In vivo efficacy of HDL-like nanolipid particles containing multivalent peptide mimetics of apolipoprotein A-I

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Abstract We have observed that molecular constructs based on multiple apoA-I mimetic peptides attached to a branched scaffold display promising anti-atherosclerosis functions in vitro. Building on these promising results, we now describe chronic in vivo studies to assess anti-atherosclerotic efficacy of HDL-like nanoparticles assembled from a trimeric construct, administered over 10 weeks either ip or orally to LDL receptor-null mice. When dosed ip, the trimer-based nanolipids markedly reduced plasma LDL-cholesterol levels by 40%, unlike many other apoA-I mimetic peptides, and were substantially atheroprotective. Surprisingly, these nanoparticles were also effective when administered orally at a dose of 75 mg/kg, despite the peptide construct being composed of l-amino acids and being undetectable in the plasma. The orally administered nanoparticles reduced whole aorta lesion areas by 55% and aortic sinus lesion volumes by 71%. Reductions in plasma cholesterol were due to the loss of non-HDL lipoproteins, while plasma HDL-cholesterol levels were increased. At a 10-fold lower oral dose, the nanoparticles were marginally effective in reducing atherosclerotic lesions. Intriguingly, analogous results were obtained with nanolipids of the corresponding monomeric peptide.

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Despite the widespread use of lipid-lowering drugs and changes in lifestyle, cardiovascular disease remains the major cause of death in developed countries. As a consequence, there has been intense interest in new therapeutic approaches, such as those that increase plasma HDL levels and/or improve the function of HDL (1–3). HDL is crucial in protecting against the buildup of fatty plaques in the arteries, which is a major contributor to cardiovascular disease (1–3). Treatments for atherosclerosis involving iv infusions of reconstituted HDL (rHDL) particles or apoA-I, the major protein component (~70%) of HDL, have provided compelling evidence for protective effects in animal models (4–8), albeit with more mixed results in humans (3, 9, 10). Indeed, while a promising opportunity exists for medical advances in combating atherosclerosis through the modulation of HDL, recent findings engendered some controversy about this approach. For example, in clinical trials with niacin or cholesterol ester transfer protein (CETP) inhibitors (dalcetrapib and torcetrapib) there was a lack of cardiovascular benefit, although total plasma HDL levels were increased (11–13). In addition, the viewpoint from epidemiological data that higher total plasma HDL levels lower the risks for cardiovascular disease was undermined by a recent meta-analysis (14). Thus, a mounting collection of information (15–17) suggests that elevating HDL levels is insufficient in protecting against atherosclerosis. This issue becomes more complex in that one must consider the importance of HDL functional properties (13, 15–17), including specific subtypes of HDL particles or specific HDL functional properties (18–20).

The use of rHDL or apoA-I as therapeutic agents may be impeded by high cost, complex manufacturing processes, and lack of oral administration. Further, their compositions...
are not conducive to traditional medicinal chemistry manipulations to optimize HDL-like function. Thus, there has been considerable interest in employing a design approach that involves shorter peptides that mimic apoA-I (21). From numerous studies on apoA-I mimetic peptides based on one or two amphiphilic α-helical segments (22–26), a wide range of peptide sequences have been found that are more-or-less effective in various aspects of apoA-I mimicry. Many of these peptides have no sequence homology to apoA-I, as they capitalize on an amphiphilic α-helical structural motif, and some are composed of all D-amino acids (21). Although in vivo efficacy has been demonstrated for certain peptides in animal models of atherosclerosis, their mechanisms of action remain the subject of active research (27–33). A leading hypothesis is that the peptides stimulate reverse cholesterol transport (RCT), in a manner similar to apoA-I, by promoting cholesterol efflux from macrophage cells. Available evidence suggests that the peptides bind to lipoproteins (especially HDLs) in vivo (29–31), and work in concert with endogenous apoA-I to improve the function of HDL (32, 33). Plasma total cholesterol lowering does not appear to be a general or primary mechanism, because some mimetic peptides that were shown to be atheroprotective in animals did not significantly affect plasma lipid levels (34, 35); further, two mimetic peptides with similar cholesterol-reducing properties had different atheroprotective effects (36). Other proposed mechanisms for these peptides include anti-inflammatory and anti-oxidant effects, as well as the binding of oxidized or pro-atherogenic lipids, such as lysophosphatidic acid (LPA), in the plasma or intestine (37–39).

