

A new HDL mimetic peptide that stimulates cellular cholesterol efflux with high efficiency greatly reduces atherosclerosis in mice

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Abstract Here, we report the creation of a single-helix peptide (ATI-5261) that stimulates cellular cholesterol efflux with K_m molar efficiency approximating native apolipoproteins. Anti-atherosclerosis activity of ATI-5261 was evaluated in LDLR^{-/-} and apolipoprotein (apo)E^{-/-} mice ~5–7 months of age, following 13–18 weeks on a high-fat Western diet (HFD). Treatment of fat-fed LDLR^{-/-} mice with daily intraperitoneal injections of ATI-5261 (30 mg/kg) for 6 weeks reduced atherosclerosis by 30%, as judged by lesion area covering the aorta (7.9 ± 2 vs. $11.3 \pm 2.5\%$ control, $P = 0.011$) and lipid-content of aortic sinus plaque (25 ± 5.8 vs. $33 \pm 4.9\%$ control, $P = 0.014$). In apoE^{-/-} mice, the peptide administered 30 mg/kg ip on alternate days for 6 weeks reduced atherosclerosis by ~45% (lesion area = 15 ± 7 vs. $25 \pm 8\%$ control, $P = 0.00016$; plaque lipid-content = 20 ± 6 vs. $32 \pm 8\%$ control, $P < 0.0001$). Similar reductions in atherosclerosis were achieved using ATI-5261:POPC complexes. Single intraperitoneal injection of ATI-5261 increased reverse cholesterol transport from macrophage foam-cells to feces over 24–48 h. In summary, relatively short-term treatment of mice with the potent cholesterol efflux peptide ATI-5261 reduced substantial atherosclerosis. This was achieved using an L-amino acid peptide, in the presence of severe hypercholesterolemia/HFD, and did not require daily injections or formulation with phospholipids when administered via intraperitoneal injection.—Bielicki, J. K., H. Zhang, Y. Cortez, Y. Zheng, V. Narayanaswami, A. Patel, J. Johansson, and S. Azhar. A new HDL mimetic peptide that stimulates cellular cholesterol

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Supplementary key words reverse cholesterol transport • macrophages • amphipathic α -helix • high density lipoprotein

Plasma HDLs are thought to protect against atherosclerosis via mechanisms related, in part, to reverse cholesterol transport (RCT) (1–3). This process involves removal of excess cholesterol from arterial foam-cells (i.e., cholesterol efflux), followed by the transport of cholesterol to the liver for excretion in feces. Over-expression of apolipoprotein (apo) A-I, the major protein of HDL, in mice enhances RCT, inhibits atherosclerosis development, and stimulates regression of plaque lesions (4–8). Similar effects can be obtained upon infusion of apoA-I-phospholipid complexes in mice and humans, suggesting HDL may be useful therapeutically (9–11).

Small peptides based on HDL apolipoproteins are being developed as alternatives to full-length recombinant proteins for therapeutic use (12–14). The mechanisms by which apolipoprotein mimetic peptides reduce atherosclerosis are not fully understood. The most widely studied 4F peptide reduces atherosclerosis in animal models via mechanisms related to binding of oxidized lipid (12, 15). The latter anti-oxidant activity greatly exceeds that of apoA-I (16). The 18A/4F peptides can stimulate cholesterol efflux from cells, albeit with far weaker activity

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Abbreviations: apo, apolipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CD, circular dichroism; CT, C-terminal; HFD, high-fat Western diet; LDLR, low-density lipoprotein receptor; RBC, red blood cells; RCT, reverse cholesterol transport.

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compared with native apolipoproteins on a molar basis (17–20). Other peptides that have been described target the macrophage cholesteryl ester cycle and LCAT activation to modulate HDL cholesterol efflux and RCT (21–23).

In contrast to anti-oxidant activity, comparatively little has been done to optimize cholesterol efflux potential of HDL mimetic peptides (19). Of particular interest is stimulating cholesterol efflux via the membrane ABCA1, which enhances RCT and protects against macrophage foam-cell formation and atherosclerosis (24, 25). Many apolipoproteins stimulate ABCA1 cholesterol efflux, suggesting common determinants may govern the efflux process (26). The nature of these determinants is not known with certainty, but class A amphipathic α -helices are thought to be involved (17, 27, 28). Apolipoproteins are largely composed of various types of amphipathic α -helices having polar and nonpolar surfaces (29). It is generally believed that two (or more) of these α -helices linked via proline are required to mediate cholesterol efflux effectively (17, 28, 30). Consequently, there is a deficiency of small peptides for stimulating cholesterol efflux with high potency. Difficulties designing peptides with secondary structure also limits scientific and clinical pursuits (31).

Here we report the design of a single-helix peptide that stimulates cholesterol efflux with high efficiency. This was achieved by engineering a class A α -helix from a short segment derived from the C-terminal domain of apoE. The synthetic peptide displayed exceptional α -helicity and solubility characteristics, stimulated cholesterol efflux similar to native proteins on a molar basis, and reduced atherosclerosis in hyperlipidemic mice.

