Identification of a Small-Molecule, Nonpeptide Macrophage Scavenger Receptor Antagonist

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ABSTRACT

Class A scavenger receptor (SR-A) antagonists may prevent the initiation of atherosclerosis, because a recent report found that SR-A/apolipoprotein E (apoE) double-knockout mice had 60% smaller lesions than apoE single-knockout littermates. We transfected human embryonic kidney (HEK) 293 cells with SR-A type I or II receptors to find small-molecule antagonists. Uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-AcLDL) showed that among common polyanionic ligands, polyinosine was the most potent, with an IC₅₀ of 0.74 μg/ml, whereas the novel compound (E)-methyl 4-chloro-1,5-dihydro-3-hydroxy-5-oxo-1-(2-thiazolyl)-2H-pyrrol-2-ylidene benzeneacacetate gave an IC₅₀ of 6.1 μg/ml (13 μM). The novel antagonist also inhibited DiI-AcLDL uptake in cultured human peripheral and rat peritoneal macrophages with IC₅₀ values of 21 μM and 17 μM, respectively. With [¹²⁵I]AcLDL as ligand for transfected HEK 293 cells, binding/uptake and degradation at 37°C for 5 h was saturable and selective. In a comparison of both types of receptor, we found no difference between the capacity of SR-AI or SR-AII for either binding or degradation. Polyinosine competed both [¹²⁵I]AcLDL binding and degradation with a Kᵢ of 1 μg/ml, whereas the novel antagonist competed with a Kᵢ of 19 μg/ml (40 μM) and 8.6 μg/ml (18 μM), respectively, for binding and degradation. Saturation binding in the presence of the ionophore monensin indicated that the novel compound behaved as a noncompetitive antagonist and perhaps as an allosteric effector. This is the first report to describe a small-molecule macrophage scavenger receptor antagonist. Utilization of this permanently transfected HEK 293 cell line will allow the identification of more potent macrophage scavenger receptor antagonists, so that their utility as therapeutics for atherosclerosis can be determined.

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ABBREVIATIONS: AcLDL, acetylated LDL; DexSO₄, dextran sulfate; DiI-AcLDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL; EMEM, Eagle’s minimal essential medium; HDL, high-density lipoprotein; HEK 293, human embryonic kidney 293 cells; LDL, low-density lipoprotein; OxLDL, oxidized LDL; poly I, polyinosine; SR-A, class A scavenger receptor; apoE, apolipoprotein E; FBS, fetal bovine serum.
longer a ligand for the LDL receptor, but becomes, instead, a ligand for the macrophage scavenger receptor (Krieger, 1992). The relative importance of SR-A to atherogenesis was demonstrated recently when it was reported that SR-A/apoE double-knockout mice developed 60% smaller atherosclerotic lesions than did single-apoE knockout littermates (Suzuki H et al., 1997). This was the first direct in vivo evidence that suggested that elevated plasma cholesterol may not be as important as arterial wall macrophages for the initiation of atherogenesis and validated the SR-A as a potential target for interventional therapy.

The known ligands of the macrophage scavenger receptor, besides the modified forms of LDL (molecular mass, 2500 kDa), consist of large, polyanionic polymers of indeterminate molecular mass (Brown et al., 1980). Some widely recognized polyanionic ligands include dextran sulfate, fucoidin, and polyinosine (poly I) and polyguanosine but not polyadenine or polycytosine. Polynucleotide binding to SR-As is determined with DiI-AcLDL (Freeman et al., 1991; Penman et al., 1991) as a fluorochrome of cells incubated in the absence of inhibitor. Determinations, and results were expressed as percent total DiI fluorescence of unlabeled cells. To determine cell-associated ligand, DiI-AcLDL fluorescence values (quadruplicate determinations) for 4 h at 37°C. During aspiration of solutions and a Locke’s buffer wash, there was minimal detachment of cells because of their enhanced attachment relative to unmodified 293 cells. Results were quantified with a CytoFluor 2350 fluorescence plate reader at 530 nm excitation/590 nm emission (PerSeptive Biosystems, Framingham, MA). Background fluorescence of unlabeled cells was subtracted from DiI-AcLDL fluorescence values (quadruplicate determinations), and results were expressed as percent total DiI fluorescence of cells incubated in the absence of inhibitor.