We recently reported on the development of branched multivalent apoA-I mimetic constructs, in which multiple copies of a modified α-helical segment derived from apoA-I were appended to a scaffold (31). In spite of their abiotic structures and distinctly different molecular topology from the linear arrangement of helical segments in the native protein, these branched, multivalent constructs bind lipids to generate HDL-like nanoparticles, exchanged into native HDLs in human plasma, promoted cellular cholesterol efflux, and induced remodeling of large mature HDLs to smaller lipid-poor (pre-B) particles. The lipid nanoparticles derived from the multivalent constructs were superior to those from either the corresponding monomeric parent peptide or a monomeric 4F reference peptide in all functional aspects tested (31). The multivalent peptides showed impressive stability toward proteolytic digestion and had long plasma half-lives in mice. As such, we hypothesized that our multivalent apoA-I mimetics would exhibit improved atheroprotection compared with the parent monomeric peptide. In this report, we describe results of chronic in vivo efficacy studies in LDL receptor-null (LDLr−/−) mice, a standard mouse model of atherosclerosis, for nanoparticle formulations of the trimeric peptide construct and the parent monomeric peptide. Administration of a nanolipid formulation of the trimeric construct, either iv or po, reduced plasma cholesterol and atherosclerotic lesions in the mice to a degree comparable with the most effective apoA-I mimetics previously reported (and administered parenterally) (21). Oral efficacy was observed in spite of the peptide segments being comprised of l-amino acids, which would make them vulnerable to rapid proteolytic degradation. This nanolipid material, containing a multivalent peptide, provides a new approach for the development of orally efficacious agents to manage atherosclerosis.

MATERIALS AND METHODS

Synthesis of multivalent constructs

All peptide native-ligation reactions involved a 1.5-fold excess of purified peptide relative to the number of thiosters in the scaffold. Ligations were performed in 200 mM MOPS buffer containing 7 M guanidine hydrochloride (Gdn·HCl), 100 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.5, at room temperature for 6–12 h. At completion of the reaction, iodoacetamide (~50-fold excess relative to Cys) was added to cap the free thiol moieties on Cys residues. After 5 min, CF3CO2H was added to quench the reaction, and the product was purified by reverse-phase HPLC. For detailed synthetic protocols for the peptide constructs, see our prior report (31).

Preparation of vesicles and peptide-lipid nanoparticles

(9R)-(+)1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was dissolved in 0.5 ml of CHCl3 in a test tube and dried into a thin film by blowing N2 gas into the tube while vortexing. The lipid film was further dried under reduced pressure overnight in a desiccator. Multilamellar vesicles (MLVs) were obtained by suspending the dried lipids into PBS [10 mM phosphate (pH 7.4), 136 mM NaCl] with vortexing and/or sonication. MLVs were typically prepared at concentrations of 10 mM. To prepare peptide-lipid nanoparticles, a stock solution of peptide in PBS was added to 10 mM MLVs at a 1:10 (helix:lipid) molar ratio, and the solutions were vigorously stirred for 24 h at 22°C. The DMPC unilamellar vesicles (ULVs) (18 mM) were made by extrusion DMPC MLVs through 0.2 μm Nuclepore track-etched membranes (Whatman) in the Avanti mini extruder, and sterile filtered before ip administration to mice.

In vivo efficacy

All procedures involving live animals were approved by the Scripps Research Institute Institutional Animal Care and Use Committee. LDLr−/− mice were fed a chow diet until they were 10 weeks old, when they were switched to a high-fat diet (HFD) (Harlan Teklad 94059). At the time that the HFD was started, peptide/DMPC nanoparticles were administered by daily ip injection or by the oral route ad libitum in the drinking water for 10 weeks in the continued presence of the HFD. Mice receiving ip injections of PBS or DMPC ULVs served as controls for the ip groups. Mice receiving drinking water containing 1% sucrose/PBS or DMPC MLVs served as controls for the oral groups. The mice were bled after an overnight fast (~15 h) after 2 weeks of treatment and at the time of harvest (10 weeks); the plasma was used to determine lipoprotein profiles, biomarkers including total cholesterol levels, triglycerides, plasma serum amyloid A (SAA), plasma 15(S)-HETE levels, and plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations.

