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Self-Assembling Cyclic D,L-α-Peptides as Modulators of Plasma HDL Function. A Supramolecular Approach toward Antiatherosclerotic Agents

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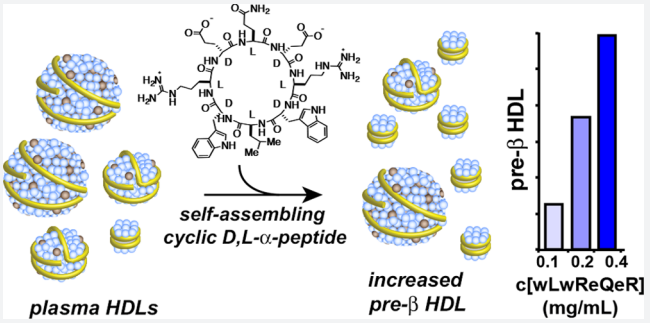
Supporting Information

ABSTRACT: There is great interest in developing new modes of therapy for atherosclerosis to treat coronary heart disease and stroke, particularly ones that involve modulation of high-density lipoproteins (HDLs). Here, we describe a new supramolecular chemotype for altering HDL morphology and function. Guided by rational design and SAR-driven peptide sequence enumerations, we have synthesized and determined the HDL remodeling activities of over 80 cyclic D,L-α-peptides. We have identified a few distinct sequence motifs that are effective *in vitro* in remodeling human and mouse plasma HDLs to increase the concentration of lipid-poor pre-beta HDLs, which are key initial acceptors of cholesterol in the reverse cholesterol transport (RCT) process, and concomitantly promote cholesterol efflux from macrophage cells. Functional assays with various control peptides, such as scrambled sequences, linear and enantiomeric cyclic peptide variants, and backbone-modified structures that limit peptide self-assembly, provide strong support for the supramolecular mode of action. Importantly, when the lead cyclic peptide c[wLwReQeR] was administered to mice (ip), it also promoted the formation of small, lipid-poor HDLs in vivo, displayed good plasma half-life (~6 h), did not appear to have adverse side effects, and exerted potent anti-inflammatory effects in an acute in vivo inflammation assay. Given that previously reported HDL remodeling peptides have been based on α-helical apoA-I mimetic architectures, the present study, involving a new structural class, represents a promising step toward new potential therapeutics to combat atherosclerosis.



INTRODUCTION

High-density lipoprotein (HDL) nanoparticles are complexes of lipids and proteins that eliminate cholesterol from the bloodstream, thereby reducing atherosclerotic plaque burden.[1−4] HDL particles are formed in vivo when apoA-I, a 243 amino acid protein consisting of ten amphiphilic α-helices, interacts with phospholipids, cholesterol, and other proteins.[5,6] HDL metabolism and remodeling is a highly dynamic process involving the constant influx, efflux, and modification of constituent proteins, cholesterol, lipids, and small-molecule components, giving rise to a spectrum of HDL particle compositions and function (Figure 1).[5,7,8] Compelling evidence for the antiatherogenicity of apoA-I, the key protein component of HDL, derives from observations that iv infusions of apoA-I or reconstituted HDL particles, or overexpression of apoA-I, exhibit atheroprotective effects, including antioxidant and anti-inflammatory properties.[9] However, the use of apoA-I directly as a therapeutic agent faces serious challenges. In particular, apoA-I is not orally bioavailable and the large amounts of protein (>3 g/single infusion) required are cost prohibitive given current production methods.[10,11]

Consequently, many research groups have devoted considerable effort to developing peptide[12−17] or other[18,19] mimetics of apoA-I. Notably, some key properties of apoA-I can be mimicked by various α-helical, amphiphilic peptides containing 18−40 amino acids. For example, the 18-residue peptide 4F is an archetypal α-helical apoA-I mimetic that has been the subject of many studies and has advanced into human clinical studies.[20,21] We recently developed branched multivalent apoA-I mimetic constructs displaying up to four copies of an α-helical peptide.[16,17] Although these constructs showed remarkable in vivo oral efficacy in a leading mouse model of atherosclerosis, we sought to advance a simpler peptide scaffold that would facilitate higher-throughput screening and more cost-effective drug candidates. Despite intense interest in α-helical peptide structures as apoA-I mimetics,[12−17] virtually no other peptide

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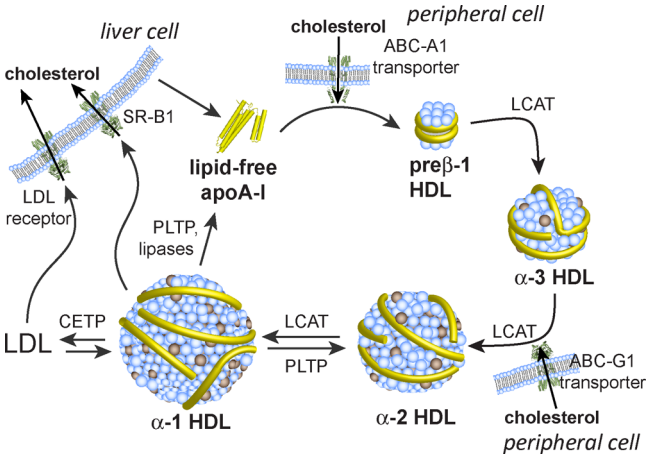

Figure 1. HDL metabolism and remodeling. HDL particles are complexes of lipids, proteins, and cholesterol that undergo constant dynamic remodeling mediated by various transporters, receptors, and enzymes. HDLs are atheroprotective in part because they exert anti-inflammatory effects and facilitate the process of reverse cholesterol transport (RCT), which removes excess cholesterol from peripheral tissues for delivery to the liver for elimination. Abbreviations: ABC, ATP-binding cassette transporter protein; apoA-I, apolipoprotein A-I; CETP, cholesteryl ester transfer protein; LCAT, lecithin−cholesterol acyltransferase; LDL, low-density lipoproteins; PLTP, phospholipid transfer protein; SR-B1, scavenger receptor B1.

