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Retention of α-helical structure by HDL mimetic peptide ATI-5261 upon extensive dilution represents an important determinant for stimulating ABCA1 cholesterol efflux with high efficiency

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ABSTRACT

ATI-5261 is a novel, single-helix peptide that stimulates cellular cholesterol efflux with high potency similar to native apolipoproteins on a molar basis. Presently we investigated structural features of the peptide that conferred cholesterol efflux activity. Analogs of ATI-5261 with amino acids arranged in reverse order or with individual arginine (R) to glutamine (Q) substitutions (i.e. R3Q, R14Q, or R23Q) stimulated ABCA1 dependent cholesterol efflux similar to ATI-5261. Consequently, neither the presence of specific positively charged residues nor their specific arrangement along the length of the peptide was necessary for mediating cholesterol efflux. Similarly, peptides composed of all D-amino acids stimulated cholesterol efflux efficiently, indicating a stereospecific component was not required for promotion of cholesterol efflux from macrophages. Removal of two or more positively charged residues (R3, 14 → Q and R3, 14, 23 → Q) however, greatly reduced the ability of ATI-5261 to mediate cellular cholesterol efflux. This was accompanied by a loss of α-helical structure upon dilution, indicating the secondary structure of individual peptide strands was important for stimulating cholesterol efflux. Surprisingly, peptides with removal of two or more positively charged residues retained the ability to bind phospholipid and adopt an α-helical structure. These data indicate that the propensity of a hydrophobic peptide to form an amphipathic α-helix is not sufficient to mediate cellular cholesterol efflux. Efficient stimulation of cholesterol efflux requires that ATI-5261 retain α-helical structure upon dilution.

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1. Introduction

High density lipoproteins (HDL) are thought to protect against the development of atherosclerosis [1,2]. This protection has been attributed, in part, to the ability of HDL to mediate reverse cholesterol transport [3–5]. Reverse cholesterol transport involves stimulation of cholesterol efflux from peripheral cells followed by the transport of cholesterol to the liver for elimination in feces. Several mechanisms of cholesterol efflux have been described, including aqueous diffusion and apolipoprotein (apo)-mediated processes [6–14]. The latter requires the membrane protein ABCA1 (i.e. ATP-binding cassette transporter A1), that transfers cellular cholesterol and phospholipid to extracellular, lipid-poor apos, such as apoA-I and E [15–22].

Recently, we demonstrated that the C-terminal (CT) lipid-binding domain of apoE was responsible for mediating cholesterol efflux via ABCA1 [18]. These studies revealed that a region of the apoE CT domain consisting of a hydrophobic segment linked to a class A α-helical motif was required for mediating cellular lipid efflux efficiently. This information was used to create a small peptide that mimics the activity of native apos to stimulate ABCA1 cholesterol efflux and reduce substantial atherosclerosis in mouse models [23]. The resulting peptide, ATI-5261, shares oligomeric properties of native apos and transitions from a self-associated form (i.e. tetramers) to low molecular weight species, including monomers of high α-helical content (~70%) in the absence of lipid [24]. The latter occurs upon extensive dilution over the active concentration range for mediating cellular cholesterol efflux, indicating peptide monomers with exposed hydrophobic surfaces are important for activity. Such a mechanism is supported by studies with crosslinking reagents that lock ATI-5261 in tetramer form and inhibit cholesterol efflux activity [25].

Presently, we utilized site-specific amino acid variants and peptidomimetics of ATI-5261 to investigate structural features of the peptide that conferred potent cholesterol efflux activity. No sequence- and/or stereo-specific requirements for mediating

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cholesterol efflux were observed with ATI-5261, consistent with previous studies of other apo mimetics [26–28]. Moreover, stimulation of cellular cholesterol efflux did not require a specific pattern of positively charged residues. However, extensive ablation of cationic character greatly reduced the α-helical content of ATI-5261 upon extensive dilution and this was associated with loss of cholesterol efflux activity. Reduction in α-helical content was not observed at high concentrations of peptides, i.e. in self-associated form, or in the presence of POPC or 50% TFE, indicating the loss of cationic residues did not disrupt the inherent nature of the peptide to form an amphipathic α-helix. These results indicate that potent stimulation of cholesterol efflux via ABCA1 is dependent on ATI-5261 retaining its α-helical structure upon extensive dilution.

2. Materials and methods

2.1. Peptides

Peptides were synthesized (Biosynthesis Inc., Lewisville, TX) using either all L- or all D-amino acids, capped with N-terminal acetyl and C-terminal amide groups, isolated by HPLC and used at a purity of >95%. Lyophilized peptides were dissolved in 10 mM phosphate buffered (pH = 7.4) saline (150 mM NaCl), referred to as PBS, or 10 mM phosphate (pH = 7.4). Peptide concentrations were determined by absorbance at 280 nm. All peptides used in these studies were highly soluble (up to 5 mg/ml concentrations normally tested) in PBS or phosphate buffer (pH = 7.4) and self-associated as tetramers, with no apparent signs of non-specific aggregation or precipitation determined upon visible inspection of solutions (over several months) or FPLC [24].