METHODS

Peptide, CD spectroscopy, lipid binding, and cholesterol efflux assays

ATI-5261 was synthesized from L-amino acids and capped with N-terminal acetyl and C-terminal amide groups (Biosynthesis Inc., TX). Lyophilized peptide (~95% pure) was routinely dissolved to ~3–4 mg/ml PBS (pH = 7.4), but stock solutions of >140 mg/ml could easily be prepared. The latter represents ~100- to 1000-fold greater solubility than current HDL mimetic peptides (22). Complexes of ATI-5261 and POPC were prepared by cholate dialysis (32). Peptide concentrations were determined by absorbance at 280 nm. Mean hydrophobicity and amphiphilicity were calculated as described (27). Circular dichroism (CD) spectroscopy was carried out on a Jasco 810 spectropolarimeter at 25°C, using lipid-free peptide in 10 mM phosphate buffer (pH = 7.4) and an average of four scans (20 nm/min/scan) (33). Cholesterol efflux activities were determined as before (27, 28). Efflux efficiency (K_m) was calculated using the Michaelis-Menten equation (Graph-Pad Prism4) and 4 h data with 0.1 to 10 μ g ATI-5261/ml and 1 to 20 μ g apoA-I/ml. Activity of lipid-free ATI-5261 to lyse human RBCs was evaluated as described (34). Percent hemolytic activity was calculated from absorbance readings at 540 nm, using maximum values obtained in the presence of 1% triton X-100.

ATI-5261 was selected over other peptides because of superior efflux efficiency and high aqueous solubility (27). For the present studies, plasma half-life of 125 I-ATI-5261 in apoE $^{-/-}$ mice and

rats (4.2 and 9.5 ± 1.4 h, respectively, $n = 4$) and uptake into plasma (rats, ~30%) following intraperitoneal injection indicated the peptide was suitable for further study.

Animals

All procedures were approved by institutional Animal Welfare Research Committee. Seven- or eight-week-old LDLR $^{-/-}$ and apoE $^{-/-}$ mice (male, C57BL/6) from Jackson Laboratories (Bar Harbor, ME) were housed in a pathogen-free environment (12-h light/dark cycle) and given free access to food and drinking water.

Atherosclerosis studies

Mice were acclimated 1 week on chow diet before receiving high-fat Western diet (HFWD) (Harlan TekLad TD.88137) to promote atherosclerosis. LDLR $^{-/-}$ mice were fed HFWD for 7 weeks, then injected (intraperitoneally) with peptide (30 mg/kg body weight) daily for 6 weeks in the continued presence of HFWD. ApoE $^{-/-}$ mice were fed the HFWD for 18 weeks before injections (intraperitoneal) of ATI-5261 or ATI-5261:POPC complexes for 6 weeks. ApoE $^{-/-}$ mice were fed chow diet during peptide intervention.

After anesthesia with isoflurane, mice were euthanized and their hearts and whole aortas were perfused with saline. Hearts were embedded in OCT compound (Tissue-Tek), frozen on dry ice, and stored until sectioning. Serial 10 μ m sections (every fifth from the middle of the ventricle until the appearance of the aortic valve and every second section from the appearance to the disappearance of the aortic leaflets) were collected on poly-D-lysine coated slides, stained with Oil Red O and hematoxylin, and counterstained with Fast Green (10). Quantification of atheromatous lesions were performed by computerized analysis (Image Pro Plus, Version 6.0; Media Cybernetics, Inc.), and expressed as average % lesion area occupied with lipid from six sections/mouse. The descending thoracic- and abdominal-aorta (up to bifurcation of common iliac arteries) were stored overnight in Histchoice fixation, split open longitudinally, and stained with Oil Red O. The percentage of aortic surface covered by atheroma was determined by computer-assisted planimetry (10).

Toxicology, lipids, and in vivo RCT

Aspartate- and alanine-aminotransferase activities (AST and ALT, respectively) in plasma were quantified by IDEXX Laboratories, Inc. (Maine) to assess liver toxicity/necrosis. Plasma total-cholesterol levels were determined using commercial kits. For aminotransferase and cholesterol measurements, blood was drawn from the retro-orbital plexus at termination of atherosclerosis studies, i.e., 2 h after final injection of ATI-5261 and ATI-5261:POPC complexes (30 mg/kg dose). Ability of ATI-5261 to stimulate RCT in vivo was assessed as described (4). The outcome of this assay was used to identify the peptide injection schedule (daily or alternate days) for atherosclerosis studies; thus RCT was evaluated in atherosclerotic mice (i.e., not exposed to 6 weeks peptide intervention).

Statistics

Data were analyzed by ANOVA and Student's *t*-test, using unequal variance and two-tail test.

RESULTS

Members of our group recently found that the C-terminal (CT) domain of apoE was a potent mediator of ABCA1 cholesterol efflux and that the entire CT domain (aa216–299) was required for activity (28). This previous study also

revealed that relatively hydrophobic segments (aa260-299) containing a class G α -helix conferred efflux efficiency, whereas the early portion (aa216-237) of the first helical segment of the CT domain possessed acidic residues endowing class A structure. Presently, we tested whether these features of hydrophobic character and class A acidic residues could be compressed into a single relatively small α -helix to create a peptide that stimulates cholesterol efflux with high efficiency. A segment corresponding to the central (aa238-266) CT region was used as a template to design the efflux peptide (Fig. 1).

