we provide cellular, biochemical, and molecular data to highlight the antioxidant and anti-inflammatory properties of AGIX-4207 to provide a framework for understanding the anti-inflammatory and antiarthritic properties of this novel agent.

**Materials and Methods**

**Cell Culture and Compound Dosing.** Cultured primary human aortic endothelial cells (HAECs) or pulmonary artery endothelial cells (HPAECs) were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and were used below passage 7. Cells were grown in endothelial growth medium-2 as previously described (Chen and Kunsch, 2004). Fresh, cryopreserved primary human peripheral blood mononuclear cells (PBMCs) were also obtained from Cambrex Bio Science Walkersville, Inc. and were grown in lymphocyte growth medium. Human fibroblast-like synoviocytes, obtained from Cell Applications, Inc. (San Diego, CA) and used below passage 6, were grown in Synoviocyte Growth Medium (Cell Applications, Inc.). U937 cells were obtained from American Type Culture Collection (Manassas, VA). All cell cultures were maintained at 37°C and 5% CO₂ in a humidified incubator. All compounds were made as a 1000 × stock in 100% DMSO. Compounds were then diluted 1:1000 in culture media (0.1% final DMSO concentration) immediately prior to addition to cells. For all experiments, 0.1% DMSO was included in all samples to control for any effects of DMSO on cellular activity. DMSO (0.1%) has no significant effect on any biological properties that we have examined in these studies.

**Determination of Cell Surface and Secreted Protein Production from Endothelial Cells.** For determination of cell surface (ICAM-1, VCAM-1, and E-selectin) and secreted protein (MCP-1, IL-6, and IL-8) levels from endothelial cells, cells were seeded at 1 × 10⁵ cells/96-well plate and grown for 16 to 24 h, at which time they were pretreated with varying concentrations of AGIX-4207 (dissolved in DMSO) such that the final concentration of DMSO was no more than 0.1% or DMSO alone for 1 h. Cells were subsequently stimulated with TNF-α (10 ng/ml) in the continued presence of the
were grown to approximately 70% confluence on glass coverslips and dried, and exposed to X-ray film. 

To fluoridate, 1 mM dithiothreitol, and 12% glycerol). Following incubation with 4 mM Tris, 60 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMFS), and 0.5% NP40. Cells were incubated for 2 h with primary rabbit polyclonal antibody directed against VCAM-1, ICAM-1, and E-selectin cell surface proteins and visualized using secondary antibodies (goat anti-rabbit or goat anti-mouse-CY2 for 1 h, both diluted at 1:10,000.

Cells were grown to 80% confluence in a 48-well plate in 0.5 ml of growth medium/well. Cells were simultaneously stimulated with TNF-α IL-1β, and IL-6 using commercially available kits (R&D Systems). Samples were measured in duplicate, and data are presented as mean ± S.D. Each experiment was repeated at least two times with similar results.

**Determination of Cytokine Levels from FLs.** Synoviocytes were grown to ~80% confluence in a 48-well plate in 0.5 ml of growth medium/well. Cells were simultaneously stimulated with TNF-α, IL-1β, and IL-6 and stimulated with various concentrations of either DMSO (0.1%) or AGIX-4207 at various concentrations for 24 h. Culture supernatants were collected and assayed by ELISA for secreted TNF-α, IL-1β, and IL-6 by ELISA using commercially available kits (R&D Systems.) Samples were measured in duplicate, and data are presented as mean ± S.D. Each experiment was repeated at least two times with similar results.

