Published in final edited form as:
Circ Res. 2010 July 23; 107(2): 217–227. doi:10.1161/CIRCRESAHA.110.216507.

Structure-function relationships of apolipoprotein A-I mimetic peptides: implications for anti-atherogenic activities of high density lipoprotein

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Abstract

Rationale—Apolipoprotein A-I (apoA-I) mimetic peptides are a promising type of anti-atherosclerosis therapy, but how the structural features of these peptides relate to the multiple anti-atherogenic functions of HDL is poorly understood.

Objective—To establish structure-function relationships of apoA-I mimetic peptides with their anti-atherogenic functions.

Methods and Results—Twenty two bi-helical apoA-I mimetic peptides were investigated *in vitro* for the capacity and specificity of cholesterol efflux, inhibition of inflammatory response of monocytes and endothelial cells and inhibition of low density lipoprotein (LDL) oxidation. It was found that mean hydrophobicity, charge, size of hydrophobic face and angle of the link between the helices are the major factors determining the efficiency and specificity of cholesterol efflux. The peptide with optimal parameters was more effective and specific towards cholesterol efflux than human apoA-I. Charge and size of hydrophobic face were also the major factors affecting anti-inflammatory properties and the presence of cysteine and histidine residues was the main factor determining anti-oxidant properties. There was no significant correlation between capacities of the peptides to support individual functions; each function had its own optimal set of features.

Conclusions—None of the peptides was equally effective in all the anti-atherogenic functions tested, suggesting that different functions of HDL may have different mechanisms and different structural requirements. The results do suggest, however, that rationalizing the design of apoA-I mimetic peptides may improve their therapeutic value and may lead to a better understanding of mechanisms of various anti-atherogenic functions of HDL.

Keywords

Mimetic peptides; high density lipoprotein; atherosclerosis

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Disclosures: None

Introduction

Atherosclerosis underlies most cases of cardiovascular disease (CVD), which is now the major cause of morbidity and mortality in developed countries. Accumulation of cholesterol in the arterial wall and vascular inflammation are in the centre of the pathogenesis of atherosclerosis, and treatments controlling delivery of cholesterol and inflammation (statins) reduce the incidence of cardiovascular disease by 30-40% [1]. There is, however, an urgent need for further reduction of the unacceptably high remaining risk of CVD. A most promising direction is complementing reduction in levels of the pro-atherogenic lipoproteins with increasing levels of the anti-atherogenic lipoprotein, high density lipoprotein (HDL), "HDL therapy" [2]. It is becoming clear that success of HDL therapy critically depends on the mechanism for elevating HDL; a straight forward and so far the most successful approach being direct infusion of exogenous HDL [3, 4]. Infusion of reconstituted HDL (rHDL), however, has considerable limitations due to high cost and requirement for intravenous delivery making it suitable mainly for acute treatment. An alternative to rHDL is apolipoprotein A-I (apoA-I) mimetic peptides.

ApoA-I mimetic peptides mimic the secondary structure of the major structural element of apoA-I, 22-mer amphipathic type A α-helix [5]. It appears that there is no requirement for a homology between the primary structure of these peptides and apoA-I; these peptides are active as long as the secondary structure of apoA-I is replicated [5, 6]. ApoA-I mimetic peptides show remarkable capacity to support cholesterol efflux, share the anti-inflammatory properties of HDL and reduce development of atherosclerosis in animal models [7-11]. ApoA-I mimetic peptides cost a fraction of the cost of rHDL; they are safe and well tolerated [12], and approaches for oral delivery are being developed [8, 13]. Perhaps the best advantage of these peptides is the ability to modify their structure to better understand the mechanisms of atheroprotective action of HDL, with a view to further improving the atheroprotective capacity of the peptides. Relatively limited research has been done to understand structure-function relationships of apoA-I mimetic peptides. The current understanding of the structure-function relationship of the peptides can be summarized as follows:

- The amphipathic α-helix of 18- 22 residues is essential for a peptide to mimic apoA-I and to be atheroprotective [14].
- There is no stereospecificity: peptides made of D-amino acids are as effective as those made of L-amino acids [10, 13, 15].
- Increasing hydrophobicity by including 2 or 4 phenylalanine residues improves the capacity of peptides to associate with lipids [16] and anti-inflammatory capacity of the peptides [8].
- Alignment of negative charges on the polar face increases cholesterol efflux [17].
- Two helixes connected through proline residue work better than a single helix in cholesterol efflux and inflammation assays [18, 19].
- Introducing asymmetry in bi-helical peptide improves its specificity in cholesterol efflux assay and reduces toxicity [20].

While the atheroprotective functions of HDL are not limited to its role in reverse cholesterol transport and inflammation, only these two functions have been tested in most studies, but never, to our knowledge, simultaneously. In the current study, we undertook a comprehensive analysis of the structure-function relationship of apoA-I mimetic peptides. We used bi-helical peptides not only because they have shown a better anti-atherosclerosis activity, but also because they offer an opportunity to mimic variability in the structure of two adjacent helices, a property increasing their similarity to apoA-I and improving

specificity of at least some their functions [18, 20]. We investigated the impact of changes in peptide mean hydrophobicity, size of hydrophobic face, charge, type of α-helix, configuration of the bridge between two helices, asymmetry of the helices and inclusion of specific residues. The impact of these changes on the capacity and specificity of cholesterol efflux, inflammatory response of monocytes and endothelium and anti-oxidant properties were studied. We endeavored to determine which structural features listed above are important for the four anti-atherogenic functions of the peptides and to establish optimal combination(s) of these features favoring each or all of these functions.

Methods

Cholesterol efflux

Cellular cholesterol was labeled by incubation in serum-containing medium with [^{3}H]-cholesterol for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37°C in serum-free medium in the presence or absence of TO-901317 (4 μmol/L). Cells were washed and incubated for another 4 h at 37°C (THP-1 cells) or 18 h (BHK-1 cells) in serum-free medium containing indicated concentrations of the peptides or lipid-free apoA-I. Where indicated, cells were fixed by incubation for 20 min with paraformaldehyde (4%) prior to the efflux experiments.

Expression of CD11b on human monocytes

Resting human monocytes were stimulated with 1μmol/L phorbol-12 myristate 13-acetate (PMA) in the presence or absence of the peptides or apoA-I final (concentration 40 μg/mL) and incubated with the FITC conjugated Ab to the active epitope of CD11b for 15min at 37°C. Cells were then fixed with 4% formaldehyde and CD11b expression was measured by flow cytometry.

Expression of VCAM-1 in mouse endothelial cells

SVEC4/VCAM-1 cells were washed and apoA-I, HDL or apoA-I mimetic peptides were added at the final concentration of 0.75 mg/ml. After 18 h incubation tissue necrosis factor (TNF-α) was added in serum-free medium to the final concentration of 10 ng/ml. Cells were incubated for 5 h and luciferase activity was measured using Bright-Glo Assay.

