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Novel Phenolic Antioxidants as Multifunctional Inhibitors of Inducible VCAM-1 Expression for Use in Atherosclerosis

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Abstract—A series of novel phenolic compounds has been discovered as potent inhibitors of TNF-α-inducible expression of vascular cell adhesion molecule-1 (VCAM-1) with concurrent antioxidant and lipid-modulating properties. Optimization of these multifunctional agents led to the identification of **3a** (AGI-1067) as a clinical candidate with demonstrated efficacies in animal models of atherosclerosis and hyperlipidemia.

Coronary artery disease (CAD), primarily as a result of atherosclerosis, remains the leading cause of death in industrialized countries. Atherosclerosis[1,2] is a multifaceted disease characterized by vascular inflammation, deposition of lipids in the arterial vessel wall, and smooth muscle cell proliferation resulting in a narrowing of the vessel passages. While drugs that target single mechanisms (e.g., the cholesterol-lowering statins) have proven to be clinically beneficial for the treatment and prevention of CAD,[3] there is an ongoing need for improved drugs that could more effectively address the multiple underlying pathological mechanisms of CAD.[4] In this Letter, we report our successful efforts to combine anti-inflammatory, lipid-modulating, and antioxidant properties into single molecules as potential treatments for CAD.

As early atherosclerotic lesions form, localized endothelial expression of vascular cell adhesion molecule-1 (VCAM-1) leads to the recruitment of monocytes to adhere to the developing lesion.[4,5] Subsequent conversion of monocytes to foamy macrophages results in the synthesis of a wide variety of inflammatory cytokines, growth factors, and chemoattractants such as monocyte chemoattractant protein-1 (MCP-1)[6] that help propagate the formation of mature atherosclerotic plaques. Cytokines, such as TNF-α and IL-1β, in turn further induce the expression of inflammatory response genes including VCAM-1 and MCP-1.[7] A recent study showed that VCAM-1 but not intercellular adhesion molecule-1 (ICAM-1) plays a dominant role in the initiation of atherosclerosis, although both VCAM-1 and ICAM-1 are upregulated in atherosclerotic lesions.[8] Inhibitors that selectively target VCAM-1 expression are likely to have potential as therapeutic agents.

A variety of agents have been reported as inhibitors of VCAM-1 expression or VCAM-1 activity/function.[9–17] Establishing pharmacological proof-of-concept for VCAM-1 modulation as a therapeutic target, a cyclic depsipeptide effectively inhibited VCAM-1 expression and reduced inflammation in a dermal model of inflammation,[11] a monoclonal antibody against VCAM-1 inhibited neointimal formation in a mouse model of arterial wall injury,[12] and a disubstituted 1,4-diazepine diminished the increase in paw thickness in a mouse model of collagen-induced arthritis.[17]

Oxidative stress is also an important contributor to atherosclerosis.[18] In particular, the facile uptake of oxidized low-density lipoprotein (LDL)[19] by macrophages helps convert the recruited monocytes to lipid-engorged macrophage foam cells that form the underlying fatty

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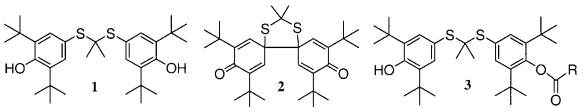
streak of atherosclerosis. Thus, anti-inflammatory compounds that selectively block inducible VCAM-1 expression and also exhibit antioxidant properties should have high potential as anti-atherosclerotic agents.

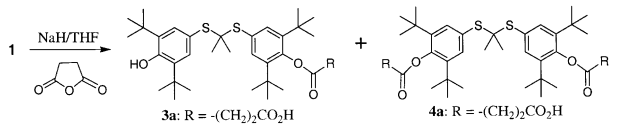
Probucol (**1**) is a modest lipid-lowering agent with powerful antioxidant properties[20,21] that effectively inhibits the oxidative modification of LDL independently of its lipid-lowering effect.[22] The absorption of probucol after oral administration is limited and variable, probably due to its high lipophilicity.[21] While probucol has demonstrated a reduction in the incidence of restenosis in patients,[23] its clinical use in CAD or any other indications is severely limited by observations that it progressively lowers the levels of high-density lipoprotein (HDL)[20] and causes significant prolongation of the QTc interval.[24,25] In addition, several potentially toxic metabolites of probucol have been identified that arise from the highly reactive spiroquinone **2**.[21]