**Electrophoretic Mobility Shift Assay.** Nuclear extracts were prepared and the assay was performed as described previously (Maury et al., 1997). Briefly, two complementary oligonucleotides containing the two NF-κB sites (underlined below) of the human VCAM-1 promoter were annealed in 50 mM Tris, pH 8.0, 100 mM NaCl, and 10 mM MgCl2. The oligonucleotide sequence was: 5′-GC-TGCCCTTTGTTTCCTGAAAGGATTTTCCTCCGTCTGCA-ACAA-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 that contained 3 μg of nuclear extract, 225 μg/ml bovine serum albumin, 3 × 10−6 cm−1-μm−1-labeled probe, 0.1 μg/ml polyinosinic/polyuridylic (polyi/d) 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 dithiothreitol, and 12% glycerol). Following incubation, the entire reaction was electrophoresed through a 5% acrylamide gel, dried, and exposed to X-ray film.

**Indirect Immunofluorescence for NF-κB p65 (RelA).** HAECs were grown to approximately 70% confluence on glass coverslips and were pretreated for 1.5 h with DMSO (0.1%) alone, 10 μM AGIX-4207, or 100 μM PDTC dissolved in DMSO and then stimulated for 15 min with 100 U/ml TNF-α. Cells were washed with PBS, fixed with phosphate-buffered formalin for 1 min, and permeabilized with antibody dilution buffer (PBS, 1% bovine serum albumin, and 0.1% NP40). Cells were incubated for 2 h with primary rabbit polyclonal antibody directed against NF-κB p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted at 1:1000 or a mouse anti-laminA/C monoclonal antibody (BD Biosciences, San Jose, CA) diluted at 1:300. Cells were washed three times for 10 min each with PBS/0.1% NP40 and incubated with either donkey anti-rabbit CY3 F(ab′)2 or donkey anti-mouse-CY2 for 1 h, both diluted at 1:10,000.

Cells were washed three times for 20 min each with PBS/0.1% NP40 and once with PBS alone. Coverslips were mounted on Permount-impregnated glass slides. Digital images were acquired with a Nikon Eclipse T-100 inverted microscope mounted with a Spot Insight Camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Analysis of mRNA Gene Expression.** For determination of the effect of AGIX-4207 on mRNA levels, HAECs were pretreated with either DMSO (0.1%) or AGIX-4207 at various concentrations for 1 h followed by coincubation with TNF-α (1 ng/ml) for 3 h. Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). The iscript kit (Bio-Rad, Hercules, CA) was used to make first strand cDNA. Quantitative real-time PCR was used to determine relative mRNA expression using the iCycler System (Bio-Rad) with genespecific primers (i.e., VCAM-1, MCP-1, and GAPDH).

**Endothelial/Monocyte Adhesion.** HAECs were seeded at a density of 2 × 10^5 cells per well of a 96-well plate and pretreated for 1 h with either DMSO (0.1%) or AGIX-4207 at various concentrations. TNF-α was then added to the cells at a final concentration of 1 ng/ml for 4 h. U937 cells were labeled by suspension in protein-free RPMI culture medium at a concentration of 5 × 10^6 cells/ml. A probe solution consisting of 1 mM Calcein AM (Molecular Probes, Eugene, OR) in DMSO was added (~5 μl/ml medium) for a final concentration of 5 μM, and the mixture was incubated at 37°C for 30 min with frequent agitation. Cells were washed three times with RPMI media and resuspended at a concentration of 5 × 10^6 cells/ml in fresh medium. Culture medium was removed from the HAEC monolayer, and the fluorescently labeled cell suspension was added to the monolayer at a concentration of 3 × 10^6 cells/microplate well. The mixture was incubated at 37°C for 30 min followed by four rinses with RPMI media. Adherent U937 cells were visualized via fluorescence microscopy. Fluorescence was measured using the Victor2 V multiplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA). Each sample condition was performed in quadruplicate, and values were expressed as mean percent adhesion ± S.D.