The engineered peptide (designated ATI-5261) possessed an α -helical content of ~ 70 – 80% in the absence of lipid, which was ~ 2 -fold higher than the original aa238-266 segment (Fig. 1). The mean hydrophobicity and hydrophobic moment of ATI-5261 were -0.22 and 0.24 , respectively, versus values of -0.34 and 0.14 for the original aa238-266 sequence. Therefore, ATI-5261 displayed greater hydrophobicity and amphiphilicity characteristics compared with the original sequence from which it was designed. The aa238-266 peptide failed to stimulate cholesterol efflux from cAMP treated macrophages (Fig. 2A),

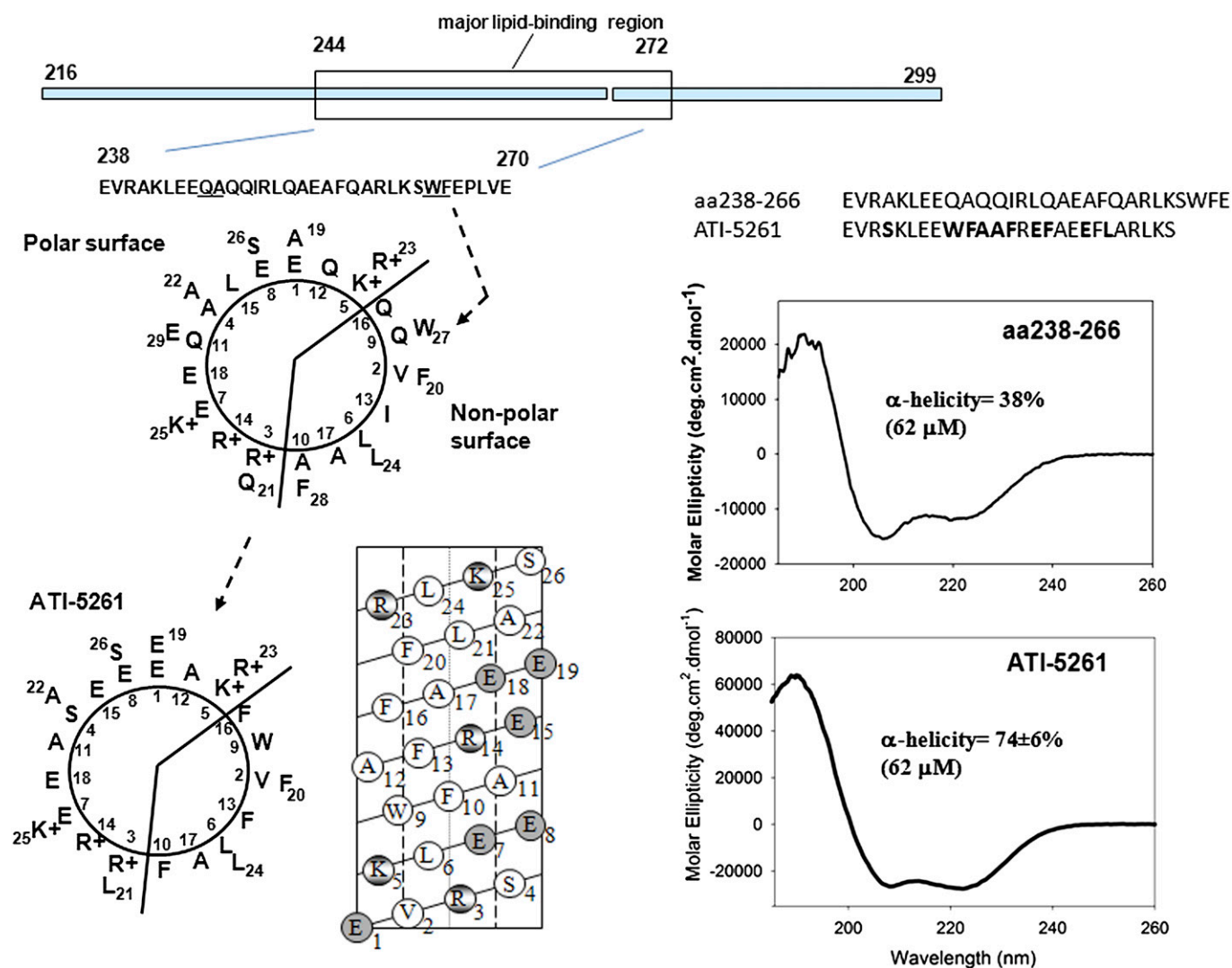


Fig. 1. Creation of α -helix peptide ATI-5261. Top bar represents the CT domain of apoE (aa216-299). The aa238-270 segment possesses features useful for constructing a model class A α -helix. These features include numerous sites for intra-helical ionic interactions (i+4) at the lipid-water interface (35) and potential for designing a favorable nonpolar surface for binding lipid. Underlined glutamine (Q) and alanine (A) residues were replaced with underlined tryptophan (W) and phenylalanine (F) to accommodate strongly hydrophobic amino acids at these sites within a shorter sequence. The polar Q21 was replaced with leucine (L) to expand nonpolar surface-area (ATI-5261-wheel and -net diagrams) and I13 replaced with phenylalanine (F) to further increase hydrophobicity. Q residues on the polar surface were replaced with smaller alanine (A) residues, generally good for α -helices. The A4 to serine (S) substitution was intended to minimize hydrophobic character of the polar surface. Glutamate (E) was used at positions 15 and 19 to create an alignment of acidic residues down the center of the polar surface, thereby endowing class A structure. E19 also created an additional site for salt-bridge formation with R23. Right side of the figure compares the original apoE CT sequence with that of ATI-5261 (amino acid changes in bold). Net-charge of the original aa238-266 sequence = 0 and net-charge of ATI-5261 = -1. Also shown are Far-UV CD spectra and % α -helicity for the original apoE CT seq. aa236-266 (top scan) vs. ATI-5261 (bottom scan, mean \pm SD, $n = 3$), using 62 μ M concentrations of peptides in 10 mM phosphate buffer (pH = 7.4).