It is well known that the strong antioxidant properties of probucol arise from the two phenol groups flanked by tertiary-butyl moieties.[21] We reasoned that suitably modified monosubstituted compounds **3** might retain the beneficial antioxidant and lipid-lowering properties of probucol through the remaining phenol group but could have an improved safety profile based on their inability to form the spiroquinone metabolite **2**. We also discovered that, in contrast to **1,** several of these monosubstituted compounds **3** were unexpectedly potent and selective inhibitors of TNF-α-inducible VCAM-1 expression on endothelial cells, with demonstrated efficacy as anti-inflammatory agents in several preclinical animal models.[26,27]

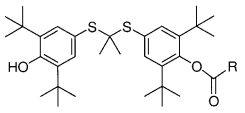
The reaction of **1** with either a suitable acid chloride or anhydride in the presence of base produces a mixture of monosubstituted **3** and disubstituted **4**, that are readily separable by typical column chromatography procedures. As exemplified in Scheme 1, treating **1** with succinic anhydride in the presence of sodium hydride produced the desired monosubstituted compound **3a**[28,29] in 28% yield after chromatography.

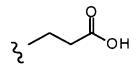
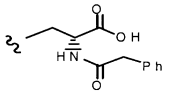
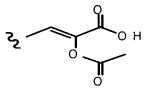
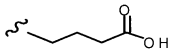
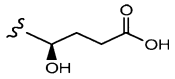
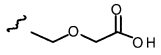
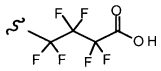
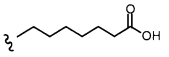
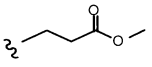
As shown in Table 1, **3a** is a surprisingly potent inhibitor of TNF-α-inducible VCAM-1 expression with an IC_{50} of 6 μM. In contrast, neither probucol (**1**) (IC_{50} > 50 μM) as previously reported,[30] nor the disubstituted ester analogue **4a** (IC_{50} > 50 μM) significantly inhibited the expression of TNF-α-inducible VCAM-1. Although **3a** has only one instead of two phenol groups, it still



Scheme 1.

Table 1. Inhibition of TNF-α-inducible VCAM-1 expression by carboxy-substituted esters[33]



Compd	R	VCAM-1 IC_{50} (μM)
3a		6
3b		> 50
3c		20
3d		17
3e		9
3f		25
3g		37
3h		100
3i		45
3j		40

acts as a chemical antioxidant and is as potent as probucol. Both **3a** and **1** showed comparable inhibitory effects (~85% inhibition at 70 μM) in preventing the oxidation of linoleic acid by 15-lipoxygenase at the same molar concentrations in an LMB (*N*-benzoylleucomethylene blue) assay.[31] Based on these highly encouraging results, additional analogues were evaluated as potential inhibitors of inducible VCAM-1 expression to further explore the structure–activity relationships of this series (Table 1).

The potency of **3a** as an inhibitor of VCAM-1 expression drops slightly when the side chain is extended by one methylene unit as in **3e** and more dramatically when either extended by 4 methylene units as in **3i** or shortened to the oxalate **3b**. Such variations in homologues have been observed in other series and can be related to lipophilicities of the individual compounds.[32] More extensive modifications of the side chain in either **3a** or **3e** through the introduction of polar substituents (**3c** and **3f**), unsaturation (**3d**), multiple fluorine atoms (**3h**), or

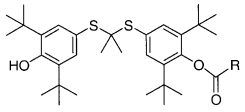
replacement of one methylene unit by an oxygen as in **3g** all reduced their ability to inhibit VCAM-1 expression. Therefore, compound **3a** probably possesses the optimal side chain length for the inhibition of VCAM-1 expression. Interestingly, **3j**, the corresponding methyl ester of **3a**, is significantly weaker as a VCAM-1 inhibitor, thereby further corroborating the important contribution of the free carboxy group in **3a** to its VCAM-1 modulating activity. See below for further discussion. Compound **3f** was prepared by acylation of **1** with (*R*)-5-oxotetrahydrofuran-2-carbonyl chloride in the presence of base followed by hydrolysis of the resulting intermediate lactone.

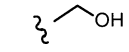
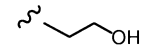
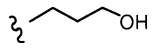
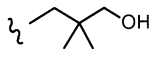
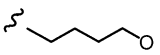
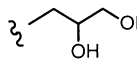
The carboxylic groups of the compounds in Table 1 could all be selectively reduced to a primary alcohol with borane in THF. As shown in Table 2 , the resulting monohydroxyesters **3k**–**3o** are all generally less potent than the corresponding carboxylic acids. However, the potency of **3m** ($IC_{50}=40$ μM) is increased 5-fold when a second hydroxyl group is introduced into its side chain as in **3p** ($IC_{50}=8$ μM). Compound **3p** was prepared by acylation of **1** with 2,3 - diacetoxy - 3 - chlorocarbonylpropionic acid methyl ester in the presence of sodium hydride and subsequent DIBAL-H reduction.