**Determination of In Vitro Antioxidant Activity.** Lipid peroxide antioxidant activity was determined by the leukomethylene blue (LMB) assay as described previously (Somers et al., 2000). Briefly, the lipid peroxide 13-(S,Z,E)-9,11-hydro-peroxyoctadecadienoic acid (13-HpODE), derived from soybean lipoxynase-catalyzed oxidation of linoleic acid, was incubated with various concentrations of test compounds in the presence of colorless N-benzoyl LMB. Reduction of 13-HpODE by antioxidants to the alcohol form results in a concomitant reduction in the oxidation of LMB that can be monitored spectrophotometrically. Data are expressed as percentage of inhibition of LMB oxidation, and average IC50 determinations were calculated from five independent experiments.

**Determination of Intracellular ROS Levels.** Basal or H2O2-stimulated intracellular ROS levels were measured using the intracellular oxidation of 2',7'-dichlorodihydrofluorescein-diacetate, H2DCF-DA (Molecular Probes). Briefly, following uptake within the cell, 2',7'-dichlorodihydrofluorescein-diacetate, a non-polar compound, is cleaved by the action of intracellular esterases into a non-fluorescent derivative, H2DCF, which is unable to translocate back across the cellular membrane and is, therefore, trapped within the cell. Upon exposure to oxidants, intracellular H2DCF is oxidized to the highly fluorescent 2',7'-dichlorofluorescein, which can be measured by an increase in fluorescence intensity at 530 nm when the sample is excited at 485 nm. Changes in fluorescent signal were measured using a fluorometer (PerkinElmer Life and Analytical Sciences). Cell preparations were pretreated with various concentrations of either DMSO (0.1%) or AGIX-4207 or probucol for 3 h, followed by cotreatment with 10 μM DCFH-DA for 30 min. Adherent cells were observed for visual signs of cell stress and/or cell death. Cells were washed with PBS, lysed in Tris-buffered saline-Tween, and 2',7'-dichlorofluorescein fluorescence was measured in an aliquot. Samples were assayed in duplicate, and each experiment was repeated several times. Data are presented as an average of each experimental sample ± S.D. Each experiment was performed in duplicate or triplicate on different days with each experiment showing similar results; however, absolute numbers varied between experiments.

**Compound Uptake Studies.** HAECs were seeded in 10-cm dishes, and at approximately 90% confluence, cells were treated with either 5 μM AGIX-4207 or 5 μM probucol dissolved in 0.1% DMSO, and cell and culture supernatant samples were collected at 0, 0.05,
0.33, 1, 15, and 24 h. At each time point, cells were washed three times with 5 ml of phosphate-buffered saline (PBS), trypsinized, 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 using the Bio-Rad assay. Compound concentrations were determined using an internal standard (ISTD) spiking technique with a struc-

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**Fig. 2.** Antioxidant activity of AGIX-4207. 

A, lipid peroxide antioxidant activity of AGIX-4207. The colorless agent LMB was incubated together with 13-HPODE and varying concentrations of AGIX-4207, probucol, or α-tocopherol. 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. Values are mean ± S.E.M., n = 6. Using a two-tailed unpaired Student’s t test, there is no significant difference in the dose-response curves between any of the treatment groups (AGIX-4207 versus probucol, p = 0.98; AGIX-4207 versus α-tocopherol, p = 0.59; probucol versus α-tocopherol, p = 0.57). B, cellular antioxidant activity of AGIX-4207. HPAECs, U937 cells, or FLSs were incubated with varying concentrations of AGIX-4207 or probucol, and ROS-mediated oxidation of the redox-sensitive dye, H₂DCF, was monitored by fluorescence spectroscopy. Values are mean ± S.E.M., n = 4 for FLSs; n = 6 for HPAECs from two independent experiments; n = 6 for U937 cells from three independent experiments. p ≤ 0.05 (compared with control) for all AGIX-4207 treatment groups in all cell types. C, HAECs were exposed to either 5 µM AGIX-4207 or probucol. At 0, 0.05, 0.33, 1, 15, and 24 h, cells were harvested, and levels of cell-associated compound were determined by high-pressure liquid chromatography/UV analysis. For the probucol-treated samples, the levels were below the lower limit of quantitation (1 µg/ml) of the assay method at all time points except for 15 and 24 h. Data are expressed as micrograms of compound per milligram of cellular protein. Data are from a single experiment and are representative of two experiments that each demonstrated similar results. Values are mean ± S.D. determined from duplicate experiments.
turally 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 (acetoni-trile) to isolate the compound and ISTD from 
the sample matrix followed by high-pressure liquid chromatogra-
phy/UV detection. The lower limit of quantitation for the assay was 
set at 1 µg/ml. Two independent studies were performed, with each 
demonstrating similar results.