Atherosclerosis in the aorta from the proximal ascending aorta to the bifurcation of the iliac artery was assessed as described previously (40). Briefly, the dissected aorta was pinned flat on black wax, stained with Sudan IV, and digitally photographed at a
fixed magnification. Total aortic areas and atherosclerotic lesion areas were calculated by using Adobe Photoshop CS4, Chromatica V, and National Institutes of Health Scion Image software. Results are reported as lesion area as a percentage of total en face aortic area. As a second assessment of atherosclerosis, lesions of the aortic root (heart sinus) were analyzed. Briefly, hearts were fixed, frozen, and sectioned on a Leica cryostat. For each aortic sinus cusp, sections were collected from the beginning of the sinus for a distance of 500 μm into the sinus. Sections (10 μm thick) were stained with oil red O and counterstained with Gill hematoxylin 1 (Fisher). Stained sections were photographed and digitized. Lesion volume in the first 500 μm of each cusp was estimated from four sections spaced at 140 μm. Lesion volume was calculated from an integration of the measured cross-sectional areas.

**Peptide plasma concentration**

Female LDLr−/− mice were administered peptide/DMPC for 10 weeks as described above. On weeks 2 and 6, the monomer and trimer concentration in the plasma of randomly selected mice was measured 2 h and 4 h after ip administration, respectively, by LC-MS in the selected ion monitoring (SIM) mode. These times correspond to the respective tmax values for the peptide agents, as determined previously (31). Details of LC-MS SIM quantification methods are described in our previously published study (31).

**Plasma biomarker (cholesterol, triglyceride, SAA, and 15-HETE) analysis**

Plasma total cholesterol and triglyceride were determined using a cholesterol Amplex assay kit (Invitrogen) and the triglyceride quantification kit (BioVision), respectively. Plasma SAA and 15-HETE levels were measured by using a SAA Mouse ELISA kit (Life Technologies) and a 15(S)-HETE EIA kit (Cayman), respectively, following the manufacturer’s instructions.

**Plasma liver enzyme activities**

At the time of harvest (10 weeks), blood (~0.5 ml) was collected by cardiac puncture into EDTA anti-coagulant-coated tubes, and centrifuged at 4°C for 10 min at 5,000 rpm. Plasma samples were stored at −80°C until analysis was conducted. Plasma ALT and AST concentrations were measured using Infinity ALT (GPT) and AST (GOT) liquid stable reagents, respectively, which is a colorimetric kinetic assay (Thermo Scientific). Assays were performed in accordance with the manufacturer’s recommendations, adjusting the reagent volumes (20 μl plasma + 200 μl reagent, 0.69 cm light pathlength of the solution in the well) for analysis of samples in 96-well flat bottom microplate format.

**Measurement of liver cholesterol and triglyceride levels**

Lipid extracts of liver tissue were assayed for cholesterol and triglyceride according to the manufacturer’s protocols using the cholesterol Amplex assay kit (Invitrogen) and the triglyceride quantification kit (BioVision), respectively. Briefly, liver tissue was homogenized in 5% NP-40 in water (1:15 w/v). Samples were slowly heated to 80°C for 10 min. Insoluble materials were removed by centrifugation (13,000 rpm, 10 min). Cholesterol and triglyceride concentrations in the supernatant were determined by the enzyme based fluorometric assays.

**Artificial micelle precipitation assay**

Peptides and peptide-DMPC nanoparticles were tested for interference on cholesterol solubility in artificially prepared micelles. Peptide-DMPC nanoparticles were prepared as described above, and purified by size-exclusion chromatography (SEC) using disposable Sephadex G-25 columns (Illustra NAP-25 columns, GE Healthcare). Solutions of peptides and purified peptide-DMPC nanoparticles were further concentrated using Amicon centrifugal filters (Millipore) with 3 K and 10 K molecular weight cut-offs, respectively. Neomycin and cholesteryamine were used as positive control compounds, as these compounds are known to inhibit intestinal cholesterol absorption by precipitating micellar lipids and sequestrating bile acids, respectively (41–44).

Artificial micelles were prepared according to a previously published method with minor modifications (45, 46). Briefly, lipids (0.5 mM cholesterol, 1 mM oleic acid, and 2.4 mM phosphatidylcholine [from egg yolk]) were dissolved in methanol in a test tube and dried into a thin film by blowing N2 gas into the tube while vortexing. The lipid film was further dried under reduced pressure overnight in a desiccator before adding PBS [10 mM phosphate (pH 7.4), 136 mM NaCl] containing 6.6 mM taurocholate salt. The suspension was sonicated for 1 h and shaken overnight at 37°C. The micelle solution was filtered through a 0.2 μm syringe filter. Each compound, or PBS as negative control, was added to the micelle solution at a volume ratio of 1:4, and incubated for 1 h at 37°C. The solution was then centrifuged at 14,000 rpm for 20 min. The supernatant was collected for the determination of cholesterol and bile acid concentrations by using a cholesterol Amplex assay kit (Invitrogen) and a total bile acids assay kit (Diazyme), respectively.