Radioligand Assay. Assays of SR-A degradation and binding/ internalization of [125I]AcLDL were adapted from previous studies (Goldstein et al., 1979, 1983; Ashkenas et al., 1993). Briefly, HEK 293 cells transfected with SR-AI or II were seeded at 10⁴ cells/ml in a 24-well dish in EMEM supplemented with 2 mM glutamine, 10% FBS, and 0.4 mg/ml geneticin. After 3 days, medium was replaced with 500 µl of fresh serum-free medium containing 2 mg/ml BSA and [125I]AcLDL at 5 µg/ml, and cells were incubated at 37°C for 5 h. After this suitable period for ligand degradation, cells were removed to a 4°C room. Supernatant was removed into trichloroacetic acid, and the mixture was centrifuged. The supernatant was chloroform extracted to isolate [125I]moniodotyrosine, the degradation product of [125I]AcLDL, and portions were counted to determine degradative activity. Blank plastic wells containing equivalent amounts of ligand without cells were incubated and processed in the same fashion to determine background ligand degradation, which was subtracted from the total counts. Nonspecific binding was determined by incubation with poly I at 100 µg/ml. To determine cell-associated ligand, cell monolayers were washed and incubated at 4°C with ice-cold buffer A containing 150 mM NaCl, 50 mM Tris-HCl, and 2 mg/ml BSA, pH 7.4, to eliminate nonspecifically bound counts. Cells were washed three times rapidly with 1 ml, incubated twice for 10 min each in 1 ml of buffer A, and then washed twice rapidly in 1 ml of buffer A without BSA. After aspiration of all wash buffer, cells were lysed in 0.1 N NaOH and removed to counting vials for determina-
tion of binding/uptake and subsequent protein determination (Pierce BCA assay). Unless otherwise noted, triplicate determinations were made for each data point within an experiment, and n equals the number of experiments performed.

Data Analysis. Data from all ligand-uptake and binding-competition assays were analyzed by nonlinear regression analysis and curve-fitting, using Graphpad Prism, version 2.0 (Graphpad Software, San Diego, CA). Binding and degradation data from saturation [125I]AcLDL experiments were analyzed by MacLIGAND (version 4.97) (Munson and Rodbard, 1980).

Results

DiI-AcLDL Assay. To characterize the specificity of the transfected receptors with known ligands, several polyanionic ligands were tested. As shown in Fig. 1A, poly I was the most effective inhibitor of DiI-AcLDL uptake, with an IC50 of 0.74 μg/ml, followed closely by fucoidin (sulfated polyfucose), with an IC50 of 4.7 μg/ml, and AcLDL, with an IC50 of 11.7 μg/ml. OxLDL was not very potent, with an IC50 of 84 μg/ml. Others (Freeman et al., 1991; Doi et al., 1993) also have seen that OxLDL has less affinity for SR-A, the acetyl LDL receptor. Unmodified LDL showed no inhibition at up to 100 μg/ml, in agreement with studies that have shown that native LDL does not bind to SR-A (Brown et al., 1980). To determine the relationship of molecular weight to inhibitory potency, a variety of molecular weights of the known SR-A ligand, dextran sulfate (DexSO4), were also tested. As shown in Fig. 1B, the ability of DexSO4 to inhibit uptake of DiI-AcLDL improved with increasing molecular weight, with DexSO4 of molecular weight <10,000 being virtually ineffective at up to 100 μg/ml. These results confirm those obtained for DexSO4 inhibition of macrophage uptake of modified LDL (Basu et al., 1979). In contrast, DexSO4 with molecular weights of 50,000 and 500,000 inhibited well and was nearly as potent as poly I, with an IC50 of 1.1 μg/ml (Fig. 1B). Screening of putative novel antagonists was performed in the same 96-well format, with transfected HEK 293 cells being incubated with DiI-AcLDL in the presence of test compounds at a concentration of approximately 30 μM. In this fashion, we identified a novel antagonist (Fig. 2) that closely matched the known inhibitors in potency, with an IC50 of 13 μM (approximately 6 μg/ml) (Fig. 3). In comparison with the polyanionic ligands, the novel antagonist was twice as potent as AcCLDL.