Results

Antioxidant Activity. Probucol is a well studied lipid 
antioxidant with low-density lipoprotein-modulating and an-
tiatherosclerotic properties (Pfuetze and Dujovne, 2000). We 
have recently described the synthesis of several series of 
probucol derivatives with biological activity (Meng et al., 
2002, 2004). One of these, AGIX-4207 (Fig. 1), is a chemically 
stable monoether derivative of probucol that demonstrates 
anti-inflammatory activity in vivo (Sundell et al., 2004). In 
the design and synthesis of AGIX-4207, modifications were 
made to probucol to retain its antioxidant properties, yet 
produce other aspects including metabolic stability, solubil-
ity, and biological activity. To confirm that the chemical 
modifications in the design of AGIX-4207 did not alter its 
antioxidant activity, we assessed its antioxidant potential in 
both cellular and cell-free systems. In vitro, AGIX-4207 dis-
played potent lipid peroxide antioxidant activity, comparable 
with both probucol and α-tocopherol (Fig. 2A). The ability of 
both probucol and α-tocopherol to function as lipid peroxide 
antioxidants confirms previously published observations 
(Wang and Quinn, 1999; Pfuetze and Dujovne, 2000). Be-
cause ROS play an important role in the pathogenesis of RA 
and other inflammatory diseases, we examined the ability of 
AGIX-4207 to inhibit intracellular levels of ROS in multiple 
cell types involved in RA including endothelial (HPAEC), 
monocytes (U937), and synovocytes (FLSs). Treatment with 
AGIX-4207 (1–10 µM) resulted in a concentration-dependent 
inhibition of basal levels of ROS (Fig. 2B). Probucol, although 
as effective an antioxidant (Fig. 2A), showed no inhibition of 
intracellular ROS. Exposure of HPAECs and U937 cells to 
hydrogen peroxide for 15 min resulted in approximately 45 
and 90% increase, respectively, in intracellular levels of ROS. 
Pretreatment with 5 µM AGIX-4207 for 1 h inhibited this 
inducible increase in ROS (data not shown). The inhibition of 
ROS in cell culture by AGIX-4207 occurs at concentrations 
that are comparable with trough plasma drug levels observed 
in animal models of RA in which AGIX-4207 demonstrated 
efficacy, and are similar to what is achieved at steady state 
upon dosing in humans (J. A. Sikorski, unpublished data).

Cellular Uptake of AGIX-4207 and Probucol. Because 
AGIX-4207 is predicted to be slightly more hydrophilic than 
probucol (Meng et al., 2004), we anticipated that it may 
exhibit altered cellular permeability relative to probucol. As 
shown in Fig. 2C, AGIX-4207 demonstrated a very rapid 
temporal increase in cellular association within the 1st h of 
incubation followed by a slower increase between 1 and 24 h. 
In contrast, probucol levels were below the quantitative limit 
of detection at all time points tested during the first 2 h of 
incubation; however, levels were detectable, although signifi-
cantly below those of AGIX-4207, at the 15- and 24-h time 
points. Despite the observation that AGIX-4207 and probucol 
display similar antioxidant activity in vitro (Fig. 2A), the 
enhanced cellular uptake of AGIX-4207 relative to probucol 
may account for the dramatic improvement in inhibition of 
intracellular ROS and inhibitory activity for redox-sensitive 
inflammatory gene expression (see below).