Oxidation of LDL

Freshly isolated LDL (final concentration 100 μg/ml) was incubated at 25°C for the indicated periods of time with $CuSO_4$ (final concentration 15 μMol/L) in the presence of the peptides or apoA-I (final concentration of 100 μg/ml) and absorption was continually monitored at 234 nm.

Results

Structure of apoA-I mimetic peptides

Twenty two apoA-I mimetic peptides were synthesized; their sequences, physicochemical properties and general features are shown in Table 1. Two peptides were used as prototypes, in order to understand how changes in their structures affect their function. The first prototype peptide was 5A (#1), which was described by us previously; it consists of two type A amphipathic α-helices connected through proline; hydrophobicity of the second helix was reduced by substituting hydrophobic amino acids with alanine [20]. Four derivatives of 5A were synthesized (peptides 19-22) to test the impact of the introduction of two aminoacids known to be anti-oxidants, cysteine and histidine, on its properties. The second prototype peptide was ELK (#2), which is made of just three amino acids, glutamic acid,

leucine and lysine [21]. Itconsists of two identical canonical type A amphipathic α-helices with 180 degree hydrophobic face and neutral net charge; helices are connected with a proline residue. ELK peptide was used to make sixteen modifications (peptides 3-18) testing the impact of modification affecting the following features: *i*) net charge, as it may affect interaction with cellular receptors and lipids; *ii*) mean hydrophobicity and size of hydrophobic face, as they may affect the interaction with lipids and cellular receptors; *iii*) type of helix and configuration of the proline bridge between the two helices, as it may affect interaction with lipoprotein particles and cellular receptors, as well as properties of complexes of peptides with lipids after they acquire the latter from cells; *iv*) asymmetry, as it was shown to affect specificity of cholesterol efflux. Some of these properties are interdependent (e.g. charge and hydrophobicity) requiring testing several peptides with combinations of these features. Peptides were tested in lipid-free form to mimic the interaction of lipid-free apoA-I with cells and to exclude the confounding effects of lipid-binding properties of the peptides and variations in size of "rHDL" particles.

Efficiency of cholesterol efflux from human monocyte cell line THP-1

To test the capacity of cholesterol efflux to the apoA-I mimetic peptides, human monocytic cells THP-1 were differentiated into macrophages, activated or not with LXR agonist TO-901317, which induces expression of ABC transporters, labeled with [^{3}H]cholesterol and incubated with various concentrations of peptides for 4 h. THP-1 cells not activated with LXR agonist contain low levels of ABC transporters, therefore, the efflux from non-activated cells was considered to represent the component of the efflux that was not mediated by these transporters. The difference between the efflux in the presence and absence of LXR agonist was therefore defined as ABCA1-mediated cholesterol efflux. The dose-dependencies of the efflux from THP-1 cells are presented in Supplementary Figures I-III. Fig. I shows the efflux from the cells activated with TO-901317, Fig. II shows the efflux from cells not-activated with TO-901317, and Fig. III shows a difference between the effluxes from activated and non-activated cells, i.e. ABCA1-dependent efflux. To quantitate cholesterol efflux, the areas under the dose dependence curves (AUC) were calculated, as well as the contribution of ABCA1 for cholesterol efflux at non-saturating concentration of 20 μg/ml. These parameters are shown in Table 2. Analysis of the structure-function relationships, as related to the capacity of the peptides to support ABCA1-dependent cholesterol efflux, allowed for the following conclusions.

Hydrophobicity and charge—The relationship between mean hydrophobicity and the capacity of the peptides to support cholesterol efflux is shown in Fig. 1 A. It appears that the relationship is characterized by a sharp peak around a mean hydrophobicity value of −0.5. Adding charges inevitably changes hydrophobicity, making it difficult to investigate the effect of the charge independently of hydrophobicity. It appears, however, that peptides carrying positive (squares) and negative (triangles) charges have lesser capacity to support cholesterol efflux and an overall neutral charge is optimal. The peptide ELK-2A2K2E was synthesized after this initial analysis, creating a peptide with neutral charge and optimal hydrophobicity. Indeed this peptide showed exceptional capacity to support cholesterol efflux, even exceeding that of apoA-I, supporting our conclusions. Four neutral charged peptides that had average hydrophobicity around −0.5, but still failed to support cholesterol efflux (Fig. 1 A) all had other features strongly detrimental for the efflux capacity, such as the inclusion of histidine and/or cysteine residues or asymmetry in ELK peptides or both (see below).

Size of hydrophobic face—Increasing the size of hydrophobic face was beneficial, as long as overall hydrophobicity and charge were maintained (ELK-2F, ELK-1F).

Type of helix—Changing the type of helix from the type A helix found in ELK to type G and Y helices, as was done for peptides ELK-3E3EK and ELK-2E2K, had a small beneficial effect on the capacity of the peptides to support cholesterol efflux.

Proline bridge—Substitution of Ala for Pro in the bridge, as was done for peptide ELK-PA, was detrimental for the efflux capacity, however, substitution of Ala-Ala for Pro (peptide ELK-P2A), which generated half of the angle generated by proline, restored the efflux capacity.

Asymmetry—Asymmetry had a significant beneficial effect for the peptide 5A as compared to the parent symmetrical peptide L37PA [20]; however, the same feature tested on ELK peptides had a strong detrimental effect (ELKA *versus* ELK).

Inclusion of Cys and His—This was tested on the derivatives of asymmetrical peptide 5A. Inclusion of Cys or His and especially Cys+His in the first (hydrophobic) helix (peptides 5A-CH1, 5A-H1 and 5A-C1) was detrimental for the efflux, whereas inclusion of these amino-acids in the second (less hydrophobic) helix (peptide 5A-CH2) was beneficial.

With few exceptions, the contribution of ABCA1 transporter for cholesterol efflux was proportional to the overall capacity of the peptides to support cholesterol efflux (Table 2 and Supplementary Figure IV), resulting in a statistically significant correlation between these two parameters ($r=0.66$, $p<0.001$), (Fig. 1 B). This finding confirms that changes in the peptide structure affected specifically the ABCA1-dependent component of the efflux.

Finally, we analyzed the effect of complexing the most active peptide, ELK-2A2K2E with phospholipid on cholesterol efflux. Complex ELK-2A2K2E/POPC was significantly more effective in supporting cholesterol efflux from THP-1 cells (Table 2, last row). Cholesterol efflux to lipidated particles is mediated by several mechanisms, including ABCG1, SR-B1 as well as by aqueous diffusion, and while the former is activated by LXR agonists, the latter are not; thus the contribution of specific transporters could not be tested using this design.