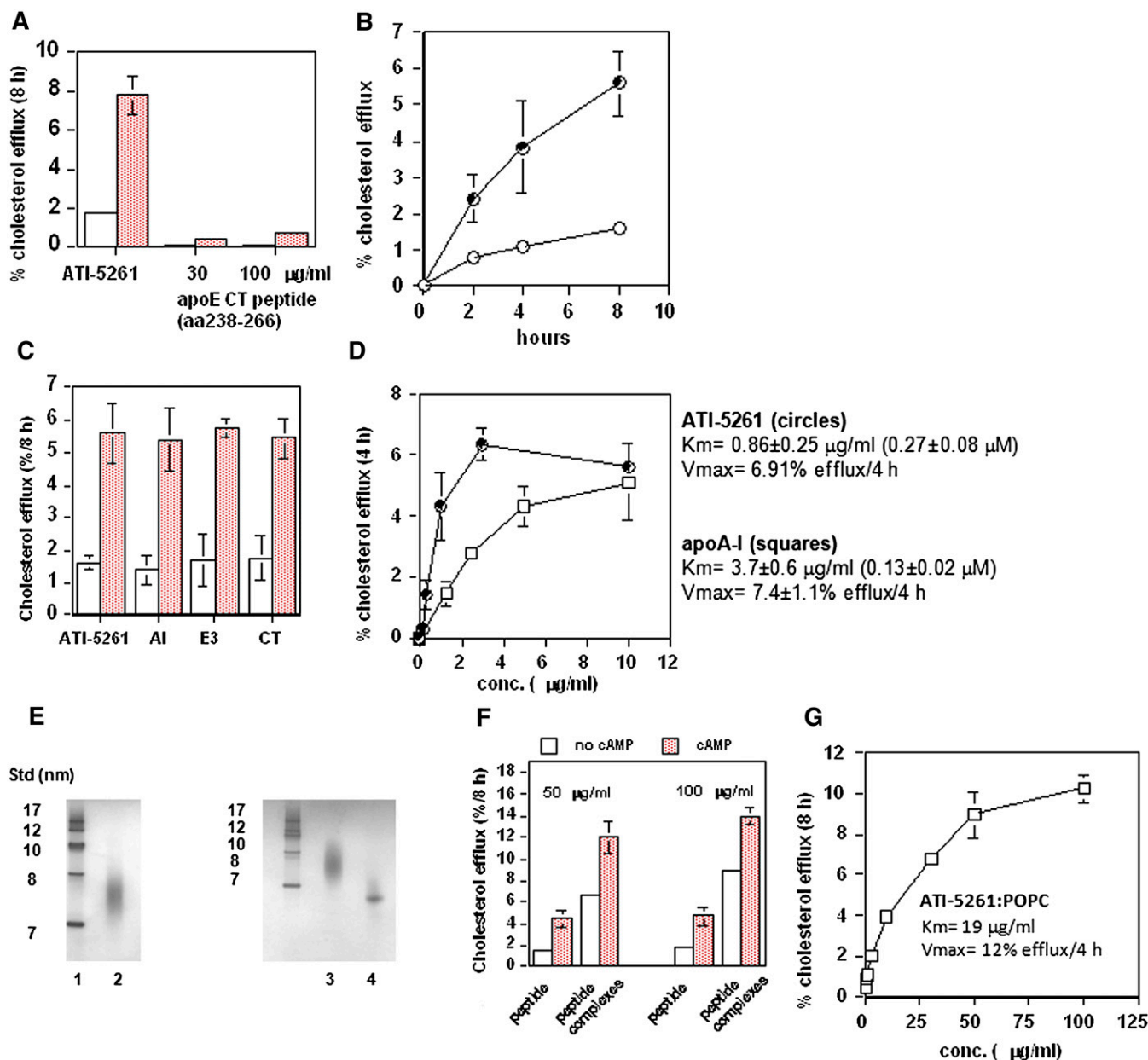


Fig. 2. In vitro cholesterol efflux activity of ATI-5261. **A:** Cholesterol efflux activity (8 h) of ATI-5261 versus original segment derived from apoE CT domain aa238-266. J774 cells were treated with (shaded bars) and without (open bars) a cAMP analog to modulate ABCA1 expression. Both peptides were capped with N-terminal acetyl and C-terminal amide groups and used in lipid-free form in serum-free medium. Results are from two experiments (performed in triplicate), comparing ATI-5261 (30 µg/ml) to the original segment at either 30 or 100 µg/ml, respectively. **B:** Time-course of [3H]cholesterol efflux to the lipid-free ATI-5261 (30 µg/ml), using J774 macrophages treated with (closed circles) and without (open) a cAMP analog. **C:** ABCA1-dependent cholesterol efflux using J774 cells treated with (shaded bars) and without (open) cAMP. A-I, E3 and CT refer to full-length apoA-I, apoE3 and the CT domain of apoE. Each acceptor was used in lipid-free form at 30 µg/ml serum-free medium. Values are means \pm SD, $n = 5$. **D:** Cholesterol efflux (cAMP treated J774 cells) to increasing concentrations of lipid-free ATI-5261 and apoA-I. Values are means \pm SD, $n = 3$. **E:** Nondenaturing gradient (4–20%) gel electrophoresis of ATI-5261:POPC complexes following standard (left gel) and short run-times (right); lane 1, standards (nm); lane 2 and 3, ATI-5261:POPC complexes (4 µg); lane 4, lipid-free ATI-5261 (4 µg). **F:** Cholesterol efflux activity of ATI-5261:POPC complexes versus lipid-free peptide, using each at 50 and 100 µg/ml (based on peptide mass). Values are means \pm SD, $n = 3$. **G:** Dependence of cholesterol efflux on concentration of ATI-5261:POPC complexes, using cAMP treated J774 macrophages. Results are from a single experiment using triplicate wells (error bars smaller than symbols when not seen) and are representative of two experiments.

consistent with previous results using peptides based on the apoE CT domain (28). In contrast, ATI-5261 stimulated high levels of cholesterol efflux from macrophages treated with cAMP and low levels of efflux in the absence of ABCA1 induction (Fig. 2A, B). This behavior was similar