Endothelial Cell/Monocyte Adherence. Because ROS 
have been implicated in mediating inflammatory pathways 
in the vasculature, including the inducible expression of ad-
hesion molecules (Kunsch and Medford, 1999), we examined 
the ability of AGIX-4207 to inhibit adhesion of U937 
monocytes to activated HAECs in vitro. Exposure of 
HAECs to TNF-α for 4 h demonstrated an approximate 15-
fold increase in adherence of U937 cells as compared with 
non-TNF-α-treated cells (Fig. 3). Pretreatment of HAECs 
with AGIX-4207 prior to TNF-α stimulation resulted in a 
concentration-dependent reduction in adherence of U937 
cells. There was no effect on cellular viability at these con-
centrations (data not shown). These data demonstrate that 
AGIX-4207 inhibits TNF-α-mediated endothelial processes 
that promote monocyte/endothelial adhesion at similar con-
centrations in which AGIX-4207 inhibits intracellular levels 
of ROS and inducible VCAM-1 expression (see below).

Adhesion Molecule and Cytokine Expression in En-
dothelial Cells. In HAECs stimulated for 4 h with TNF-α,
cotreatment with AGIX-4207 (1–20 µM) resulted in a concen-
tration-dependent inhibition of cell surface VCAM-1 expres-
sion (Fig. 4A). The IC_{50} value for inhibition was approxi-
mately 8 µM. In similar experiments, AGIX-4207 was 
effective at inhibiting the cell surface expression of VCAM-1 
in human microvascular endothelial cells as well as HPAECs 
at approximately the same concentrations (data not shown). 
At similar concentrations, AGIX-4207 did not inhibit TNF-α-
inducible ICAM-1 expression. TNF-α-inducible E-selectin 
cell surface expression was inhibited by approximately 50% 
at the highest concentration tested (20 µM). The TNF-α-
inducible expression of the cytokines MCP-1, IL-6, and IL-8 
in HAECs was also inhibited in a concentration-dependent 
manor by AGIX-4207 (Fig. 4B) with IC_{50} values ~13, 8, and 
6 µM for MCP-1, IL-6, and IL-8, respectively.

To investigate whether the inhibition of expression of en-
dothelial inflammatory genes occurred at the level of gene 
expression, we examined mRNA accumulation by quantita-

![Fig. 3. AGIX-4207 inhibition of monocyte adhesion. HAECs were pre-
treated with varying concentrations of AGIX-4207 for 1 h prior to stim-
ulation with TNF-α for 4 h. Cells were washed, and fluorescently labeled 
U937 cells were incubated for 30 min, washed, and adherent cells were 
quantified by fluorometry. Results are expressed as percent adhesion 
relative to cultures treated with TNF-α alone. The data shown are repre-
sentative from several independent experiments with similar results; 
data are expressed as mean ± S.D., n = 3. *p ≤ 0.05.
itive real-time PCR. mRNAs for VCAM-1 and MCP-1 were inducible by TNF-α after 3 h; however, pretreatment with AGIX-4207 resulted in inhibition of only VCAM-1 mRNA, but not MCP-1 (Fig. 5) nor ICAM-1 (data not shown). Despite the lack of effect of AGIX-4207 on MCP-1 mRNA expression, secreted MCP-1 protein levels were decreased in the culture media. These observations imply that AGIX-4207 may exert regulation at both inhibition of TNF-α-inducible gene expression (i.e., VCAM-1) and also at a translational or post-translational level (i.e., MCP-1).