Since there is no carboxylic group in **3p**, the carboxylic group in the compounds in Table 1 is clearly not absolutely necessary for the inhibition of VCAM-1 expression. Rather, the carboxylic group in **3a** or the dihydroxy substituents in **3p** likely bring these compounds into the right lipophilicity range required for activity in the VCAM-1 assay.

The ability of **3a** to inhibit TNF-α-inducible VCAM-1 expression at low micromolar levels prompted us to select it as a prototype for additional testing in order to better define its selectivity and potential for in vivo efficacy. In contrast to its potent inhibition of VCAM-1 expression, **3a** showed no effect on inducible ICAM-1 expression at concentrations up to 10 μM and exhibited reduced potency for inhibiting TNF-α-inducible E-selectin ($IC_{50}=25$ μM) expression. However, **3a** was a potent inhibitor of inducible MCP-1 expression ($IC_{50}=10$ μM). MCP-1 is also highly expressed in human atherosclerotic lesions.[34] Also, **3a** inhibited the proliferation of human aortic smooth muscle cells (~50% inhibition at 5 μM), whereas probucol exhibited no significant effect at concentrations up to 100 μM. Smooth muscle cell proliferation contributes to late-stage formation of atherosclerosis.

Table 2. Inhibition of TNF-α-inducible VCAM-1 expression by hydroxyl-substituted esters[33]



Compd	R	VCAM-1 IC_{50} (μM)
3k		30
3l		30
3m		40
3n		110
3o		55
3p		8

Compound **3a** is stable in solution (85:15 acetonitrile: water) for weeks at room temperature, and **3a** is also stable to mild acid or base and can be stored in the solid state without change for extended periods (years). This extended stability is presumably attributable to the highly hindered ester moiety in **3a**. Preclinical animal studies and human clinical trials also showed that **3a** was not significantly metabolized to probucol. For example, **3a** was the predominant component in plasma following oral administration of [^{14}C]-**3a** in both the dog and rat. Following administrating a single oral dose of [^{14}C]-**3a** to six healthy male volunteers, most of the radioactivity in plasma (94.37–97.84%) was unchanged **3a**. Thus, **3a** is not a prodrug of probucol, and the biological efficacy of **3a** is completely attributable to the parent molecule.

In summary, **3a** exhibits many of the in vitro properties desirable in a molecule to treat atherosclerosis. It functions as a potent antioxidant, selectively and potently inhibits inducible VCAM-1 and MCP-1 expressions, and inhibits human aortic smooth muscle cell proliferation. Indeed, while details of its pharmacological properties will be reported separately,[26,27] **3a** was well-tolerated in animals and lowered LDL cholesterol in mice, hamsters, rabbits and monkeys with neutral or elevating effects on HDL levels after oral dosing.[27] Moreover, **3a** also inhibited the progression of atherosclerosis in the aorta by 94% after oral dosing in cholesterol-fed rabbits and 43–66% in LDL receptor-knockout mice.[26,27] Based on these results, **3a** (AGI-1067) was selected for clinical evaluation. Recently, Phase II studies have demonstrated that AGI-1067 has beneficial effects on the prevention of restenosis in patients without prolongation of the QTc interval.[25]

Acknowledgements

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28. TLC (silica gel, 60:40 ether/hexane + 10 drops HOAc, R*f* = 0.35); Flash Column Chromatography (silica gel, 70:30 hexane:ether to 100% ether); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.61 (s, 2H), 7.43 (s, 2H), 5.38 (s, 1H), 2.97 (t, *J* = 6.8 Hz, 2H), 2.76 (t, *J* = 6.8 Hz, 2H), 1.45 (s, 8H), 1.42 (s, 16H), 1.32 (s, 18H); white to off-white solid, mp 132–134 °C.
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33. Human aortic endothelial cells (90–95% confluent) are incubated with compounds in DMSO and 100 U/mL TNF-α overnight. Upon washing the cells are incubated with anti-VCAM-1 monoclonal antibody and then an anti-mouse IgG/HRP-conjugate. Color is developed using TMB substrate and the reaction is stopped with 2 N sulfuric acid. Spectrophotometric readings are then taken at wavelength 450 nm. For a more detailed procedure, see ref 16.
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