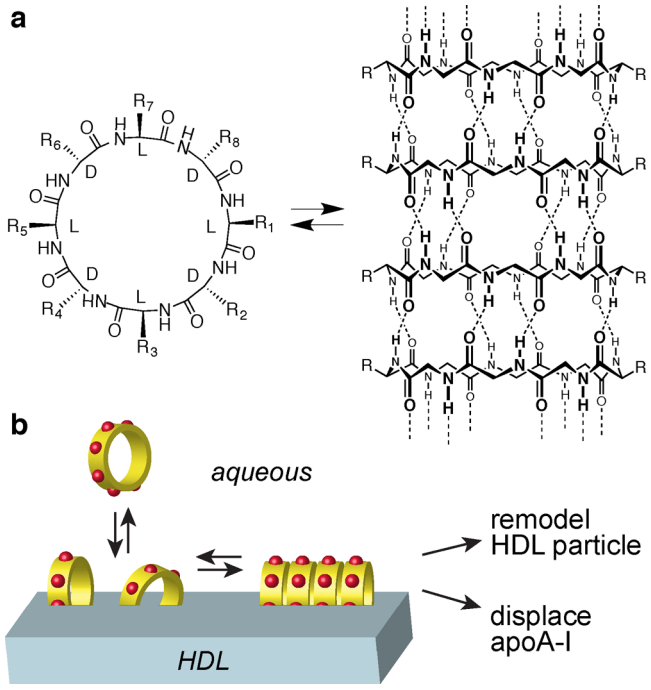

Figure 2. Structure and function of self-assembled cyclic D,L-α-peptide nanotubes. (a) Chemical structure of a generic cyclic D,L-α-peptide and corresponding peptide nanotube. Self-assembly of cyclic D,L-α-peptides involves the peptides adopting a beta-sheet-like, hydrogen-bonded architecture in which side chains decorate the outside of the nanotube surface. In the nanotube, some side chains have been removed for clarity. (b) Appropriately designed, amphiphilic cyclic D,L-α-peptides can insert into membranes and generate lipid-interacting nanotubular ensembles.

architectures have been studied in this area of research.[22] Herein, we describe a novel chemotype for modulating HDL morphology and function based on small six- or eight-residue self-assembling cyclic D,L-α-peptides.

Cyclic peptides with an even number of alternating D- and L-α-amino acids can adopt flat, ring-shaped conformations in which the backbone amide groups are oriented perpendicular to the side chains and the plane of the ring (Figure 2).[23−25] Under conditions that favor hydrogen bonding, such as adsorption onto lipid droplets or membranes, cyclic D,L-α-peptides can stack to form hollow, β-sheet-like tubular structures with the amino acid side chains positioned on the outside surface of the nanotube.[23−28] Importantly, the self-association is reversible, such that a dynamic assembly process takes place,[29] in which ensembles can be formed according to the specific lipid environment at hand. We considered that such dynamic supramolecular peptide structures with inherent structural adaptability might effectively interact with HDLs (which are also compositionally and morphologically dynamic). It is known that helical, amphiphilic apoA-I mimetic peptides function by binding to the surface of lipoprotein particles.[16,30] Analogously, previous studies have shown that amphiphilic cyclic D,L-α-peptide nanotubes can bind and functionally impact biological membranes.[26,28] The multivalent amino acid side chains present on the self-assembled peptide nanotube surface have certain similarities to the side chain display in α-helices (Figure S1). The distance between neighboring side chains in a peptide nanotube (α-carbon distance of 4.7−5.1 Å)[31] is similar to that between the i and i + 3 residues in an α-helix (α-carbon distance of 5.0−5.3 Å). Therefore, it seemed possible to utilize cyclic D,L-α-peptides to create lipid particle-interacting nanotubes that modulate HDL morphology and function, through dynamic HDL nanoparticle remodeling.[6,7,32,33]

RESULTS AND DISCUSSION

A major objective in developing HDL-focused therapeutics is to promote cholesterol efflux by remodeling mature HDL nanoparticles into lipid-poor, pre-beta HDL nanoparticles, the latter of which are key initial acceptors of cholesterol in the reverse cholesterol transport (RCT) process.[34,35] In fact, lipid-poor HDL levels correlate better with the cholesterol efflux capacity of human sera than either total HDL-cholesterol or apoA-I levels.[34,35] Toward that goal, we embarked on a program to design, select, and assess the effectiveness of self-assembling cyclic D,L-α-peptides in remodeling plasma HDL particles in vitro.

To test the hypothesis that HDL morphology and function could be modulated by using nanotubes derived from amphiphilic cyclic D,L-α-peptides, we carried out several iterations of design, synthesis, and initial screening for effectiveness in generating pre-beta HDL particles (Figure 3, see Table S1 for a full list of peptide sequences screened). We examined cyclic D,L-α-peptides for their effectiveness in remodeling mature human plasma HDLs into lipid-poor HDLs (Figure 3 and Figure 5a). Briefly, the testing method involved incubating human plasma with a peptide and then determining the level of pre-beta HDL in the sample by using an ELISA assay.[36] We confirmed the results of the ELISA assays and the dose dependence of the peptide effects by using Western blots for human apoA-I (Figure S2 and Figure S3).[36] Figure 3 provides the pre-beta HDL formation activities of a subset of cyclic D,L-α-peptides chosen for usefulness in

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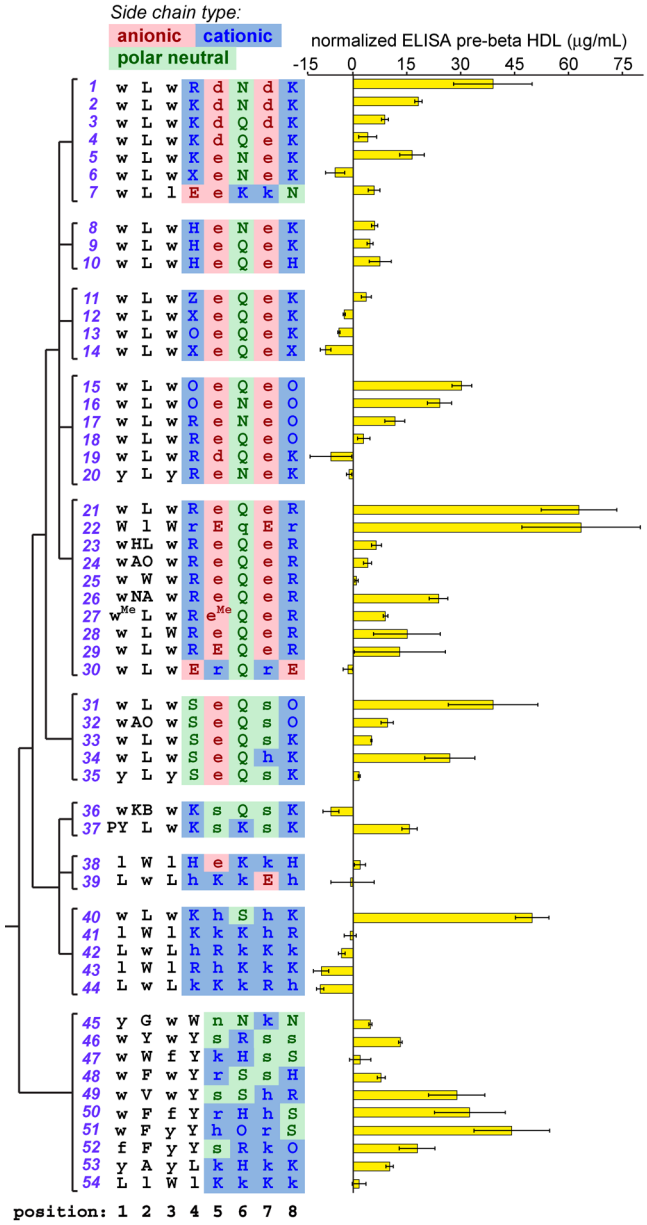