Inhibition of Cytokine Release from Activated FLSs. FLSs are a major cell type that comprise the invasive and hyperplastic synovium (pannus) in RA. FLSs have been shown to produce cytokines and matrix-degrading proteases that are found in abundance in synovial tissue from patients with RA. Treatment of FLSs with either recombinant IL-1β or TNF-α alone resulted in a marginal increase in release of MCP-1, IL-6, and IL-8 into the culture medium (data not shown). However, a mixture of TNF-α and IL-1β resulted in a significant induction in the release of these inflammatory mediators (Fig. 6). Cotreatment with AGIX-4207, TNF-α, and IL-1β caused a modest concentration-dependent inhibition in the release of all three cytokines from activated FLSs. At the highest concentration of AGIX-4207 tested (8 μM), there was a 42.3, 50.4, and 31% inhibition in MCP-1, IL-6, and IL-8, respectively. To our knowledge, these are the first observations of antioxidant inhibition of cytokine release from activated FLSs and suggest that, like endothelial cells and monocytes, redox mechanisms may govern cytokine-inducible expression of cytokines in FLSs as well.

AGIX-4207 Inhibits LPS-Stimulated Cytokine Release from hPBMCs. Infiltrating monocytes, and the cytokines released from them, contribute to the pathogenesis of a variety of inflammatory conditions, especially RA. Because

![Fig. 4. Effect of AGIX-4207 on endothelial cell adhesion molecule and cytokine expression. HAECs were exposed to varying concentrations of AGIX-4207 simultaneously with TNF-α for 4 h. A, relative levels of the cell surface adhesion molecules VCAM-1, ICAM-1, and E-selectin were determined by ELISA, and data are expressed as percent maximal induction. B, relative levels of the secreted cytokines MCP-1, IL-6, and IL-8 were determined by ELISA. All data are representative of multiple experiments each yielding similar results. Values are mean ± S.E.M., n = 8. *, statistical significance relative to untreated samples, where p < 0.05.](downloaded from jpet.aspetjournals.org at ASPET Journals on January 8, 2018)
IC50 values were 1.0, 1.1, and 1.3 at the highest concentration tested, AGIX-4207 reduced a known inhibitor of NF-κB treated for 1 h with either the thiol antioxidant PDTC (a redox-sensitive transcription factor, NF-κB, treatment with AGIX-4207 had no effect.

antioxidants have previously been shown to inhibit cytokine production from monocytes (Haddad and Land, 2002; Hsu and Wen, 2002), we evaluated if the inhibitory effect of AGIX-4207 on cytokine production that was observed in ECs and FLSs extended to PBMCs. As shown in Fig. 7, AGIX-4207 inhibited, in a concentration-dependent manner, LPS-induced levels of TNF-α, IL-1β, and IL-6 in the culture media. At the highest concentration tested, AGIX-4207 reduced LPS-activated cytokine levels to basal levels. Approximate IC50 values were 1.0, 1.1, and 1.3 μM for TNF-α, IL-1β, and IL-6, respectively. In contrast, probucol demonstrated no inhibitory activity on any of these cytokines at any of the tested doses. At the highest concentrations tested of both AGIX-4207 and probucol, there was no cytotoxic effect as assessed by lactate dehydrogenase release.

Effects on TNF-α-Inducible Nuclear Translocation of NF-κB. The regulation of several of the adhesion molecules and cytokines evaluated in this study is mediated, in part, via the redox-sensitive transcription factor, NF-κB (Baldwin, 1996). Therefore, to determine whether this important transcriptional activator is a target of action, we examined the ability of AGIX-4207 to inhibit TNF-α-inducible NF-κB activation. Nuclear extracts were prepared from HAECs pretreated for 1 h with either the thiol antioxidant PDTC (a known inhibitor of NF-κB nuclear translocation) or AGIX-4207 and stimulated for 1 h with TNF-α. NF-κB DNA binding activity was assessed by electrophoretic mobility shift assay (EMSA) using a double-stranded DNA oligonucleotide probe containing the two NF-κB binding sites from the VCAM-1 promoter. NF-κB-specific binding was assessed by competition of binding activity by excess unlabeled probe, lack of effect of competition with a probe containing a mutant copy of the NF-κB binding element, and absence of binding of nuclear extracts to the VCAM-1 mutant probe (data not shown). As shown in Fig. 8A, treatment with TNF-α resulted in the induction of two bands representing NF-κB-specific DNA binding activity. Treatment with 25 μM PDTC significantly reduced NF-κB binding activity; however, treatment with 25 μM AGIX-4207, a concentration more than three times the IC50 for inhibition of inflammatory gene expression in ECs, failed to inhibit NF-κB binding. In addition, the effect of AGIX-4207 on TNF-α-induced nuclear translocation of NF-κB as assessed by indirect immunofluorescence microscopy confirmed the results obtained by EMSA (Fig. 8B). Although PDTC inhibited the nuclear accumulation of the RelA (p65) subunit of NF-κB, treatment with AGIX-4207 had no effect.