**Characterization of gut flora by quantitative real-time PCR**

Quantitative real-time PCR (qPCR) was performed with universal and group-specific bacterial primers (see supplementary Table I for primer sequences) targeting the 16S rRNA genes of two major intestinal bacterial phyla, Bacteroidetes and Firmicutes (47–49). Bacterial genomic DNA was extracted from snap-frozen cecum and colon contents by using FastDNA Spin kit feces (MP Bio) according to the manufacturer’s instructions. The abundance of total and specific intestinal bacterial groups (Bacteroidetes, Firmicutes) was measured by qPCR by using universal and group-specific 16S rRNA gene primers (supplementary Table I) and the iTaq Universal SYBR Green Supermix (Bio-Rad). We performed qPCR on a CFX Connect real-time PCR detection system (Bio-Rad). Bacterial DNA was quantified using standard curves constructed with reference bacteria (supplementary Table I) specific for each bacterial group analyzed.

**Statistical analysis**

Data are expressed as the mean ± SD. The difference between groups was assessed by using the one-way ANOVA with a post hoc Tukey-Kramer test, with Prism software (version 5.0d). We considered P < 0.05 as statistically significant.

**RESULTS**

**Peptide agents and HDL-like nanoparticles**

The amino acid sequence used in our study (31) was a conservatively modified derivative of helix 10 of human apoA-I (residues 221–241), which is required in the native protein for lipid binding and cholesterol efflux. A Cys-Gly dipeptide was appended to the N terminus to allow for synthesis of the multivalent constructs via native chemical ligation. We made two amino acid substitutions by replacing Val-227 with Ala to increase amphipathicity and Tyr-236...
Encouraged by these results and the impressive in vitro proteolytic stability of the trimer nanoparticles (31), we carried out further cholesterol-reduction studies by using an oral route of administration. The peptide/DMPC nanoparticles were provided ad libitum in the drinking water, by dissolving them in PBS at concentrations such that each mouse would receive on average a 75 mg/kg dose of peptide over the course of 1 day. Fresh water was prepared every 2 days, and we confirmed by HPLC analysis that the peptide materials did not degrade in PBS over this time period. After 2 weeks of oral administration, the trimer nanoparticles (n = 10) reduced plasma total cholesterol levels by 43%, compared with the PBS control (n = 15) (Fig. 2A). As with the ip administration, SEC lipoprotein fractionation indicated that VLDL and LDL, but not HDL, levels were reduced in the animals (Fig. 2B). On the contrary, peptide treatment caused an increase in observed HDL-cholesterol levels compared with the PBS control (Fig. 2B, inset). An additional control group administered DMPC liposomes in the drinking water (n = 8) had unchanged plasma total cholesterol levels compared with the PBS group (Fig. 2). At a 10-fold lower oral dose (∼7.5 mg/kg/day) over a 2 week period, the trimer nanoparticles (n = 7) reduced total plasma cholesterol by 19% compared with the PBS control (n = 15) (Fig. 2).

A nanolipid formulation of the corresponding monomeric peptide was also studied according to the above in vivo protocol. Two weeks of daily 40 mg/kg ip injections of monomer nanoparticles (n = 5) reduced plasma total cholesterol levels by a surprising 30%, compared with the PBS control (n = 5 or 7) (supplementary Fig. 1), despite having an inferior plasma residency compared with the trimer (area under the curve = 110 ± 10 and 500 ± 40 μM·h for monomer and trimer, respectively) (31). Even more surprising, oral administration of the monomer nanoparticles (n = 10) for 2 weeks reduced plasma total cholesterol levels by 32% compared with the PBS control (n = 15) (Fig. 2A). It is remarkable that oral administration of the monomer formulations effectively diminished cholesterol levels in vivo, considering that the simple linear peptide synthesized from all L-amino acids would be expected to undergo rapid digestion in the gut. Plasma pharmacokinetic (PK) levels of both the monomer and trimer peptide were below the limit of detection (0.5 and 0.2 μM for monomer and trimer, respectively) during the oral administration studies.