Verification of Novel Antagonist in Macrophages. To verify the use of this transfected HEK 293 cell assay, other cell types that normally express SR-A, such as rat peritoneal macrophages and human macrophages, were also tested. Cultured human macrophages were allowed to mature for 2 weeks, during which time spontaneously floating cells were subcultured into 96-well dishes, where they quickly attached, and were maintained as confluent cultures until assayed as described. We confirmed the novel compound's activity against cultured human macrophages and demonstrated an IC50 of 21 μM (Fig. 3). Because animal models of atherosclerosis ultimately will be used to verify in vivo activity of any novel compound, SR-A antagonist activity against rat peritoneal macrophages was also determined. Figure 3 also shows the inhibition of DiI-AcLDL uptake into rat macrophages with an IC50 of 17 μM, comparable to those obtained with the transfected HEK 293 cells and with human macrophages.

Radioligand Assay. To determine saturability of SR-A receptors in transfected HEK 293 cells, cells were incubated at 37°C with increasing concentrations of [125I]AcLDL up to 50 μg/ml in serum-free medium. Data from representative assays are shown in Figs. 4 and 5. Binding/uptake and degradation of [125I]AcLDL in the transfected 293 cells were saturable processes that showed high affinity and a low nonspecific component. Replicate experiments and MacLIGAND analysis determined an apparent dissociation constant (Kd) of 11.4 μg/ml with a Bmax of 6525 ng/5 h/mg protein for binding/uptake.
uptake (Fig. 4) and an apparent $K_D$ of 5.1 $\mu$g/ml with a $B_{\text{max}}$ of 2680 ng/5 h/mg protein for degradation (Fig. 5). Calculated on a nanomolar basis, using 514 kDa as the molecular mass of the labeled apolipoprotein B (Snyder et al., 1994), the values are an apparent $K_D$ of 22.3 ± 2.3 nM with a $B_{\text{max}}$ of 12.7 ± 1.3 pmol/5 h/mg protein for binding/uptake and an apparent $K_D$ of 10 ± 1.5 nM with a $B_{\text{max}}$ of 5.2 ± 0.5 pmol/mg protein for degradation. These values compare favorably with those reported previously in the literature for both normal cells and for those expressing transfected receptors (Goldstein et al., 1979; Freeman et al., 1991; van Berkel et al., 1991; Ashkenas et al., 1993). The apparent $K_D$ values listed above should be viewed with caution, however, because previous authors have noted that these binding studies are not conducted under equilibrium conditions, but, rather, are conditions of steady-state (see Ashkenas et al., 1993). In addition, because the ligand is large (2500 kDa) relative to the receptor (220 kDa), there may be steric hindrance as saturation is approached, causing curvilinear Scatchard plots (Chappell et al., 1991). We have noted this effect in the transfected cells, yet MacLIGAND analysis usually gives only a single-site fit. Nonetheless, it is felt that these apparent $K_D$ values are comparable for both degradation and binding/uptake (Goldstein et al., 1979; Ashkenas et al., 1993).