Discussion

AGIX-4207 is a derivative of the drug probucol, a well studied phenolic antioxidant that has been used for the treatment of cardiovascular disease. Introduction of an acetic acid side chain onto probucol renders AGIX-4207 slightly more hydrophilic (Meng et al., 2004). As a result, this study demonstrates that AGIX-4207 exhibits significantly enhanced cellular uptake or penetration relative to probucol. It has been suggested that specific proton cotransport mechanisms exist for monocarboxylic acid forms of drugs (Ogihara et al., 1996). Therefore, the enhanced cellular accumulation of AGIX-4207 relative to probucol may be due to active transport mechanisms via the carboxylic acid moiety. Nevertheless, although the exact mechanism of cellular uptake is not known, AGIX-4207 readily accumulates within cells at a much greater rate than probucol.

Interestingly, although one of the redox centers of probucol is lost by the addition of the acetic acid side chain, this modification does not change the antioxidant capacity of AGIX-4207; probucol and AGIX-4207 display equivalent cell-free lipid peroxide antioxidant activity. However, AGIX-4207 is more potent than probucol at reducing intracellular ROS levels in multiple cell types, likely due to the enhanced cellular uptake. The assay used for determination of intracellular ROS in this study does not discriminate between the many possible species of reactive oxygen. Therefore, further studies are needed to address the precise ROS that is/are inhibited by AGIX-4207 and to determine whether this class of antioxidant compounds acts to scavenge ROS or to modulate the activity and/or levels of key proteins involved in redox homeostasis.

Because AGIX-4207 had potent cell-free antioxidant activity and inhibited intracellular ROS levels, we examined its ability to inhibit several redox-sensitive inflammatory pathways in cell types relevant to the pathogenesis of RA. In ECs, PBMCs, and FLSs, AGIX-4207 inhibited the inducible expression of key inflammatory proteins at similar concentrations that were reached in plasma in the rat adjuvant arthritis model (Sundell et al., 2004) and upon oral dosing in humans (data not shown). AGIX-4207 displayed a preferential inhibition of the leukocyte adhesion molecule VCAM-1 in comparison with ICAM-1 or E-selectin. This observation is consistent with the notion that VCAM-1 is more sensitive to...
modulation by redox signals than is either ICAM-1 or E-selectin (Marui et al., 1993). The inducible expression of the cytokines MCP-1, IL-6, and IL-8 was also inhibited by AGIX-4207 in HAECs and FLSs. In addition, AGIX-4207 inhibited the release of IL-6, TNF-α, and IL-1β from activated PBMCs. These cytokines and chemokines are critically important in leukocyte recruitment, and there is a wealth of data on the potential role of these cytokines and their receptors in the pathogenesis of RA. Inhibition of their inducible expression may be one of the main mechanisms responsible for the antiarthritic properties of AGIX-4207.

In ECs, the inhibition of TNF-α-inducible VCAM-1 cell surface protein by AGIX-4207 was accompanied by inhibition of steady-state levels of mRNA for VCAM-1. In contrast, whereas AGIX-4207 inhibited TNF-α-inducible MCP-1 protein from HAECs, this was not reflected at the level of mRNA for this gene. This suggests that although the mechanism of inhibition by AGIX-4207 on EC gene expression may occur at the transcriptional level for some genes (i.e., VCAM-1), it is also apparent that it exerts post-transcriptional effects on others (i.e., MCP-1). Further experimentation is needed to fully understand the specific nature of the post-transcriptional effect on inhibition of MCP-1 expression.