Figure 3. In vitro effectiveness of selected cyclic D,L-α-peptides in increasing the levels of pre-beta HDLs in human plasma. Pre-beta HDLs are key acceptors of cholesterol in reverse cholesterol transport. Not all sequences screened are shown here; see Table S1 for a full list of peptides screened and corresponding activities. Peptides were incubated with human plasma in vitro for 1 h, after which pre-beta HDL levels were measured using ELISA. Peptides are grouped based on sequence homology. The data are shown as mean ± SD. Capital letters represent L-amino acids; small letters represent D-amino acids. Abbreviations: Z, 2,3-diaminopropionic acid; X, 2,4-diaminobutyric acid; O, ornithine; HL, homoleucine; AO, 2-aminooctanoic acid; NA, 2-naphthylalanine; KB, Nε-benzyl lysine; PY, 3-pyridylalanine; ^{Me}, N-methylated amino acid.

developing a SAR; the ranked activities of all peptides screened are given in Table S1.

The peptide library was divided roughly equally between peptides that were (i) overall cationic (43 peptides screened) or (ii) overall neutral and bearing a charge distribution similar to that of class A α-helices found in plasma apolipoproteins (38 peptides screened).[37] Class A α-helices have an amphiphilic structure in which the cationic residues are clustered at the polar/nonpolar interface and the anionic residues are near the center of the polar region. Therefore, many cyclic D,L-α-peptides in our panel were designed to display cationic Arg, Lys, Orn (O), diaminobutyric acid (X), or diaminopropionic acid (Z) residues at the polar/nonpolar interface, and negatively charged Asp or Glu residues near the center of the polar face; this charge distribution is exemplified by peptides **1**−**6** and **8**−**29** (Figure 3, see Table S1 for a full list of the 81 peptide sequences screened, along with the corresponding activities). Cyclic peptides bearing this pattern of charged residues can self-assemble into nanotubular structures that position charged residues along the nanotube, reminiscent of the pattern along the belt-like structures of class A α-helices in natural apolipoproteins. Interestingly, we found that a larger proportion of the most effective compounds were of this charge-neutral, class A sequence class. Additionally, despite representing slightly less than half of the overall number of peptides, such "class A" sequences comprised 10 of the top 15 most active cyclic peptides (Table S1). The library encompassed peptides containing two to five hydrophobic residues, and nearly all the peptides were amphiphilic (with hydrophobic residues clustered together). Whereas most of the peptides screened (73 sequences) were eight-residue peptides, our preliminary results suggest that six-residue peptides (8 sequences screened) also have the potential to promote plasma HDL remodeling (see Supporting Information).

The SAR studies identified a pronounced sequence dependence for efficient plasma HDL remodeling. Early in our screening campaign, we identified peptides **1** and **15** as effective HDL remodeling sequences, so a number of peptides closely related to those were prepared (peptides **1**−**30**). These cyclic D,L-α-peptides are characterized by an amphiphilic topology with a hydrophobic trp-Leu-trp (wLw) segment at amino acid positions 1−3, positively charged amino acids at interfacial positions 4 and 8, negatively charged amino acids at positions 5 and 7, and a polar neutral residue at position 6.

In examining analogues of peptide **15**, we identified **21**, which had Arg residues at both cationic positions (Figure 4), as an effective promoter of pre-beta HDL formation. We made several analogues of **21** designed to be more hydrophobic (and presumably have a higher affinity for lipoprotein particles) by replacing the Leu residue with homoleucine (**23**), 2-amino-octanoic acid (**24**), Trp (**25**), or naphthylalanine (**26**). All of these analogues proved to be less active than peptide **21**. In some cases, subtle changes in sequence had pronounced functional effects. For example, while **21** differs from **18** only by replacement of a single Orn with Arg, both of which are cationic, **18** was essentially inactive but **21** was the most active peptide screened. This observation is consistent with previous studies where seemingly small sequence differences at the peptide monomer level were amplified through the multivalent side-chain presentation of the tubular ensemble.[26−28]

We included several peptides in our screening to provide mechanistic insight. Peptide **22** is the enantiomer of **21**; the HDL remodeling activity of these two compounds was nearly identical, indicating that the mechanism of action does not involve specific ligand-type interactions between the cyclic peptides and chiral molecules in the plasma, such as proteins. We examined c[wLwErQrE] (**30**), in which the pattern of charged residues is swapped compared to that in c-[wLwReQeR] (**21**) (and the other neutral, class A sequences). The plasma remodeling activity for **30** was dramatically reduced