In replicate experiments to date with SR-AI-transfected cells, [125I]AcLDL binding/uptake (cell-associated counts) amounted to an average of 1660 ± 60 ng/mg protein ($n = 65$ experiments) when assays were performed at a final ligand concentration of 5 $\mu$g/ml. Similarly, degradation values (supernatant counts) averaged 1010 ± 60 ng/mg protein ($n = 65$). In a limited, direct comparison of both types of scavenger receptor activity ($n = 18$ experiments), we tested either the SR-AI or SR-AII permanently transfected 293 cells. As shown in Fig. 6, there was no statistically significant difference noted between the two receptor types. Therefore, all other results reported here were performed with the type I transfected cells.

**Activity of Known Ligands.** As with the fluorescence-based assay, known SR-A ligands were used to characterize further the specificity of the transfected HEK 293 cell recep-
Fig. 6. Comparison of SR-AI with SR-AII activity in transfected HEK 293 cells. Transfected cells in 24-well dishes were incubated for 5 h at 37°C with [125I]AcLDL at a final concentration of 5 μg/ml, degradation counts were determined by processing aliquots of supernatant, and cell-associated counts were obtained from well-washed cell monolayers (see Experimental Procedures). Degradation (D) averaged 1122 ± 176 ng/5 h/mg protein for SR-AI and 1191 ± 126 ng/5 h/mg protein for SR-AII (n = 18 experiments). Binding (B) averaged 1895 ± 186 ng/5 h/mg protein for SR-AI and 1767 ± 133 ng/5 h/mg protein for SR-AII (n = 18 experiments). Differences between receptors were not statistically significant (Student’s t test).

Fig. 7. Inhibition of [125I]AcLDL binding and degradation in transfected HEK 293 cells by polyanionic ligands. Transfected cells in 24-well dishes were incubated for 5 h at 37°C with [125I]AcLDL at a final concentration of 5 μg/ml, in the presence of increasing concentrations of known polyanionic macrophage scavenger receptor ligands. The inhibition curves are expressed as percentage total binding/uptake (A) or percentage of total degradation (B) of cells incubated in the absence of competitor. Curves are averaged data ± S.E.M. from three experiments performed in triplicate.

Fig. 8. Inhibition of [125I]AcLDL degradation but not binding in transfected HEK 293 cells by trifluoperazine. Assays were performed as in Fig. 7. Results show that trifluoperazine is effective against degradation of [125I]AcLDL with a Ki of 9 μM.
ligand concentration of 5 μg/ml, the novel antagonist inhibited 4°C binding in the transfected HEK 293 cells with an average $K_i$ of 5.8 μM (Fig. 10A), which showed 7-fold greater affinity than at 37°C (Fig. 9). However, inhibition at 4°C was less efficacious than at 37°C. Similar binding inhibition results were obtained at 4°C for the large polyanion, poly I, which gave a $K_i$ of 73 ng/ml, or a 15-fold greater affinity than at 37°C (Fig. 7), while retaining approximately 80% efficacy. Therefore, temperature influenced the inhibitory properties of the small-molecule antagonist to a much greater extent than was seen for the large polyanion, and, therefore, we preferred to perform experiments at physiological temperatures.

We also tested the binding of [125I]AcLDL to the transfected HEK 293 cells in the presence of the ionophore monensin to inhibit ligand degradation and measure ligand binding at 37°C without the uptake component. Previous investigators have proposed the importance of performing experiments at 37°C to maintain the only known ligand-binding site by maximizing hydrophobic interactions within the collagenous and α-helical coiled-coil domains (Doi et al., 1993). Degradation of [125I]AcLDL ligand was inhibited >95% with 10 μM monensin. Replicate experiments and MacLIGAND analysis of [125I]AcLDL binding determined an apparent $K_D$ of 1.9 nM with a $B_{max}$ of 2726 fmol/5 h/mg protein (Fig. 11), values that show nearly 12-fold greater affinity and 80% less binding/uptake, respectively, compared with values obtained in the absence of monensin (Fig. 4). We repeated these saturation experiments with monensin in the presence of the novel antagonist in an attempt to determine the nature of the competitive inhibition. As shown in Fig. 12, MacLIGAND analysis of the binding data obtained in the presence of 50 μM novel antagonist determined that the $B_{max}$ of 1964 fmol/5 h/mg protein was decreased 28%, whereas the $K_D$ of 2.1 nM remained the same, evidence for a noncompetitive type of inhibition.