to native apolipoproteins (Fig. 2C). The peptide stimulated cholesterol efflux at concentrations where apoA-I was largely ineffective (Fig. 2D), reaching maximal efflux at 3 µg peptide/ml (Fig. 2D). As a result, ATI-5261 stimulated cholesterol efflux with a K_m molar efficiency approxi-

maturing apoA-I (Fig. 2D). These K_m values were similar to those previously reported for apoA-I, E, and the CT-domain of apoE (28).

Complexes of ATI-5261:POPC (7–8 nm) possessed ~4-fold greater cholesterol efflux capacity than the lipid-free peptide (Fig. 2E, F). This was associated with an increased V_{max} for activity obtained with complexes versus the lipid-free peptide (Fig. 2G). The former was likely attributed to phospholipid and ABCA1-independent mechanisms. However, cholesterol efflux to complexes increased substantially with cAMP treatment of cells, suggesting a portion of efflux was attributed to ABCA1. This was verified using ABCA1 expressing HeLa cells (16 ± 0.4 vs. $22 \pm 3\%$ efflux/24 h from nonexpressing and ABCA1-expressing cells, respectively, $50 \mu\text{g}$ complexes/ml, $n = 3$). The ABCA1 component of efflux was not attributed to contamination of the complexes with lipid-free peptide (Fig. 2E, right). Therefore, ATI-5261 formulated with POPC was not prevented from mediating ABCA1 efflux, as expected for apoA-I on HDL (36). The peptide-POPC formulation, however, stimulated cholesterol efflux with lower efficiency versus lipid-free ATI-5261, as judged by a higher K_m for activity (Fig. 2G).

Daily intraperitoneal treatments of atherosclerotic LDLR^{-/-} mice with lipid-free ATI-5261 for 6 weeks reduced atherosclerosis by ~30% (Fig. 3). Total-cholesterol concentrations in plasma were similar in control and peptide treated mice at termination (2552 ± 586 and 2809 ± 436 mg/dl, respectively), indicating that ATI-5261 reduced substantial atherosclerosis in the presence of severe and persistent hypercholesterolemia.