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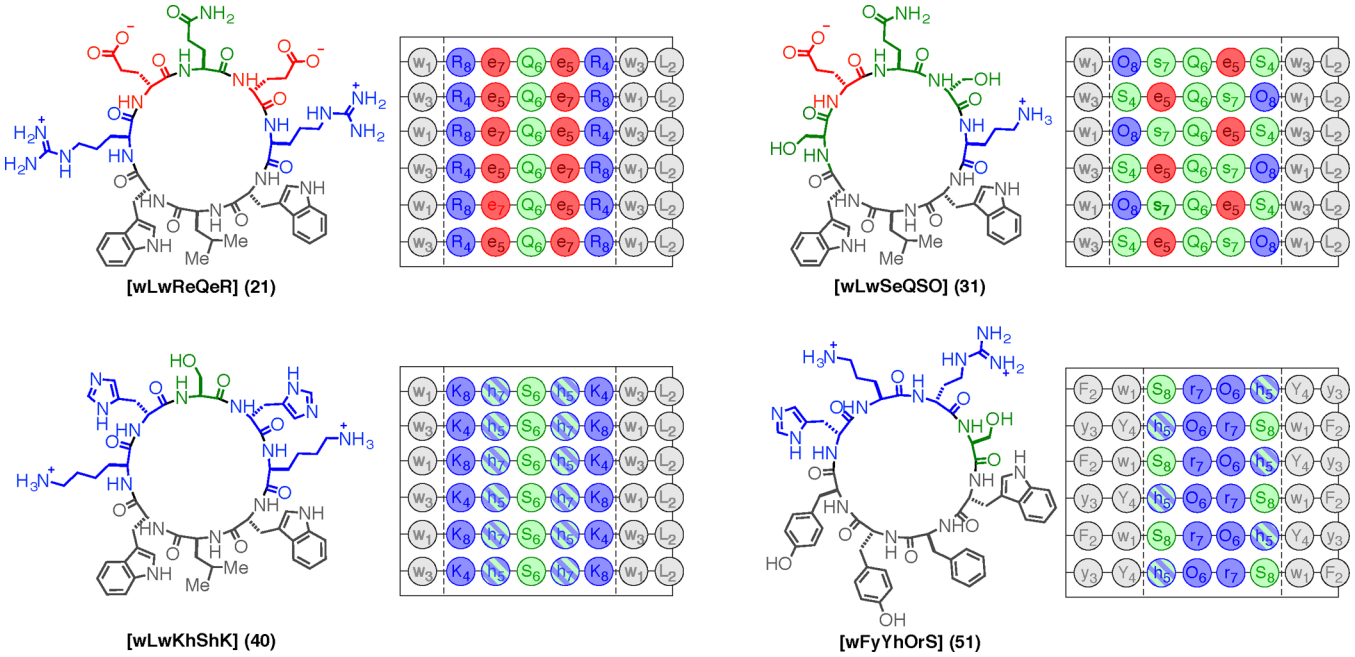

Figure 4. Structures of lead peptides identified by the HDL remodeling ELISA. For each compound, the molecular structure is shown on the left. On the right, a peptide nanotube containing a stack of six peptides is shown as a cylinder cut down the long axis of the hydrophobic face and flattened. Dotted lines mark the hydrophobic/hydrophilic interface. Note that cyclic D,L-α-peptides assemble in an antiparallel fashion, as shown in the cylindrical structures. In reality, the peptides would not necessarily stack in the shown relative rotations having the hydrophobic and hydrophilic residues clustered. Cationic residues are colored blue, anionic residues are red, and polar neutral residues are green. His residues are colored as blue/green hatch because His can be positively charged or neutral since the imidazole pK_a is close to physiological pH.

compared to **21** (Figure 3), despite the same amino acids being present. These findings support the identified charge distribution in **21** as being mechanistically important. We also found that peptide nanotube formation is required for remodeling activity by assaying peptide **27**, a backbone N-methylated analogue of **21**. Such methyl group incorporation limits cyclic peptide self-assembly into dimeric complexes by rendering one face of the peptide incapable of intersubunit hydrogen bonding (Figure S4).[38] The efficacy of this N-methylated peptide was greatly diminished compared to **21**. Similarly, HDL remodeling activity was reduced for peptides **28** and **29**, which have the same sequence as **21**, but one of the D-amino acids switched to L-chirality to disrupt the alternating D,L-topology and reduce the propensity for nanotube formation. Taken together, our studies support a mechanism involving self-assembly of the active cyclic peptides into supramolecular nanotubular complexes.

One of the analogues of peptide **15** with high HDL remodeling activity was sequence **31** (Figure 4), in which there were only two charged residues instead of four, but the sequence would remain charge neutral overall at physiological pH. In exploring analogues of **31**, we noted the good HDL remodeling activity for potentially cationic **34** compared to **33**, so it seemed appropriate to examine a series of cationic sequences. Peptides **36**−**44** range from an overall charge of +2 to +5. Only two of these peptides (sequences **37** and **40**) promoted HDL remodeling. Notably, peptide **40** was the only cationic sequence tested that contained a Trp-Leu-Trp hydrophobic region (Figure 4), and a similar sequence consisting of 3-pyridylalanine-Leu-Trp was present in **37**. Although we did not survey the sequence space for cationic sequences as extensively as for the class A neutral sequences, based on these data it seems likely that compounds with improved activity could be obtained, especially those having a Trp-Leu-Trp hydrophobic region and a charge around +3.

Our initial screens did not identify active sequences having two or five hydrophobic residues. However, we did find some active compounds that contained four hydrophobic amino acids, in addition to the peptides containing three hydrophobes discussed above. Sequences **45**−**54** all contain four hydrophobic residues and are cationic overall, ranging from overall charge of +1 to +4. Among these compounds, the HDL remodeling activity was highest for **49**−**51**, which contain one or two cationic residues and one or two His residues. Sequences containing just one cationic amino acid (**45** and **46**) or three or four cationic residues (**52**−**54**) were less active. All three of **49**−**51** had a similar hydrophobic region consisting of Trp-(Val or Phe)-aromatic-Tyr.

Considering the library as a whole, moderate levels of hydrophobicity were preferable for HDL remodeling activity. Nearly all of the most effective peptides (14 of the top 15 sequences) contained 3 or 4 hydrophobic residues, as opposed to those having 2 or 5 hydrophobic residues (one caveat is that relatively few peptides with 2 or 5 hydrophobic positions were screened). Moreover, the series of peptides **21**−**26** indicated that the least hydrophobic peptide of the group, **21**, was most effective in remodeling HDL. These findings may point to a requirement for the cyclic peptides to dissociate from the lipoprotein particles or only transiently interact with them, analogously to the requirement for apoA-I to exchange on and off HDL particles to protect against atherosclerosis.[39]

For each lead cyclic peptide, Figure 4 shows the putative nanotube wheel diagrams of a stack of six peptides, assuming the peptides orient to maximize hydrophobic clustering. It is notable that all four sequences contain aromatic Trp or Tyr residues at the interface positions of the hydrophobic region. At