### In vivo reduction of atherosclerotic lesions

The results in our cholesterol-reduction studies suggested that the trimer and monomer nanolipids would have potential as atheroprotective agents. Thus, we sought to establish the effect of the peptide/DMPC nanoparticles on the development of atherosclerotic plaques in the LDLr−/− mice. Groups of animals were treated for 10 weeks via daily ip injections (∼40 mg/kg) or oral administration ad libitum in the drinking water with nanolipid formulations of trimer and monomer (∼75 mg/kg/day). The 40 mg/kg value for the ip route is an average daily

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**In vivo reduction of plasma cholesterol**

To determine the efficacy of the peptide-containing nanolipids in an animal model of atherosclerosis, we carried out a series of atheroprotection studies with the LDLr−/− mouse model, which has a human-like lipoprotein profile and is a widely used animal model for this purpose (50, 51). The mice were fed a chow diet until ∼10 weeks of age, at which time they were switched to a HFD (1.25% cholesterol, 15.8% fat, and no cholate), whence treatments with the peptide/DMPC nanoparticles were commenced. After 2 weeks of daily 40 mg/kg ip injections, treatment with the trimer nanoparticles (n = 5 or 8) reduced plasma total cholesterol levels by 30–40% compared with the PBS control (n = 5 or 7), as reported in our prior publication (31) (supplementary Fig. 1). When the mice dosed for 2 weeks with the trimer nanoparticles were kept for an additional 2 weeks without any further treatment (“wash out”), their plasma cholesterol levels rose to match those of the PBS control group (supplementary Fig. 1A). Thus, the 23-mer peptide construct had just a short-term reversible effect on plasma cholesterol levels. As determined by SEC fractionation of pooled plasma samples, the reductions in plasma total cholesterol stemmed mainly from reduced levels of VLDLs and LDLs (supplementary Fig. 1C).

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**Fig. 1.** Schematic illustrations of the trimeric apoA-I mimetic construct and the parent monomer peptide. In the final test compounds, the sulfur atom of the N-terminal Cys residues was capped with CH₂C(O)NH₂.
Fig. 2. Oral administration of the trimer and monomer DMPC nanoparticles reduced plasma VLDL and LDL levels in 10-week-old female LDLr−/− mice fed a HFD. A: Two week daily 75 mg/kg oral administration (ad libitum in the drinking water) of the trimer/DMPC nanoparticles (n = 10) or monomer/DMPC nanoparticles (n = 10) reduced the plasma total cholesterol levels of the mice compared with PBS (n = 15) or DMPC ULV (n = 8) controls. A 10-fold lower dose of the trimer/DMPC nanoparticle also significantly lowered plasma cholesterol levels, albeit to a lesser degree. Individual data points are shown along with the mean ± SD; ns, not significantly different; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 by one-way ANOVA, post hoc Tukey-Kramer test. B: Pooled plasma from all mice in the PBS, monomer, and trimer groups was fractionated via SEC to analyze the lipoprotein profile. The majority of cholesterol reductions observed for the peptide-treated animals were due to decreases in the VLDL and LDL levels, as indicated by the SEC 280 nm absorbance trace (top) and a determination of cholesterol levels in the SEC fractions (bottom). HDL-cholesterol levels were higher in the peptide-treated groups than in the PBS control group (see inset).

dose over the 10 week period, accounting for dosing the animals with a constant daily quantity of peptide (not adjusted for mouse body weight). A schematic diagram of the study design is presented in Fig. 3. On weeks 2 and 6 of the study, the trimer and monomer concentration in the plasma of randomly selected mice was measured 4 h or 2 h, respectively, after ip administration (Fig. 4). These times correspond to the respective t\textsubscript{max} values for the peptide agents, as determined previously (31). The observed peptide concentrations on weeks 2 and 6 were 22 ± 6 μM (n = 5) and 22 ± 3 μM (n = 10) for the trimer and 12 ± 4 μM (n = 5) and 12 ± 3 μM (n = 10) for the monomer, respectively; these values were consistent with our earlier PK studies (31). At the completion of the study, whole aorta atherosclerotic lesions were reduced in the ip cohort by 49% for the monomer nanoparticles (n = 12) and by 55% for the trimer nanoparticles (n = 15) compared with the PBS control (n = 10) (Fig. 5; see supplementary Fig. II for all whole aorta images). Similarly, lesion volumes in the aortic sinus were reduced by 50 and 61% by daily ip dosing with the monomer and trimer nanoparticles, respectively (Fig. 5; see supplementary Fig. III for all aortic sinus cross-section images). No significant differences were observed between the four groups regarding food intake, water intake, spleen weight, liver weight, whole animal weight (supplementary Fig. IV), or plasma liver enzyme levels (supplementary Fig. V), consistent with an absence of toxicity.