ATI-5261 stimulated macrophage RCT in apoE^{-/-} mice over 24–48 h, as judged by an increase in fecal [3H]sterol secretion (Fig. 4A). The latter suggested ATI-5261 may reduce atherosclerosis if provided at 48 h intervals, as opposed to daily injections. This was tested by treating apoE^{-/-} mice with either daily ip injections of ATI-5261 (15 mg/kg) or injections every other day (30 mg/kg) for 6 weeks. Both protocols significantly reduced plaque lesions (Fig. 4B), producing 20 and 47% reductions in atherosclerosis with daily and alternative-day injection

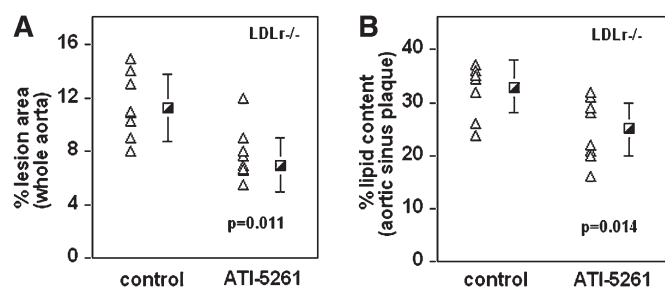


Fig. 3. Anti-atherosclerosis effects of ATI-5261 in LDLR^{-/-} mice. Male mice (8 weeks of age) were fed a HFD for 7 weeks. The mice subsequently received daily intraperitoneal injections of saline (vehicle control) or lipid-free ATI-5261 peptide (30 mg/kg) for 6 weeks in the continued presence of HFD. A: Area of whole-aorta covered with plaque-lesions at termination. B: Lipid content of aortic-sinus plaque using Oil Red O. Individual data points shown with means \pm SD, $n = 8$ mice per group.

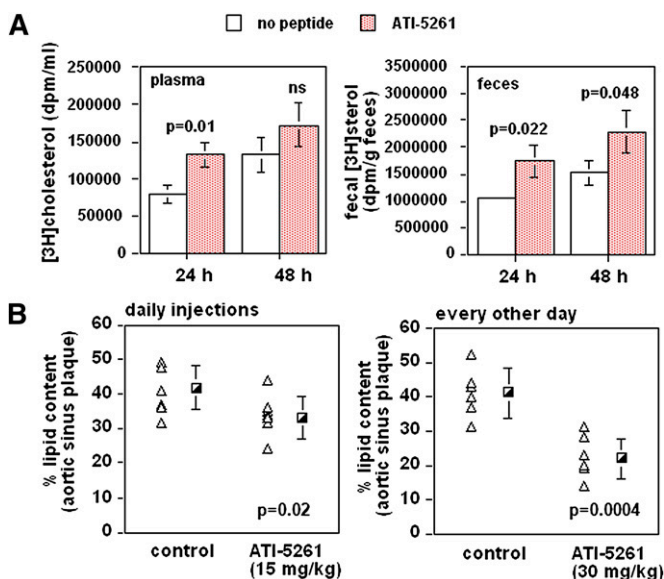


Fig. 4. ATI-5261 enhances macrophage RCT and reduces atherosclerosis in apoE^{-/-} mice. A: To assess RCT, atherosclerotic (6 months) male mice were injected intraperitoneally with [3H]cholesterol-labeled J774 macrophage foam-cells plus lipid-free ATI-5261 at a dose of 20 mg/kg (shaded bars) or with the [3H]foam-cells alone (open bars). For the former, [3H]foam-cells and peptide were injected together. Left: Appearance of [3H]cholesterol in plasma following treatment. Right: [3H]sterol per gram of feces collected between 0–24 h (24 h) and 24–48 h (48 h) intervals. Values are means \pm SD ($n = 5$). Vehicle-control plasma dpm/ml corresponded to 5.4 ± 0.82 and $8.9 \pm 1.6\%$ of injected [3H] at 24 and 48 h, respectively; ATI-5261 plasma dpm/ml to 8.9 ± 1.1 and $11.6 \pm 1.9\%$; control versus ATI-5261 [3H]sterol 1.11 ± 0.16 versus $1.65 \pm 0.28\%$ of total injected [3H] at 48 h. Results representative of two experiments. B: Impact of ATI-5261 on atherosclerosis: male apoE^{-/-} mice were fed HFD for 18 weeks, then injected intraperitoneally with ATI-5261 once daily (left) or on alternate days (right) to maintain equivalent peptide delivery (18 mg) over 6 weeks. Each treatment group had its own vehicle control, as the number of total injections differed (42 vs. 21 injections, respectively). Lipid content of aortic-sinus plaque is shown versus vehicle controls. Values are means \pm SD, $n = 8$ mice per group. $P = 0.0017$ for 30 mg/kg dose every other day versus 15 mg/kg daily.

schedules, respectively. The latter was verified in a second apoE^{-/-} mouse study (18 weeks HFD), where 10 mg/kg injected ip on alternate days (6 weeks) reduced the lipid content of aortic-sinus plaque (26 ± 5 vs. 36 ± 4 control, respectively, $n = 8$ /group, $P = 0.00088$) in continued presence of HFD.