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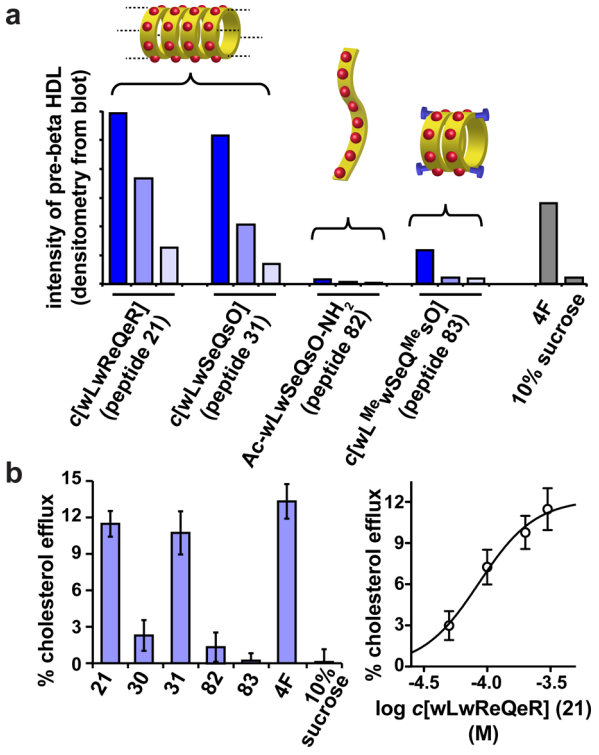

Figure 5. Cyclic D,L-α-peptides can promote HDL remodeling and cholesterol efflux in vitro. (a) Selected cyclic D,L-α-peptides enriched the level of pre-beta HDLs by remodeling human plasma HDL particles in a dose-dependent fashion in vitro. The peptides were assayed at 0.4, 0.2, and 0.1 mg/mL (~360, 180, and 90 μM) with analysis via Western blotting. Peptide 4F was used as a positive control at 0.1 mg/mL. The "10% sucrose" lane is a negative control. O, ornithine. (b) Selected peptides promoted cellular cholesterol efflux in vitro. In the left panel, all compounds were assayed at 0.3 mg/mL (~270 μM cyclic peptide) in human plasma. In the right panel, **21** was assayed over a range of concentrations. Efflux was measured from mouse macrophage J774 cells incubated with 2% apoB-depleted plasma samples for 4 h. Values are shown as mean ± SD of samples in quadruplicate, and are given relative to the vehicle-treated plasma sample as 0% efflux.

first glance, sequences **21** and **31** appear to differ in that **21** contains cationic amino acids at both sides of the polar region, whereas **31** has a cationic residue on one side and an anionic residue near the other side. However, due to the preference of cyclic D,L-α-peptides to stack in an antiparallel fashion, the nanotube structures of these two peptides are actually rather similar, with the main difference being the higher density of charged amino acids in **21**. Therefore, all four sequences (**21**, **31**, **40**, **51**) give rise to nanotubes in which cationic residues are found at the interface positions of the polar region. Peptides **21** and **40** have cationic residues at every interfacial position, whereas **31** and **51** have cationic residues at every other interfacial site. The nanotube formed by peptide **51** is somewhat different from the others, having only four polar residues and a stripe of cationic residues along the center of the polar region.

Cellular cholesterol efflux is a critical component of the antiatherogenicity of HDL.[8,40,41] Thus, we evaluated charge-neutral cyclic D,L-α-peptides **21** and **31** for promoting cellular cholesterol efflux from mouse macrophage J774 cells. Although cationic peptides **40** and **51** promoted more pre-beta HDL formation than **31**, sequences **21** and **31** were less cytotoxic than the cationic sequences, consistent with our prior experience with antimicrobial peptides. Specifically, **40** and **51** were more hemolytic and more toxic to mammalian cells in culture than **21** and **31** (Table 1).

To measure cholesterol efflux mediated by **21** and **31**, cholesterol-laden cells were incubated with peptide-treated plasma for 4 h, after which the level of cholesterol effluxed to the media was measured.[42] The cyclic D,L-α-peptide-treated plasma markedly promoted cholesterol efflux, and **21** showed a clear concentration dependency (Figure 5b). The 4F peptide, employed as a positive control, was slightly more effective than **21** and **31** in promoting cholesterol efflux, consistent with the somewhat higher levels of pre-beta HDL it caused compared to the cyclic peptides at the same concentration. The cyclic D,L-α-peptides did not appear to be toxic to the macrophage cells over the concentration range tested (up to 300 μM). It is noteworthy that when the efflux experiment was conducted with just the peptide agents as cholesterol acceptors, instead of peptide-treated plasma, cholesterol efflux was not increased compared to vehicle controls. In other words, the cyclic peptides were not sufficient cholesterol acceptors by themselves to promote efflux from the J774 cells. This is consistent with our observation that the cyclic peptides did not form peptide–lipid nanoparticles upon incubation with liposomes, unlike α-helical apoA-I mimetic peptides. Together, these findings suggest that the enhanced efflux capacity caused by the cyclic peptides is due to the increased concentration of pre-beta HDL in the plasma that results from peptide-mediated plasma HDL remodeling.

To further support the mechanistic insight gained from our initial screen, we tested several analogues of the lead compounds in the cholesterol efflux assay. Notably, the cholesterol efflux activity of **30**, in which the positions of the Arg and Glu residues were swapped, was dramatically reduced compared to that of **21** (Figure 5b), again supporting the importance of cationic residues at the interfacial region of the peptide. We established that the cyclic structure was required

Table 1. Bioactivity and Physical Data for Lead Peptides Identified by HDL Remodeling ELISA

				cytotoxicity LD_{50} (μM)			
no.	sequence	theor p*I* value[a]	pre-beta HDL[b] (μg/mL)	NCI	MCF7	SKOV-3	hemolysis HD_{50} (μM)
21	wLwReQeR	6.14	81 ± 11	36	>40	>40	>660 (4%)[c]
31	wLwSeQsO	6.00	58 ± 12	>40	>40	>40	>500 (3%)
40	wLwKhShK	10.00	68 ± 5	7.8	11	8.5	145
51	wFyYhOrS	9.70	63 ± 10	7.2	18	6.9	98

[a]Isoelectric point (p*I*) values were calculated by using the online ExPASy bioinformatics resource (http://web.expasy.org/compute_pi/). [b]Concentration of pre-beta HDL generated by the peptide after incubation with human plasma, as determined by ELISA. [c]Values in parentheses are the percent of hemolysis at the concentration listed (highest concentration tested).

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for activity by preparing two derivatives of peptide **31**. First, we assayed linear peptide Ac-wLwSeQsO-NH_2 (**82**), which failed to remodel plasma HDL or promote cholesterol efflux (Figure 5), even though it retains amphiphilicity. Second, we confirmed that peptide nanotube formation is require for plasma HDL remodeling activity and promotion of cholesterol efflux by assaying backbone N-methylated analogue c[wL^{Me}wSeQ^{Me}sO] (**83**). As with unstackable methylated peptide **27**, the efficacy of this N-methylated peptide was greatly diminished in both plasma HDL remodeling and cholesterol efflux.