In the oral cohort, the trimer/DMPC nanoparticles (n = 10; ~75 mg/kg/day) reduced the development of whole aorta atherosclerotic lesions by 58% compared with the PBS control (n = 18) and by 55% compared with the DMPC liposome control (n = 8) (Fig. 6; see supplementary Fig. VI for all whole aorta images). Lesion volumes in the aortic sinus were strikingly reduced, by 71 and 64% compared with the PBS and DMPC controls, respectively (Fig. 6; see supplementary Fig. VII for all aortic sinus cross-section images). Surprisingly, the monomer/DMPC nanoparticles (n = 10; ~75 mg/kg/day) likewise reduced whole aorta and aortic sinus lesions compared with the PBS and DMPC liposome controls by 49–74% (Fig. 6, supplementary Figs. VI, VII). No significant differences were observed between the four groups regarding food intake, water intake, spleen weight, whole animal weight, or plasma liver enzyme levels; however, liver weights were increased in the orally dosed peptide-treated groups (supplementary Figs. VIII, IX). To determine whether the increased liver weights were due to an accumulation of lipids in the liver, we measured cholesterol and triglyceride levels in liver homogenates from the mice. Interestingly, the levels of both lipids were reduced in the peptide-treated animals compared with the controls (supplementary Fig. X). Plasma PK levels of both the monomer and trimer peptide were below the limit of detection (0.5 and 0.2 μM for monomer and trimer, respectively) during these studies.

To further define the oral efficacy of the trimeric construct, we carried out a 10 week drinking water study at a 10-fold lower dose (n = 8; ~7.5 mg/kg/day). In this group, 2 week plasma cholesterol levels were reduced by 24% (Fig. 2), 10 week whole aorta lesion areas were not significantly changed (Fig. 6), and 10 week aortic sinus lesion volumes were reduced by 50% compared with the controls (Fig. 6). In the low-dose trimer group, no significant differences were
We have previously conducted research to systematically evaluate the importance of multivalency in the function of synthetic apoA-I-like materials, especially their effects on native HDL particle distribution, cholesterol disposition, and in vivo stability (31). Our positive results with certain multivalent peptide constructs encouraged us to undertake in vivo efficacy studies in LDL receptor-deficient mice, a standard animal model observed from controls regarding food intake, water intake, spleen weight, whole animal weight, or liver weight (supplementary Fig. VIII).

**Analysis of plasma biomarkers from chronic efficacy studies**

To explore the mechanism of action of the peptides, we determined the levels of several plasma markers taken after 10 weeks of treatment. Interestingly, plasma cholesterol and triglyceride levels were reduced by 10 weeks of ip peptide nanoparticle administration, but no significant differences were observed for these lipids in the orally treated animals (Fig. 7), despite both routes reducing the development of atherosclerotic lesions. Thus, both routes of administration initially reduced plasma cholesterol levels (after 2 weeks of treatment) (Fig. 2, supplementary Fig. I), but by 10 weeks of treatment, the cholesterol levels in only the ip group remained depressed relative to controls. The plasma SAA level, which is an indication of systemic inflammation, was significantly reduced by oral administration of both the peptide nanoparticles (Fig. 7). In the ip treatment regime, the monomer nanoparticles significantly reduced the plasma SAA level compared with DMPC, but the reduction brought about by the trimer nanoparticles was not statistically significant (supplementary Fig. XI). The level of plasma 15(S)-HETE, an oxidized metabolite of arachidonic acid, was not affected by either ip or oral administration of the peptide nanoparticles (Fig. 7, supplementary Fig. XI).

**DISCUSSION**

We have previously conducted research to systematically evaluate the importance of multivalency in the function of synthetic apoA-I-like materials, especially their effects on native HDL particle distribution, cholesterol disposition, and in vivo stability (31). Our positive results with certain multivalent peptide constructs encouraged us to undertake in vivo efficacy studies in LDL receptor-deficient mice, a standard animal model. 

**Fig. 3.** Schedule of in vivo studies. Female LDLr<sup>−/−</sup> mice were used as the animal model.

**Fig. 4.** Plasma concentrations of trimer and monomer species on weeks 2 and 6 of 10 week ip plaque prevention studies. The monomer and trimer concentration in the plasma of randomly selected mice was measured by LC-MS SIM 2 h or 4 h after ip injection, respectively. These times correspond to the respective <i>t</i><sub>max</sub> values for the peptide agents, as determined previously (31). Data are shown as mean ± SD.
model of atherosclerosis. The work reported herein explored such peptide-based HDL-like nanoparticles in LDLr−/− mice fed a HFD for effectiveness in reducing plasma cholesterol and protecting against atherosclerosis.