ATI-5261:POPC complexes administered on alternate days also reduced (~40–45%) atherosclerosis in apoE^{-/-} mice (Fig. 5). This effect was similar to the lipid-free peptide. The complexes also reduced atherosclerosis to the same extent as the lipid-free peptide in LDLR^{-/-} mice (% lipid content of aortic-sinus plaque = 25 ± 6 , 26 ± 5 vs. 32 ± 5 for lipid-free ATI-5261, ATI-5261:POPC complexes, and vehicle control, respectively, $P = 0.044$ complexes vs. control, $n = 8$ per group). At the end of treatment, total cholesterol in plasma of apoE^{-/-} mice was similar in all groups (916 ± 62 , 875 ± 62 , 816 ± 40 mg/dl, free peptide, complexes, and vehicle control, respectively) and plasma aminotransferase activities were

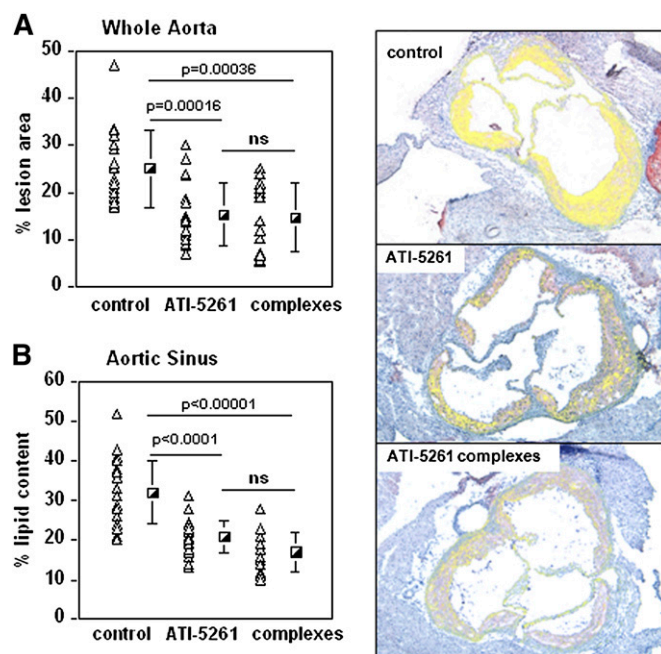


Fig. 5. Anti-atherosclerosis effects of ATI-5261 and ATI-5261:POPC complexes. Male apoE^{-/-} mice (7 weeks of age) were fed a HFWD for 18 weeks. Mice then received intraperitoneal injections of either saline (control, *n* = 18), ATI-5261 (*n* = 19), or ATI-5261:POPC complexes (*n* = 14) on alternate days for 6 weeks (chow diet). A dose of 30 mg/kg was used per injection for both lipid-free ATI-5261 and ATI-5261:POPC complexes, based on peptide mass. **A:** Percentage of whole aorta covered with plaque lesions. **B:** Lipid-content of aortic-sinus plaque at termination of treatment, shown as data from individual mice with means ± SD. Representative cross-sections of aortic tissue stained with Oil Red O shown vertically on right (top to bottom as saline controls, lipid-free ATI-5261 peptide and ATI-5261:POPC complexes).

within normal range (ALT values ranged from 35–60, 29–46, and 25–39 and AST values 71–113, 50–98, and 61–106 for free peptide, complexes, and vehicle control, *n* = 4 mice/group). No differences were observed between groups regarding food- and water-intake and animal- and organ-weights (data not shown). Moreover, the lipid-free peptide exhibited negligible hemolytic activity toward human RBC, i.e., similar to background PBS readings (% RBC lysis = 0 ± 0, 0.03 ± 0.06, 0.23 ± 0.19, 0.03 ± 0.06, 0.02 ± 0.06, 0.03 ± 0.06, 0.11 ± 0.19 at peptide concentrations of 0.025, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mg/ml, respectively, means ± SD, *n* = 3), consistent with its modest amphiphilicity and dependence on ABCA1 for efflux activity (19).

DISCUSSION

ATI-5261 constitutes a novel peptide that stimulates cellular cholesterol efflux with high potency. The cholesterol efflux response saturated with increasing concentrations of lipid-free ATI-5261, displaying a hyperbolic relationship typical of apoA-I and E (28). This relationship is indicative of a cooperative process that governs cholesterol efflux stimulation. Noteworthy, ATI-5261 lacks proline residues

and tandem repeats, indicating that efficient stimulation of cholesterol efflux is not dependent on multiple α -helices as previously thought.

ATI-5261 was engineered by introducing amino acid substitutions to a “template” segment derived from the CT domain of apoE. Negatively charged glutamine residues were introduced into this segment at positions 15 and 19 (endows class A structure), together with hydrophobic amino acids (A, W, F, and L) at various positions indicated in Fig. 1. These changes were accompanied by a marked increase in α -helicity, hydrophobic moment, and hydrophobicity. The former is consistent with secondary structure being a key determinant for efflux activity (17, 37). The molecular determinants governing ATI-5261 structure and activity are not fully understood at present. However, we have found that ATI-5261 apparently does not form high molecular weight aggregates in solution (data not shown), as previously described for peptide 4F (22). This suggests that numerous factors may influence the cholesterol efflux activity of HDL mimetic peptides, including secondary structure, physical state of the peptide in solution, as well as charge and/or hydrophobic characteristics. Consequently, identifying determinants that confer cholesterol efflux activity likely requires detailed and systematic mutagenesis experiments that take into account changes in the biophysical properties, aggregation tendency, and α -helical behavior of peptides (19).