To ascertain if the cyclic D,L-α-peptides would function in vivo to modulate HDLs, we administered **21** to mice (BALB/c, n = 3) via intraperitoneal (ip) injection at a dose of 20 mg/kg. We observed in each mouse a marked increase in the level of pre-beta HDL, consistent with the in vitro assays (Figure 6a). The effects persisted for up to 8 h from the preinjection time point, and this rather long period of action in vivo was supported by a pharmacokinetic study for **21** in mice (BALB/c, n = 3). After ip administration, the peptide had a plasma half-life of approximately 6 h (Figure 6b and Figure S5), consistent with the high proteolytic stability of cyclic D,L-α-peptides.[26,43]

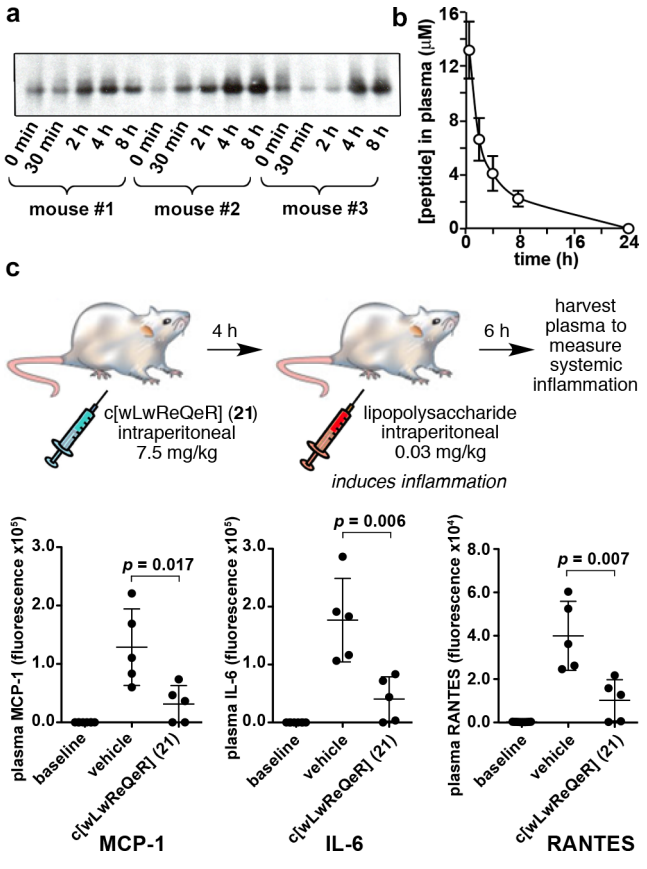

Figure 6. Cyclic D,L-α-peptide **21** exerts promising activity in vivo. (a) Peptide **21** increased the level of pre-beta HDLs after intraperitoneal injection (20 mg/kg) to male BALB/c mice (n = 3), as determined by Western blotting for mouse apoA-I. (b) The pharmacokinetic half-life of the peptide in vivo was several hours in mice. (c) Peptide **21** suppressed LPS-induced inflammatory responses in vivo (BALB/c mice, n = 5 per group), as indicated by reduced stimulation of certain cytokines. Systemic inflammatory responses were induced by ip injection of the endotoxin lipopolysaccharide (LPS). Peptide **21** (7.5 mg/kg) or vehicle was administered 4 h prior to LPS challenge; plasma samples were taken 6 h post challenge. Baseline refers to animals that did not receive LPS challenge. Data are shown as mean ± SD. p values were determined by a Student's unpaired two-tailed t-test. MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6; RANTES, regulated on activation, normal T cell expressed and secreted.

An important aspect of atheroprotection for HDLs is their anti-inflammatory properties. To test if active cyclic D,L-α-peptides could augment the anti-inflammatory properties of HDLs in vivo, we carried out a widely used model of acute inflammation in which systemic inflammatory responses are induced by ip injection of the endotoxin lipopolysaccharide (LPS).[44,45] Indeed, treatment with peptide **21** significantly protected mice from LPS-mediated induction of key biomarkers of inflammation, cytokines MCP-1, IL-6, and RANTES (Figure 6c).

CONCLUSION

Our results establish a new class of molecules that can enhance the function of HDLs to promote reverse cholesterol transport. Certain cyclic D,L-α-peptides, namely **21**, **31**, **40**, and **51**, represent distinct sequence motifs that markedly promoted the formation of pre-beta HDLs in human plasma. Each of these motifs may serve as a starting point for future optimization, such as by using focused combinatorial libraries.[46]

Previously studied apolipoprotein mimetic peptides can package lipids into HDL-like nanoparticles as an aspect of their mode of action.[12−17] In contrast, the cyclic D,L-α-peptides did not form peptide−lipid nanoparticles, as determined by FPLC and liposome clearance assays. We speculate that the cyclic peptides displace apoA-I from HDL particles to generate lipid-free or lipid-poor apoA-I,[32,33] which can then accept cellular lipids or cholesterol. If true, the cyclic D,L-α-peptides would be better described as modulators of HDL function (by increasing the concentration of the pre-beta subset of HDL) rather than as apoA-I mimetic peptides. Our finding that the cyclic peptides alone do not promote cellular cholesterol efflux, but cyclic peptide treated plasma does promote cholesterol efflux, supports this hypothesis. HDL modulation by cyclic D,L-α-peptides could offer a new approach for studying the stability, remodeling, and function of HDL particles, and for preparing new compositions of HDL particles.

Several interesting mechanistic questions remain to be answered. Do the cyclic D,L-α-peptides stay associated with HDLs, or only transiently interact to modulate their morphology and function? Are the peptides selective for lipoprotein particles vs cellular membranes in vivo? Are there important differences in the function of HDLs that have been remodeled by cyclic D,L-α-peptides compared to native HDLs? Ongoing studies are aimed at clarifying these important issues. Cyclic D,L-α-peptides are likely just one example of a suitable amphiphilic peptide architecture, and it is worth exploring other amphiphilic polymers, peptoids, dendrimers, etc.,[47−50] as remodelers of plasma HDL. It is conducive for further structure−activity optimization that self-assembling cyclic D,L-α-peptides can be derived from a large sequence space of natural and unnatural amino acids. Because such cyclic peptides are generally proteolytically stable and easy to synthesize, our results with these low-molecular-weight compounds offer an attractive new approach toward potential therapeutic agents for atherosclerosis.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental methods, Table S1, and Figures S1−S5 (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Joy, T. R. Novel HDL-based therapeutic agents. *Pharmacol. Ther.* **2012**, *135*, 18−30.