Specificity of cholesterol efflux from human monocyte cell line THP-1

The amphipathic nature of the peptides is essential for their capacity to support cellular cholesterol efflux and form lipoprotein particles; however, it can potentially cause cytotoxicity by damaging the plasma membrane [15]. To analyze the contribution of the potentially cytotoxic "non-specific" efflux, we compared cholesterol efflux to the peptides at saturating concentration (80 μg/ml) from live THP-1 cells and cells fixed with paraformaldehyde, a method we used previously to analyze cytotoxic properties of peptides 5A and L37PA [20]. The data for cholesterol efflux are shown in Supplementary Figure V, the absolute values of non-specific efflux (i.e. efflux from fixed cells) is shown in Fig. 2 A and the "specificity" of the efflux (i.e. efflux from live cells minus efflux from fixed cells divided by the efflux from live cells x100%) is shown in Fig. 2 B. Analyzing features of the peptides responsible for the high non-specific efflux, we excluded from consideration peptides with low overall capacity to support efflux from live cells (marked with arrows in Fig. 2). The rationale for this exclusion was that analyzing efflux properties of the peptides that do not support total cholesterol efflux would not provide meaningful information about specificity of the efflux. Two features of the peptides associated with the high level of non-specific efflux were the following:

1. Net positive charge of the peptide (peptides with charge ≥ +2 denoted with fine cross-hatched bars in Fig. 2)

2. Replacement of the proline a bridge with a single alanine (peptide ELK-PA denoted with coarse cross-hatched bar in Fig. 2)

Thus, these two features should be avoided to avert toxicity of the peptides. The analysis also pointed to the two peptides with exceptional specificity, ELK-2A2K2E and ELK-1W; their specificity surpassing that of apoA-I. However, while the former peptide was very active in ABCA1-dependent cholesterol efflux, the latter had a modest capacity for the ABC-dependent efflux, indicating that peptide ELK-1W may interact with alternative transporters or receptors that promote cholesterol efflux.

Cholesterol efflux from BHK and BHK/ABCA1 cells

Although it is likely that the cholesterol efflux observed from the THP-1 cells was due to ABCA1, because only lipid-free peptides were used, it is possible that other ABC transporters, such as ABCG1, could also be contributing to the measured cholesterol efflux. An alternative model was, therefore, also used to assess the capacity and specificity of the peptides towards cholesterol efflux. BHK cells stably transfected with ABCA1 were compared to parent BHK cells, which do not have significant levels of endogenous ABCA1 or ABCG1[22]; thus the difference between the two cell lines was defined as ABCA1-dependent cholesterol efflux.

With two exceptions, peptides ELK-2A and 5A-CH2, the efficiency of the peptides toward specifically ABCA1-dependent efflux closely followed the ABCA1-dependent efflux observed from THP-1 cells (Fig. 3 A). There was a significant correlation between the ABCA1-dependent efflux from BHK cells and ABC-dependent efflux from THP-1 cells (rank order correlation: $r=0.75$, $p<0.0001$) (Fig. 3 B). The relationships between structural features of the peptides and efficiency of the efflux from BHK-ABCA1 cells was similar to that of THP-1 cells, except that relationship between efflux and mean hydrophobicity peaked at a slightly higher value of −0.4 (not shown). Thus, cholesterol efflux to lipid-free peptides primarily reflects ABCA1-dependent efflux.

Anti-inflammatory properties: monocytes

We have previously demonstrated that apoA-I and HDL are capable of reducing the expression of a key adhesion molecule, CD11b, on human monocytes in response to activation with a number of pro-inflammatory stimuli [23]. We tested the capacity of the peptides to mimic this property of apoA-I on human monocytes activated with PMA in the presence of the peptides or apoA-I (final concentration 40 μg/ml). The expression of CD11b was assessed by flow cytometry and is shown in Fig. 4 A. All peptides, with the exception of ELK-1F and ELK-2A, inhibited the expression of CD11b on activated human monocytes; there was a 6-fold difference in the magnitude of inhibition between the most and the least efficient peptides. Analysis of structure-function relationships as related to the inhibition of CD11b expression led to the following conclusions:

1. The structural feature having the most impact on the capacity of peptides to inhibit CD11b expression was asymmetry: all three asymmetrical peptides, 5A, ELKA and ELKA-CH2, were most active in inhibition of CD11b expression on monocytes.
2. Second feature having considerable impact on the capacity of the peptides to inhibit CD11b expression was the size of the hydrophobic face. An increase of the size of hydrophobic face over 180° was detrimental for the anti-inflammatory property of the peptides (peptides ELK-2F, ELK-1F and ELK-2A).
3. Third feature affecting this anti-inflammatory property was charge. Two or more additional positive charges were detrimental (peptides ELK-2A, ELK-1A and

ELK-1L1K); however, additional negative charges were neither detrimental nor beneficial (peptides ELK-1W, ELK-3E3LK and ELK-3E3K3A).

4. Changing the type of helix to type G or Y was detrimental for the anti-inflammatory capacity of the peptides (peptides ELK-2E2K and ELK-3E3EK).

5. Introduction of Cys and His residues or manipulation with proline bridge between the two helices had limited impact on the anti-inflammatory properties of the peptides.

Thus, the optimal structural features of an effective anti-inflammatory peptide are an asymmetrical pair of type A α-helices with hydrophobic face less than 180° and with neutral or negative charge.

We also analyzed the effect of complexing of the most active peptide, ELK-CH2, with phospholipid on its capacity to inhibit CD11b expression. Complex ELK-CH2/POPC had a higher capacity to inhibit CD11b expression compared to lipid-free peptide (Fig. 4 A). Similar effects were observed for the peptide, 5A (not shown).

Anti-inflammatory properties: endothelium

HDL and apoA-I affect expression of adhesion molecules on endothelial cells and this function may contribute significantly to the anti-inflammatory properties of HDL [24]. To test the capacity of the peptides to mimic anti-inflammatory function of apoA-I towards endothelium we used a mouse endothelial cell line (SVEC4) stably transfected with luciferase under control of human VCAM-1 promoter. It was originally suggested that while apoA-I reconstituted with phospholipid or native HDL were potent inhibitors of VCAM-1 expression, lipid-free apoA-I may not be as effective ascribing endothelial anti-inflammatory property of HDL to its lipid constituencies [25]. This was not confirmed in our studies, with mouse endothelial cells: lipid-free apoA-I was just as an effective inhibitor of VCAM-1 expression as HDL, inhibiting 90% of VCAM-1 expression (HDL inhibited VCAM-1 expression by 95%, $p>0.05$ *versus* apoA-I) (Fig. 4 B). Peptides were tested in lipid-free form; cells were activated with TNF-α and incubated with apoA-I, HDL or the peptides at the final concentration of 0.75 mg/ml, which was found to be a non-saturating concentration of the peptides for this response. Analysis of structure-function relationships as related to the inhibition of VCAM-1 expression is shown in Fig. 4 B and led to the following conclusions:

1. Increased size of hydrophobic face (peptides ELK-2A, ELK-1F, ELK-2F) was beneficial for the inhibition of the VCAM-1 expression

2. Negative charge (peptides ELK-1W, ELK-3E3LK, ELK-3E3K3A) was detrimental for the inhibition of VCAM-1 expression.

3. Inclusion of a combination of Cys+His residue was detrimental independently of their location (peptides 5A-CH2, 5A-CH1), while inclusion of Cys residue into the first helix of asymmetrical peptide was beneficial (peptide 5A-C1).