**Discussion**

The novel antagonist gave qualitatively similar dose/response curves in the transfected 293 cells for both the DiI-AcLDL- and [125I]AcLDL-binding assays, with IC$_{50}$ and $K_i$ values that were close to those of AcLDL and fucoidin.
thermore, the antagonist was an effective inhibitor of SR-A activity in both human monocyte-derived macrophages and in rat peritoneal macrophages. These data suggest that novel antagonists that are identified by transfected cells expressing SR-A's can be anticipated to maintain a functional antagonism in macrophages. In addition, both binding/uptake and degradation of 125I-AcLDL were sensitive to the novel antagonist, whereas weak bases such as trifluoperazine only inhibited degradation. These data suggest an antagonism at the level of the receptor, which is similar to the known polyanionic ligands. Although these results demonstrate that a small-molecule SR-A antagonist is possible, more development obviously is needed to attain more potency, as well as bioavailability and tolerability, before testing in animal models of atherosclerosis.

Nevertheless, it is not clear that we have identified an antagonist that competes at the level of the collagen-like binding site for AcLDL. Evidence from truncation mutants or from point mutations of the SR-A established that the collagenous domain is necessary for AcLDL binding (Acton et al., 1993; Doi et al., 1993). As few as six Gly-X-Y repeats of the collagenous domain have been made into a functional, synthetic SR-A receptor, although AcLDL binds with diminished affinity (Tanaka et al., 1996). However, the collagenous domain may not be the only site to which ligands bind or the only site necessary for ligand binding. Mutational analysis of the α-helical coiled-coil domain also has shown a necessary role for this domain in SR-A function and in ligand dissociation (Doi et al., 1994; Suzuki K et al., 1997). The discovery that the collagenous domain of the SR-A lies alongside the α-helical coiled-coil region at physiological pH (Resnick et al., 1996) also implies a broader potential binding site than what had been predicted formerly by molecular and biochemical analysis alone (Krieger, 1992; Krieger and Herz, 1994). Perhaps many small molecules would have to interact at these regions to block with the same efficacy as a larger, multivalent polyanionic ligand. It is possible that our novel antagonist may have binding sites within the α-helical coiled-coil region as well as within the collagenous domain. Alternatively, it could prevent proper interaction between the two domains. An allosteric type of inhibition could explain our findings of noncompetitive inhibition by the novel antagonist.