We had observed enhanced proteolytic and in vivo stability for the trimer construct, which would be expected to provide a functional advantage over the monomer (31). Nevertheless, we decided to conduct parallel studies with both constructs to have a basis for comparison. This experimental approach required scaling up the synthesis of the trimer and monomer to supply gram quantities for the chronic in vivo efficacy studies. We were surprised to find that both nanoparticles, administered ip as nanolipid formulations, were similarly efficacious in lowering plasma cholesterol and reducing the development of atherosclerosis. Remarkably, we also found that the nanoparticles of both peptides were effective when administered orally ad libitum in drinking water, despite having undetectable plasma concentrations. Thus, plasma level and duration are apparently not important determinants of anti-atherogenic activity for these peptides, at least under the experimental conditions employed. These findings point to the possibility of two different in vivo mechanisms of action, one based in the plasma compartment and one based in the gastrointestinal (GI) tract. In this regard, recent studies (37, 38, 52, 53) involving the 4F and 6F apoA-I mimetic peptides suggest that the intestine may be a key site of action.

An important observation of our studies is that the multivalent construct and monovalent peptide, which contain a peptide sequence that is completely unrelated to the apoA-I mimetic 4F peptide, behave so similarly to 4F in terms of in vivo efficacy in the absence of measurable plasma concentrations. Our findings provide further evidence
VLDL-cholesterol lowering may not require direct binding of the peptide material to those lipoproteins. Some or all of the observed reductions in atherosclerotic lesions may result from plasma lipid lowering. However, it is difficult to reconcile this hypothesis with the intriguing observation that the oral route of administration was similarly atheroprotective to the ip route, despite cholesterol and triglyceride levels only being lowered at the early (2 week) time point for the orally dosed groups. We are working to understand the different time courses of lipid lowering promoted by the peptide agents when administered by different routes. A related question is whether other apoA-I mimetics, when given orally, lower LDL-cholesterol levels in the initial few weeks of administration but not over longer time periods.

A second difference between the peptides described here and the 4F/6F peptides is their disparate modulatory effect on oxidized fatty acids in vivo. Recently emerging data for 4F/6F have pointed to a mechanism of atheroprotection in which the peptides modulate the level of oxidized phospholipids and fatty acids to reduce inflammation (37, 53, 59). Consistent with this hypothesis, these peptides were found to reduce markers of systemic inflammation, such as SAA, and the levels of oxidized lipids and fatty acids, such as 15-HETE, in the intestines and plasma (37, 53, 59). Although our monomer and trimer peptide nanoparticles did reduce the plasma level of SAA (both peptides reduced SAA levels by oral administration, while
only the monomer caused a reduction by ip injection), plasma 15(S)-HETE was not affected (Fig. 7). Future mechanistic studies will be needed to establish the affinity and potential modulatory effects of our peptides on oxidized phospholipids or fatty acids, to clarify the similarities and differences in their function with that of other apoA-I mimetic peptides.

It is a striking result that our trimer and monomer peptides, synthesized from l-amino acids, exhibited marked in vivo efficacy when administered orally. Especially surprising was the frank anti-atherosclerotic efficacy of the monomeric peptide, because it is not resistant to degradation, unlike the trimer (31). Basically, the monomer, as a simple linear peptide comprised of native l-amino acids, would not be expected to survive in the GI environment. However, there are previous suggestions from the literature that apoA-I mimetic peptides comprised of l-amino acids can exert biological effects on oral administration. The 4F peptide, when administered orally together with niclosamide as means of protecting the peptide from degradation, improved the HDL-inflammatory index in apoE-null mice (60). Even in the absence of niclosamide, a relatively high dose of 4F (100 mg/kg) administered to mice in chow significantly decreased plasma LPA levels (61). Most recently, the 6F peptide, produced in transgenic tomatoes and fed to LDLr−/− mice fed a HFD, resulted in improved biomarkers, lower levels of plasma cholesterol, and a reduction in atherosclerotic lesions (52, 53). The lipid nanoparticle formulation used in the present work may have played a role in protecting our peptides from destruction in the GI tract.

One factor that could be responsible for the observed similarity in efficacy of our trimer- and monomer-based nanoparticles, despite the trimer exhibiting better PK properties (31), is the dosing regimen. Perhaps, the doses used in our studies were sufficiently high to mask differences in efficacy, that is, the animals may have been overdosed. To investigate this possibility, we evaluated the trimer and monomer nanoparticles at a 10-fold lower dose (7.5 mg/kg ip) in 2 week-dosing experiments. As expected, the magnitude of cholesterol reduction was less at the lower dose (Fig. 2); however, both agents again reduced plasma cholesterol levels to a similar degree, suggesting that overdosing was not a factor at the 75 mg/kg dose level.