4. Hydrophobicity, changing helix type, disruption of the proline bridge and asymmetry had limited impact on the capacity of the peptides to inhibit VCAM-1 expression.

Thus, to be effective in inhibition of VCAM-1 expression the peptide ideally should have a larger hydrophobic face, positive or neutral charge, and may contain a Cys residue. We also analyzed the effect of complexing of the most active peptide, ELK-2A, with phospholipid on its capacity to inhibit VCAM-1 expression. Unexpectedly, complex ELK-2A/POPC did not inhibit VCAM-1 expression (Fig. 4 B). Complexing ELK-2A with another phospholipid,

DMPC, partially restored the capacity of the peptide to inhibit VCAM-1 expression (Fig. 4 B). Similar effects were observed for the peptide, 5A (not shown).

Anti-oxidant properties

The anti-oxidant properties of the peptides were assessed in an LDL oxidation assay. Human plasma LDL was incubated in the presence of Cu^{++} and apoA-I mimetic peptides or apoA-I (final concentration 100 μg/ml); time-course of diene formation was monitored by measuring absorption at 234 nm. Duration of lag phase and maximum diene formation were used to quantitate the rate of LDL oxidation as described by Pinchuk et al [26]. The time-course curves for LDL oxidation are shown in supplementary Fig. VI, and the rates of LDL oxidation are shown in Fig. 5. All peptides, with the exception of ELK-1K1A1E, ELK-1F and ELK-1L1K inhibited oxidation of LDL by Cu^{++}; there was a 5-fold difference in the magnitude of inhibition between the most and least effective peptides. Analysis of structure-function relationships as related to the inhibition of LDL oxidation led to the following conclusions:

1. As expected, presence of Cys and/or His residue (peptides 5A-CH1, 5A-C1, 5A-H1, 5A-CH2, ELKA-CH2) significantly increased the anti-oxidant capacity of the peptides. The exact position of the residues had limited impact.
2. Unexpectedly, changes affecting secondary structure of the peptides had beneficial effect: asymmetrical peptides, peptides comprising type G and type Y helices (peptides ELK-3E3EK and ELK-2E2K), as well as peptides with modification of the proline bridge (peptides ELK-P2A and ELK-PA) were all better anti-oxidants. Most peptides were better anti-oxidants than apoA-I.
3. Charge, hydrophobicity and size of hydrophobic face had limited impact on anti-oxidant capacity of the peptides.

Thus, an effective anti-oxidant peptide should contain Cys residue and preferably contains a non- type A α-helix, such as type G or type Y.

We also analyzed the effect of complexing of the most active peptide, 5A-CH1, with phospholipid on its capacity to inhibit LDL oxidation. Complex 5A-CH1/POPC had a higher capacity to inhibit LDL oxidation compared to lipid-free peptide (Fig. 5).

Relationships between different anti-atherogenic properties of the peptides

The finding that different apoA-I mimetic peptides have a wide range of efficiencies towards various anti-atheroghenic properties made it possible to investigate if any of these functions are related to each other. We found, however, no significant positive relationship for the various assays. In fact, the analysis of the structural features shows that features beneficial for one function may be detrimental for another (Table 3). For example, increased size of hydrophobic face was beneficial for cholesterol efflux, but detrimental for the monocyte anti-inflammatory function. Maintaining the proline bridge was essential for the efflux, but was detrimental for the anti-oxidant function and did not affect monocyte anti-inflammatory function. While several peptides were superior to apoA-I for some of the individual functional assays, none of them was better than apoA-I in all tested functional assays. These findings are consistent with a suggestion that the various functions of apoA-I may have different structural requirements and are determined by different regions of the protein.

Discussion

In this study we analyzed the structure-function relationship of 22 bi-helical apoA-I mimetic peptides to identify structural features of these peptides that enable them to better mimic the

various anti-atherogenic functions of HDL. We also aimed at establishing if the various anti-atherogenic properties of apoA-I are dependent upon the same structural features, and if so, whether they share the same underlying mechanism.

The critical features of the peptides for cholesterol efflux capacity and ABCA1 specificity were hydrophobicity, size of the hydrophobic face, charge, and angle between two helices. Some of these features, such as requirement for a proline bridge [18, 19] or size of hydrophobic face [8], were previously investigated and our data are consistent with the results of these studies; however, this is the first systematic analysis of multiple structural modifications on cholesterol efflux. Following this analysis, we were able to synthesize a peptide combining all beneficial features required for cholesterol efflux, ELK-2A2K2E. This peptide was more effective than apoA-I in capacity and specificity of cholesterol efflux, thus supporting our findings. As was shown for the experiment with ABCA1 transfected cells, the optimal structural features most likely enable these peptides to interact with ABCA1 and for triggering any downstream events that are necessary for cholesterol efflux. An unresolved issue among factors affecting cholesterol efflux is requirement for an asymmetry of the peptides. We have previously shown that introduction of asymmetry in bi-helical peptide L37PA resulted in dramatic improvement of specificity, with only modest reduction in overall efflux capacity [20]. We attempted to introduce such an asymmetry into ELK-2A2K2E peptide (peptide ELKA) expecting a peptide with even better cholesterol efflux specificity. However, this peptide had very low overall capacity to support cholesterol efflux; the reasons for this are yet to be established.

Anti-oxidant property of the peptides strongly depend on the presence of particular amino acids, such as histidine and cysteine, a finding consistent with that of Jia et al [27]. Presence of these amino-acids and enhanced anti-oxidant capacity have also been proposed to be behind the anti-atherogenic properties of apoA-I_{Milano} and apoA-I_{Paris} [28]. As expected, the other physico-chemical properties of the peptides had limited impact on anti-oxidant capacity, but unexpectedly changes disrupting "apoA-I – like" secondary structure, such as changing helix type or removing proline bridge were beneficial. Possibly these changes alter the binding of these peptides to LDL, as was suggested by Getz et al [19].