It has been acknowledged that binding kinetics for the SR-A are complicated, and there well could be positive cooperativity displayed by a small-molecule antagonist, as additional molecules bind to a stretch of positively charged residues within the putative binding domain (Acton et al., 1993; Doi et al., 1993; Tanaka et al., 1996). Analysis of SR-A binding is also complicated by possible steric hindrance as a very large ligand binds to a receptor that is clustered in coated pits for the purpose of receptor-mediated endocytosis (Chappell, 1991). An allosteric effector would engender an additional level of complexity to these studies. The loss of efficacy by the novel antagonist noted in ligand-binding experiments performed at 4°C may be a result of the physical nature of the charged, collagenous binding domain. This region is temperature-sensitive, because collagen is more soluble at 4°C, whereas it aggregates at 37°C because of strong hydrophobic interactions (Doi et al., 1993). Site-directed mutagenesis of a critical lysine in this region, analogous to Lys335 in humans, is enough to severely abrogate 125I-AcLDL binding at 37°C, presumably because the mutant receptor cannot form the correct binding array to display its positive charges. The above mutated receptor bound 125I-AcLDL at 4°C, however, because a decreased temperature would cause disaggregation of the tight trimer formation, extending the distance between the helices. This altered shape then may have displayed appropriate charges to accommodate the AcLDL particle or large polyanionic molecules for binding at 4°C. However, a small-molecule antagonist of limited molecular mass may not be able to find a suitable binding pocket among the disordered helices, which may only need to change shape to a small extent. As a consequence of the above results, those investigators have emphasized the importance of performing binding studies at 37°C versus 4°C (Doi et al., 1993). A recent report has expanded on the above-cited studies with a broader mutational analysis of basic residues along the entire collagenous domain (Andersson and Freeman, 1998). In this new study, multiple charged amino acid interactions within both proximal and distal regions of the receptor were demonstrated to be required for ligand binding. These mutated residues appeared to be critical for stabilizing receptor conformation at physiological temperatures. As recognized by Andersson and Freeman (1998), multiple interactive residues within the collagenous domain may be essential for cooperativity with the α-helical coiled-coil domain to adopt a closed-jackknife conformation that seems essential for binding (Resnick et al., 1996). A small-molecule allosteric effector could interact among these residues to open the jackknife conformation and inhibit ligand binding. It is expected that the availability of a purified preparation of secreted SR-A would be extremely useful to pursue these questions pertaining to receptor-ligand interactions (Resnick et al., 1996; Andersson and Freeman, 1998).

Plaque regression is a function of the dynamic balance
among initiation, progression, stabilization, and removal of plaque constituents (Schwartz et al., 1992). Although lowering of plasma cholesterol and LDL cholesterol has demonstrated significant patient benefits in a number of clinical trials, the magnitude of angiographic regressive changes is relatively small despite aggressive lipid-lowering regimens. Because atherosclerosis is a progressive disease initiated early in life, even subjects with normal LDL-cholesterol levels eventually will be at risk. Likewise, subjects with normal LDL-cholesterol levels but low high-density lipoprotein (HDL)-cholesterol levels are now thought to be at risk. There is an emerging need for alternative or complementary therapeutic regimens, such as scavenger receptor antagonists, which target a pivotal cellular and molecular mechanism involved in initiation and progression of atherosclerosis. These new modalities will augment the success already achieved with cholesterol-lowering agents alone.

One hypothesis is that scavenger receptor antagonists will prevent the formation of macrophage-derived foam cells and the development of the fatty streak, the initial lesion of atherosclerosis. With the plethora of macrophage receptors that are available to bind modified LDLs such as Fc×RII-B2 (Stanton et al., 1992), CD36 (Endemann et al., 1993), SR-B1 (Acton et al., 1994), CD68 (Ramprasad et al., 1995), and LOX-1 (Sawamura et al., 1997), elimination of only the SR-A class of receptor has been shown to be of benefit in knock-out animal models of atherosclerosis (Suzuki K et al., 1997; Sakaguchi et al., 1998). Furthermore, elimination of SR-A may account for the loss of as much as 80% of the degradation of modified LDL, as observed in vitro with isolated peritoneal macrophages from these knock-out animals (van Berkel et al., 1998). If we can block lipid accumulation within macrophage-derived foam cells by utilizing scavenger receptor antagonists, we may retard plaque progression and reduce vulnerability by initiating plaque regression through the process of “reverse cholesterol transport” to acceptor HDL particles (Brown and Goldstein, 1983). This, therefore, may inhibit further cholesteryl ester accumulation, arrest the progression of atherosclerotic lesions, and favor their regression. Even advanced lesions can regress over time with dietary changes and therapeutic intervention. Macrophage scavenger receptor antagonists may hasten plaque regression in patients that need it most, such as those suffering from ischemic heart disease or stroke, where further lipid accumulation within arterial macrophages would decrease further luminal diameter and possibly precipitate a fatal event. Implications for cholesterol deposition in atherosclerosis. Annu Rev Biochem 52: 223–261.

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