(2) Gadi, R.; Amanullah, A.; Figueredo, V. M. HDL-C: Does it matter? An update on novel HDL-directed pharmaco-therapeutic strategies. *Int. J. Cardiol.* **2013**, *167*, 646−655.

(3) Kontush, A.; Chapman, M. J. *High-density lipoproteins. Structure, metabolism, function and therapeutics*; Wiley: Hoboken, NJ, 2012.

(4) Schaefer, E. J., Ed. *High density lipoproteins, dyslipidemia, and coronary heart disease*; Springer: New York, NY, 2010.

(5) Asztalos, B. F.; Tani, M.; Schaefer, E. J. Metabolic and functional relevance of HDL subspecies. *Curr. Opin. Lipidol.* **2011**, *22*, 176−185.

(6) Lewis, G. F.; Rader, D. J. New insights Into the regulation of HDL metabolism and reverse cholesterol transport. *Circ. Res.* **2005**, *96*, 1221−1232.

(7) Rosenson, R. S.; Brewer, H. B., Jr.; Chapman, M. J.; Fazio, S.; Hussain, M. M.; Kontush, A.; Krauss, R. M.; Otvos, J. D.; Remaley, A. T.; Schaefer, E. J. HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. *Clin. Chem.* **2011**, *57*, 392−410.

(8) Rothblat, G. H.; Phillips, M. C. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr. Opin. Lipidol.* **2010**, *21*, 229−238.

(9) Tardif, J. C. Emerging high-density lipoprotein infusion therapies: fulfilling the promise of epidemiology? *J. Clin. Lipidol.* **2010**, *4*, 399−404.

(10) Kim, J.-S.; Kang, Y.; Son, K.-H.; Choi, S.-M.; Kim, K.-Y. Manufacturing and shelf stability of reconstituted high-density lipoprotein for infusion therapy. *Biotechnol. Bioprocess Eng.* **2011**, *16*, 785−792.

(11) Schmidt, H. H.; Haas, R. E.; Remaley, A.; Genschel, J.; Strassburg, C. P.; Buttner, C.; Manns, M. P. In vivo kinetics as a sensitive method for testing physiologically intact human recombinant apolipoprotein A-I: comparison of three different expression systems. *Clin. Chim. Acta* **1997**, *268*, 41−60.

(12) Gordon, S. M.; Davidson, W. S. Apolipoprotein A-I mimetics and high-density lipoprotein function. *Curr. Opin. Endocrinol., Diabetes Obes.* **2012**, *19*, 109−114.

(13) Osei-Hwedieh, D. O.; Amar, M.; Sviridov, D.; Remaley, A. T. Apolipoprotein mimetic peptides: mechanisms of action as anti-atherogenic agents. *Pharmacol. Ther.* **2011**, *130*, 83−91.

(14) Navab, M.; Shechter, I.; Anantharamaiah, G. M.; Reddy, S. T.; Van Lenten, B. J.; Fogelman, A. M. Structure and function of HDL mimetics. *Arterioscler., Thromb., Vasc. Biol.* **2010**, *30*, 164−168.

(15) Leman, L. J.; Maryanoff, B. E.; Ghadiri, M. R. Molecules That Mimic Apolipoprotein A-I: Potential Agents for Treating Atherosclerosis. *J. Med. Chem.* **2014**, *57*, 2169−2196.

(16) Zhao, Y.; Imura, T.; Leman, L. J.; Curtiss, L. K.; Maryanoff, B. E.; Ghadiri, M. R. Mimicry of high-density lipoprotein: functional peptide-lipid nanoparticles based on multivalent peptide constructs. *J. Am. Chem. Soc.* **2013**, *135*, 13414−13424.

(17) Zhao, Y.; Black, A. S.; Bonnet, D. J.; Maryanoff, B. E.; Curtiss, L. K.; Leman, L. J.; Ghadiri, M. R. In vivo efficacy of HDL-like nanolipid particles containing multivalent peptide mimetics of apolipoprotein A-I. *J. Lipid Res.* **2014**, *55*, 2053−2063.

(18) Damiano, M. G.; Mutharasan, R. K.; Tripathy, S.; McMahon, K. M.; Thaxton, C. S. Templated high density lipoprotein nanoparticles as potential therapies and for molecular delivery. *Adv. Drug Delivery Rev.* **2013**, *65*, 649−662.

(19) Thaxton, C. S.; Daniel, W. L.; Giljohann, D. A.; Thomas, A. D.; Mirkin, C. A. Templated spherical high density lipoprotein nanoparticles. *J. Am. Chem. Soc.* **2009**, *131*, 1384−1385.

(20) Watson, C. E.; Weissbach, N.; Kjems, L.; Ayalasomayajula, S.; Zhang, Y.; Chang, I.; Navab, M.; Hama, S.; Hough, G.; Reddy, S. T.; Soffer, D.; Rader, D. J.; Fogelman, A. M.; Schecter, A. Treatment of patients with cardiovascular disease with L-4F, an apo-A1 mimetic, did not improve select biomarkers of HDL function. *J. Lipid Res.* **2011**, *52*, 361−373.

(21) Bloedon, L. T.; Dunbar, R.; Duffy, D.; Pinell-Salles, P.; Norris, R.; DeGroot, B. J.; Movva, R.; Navab, M.; Fogelman, A. M.; Rader, D. J. Safety, pharmacokinetics, and pharmacodynamics of oral apoA-I mimetic peptide D-4F in high-risk cardiovascular patients. *J. Lipid Res.* **2008**, *49*, 1344−1352.

(22) Navab, M.; Anantharamaiah, G. M.; Reddy, S. T.; Hama, S.; Hough, G.; Frank, J. S.; Grijalva, V. R.; Ganesh, V. K.; Mishra, V. K.; Palgunachari, M. N.; Fogelman, A. M. Oral small peptides render HDL antiinflammatory in mice and monkeys and reduce atherosclerosis in ApoE null mice. *Circ. Res.* **2005**, *97*, 524−532.

(23) Brea, R. J.; Reiriz, C.; Granja, J. R. Towards functional bionanomaterials based on self-assembling cyclic peptide nanotubes. *Chem. Soc. Rev.* **2010**, *39*, 1448−1456.

(24) Hartgerink, J. D.; Clark, T. D.; Ghadiri, M. R. Peptide nanotubes and beyond. *Chem. - Eur. J.* **1998**, *4*, 1367−1372.

(25) Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. Self-assembling organic nanotubes based on a cyclic peptide architecture. *Nature* **1993**, *366*, 324−327.