Anti-inflammatory properties of the peptides were investigated in two models, related to the expression of adhesion molecules on monocytes and endothelium. The anti- inflammatory effect of HDL to these two cell types, however, likely involves different mechanisms. The anti-inflammatory effect of HDL on monocytes is fast, short lived and requires low concentration of apoA-I [23], whereas the response of endothelial cells is slow, long lasting and requires high levels of HDL [29]. These differences suggest different mechanisms responsible for the anti-inflammatory effects of HDL in these cell types. It is therefore not surprising that the structural requirements for anti-inflammatory effect of the different peptides in these two models did not overlap and in fact were almost opposite. Peptides active in inhibiting monocyte CD11b were asymmetrical peptides with a smaller hydrophobic face and a negative charge. In contrast, peptides active in inhibiting expression of endothelial VCAM-1 had relatively large hydrophobic faces and were positively charged. As the mechanisms of the anti-inflammatory effects of apoA-I on monocytes and endothelial cells are not known, it is premature to speculate on how these structural features are translated into differences in the expression of adhesion molecules.

Another interesting finding of this study was that different atheroprotective functions of the peptides were determined by different structural features. No consistent correlation was found between the capacities of the peptides to mediate the various functions. No specific structural feature equally benefitted all functions. Furthermore, time and dose dependencies of the effects of the peptides and apoA-I on specific functions varied dramatically from one

function to another: it took under 15 min to inhibit the expression of CD11b and almost 24 h to inhibit expression of VCAM-1. The saturating concentration of the peptides significantly differed for the different assays. Approximately 20 μg/ml of the peptides was required for maximum cholesterol efflux, whereas 100 μg/ml was needed for anti-oxidant capacity and over 750 μg/ml for inhibition of VCAM-1 expression in endothelium. These findings suggest that different anti-atherogenic functions of apoA-I have different mechanisms. This is consistent with findings of Wool et al [18], who demonstrated that modification of the peptides that favors HDL remodeling have negative impact on anti-oxidant function. Although a number of peptides were better than apoA-I in supporting individual functions, none of them could match the versatility of apoA-I when all the functions were taken into consideration. Possibly, different parts of apoA-I are responsible for different anti-atherogenic functions and mimicking just one or two structural features of apoA-I is insufficient to create a peptide active in the many anti-atherogenic facets of apoA-I. Although it may, therefore, be difficult to duplicate all of the biological properties of apoA-I in a single peptide of limited length, it may be possible to use a combination of peptides, an option that is currently being tested. The uncoupling, in the peptides, of the different anti-atherogenic properties of HDL, however, creates a unique opportunity to investigate the relative contribution of the different anti-atherogenic activities of HDL by testing them in animal models of atherosclerosis.

It is important to recognize several potential limitations of this study. First, HDL constituents other than apoA-I most likely also contribute to the anti-atherogenic properties of HDL. Size of HDL affects its ability to support cholesterol efflux [30], paraoxonase has a significant contribution to the anti-oxidant function [31], phospholipids may contribute to the anti-inflammatory effects of HDL to endothelium [25] and various pro- and anti-inflammatory factors carried on HDL may contribute to the HDL anti-inflammatory effects [32]. Furthermore, a number of HDL functions were not investigated in this study, such as anti-thrombotic activity, suppression of apoptosis, regulation of endothelial function, insulin secretion and glucose oxidation. Although these functions may contribute to the anti-atherogenic properties of HDL, most available data suggest that involvement of HDL in cholesterol efflux, inflammation and oxidation are the major determinants of its atheroprotective potential and therefore are the main targets of "HDL therapy". Finally, peptides were examined in their lipid-free form. This was a deliberate strategy, as the physico-chemical properties of the peptides will have a significant impact on the lipid content of rHDL particles assembled with the peptides, the size of these particles and on the number of peptide molecules per particle. These factors may have a significant confounding influence on many anti-atherogenic properties of the peptides. The lipid-free peptides may also better simulate the process by which apoA-I interacts with cells and lipid-free peptides were shown to be as effective as their phospholipid complexes in preventing atherosclerosis in vivo [33]. It is likely, however, that these peptides, like apoA-I, will readily acquire *in vivo* various lipids and/or will recombine with endogenous HDL; therefore, future *in vivo* studies will be needed to fully assess the effect of these peptides on atherosclerosis. Lipidated peptides (rHDL) would also interact with endogenous HDL and undergo remodeling and therefore would not be a better representation of the *in vivo* situation than lipid-free apoA-I. Acknowledging however that *in vivo* apoA-I mimetic peptides are likely to be lipidated, we examined the effect of complexing the most active peptides with phospholipid on their activity. As expected, lipidation increased peptide cholesterol efflux capacity, most likely by complementing ABCA1-dependent efflux with the efflux through other pathways. Lipidation further improved peptide capacities to prevent LDL oxidation and to inhibit CD11b expression on monocytes. Surprisingly, lipidation had a profound effect on the capacity of peptides to inhibit VCAM-1 expression in endothelium. While lipidation of apoA-I into HDL did not alter its anti-inflammatory capacity, lipidation of the peptides with DMPC reduced this capacity and lipidation with POPC abolished it. *In vivo*, however,

lipidated peptide 5A did inhibit expression of VCAM-1 in rabbit arteries [34]. Clearly, this property of the peptides may be affected by lipid constituency.

In summary, by examining a panel of amphipathic bi-helical peptides, it was found that the different anti-atherogenic features of these peptides requires different structural features. This is relevant to the design of apoA-I mimetic peptides for HDL therapy and may lead to new insights into what properties of HDL are the most relevant for its ability to reduce cardiovascular disease.

Novelty and Significance

What is Known?

- Apolipoprotein A-I (apoA-I) mimetic peptides are active in a number of anti-atherogenic functions of high density lipoprotein (HDL) and can protect against atherosclerosis
- Structurally, ApoA-I mimetic peptides reproduce secondary structure of apoA-I, an array of 22-mer amphipathic α-helices, without close homology to the apoA-I primary structure
- Knowledge on structure-function relationship of the peptides is limited to the facts that peptides with two helices work better than single helix peptides and that increasing peptide hydrophobicity improves their functionality

What New Information Does This Article Contribute?

- Using a panel of related peptides, we established optimal structural requirements for the activity of the peptides in cholesterol efflux, anti-inflammatory and anti-oxidant actions
- Different anti-atherogenic properties of apoA-I have different, and sometimes conflicting, structural requirements pointing to independent mechanisms
- Different parts of apoA-I may be responsible for different anti-atherogenic functions and it may not be possible to combine all the features in one peptide of limited length

ApoA-I mimetic peptides are a promising therapeutic approach attempting to reproduce the anti-atherogenic properties of HDL. These peptides support cholesterol efflux and have anti-inflammatory and anti-oxidant activities. Structurally, they are comprised of one or two 18-22 mer canonical amphipathic α-helices. ApoA-I, however, is comprised of "almost" canonical, as well as "imperfect" α-helices, and such mixture may be required for the variety of anti-atherogenic functions of apoA-I. By changing the structure of one or both helices in bi-helical peptides, we were able to "fine tune" the peptides to be more active and more specific in individual functions compared with apoA-I. However, structural requirements for the individual functions were often conflicting with each other, and although a number of peptides have high activity in all assays, we were unable to design a peptide that was more active than apoA-I in all its functions. These findings provide a rationale for designing more complex formulations that would combine multiple anti-atherogenic activities. We have also designed a number of peptides that are active in one, but inactive in other anti-atherogenic functions, providing a powerful tool to study the relative contribution of various HDL functions to its overall antiatherogenic capacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding: This study was supported by a grant from the National Heart Foundation of Australia (G 07M 3165) to DS and JCD and Postgraduate Award from the National Health and Medical Research Council of Australia to WD. DS and JCD are Fellows of the National Health and Medical Research Council of Australia. Research done by ATR was supported by intramural research funds from the NIH. AAS was supported by the Danish Agency for Science, Technology and Innovation.