Peptide 18A mimics class A amphipathic α -helices of apoA-I (12). This peptide stimulates cholesterol efflux from ABCA1 expressing cells, but high concentrations are required for activity compared with apolipoproteins on a molar basis (17, 20). It is often discussed that 18A is unable to inhibit atherosclerosis development in mice, contrasting its D-4F and 5F analogs (12, 22, 23). However, 18A stimulates cholesterol efflux in vitro similar to its 4F family members (20). This suggests that anti-atherosclerosis activity of these peptides does not correlate with a cholesterol efflux mechanism. Rather, the anti-atherosclerosis activity of D-4F appears to correlate with anti-oxidative function (12, 16, 23).

Peptides 4F and 5F display efficacy for inhibiting lesion formation in C57BL/6J mice and/or LDLR^{-/-} and apoE^{-/-} mice under conditions where 2–8% of the aortic surface is covered with plaque, i.e., when peptide is administered 4 to 6 months (15, 38, 39). Moreover, D-4F inhibits rapidly developing lesions in aortic vein-graft segments (40) and stimulates lesion regression (6 months treatment) in old (12 months) apoE^{-/-} mice on chow diet when given at low doses in the presence of a statin (% lesion area = 2.5 vs. 1.7% with D-4F+paravastatin) (39). Similar results can be obtained in apoE^{-/-} mice 9.5 months of age (chow diet) having substantial atherosclerosis, following treatment (6 months) with L-4F (0.2 mg/day/mouse) +paravastatin+salicylanilide anti-parasitic drug (41).

In the present studies, we found that ATI-5261 is able to reduce substantial atherosclerosis in mice when administered alone and over a short period of time. This was achieved with ~18 mg (total over 6 weeks) of ATI-5261/mouse (Fig. 4B), representing half the administered L-4F

used over 6 months in atherosclerotic apoE^{-/-} mice (41). Our studies adopted dietary and treatment protocols similar to those used in studies of ETC-216, i.e., apoA-IMilano-phospholipid complexes (10). This comparison revealed ATI-5261 reduced atherosclerosis to roughly the same extent as ETC-216 in the apoE^{-/-} mouse model. Moreover, ATI-5261 did not exhibit hemolytic activity and was highly soluble in aqueous buffers, suggesting the peptide may be useful for acute treatment of atherosclerosis and/or as a chronic therapy.

The underlying mechanism(s) by which ATI-5261 exerts its anti-atherosclerosis effects *in vivo* are incompletely understood at present. The peptide was designed to stimulate cholesterol efflux with high potency and thus is likely to be efficiently lipidated *in vivo*. Thus the lipid-free peptide administered intraperitoneally likely associates with endogenous phospholipid before entering the plasma compartment. This may improve peptide pharmacodynamics and explain why the lipid-free peptide reduced atherosclerosis to the same extent as ATI-5261:POPC complexes in the present studies. Therefore, it is currently not known whether the lipid-free peptide would reduce atherosclerosis to the same extent as ATI-5261:POPC complexes if administered intravenously, which represents one potential route for administration in humans. Interestingly, ATI-5261 proved highly effective at reducing atherosclerosis when administered every other day. This could reflect peptide mechanism-of-action and/or increased stress associated with daily injections/handling of mice, which may influence response to treatment.

Complexes of ATI-5261:POPC stimulated ABCA1 cholesterol efflux, providing evidence the peptide bound to lipid was not prevented from interacting with membranes of ABCA1 expressing macrophages. The latter could involve peptide exchanging from the complexes to the cell surface. Moreover, spiking of mouse serum with peptide (2:1 mol ratio relative to apoA-I) increased by ~40% cholesterol efflux activity of the subsequently diluted serum (1% final) from cAMP treated J774 cells (4.2 ± 0.6 vs. $5.9 \pm 1\%$ /4 h control vs. ATI-5261), but had little impact on cholesterol efflux from J774 cells not induced for ABCA1 response (2.2 ± 0.16 vs. $2.4 \pm 0.3\%$ /2 h for control vs. ATI-5261). These results suggest ATI-5261 is capable of exerting effects on cholesterol efflux in a biological milieu in the presence of lipid and align with the peptide increasing macrophage RCT/fecal [3H]sterol secretion in mice. With regards to the latter, ATI-5261 and [3H]foam-cells were injected simultaneously via the intraperitoneal injection route for assessment of RCT. Therefore, it will be important to test whether a similar response occurs following intravenous infusion of peptide or using different methods of delivery. In addition, ATI-5261 may bind oxidized lipid and thereby exert anti-oxidant effects (42). It is also feasible that the anti-atherosclerosis effects of ATI-5261 could be independent of lipid transport, perhaps involving ABCA1-mediated activation of JAK2/STAT3 signaling to suppress inflammation (43).

In summary, ATI-5261 possesses potent cholesterol efflux activity, reduces substantial atherosclerosis in

mice, and shows greatly improved α -helicity/solubility characteristics, suggesting the peptide may be useful therapeutically. Obviating the need for full-length apolipoproteins/peptide formulations simplifies production, reduces costs, and may enable wide-spread applications of HDL mimetic technologies. More detailed mechanistic studies of ATI-5261 may prove useful for optimizing dose-regimens and biomarkers for human efficacy studies.

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