(26) Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. Antibacterial agents based on the cyclic D,L-alpha-peptide architecture. *Nature* **2001**, *412*, 452−456.

(27) Montero, A.; Gastaminza, P.; Law, M.; Cheng, G.; Chisari, F. V.; Ghadiri, M. R. Self-assembling peptide nanotubes with antiviral activity against hepatitis C virus. *Chem. Biol.* **2011**, *18*, 1453−1462.

(28) Horne, W. S.; Wiethoff, C. M.; Cui, C.; Wilcoxen, K. M.; Amorin, M.; Ghadiri, M. R.; Nemerow, G. R. Antiviral cyclic D,L-alpha-peptides: targeting a general biochemical pathway in virus infections. *Bioorg. Med. Chem.* **2005**, *13*, 5145−5153.

(29) Kobayashi, K.; Granja, J. R.; Ghadiri, M. R. Beta-sheet peptide architecture: measuring the relative stability of parallel vs. antiparallel beta-sheets. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 95−98.

(30) Lu, S. C.; Atangan, L.; Won Kim, K.; Chen, M. M.; Komorowski, R.; Chu, C.; Han, J.; Hu, S.; Gu, W.; Veniant, M.; Wang, M. An apoA-I mimetic peptibody generates HDL-like particles and increases alpha-1 HDL subfraction in mice. *J. Lipid Res.* **2012**, *53*, 643−652.

(31) Hartgerink, J. D.; Granja, J. R.; Milligan, R. A.; Ghadiri, M. R. Self-assembling peptide nanotubes. *J. Am. Chem. Soc.* **1996**, *118*, 43−50.

DOI: 10.1021/acscentsci.7b00154
ACS Cent. Sci. 2017, 3, 639−646

(32) Gao, X.; Yuan, S.; Jayaraman, S.; Gursky, O. Differential stability of high-density lipoprotein subclasses: Effects of particle size and protein composition. *J. Mol. Biol.* **2009**, *387*, 628−638.
(33) Guha, M.; Gao, X.; Jayaraman, S.; Gursky, O. Correlation of structural stability with functional remodeling of high-density lipoproteins: The importance of being disordered. *Biochemistry* **2008**, *47*, 11393−11397.
(34) Kane, J. P.; Malloy, M. J. Prebeta-1 HDL and coronary heart disease. *Curr. Opin. Lipidol.* **2012**, *23*, 367−371.
(35) de la Llera-Moya, M.; Drazul-Schrader, D.; Asztalos, B. F.; Cuchel, M.; Rader, D. J.; Rothblat, G. H. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler., Thromb., Vasc. Biol.* **2010**, *30*, 796−801.
(36) Troutt, J. S.; Alborn, W. E.; Mosior, M. K.; Dai, J.; Murphy, A. T.; Beyer, T. P.; Zhang, Y.; Cao, G.; Konrad, R. J. An apolipoprotein A-I mimetic dose-dependently increases the formation of pre-beta1 HDL in human plasma. *J. Lipid Res.* **2008**, *49*, 581−587.
(37) Segrest, J. P.; Jones, M. K.; De Loof, H.; Brouillette, C. G.; Venkatachalapathi, Y. V.; Anantharamaiah, G. M. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J. Lipid Res.* **1992**, *33*, 141−166.
(38) Clark, T. D.; Buriak, J. M.; Kobayashi, K.; Isler, M. P.; McRee, D. E.; Ghadiri, M. R. Cylindrical beta-sheet peptide assemblies. *J. Am. Chem. Soc.* **1998**, *120*, 8949−8962.
(39) Borja, M. S.; Zhao, L.; Hammerson, B.; Tang, C.; Yang, R.; Carson, N.; Fernando, G.; Liu, X.; Budamagunta, M. S.; Genest, J.; Shearer, G. C.; Duclos, F.; Oda, M. N. HDL-apoA-I exchange: rapid detection and association with atherosclerosis. *PLoS One* **2013**, *8*, e71541.
(40) Rader, D. J.; Alexander, E. T.; Weibel, G. L.; Billheimer, J.; Rothblat, G. H. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J. Lipid Res.* **2009**, *50*, S189−S194.
(41) von Eckardstein, A.; Nofer, J.-R.; Assmann, G. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arterioscler., Thromb., Vasc. Biol.* **2001**, *21*, 13−27.
(42) Sankaranarayanan, S.; Kellner-Weibel, G.; de la Llera-Moya, M.; Phillips, M. C.; Asztalos, B. F.; Bittman, R.; Rothblat, G. H. A sensitive assay for ABCA1-mediated cholesterol efflux using BODIPY-cholesterol. *J. Lipid Res.* **2011**, *52*, 2332−2340.
(43) Dartois, V.; Sanchez-Quesada, J.; Cabezas, E.; Chi, E.; Dubbelde, C.; Dunn, C.; Granja, J.; Gritzen, C.; Weinberger, D.; Ghadiri, M. R.; Parr, T. R., Jr. Systemic antibacterial activity of novel synthetic cyclic peptides. *Antimicrob. Agents Chemother.* **2005**, *49*, 3302−3310.
(44) Morrison, D. C.; Duncan, R. L., Jr.; Goodman, S. A. In vivo biological activities of endotoxin. *Prog. Clin. Biol. Res.* **1985**, *189*, 81−99.
(45) Li, P.; Allen, H.; Banerjee, S.; Franklin, S.; Herzog, L.; Johnston, C.; McDowell, J.; Paskind, M.; Rodman, L.; et al. Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. *Cell* **1995**, *80*, 401−411.
(46) Redman, J. E.; Wilcoxen, K. M.; Ghadiri, M. R. Automated Mass Spectrometric Sequence Determination of Cyclic Peptide Library Members. *J. Comb. Chem.* **2003**, *5*, 33−40.
(47) Horne, W. S. Peptide and peptoid foldamers in medicinal chemistry. *Expert Opin. Drug Discovery* **2011**, *6*, 1247−1262.
(48) Olsen, C. A. Peptoid-peptide hybrid backbone architectures. *ChemBioChem* **2010**, *11*, 152−160.
(49) Zuckermann, R. N.; Kodadek, T. Peptoids as potential therapeutics. *Curr. Opin. Mol. Ther.* **2009**, *11*, 299−307.
(50) Palmer, L. C.; Stupp, S. I. Molecular self-assembly into one-dimensional nanostructures. *Acc. Chem. Res.* **2008**, *41*, 1674−1684.

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ACS Cent. Sci. 2017, 3, 639−646