Abbreviations

ApoA-I	apolipoprotein A-I
AUC	area under the curve
DMPC	dimyrisoylphosphatidyl choline
CVD	cardiovascular disease
HDL	high density lipoprotein
LXR	liver X receptor
PMA	phorbol-12 myristate 13-acetate
POPC	palmitoyloleoyl phosphatidyl choline
TNF-α	tissue necrosis factor

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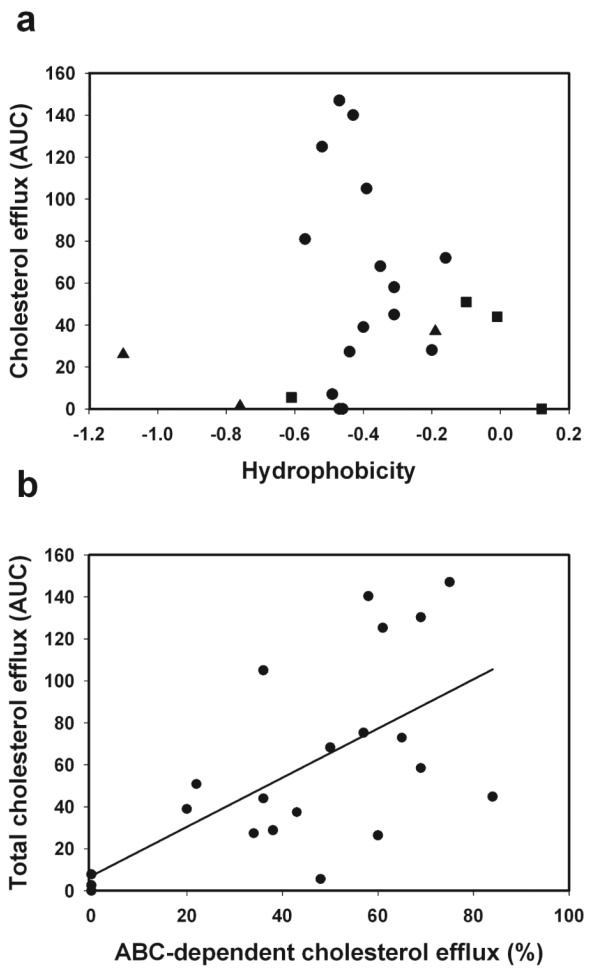

Fig. 1. Dependence of the capacity of the peptides to support cholesterol efflux on mean hydrophobicity and charge of the peptides (a) and relationship between cholesterol efflux capacity and contribution of ABC transporters (b)

Data for cholesterol efflux capacity and specificity are taken from Table 2 and data for hydrophobicity and charge are taken from Table 1. Squares denote positively charged peptides; triangles denote negatively charged peptides.

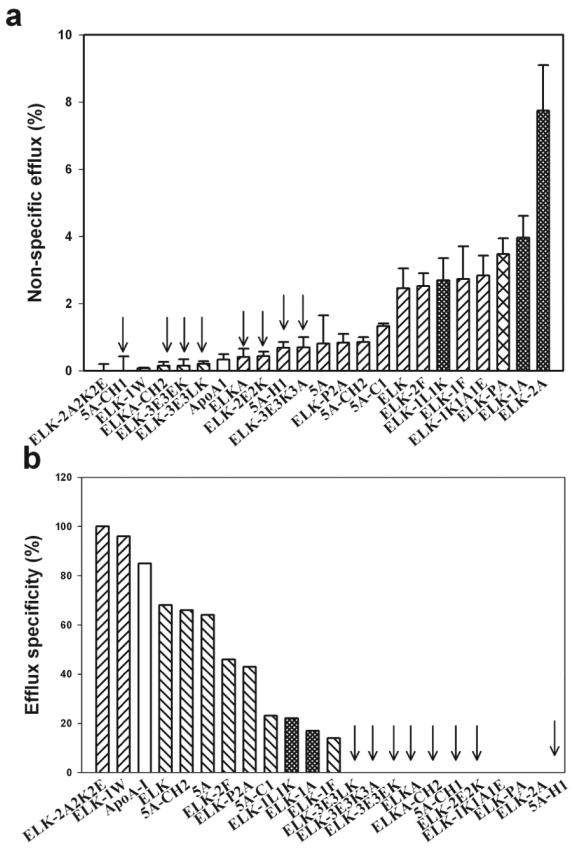

Fig. 2. Specificity of cholesterol efflux from THP-1 cells

Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium; concentration of the peptides was 80 μg/ml. Fine cross-hatched bars denote peptides with charge ≥ +2; coarse cross-hatched bar denotes peptides with A for P substitution.

a – Efflux from the fixed cells

b - Contribution of the specific efflux (efflux from live cells to the total efflux (efflux from live cells – efflux from fixed cells/efflux from live cells x100%). Negative values are shown as "0".