Although we have not identified the precise molecular target for AGIX-4207 that would explain its anti-inflammatory properties, we have ruled out an effect on the pleiotropic transcription factor NF-κB. This transcription factor regulates the inducible expression of a wide variety of inflammatory and proliferative genes (Balchin, 1996) by multiple activation signals. NF-κB is considered the prototypic redox-sensitive transcription factor. It has been shown that ROS activate, whereas antioxidants inhibit, the activation of NF-κB (Schreck et al., 1991). Our results demonstrate that unlike the thiol antioxidant PDTC, which is a well known inhibitor of TNF-α-mediated NF-κB activation in ECs (Staal et al., 1990; Schreck et al., 1992; Marui et al., 1993), AGIX-4207 does not inhibit TNF-α-inducible nuclear translocation of NF-κB as assessed by both EMSA and immunofluorescence. The lack of effect on NF-κB nuclear translocation has been reported previously with other antioxidant pharmacologic agents (Gerritsen et al., 1995; Wolle et al., 1996; Kunsch et al., 2004). In further attempts to understand the molecular mechanism of AGIX-4207, we conducted a systems biology approach termed Biologically Multiplexed Activity Profiling (Kunkel et al., 2004; Plavec et al., 2004). In this analysis, the effect of AGIX-4207 on more than 30 multiplexed biological readouts in four independent and contextually unique cellular systems was evaluated and compared with a reference database of greater than 50 known anti-inflammatory compounds. These included, among others, inhibitors of MAP kinase pathways, cyclooxygenase, lipooxygenase, phosphatase pathways, glucocorticoids, HMG CoA reductase inhibitors, and cytokine antagonists. AGIX-4207 demonstrated no statistically significant similarities to any compounds in this reference collection (C. Kunsch, unpublished data), suggesting that the mechanism of action of AGIX-4207 is unique relative to many well known anti-inflammatory compounds. Further studies will be required to identify which redox-sensitive signaling pathways are targeted by AGIX-4207 in the regulation of inflammatory gene expression.

In summary, AGIX-4207 displays potent intracellular antioxidant activity and broad anti-inflammatory activity in a variety of cell types. AGIX-4207 selectively inhibits the inducible expression of redox-sensitive inflammatory genes on activated ECs and inhibits the release of multiple cytokines and chemokines from FLSs and PBMCs. The
selective inhibition can be explained, at least in part, by the lack of effect on nuclear translocation of NF-κB. Since NF-κB regulates a multitude of immune response genes, AGIX-4207 should not act as a global immunosuppressive compound. Furthermore, using a systems biology approach, AGIX-4207 displays biological activity that is distinct from multiple known anti-inflammatory compounds, suggesting that the mechanism of action of AGIX-4207 is unique. Taken together, the properties of this novel antioxidant and anti-inflammatory compound provide a mechanistic framework for understanding its antiarthritic activity. These studies provide support for the notion that vascular protection, through maintenance of redox homeostasis and suppression of inflammatory genes, offers an attractive therapeutic approach not only for the treatment of cardiovascular disease but also for other chronic inflammatory disorders such as RA.

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References


Fig. 8. Effect of AGIX-4207 on NF-κB activation. HAEcs were exposed to either PDTC (100 μM) or AGIX-4207 (10 μM) for 1 h followed by the addition of TNF-α for 1 h. Nuclear extracts were prepared and evaluated by EMSA for NF-κB-specific DNA binding (A) or evaluated by indirect immunofluorescence for nuclear staining of the p65(RelA) subunit of NF-κB (B). Data are representative from at least two independent experiments.