We observed some surprising effects of DMPC treatment compared with the PBS controls when the agents were administered by ip injection. At the two-week time-point, the DMPC unilamellar vesicles modestly increased the level of plasma total cholesterol compared with PBS controls (supplementary Fig. I), although there was no difference between these groups in plasma cholesterol or triglyceride levels after 10 weeks (Fig. 7). Further, ip DMPC apparently reduced the development of en face aortic lesions in the LDLr−/− mice, while the development of aortic root lesions was not affected by ip DMPC treatment (Fig. 5). In the analogous studies involving oral administration, DMPC MLVs did not affect the development of lesions or plasma lipid levels to any degree compared with PBS controls. There are some previous indications that DMPC can reduce the development of atherosclerosis in vivo (62, 63). In view of these data, it is possible that the lipid component of the nanoparticles is contributing somewhat to the observed atheroprotective effects. However, these contributions would appear to be minimal, based on the absence of effects in the aortic root upon ip administration and a lack of any effects with oral administration.

Liver weights increased in the peptide-treated animals (supplementary Figs. IV, VIII), without an associated increase in plasma liver enzymes (supplementary Figs. V, IX). In light of the reduced plasma total cholesterol levels in these animals, we determined lipid levels in liver tissue samples to test the hypothesis that excess cholesterol had been removed from plasma and deposited in the liver. Instead, we found that cholesterol and triglyceride levels were lower in the livers of treated animals (supplementary Fig. X). Enlarged livers can be a sign of inflammation; however, the treated animals exhibited generally reduced levels of plasma SAA, which indicates lower systemic inflammation. It remains unclear why liver size was increased by treatment with the peptide nanoparticles.

It is important to understand the possible mechanisms behind the cholesterol lowering and atheroprotection. Previously, we showed that our peptides promote remodeling of HDLs to lipid-poor particles in vitro and in vivo (parenteral dosing), and induce cellular cholesterol efflux in vitro (31). Therefore, the mechanism of anti-atherogenicity could involve the promotion of RCT, at least in part. However, in the case of oral administration, it is difficult to reconcile a RCT mechanism operating in the vascular system with the virtually undetectable plasma concentrations of the peptides. Further, the monomeric peptide was 3-fold less effective than the trimeric construct in promoting cholesterol efflux in vitro (31), but both agents were similarly efficacious in vivo in preventing atherosclerosis. An alternative consideration is that the peptides act in the intestine, as suggested previously for the 4F and 6F peptides (37, 38, 52, 53), and one can propose several possible mechanisms. A first possibility is that the peptide nanoparticles are remodeling the maladaptive composition of the gut microbiota associated with a HFD (64–73) to yield a rebalanced bacterial community that would ultimately reduce the progression of atherosclerosis. To test this hypothesis, we measured by qPCR the number of 16S rRNA gene copies (47–49) for a number of representative gut bacterial strains in both the vehicle-treated and peptide nanoparticle-treated animals (supplementary Table I). No significant differences were observed between groups (supplementary Fig. XII), indicating that modulation of the microbiota is unlikely to be a meaningful cause. A second possibility is that the peptide nanoparticles prevent the absorption of dietary cholesterol, such as by precipitating cholesterol or bile acids in the gut (74). To explore this hypothesis, albeit indirectly, we prepared synthetic micelles containing cholesterol and bile acids designed to mimic the micelles found in the intestine (45, 46), and mixed these synthetic micelles with the peptide nanoparticles. However, we did not observe any
significant precipitation of cholesterol or bile acids from solution (supplementary Fig. XIII). A third possibility is that the peptide nanoparticles could be imparting anti-inflammatory effects by various means. For example, our nanolipids could be reducing the levels of certain pro-atherogenic lipids in the intestine, such as LPA, as has been suggested for 4F (37, 38) and 6F (52, 53). We are currently working to explore this hypothesis. Ultimately, the peptide nanoparticles may function through multiple mechanisms, including ones not mentioned above, that depend on the route of administration.

In summary, we have evaluated the in vivo efficacy of novel apoA-I mimetics, packaged as HDL-like nanolipids, in lowering non-HDL cholesterol levels and preventing the development of atherosclerosis in LDL receptor null mice. Intriguingly, the nanoparticles were markedly effective when administered both intraperitoneally and orally, despite the peptides being composed of l-amino acids. apoA-I mimetics continue to hold promise for combating atherosclerosis, and our results provide a potential avenue to develop novel therapeutic agents in this arena.

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