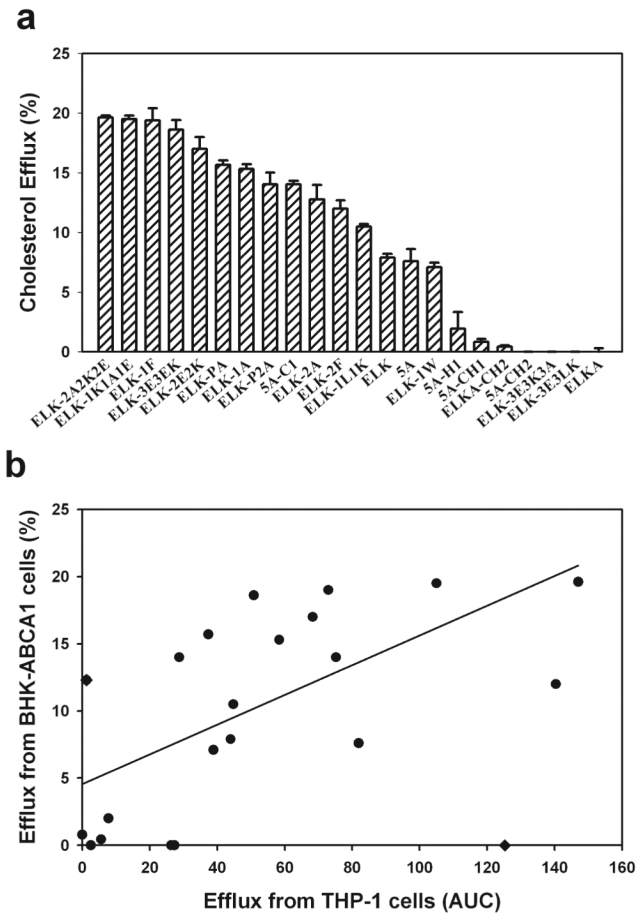

Fig. 3. Cholesterol efflux from BHK/ABCA1 cells

Cholesterol efflux was expressed as the proportion of [^{3}H]cholesterol transferred from cells to medium; concentration of the peptides was 20 μMol/ml (or approximately 90 μg/ml).
a – ABCA1-dependent efflux from BHK cells. Data presented are a difference between the efflux from BHK/ABCA1 cells and BHK/mock cells. Means ± SEM are presented.
b - Correlation between the ABC-dependent efflux from THP-1 and ABCA1-dependent efflux from BHK cells. Two peptides, ELK-2A and 5A-CH2, shown as ◆, were excluded from the analysis of correlations.

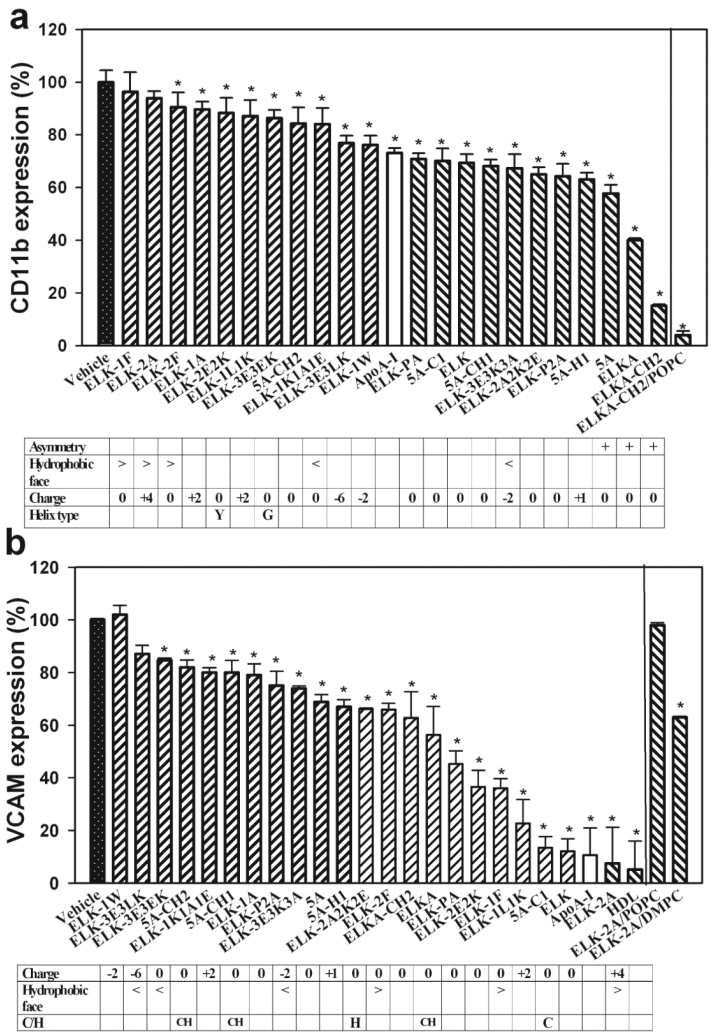

Fig. 4. The effect of peptides on anti-inflammatory properties

a - CD11b expression in human monocytes. CD11b expression was measured by flow cytometry; results were expressed as percentage of the CD11b expression compared to cells stimulated with PMA in the presence of a vehicle; concentration of peptides was 40 μg/mL. Means ± SEM are presented; *p<0.01 (*versus* vehicle). Table shows peptide properties that are likely to influence CD11b expression.

b - VCAM-1 expression in mouse endothelial cells, SVEC4/VCAM-1. Data were expressed per milligram of cellular protein and related to the luciferase activity in cells incubated with a vehicle instead of the peptides; concentration of peptides was 0.75 mg/mL. Means ± SEM are presented; *p<0.01 (*versus* vehicle). Table shows peptide properties that are likely to influence VCAM-1 expression.

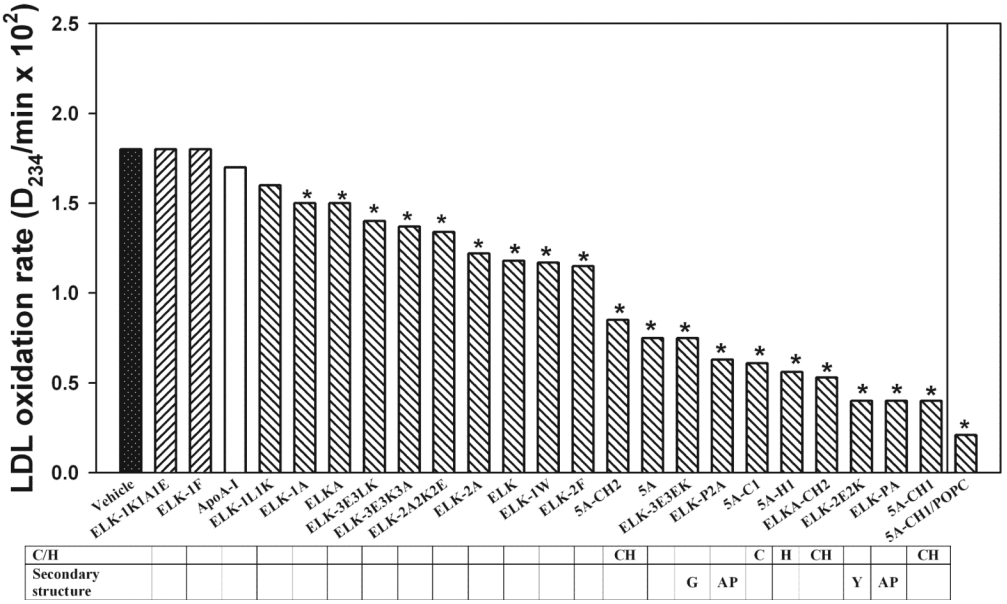

Fig. 5. The effect of peptides on LDL oxidation

Concentration of peptides was 100 μg/mL. Rate of oxidation was calculated as maximum absorbance divided to the length of the lag period. *p<0.01 (calculated from comparing the time-dependence curves presented in the Supplementary Figure VI). Table shows peptide properties that are likely to influence anti-oxidant properties. G – G-helix, Y-Y-helix, AP-substitution of A for P.

Table 1

Sequences and structural features of apoA-I mimetic peptides

#	Peptide	Sequence	Mean Hydrophobicity	Charge	Key features
1	5A	DWLKAFYDKVAEKLKEAF-P-DWAKAAYDKAAEKAKEAA	−0.57	0	A symmetrical
2	ELK	EKLKELLEKLLEKLKELL-P-EKLKELLEKLLEKLKELL	−0.4	0	Canonical Type A helix with 180 degree hydrophobic face and 0 net charge
3	ELK-3E3LK	EELKEKLEELKEKLEEKL-P-EELKEKLEELKEKLEEKL	−1.1	−6	3 x (K-E, L-K) substitutions. Decreased hydrophobic face and 3 additional negative charges per helix
4	ELK-3E3K3A	EELKAKLEELKAKLEEKL-P-EELKAKLEELKAKLEEKL	−0.76	−2	3 x (K-E, E-A, L-K) substitutions. Decreased hydrophobic face and 1 additional negative charges per helix
5	ELK-2A	EKLKALLEKLLAKLKELL P-EKLKALLEKLLAKLKELL	0.12	+4	2 x E-A substitutions. Increased hydrophobic face and 2 negative charges per helix less
6	ELK-1W	EWLKELLEKLLEKLKELL-P-EWLKELLEKLLEKLKELL	−0.19	−2	K-W substitution. 1 positive charges per helix less
7	ELK-2F	EKFKELLEKFLEKFKELL-P-EKFKELLEKFLEKFKELL	−0.43	0	2x (L-F) substitutions. Increased hydrophobic face
8	ELK-1L1K	EKLKELLEKLLELLKKLL-P-EKLKELLEKLLELLKKLL	−0.01	+2	K-L and E-K substitutions. 1 negative charge per helix less
9	ELK-1K1A1E	EKLKELLEKLKAKLEELL-P-EKLKELLEKLKAKLEELL	−0.39	0	L-K, E-A and K-E substitutions. Decreased hydrophobic face
10	ELK-1A	EKLKELLEKLLAKLKELL-P-EKLKELLEKLLAKLKELL	−0.1	+2	E-A substitution. 1 negative charge per helix less
11	ELK-1F	EKFKELLEKLLEKLKELL-P-EKFKELLEKLLEKLKELL	−0.35	0	L-F substitution Increased hydrophobic face
12	ELK-2A2K2E	EKLKAKLEELKAKLEELL-P-EKLKAKLEELKAKLEELL	−0.47	0	2 x (E-A, L-K and K-E) substitutions. optimal hydrophobicity and charge
13	ELK-3E3EK	EELKELLKELLKKLEKLL-P-ELKELLKELLKKLEKLL	−0.31	0	3 x (K-E, E-K) substitutions. G-helix

#	Peptide	Sequence	Mean Hydrophobicity	Charge	Key features
14	ELK-2E2K	EELKKLLEELLKKLKELL-P-EELKKLLEELLKKLKELL	−0.31	0	2 x (K-E, E-K) substitutions. Y-Helix
15	ELK-PA	EKLKELLEKLLEKLKELL-A-EKLKELLEKLLEKLKELL	−0.2	0	A-P substitution in the link
16	ELK-P2A	EKLKELLEKLLEKLKELL-AA-EKLKELLEKLLEKLKELL	−0.16	0	2A-P substitution in the link
17	ELKA	EKLKAKLEELKAKLEELL-P-EKAKAALEEAKAKAEELA	−0.49	0	ELK-2A2K2E peptide with 5A substitution in second helix.
18	ELKA-CH2	EKLKAKLEELKAKLEELL-P-EHAKAALEEAKCKAEELA	−0.46	0	ELKA peptide with C+H substitution in the second helix.
19	5A-CH1	DHLKAFYDKVACKLKEAF-P-DWAKAAYDKAAEKAKEAA	−0.47	0	C+H substitution in the first helix
20	5A-CH2	DWLKAFYDKVAEKLKEAF-P-DHAKAAYDKAACKAKEAA	−0.52	0	C+H substitution in the second helix
21	5A-C1	DWLKAFYDKVACKLKEAF-P-DWAKAAYNKAAEKAKEAA	−0.44	0	C substitution in the first helix
22	5A-H1	DHLKAFYDKVAEKLKEAF-P-DWAKAAYDKAAEKAKEAA	−0.61	+1	H substitution in the first helix

Table 2

Efficiency of cholesterol efflux from THP-1 cellsand contribution of ABC A1 transporter.

Peptide	Cholesterol efflux efficiency (AUC)	Contribution of ABCA1-dependent cholesterol efflux (%)
ELK-2A2K2E	147.0	75
ELK-2F	140.3	58
ApoA-I	130.3	69
5A-CH2	125.3	61
ELK-1K1A1E	105.0	36
5A	82.0	64
ELK-P2A	72.9	65
ELK-1F	68.3	50
ELK-2E2K	58.4	69
ELK-1A	50.8	22
ELK-3E3EK	44.8	84
ELK-1L1K	44.0	36
ELK	38.9	20
ELK-1W	37.4	43
ELK-PA	28.7	38
5A-C1	27.3	34
ELK-3E3LK	26.3	60
ELKA	7.7	0
5A-H1	5.6	48
ELKA-CH2	2.6	0
ELK-3E3K3A	1.3	0
ELK-2A	0	0
5A-CH1	0	0
ELK-2A2K2E/POPC	381.6	ND

Table 3

Structural features responsible and individual anti-atherogenic properties of the peptides.

Function	Hydrophobicity	Size of hydrophobic face	Charge	Maintaining proline bridge	Type of helix	Inclusion of Cys/His residues	Asymmetry
Efficiency of cholesterol efflux	Optimal (−0.5)	Increased size is beneficial	Neutral	Essential	Limited effect	Detrimental in the first helix, beneficial in the second helix	Beneficial in 5A, detrimental in ELK
Specificity of cholesterol efflux	Limited effect	Limited effect	Neutral or negative	Essential	Limited effect	Limited effect	Beneficial in 5A, detrimental in ELK
Anti-inflammatory -monocytes	Limited effect	Increased size is detrimental	Neutral or negative	Limited effect	Changing to G or Y is detrimental	Limited effect	Beneficial
Anti-inflammatory -endothelium	Limited effect	Increased size beneficial	Neutral or positive	Limited effect	Limited effect	C+H detrimental, C beneficial	Limited effect
Anti-oxidant	Limited effect	Limited effect	Limited effect	Detrimental	Changing to G or Y is beneficial	Beneficial	Limited effect