2.2. CD spectroscopy

Circular dichroism (CD) spectroscopy was carried out on a Jasco 810 spectropolarimeter at 25 °C, using lipid-free peptides in 10 mM sodium phosphate buffer (pH = 7.4) and averaging 4 scans (20 nm/min/scan) per run as described [24]. Samples were diluted in this same buffer to assess the α-helical content of peptides at various concentrations. Where indicated, peptides were prepared in 10 mM phosphate (pH = 7.4) plus 50% TFE or complexed with POPC to form small 7–8 nm sized particles to assess the ability of specific variants to adopt an α-helical structure [23].

2.3. Cell-culture and measurement of cholesterol efflux activity

J774 mouse macrophages were plated onto 24-well culture plates and labeled with [^{3}H] cholesterol (1 μCi/ml) in RPMI-1640 with 1% FBS for 48 h. A cAMP analogue (cpt-cAMP) was added (0.3 mM final concentration) to upregulate ABCA1 expression. Cells were extensively rinsed with serum-free RPMI-1640 medium followed by an extended rinse (2 h) with RPMI-1640 containing 0.2% bovine serum albumin (BSA). Lipid-free peptides were added to cells in serum-free RPMI-1640 medium to initiate cholesterol efflux. The amount of [^{3}H] cholesterol appearing in the medium was expressed as a percentage of the radioactivity initially present in cells at time zero [6,7,17,18]. The background release of [^{3}H] cholesterol to serum-free medium alone was subtracted from the values obtained with lipid-free peptides.

2.4. Lipid binding

A turbid solution of dimyristoylphosphatidylcholine (DMPC) was used to assess the capacity of peptides to solubilize phospholipid [17]. The DMPC was used at a final concentration of 0.16 mg/ml PBS (pH = 7.4). The mass ratio of DMPC to peptides was 2:1 or 4:1. The absorbance (400 nm) of samples was monitored continuously over 20 min at 25 °C.

2.5. Statistics

Where appropriate, data were expressed as means ± SD of at least 3 independent experiments and statistical analyses performed using Student's *t*-test.

3. Results

Sequence variants of ATI-5261 were used in the present study to identify features of the peptide that conferred cholesterol efflux activity. Reversing the sequence of ATI-5261 altered the specific positions of positively charged residues, but overall class A structure and amphipathic nature were retained (Fig. 1A). Despite the altered pattern of charged residues, the reverse sequence form of ATI-5261 stimulated cholesterol efflux in an ABCA1 dependent manner (Fig. 1B). Maximum levels of cholesterol efflux were achieved using 3 μg peptide/ml, indicating the reverse sequence form of ATI-5261 was a potent stimulator of cholesterol efflux (Fig. 1C, top right) like ATI-5261 [23]. Similarly analogs of ATI-5261 composed of all D-amino acids stimulated cholesterol efflux similar to the L-amino acid form of ATI-5261 (Fig. 1C, bottom vs. top panels), indicating there was no stereospecific requirement for mediating cholesterol efflux via ABCA1.

To further explore the contribution of specific charged residues to cholesterol efflux, R → Q variants of ATI-5261 were designed to ablate cationic character (Fig. 2A). Use of single R → Q substitutions at specific positions along the length of ATI-5261, i.e. R3Q, R14Q, or R23Q, did not alter cholesterol efflux activity compared to the parent ATI-5261 peptide (Fig. 2B). High-levels of cholesterol efflux were observed using 3 μg/ml peptides, consistent with the behavior of ATI-5261. This was verified using several of the single R → Q variants, which stimulated cholesterol efflux in a highly efficient manner similar to ATI-5261 (Km = 0.73, 0.95, 0.94 μg/ml, for R14Q, R23Q, and ATI-5261 respectively).

In contrast to the results obtained with the single R → Q substitutions, peptides with double (R3, 14 → Q) or triple (R3, 14, 23 → Q) glutamine substitutions had greatly reduced cholesterol efflux activity (Fig. 2). Reductions in absolute levels of ABCA1-dependent cholesterol efflux (Fig. 2C) as well as efflux efficiency (Fig. 2D) were observed with loss of two or more positively charged residues. The latter was associated with a marked decrease in the ability of the R14, 23 → Q and R3, 14, 23 → Q variants to mediate cholesterol efflux over the active concentration range (<10 μg/ml) characteristic of ATI-5261. This produced an increase in the Km values for cholesterol efflux activity of the double and triple R → Q variants, indicative of poor efflux efficiency (Fig. 2D). Interestingly, the ability of R → Q peptides to bind purified phospholipid was increased (Table 1 and Fig. 3). Nearly all the R → Q variants tested displayed a tendency for increased ability to clear turbid solutions of DMPC, with more dramatic effects seen using the double and triple R → Q variants (Fig. 3A and B). Similar results with peptides were obtained using the acidic phospholipid DMPG in clearance assays (data not shown). Therefore, the increased lipid-binding affinity of the R → Q peptides appeared to be unrelated to charge effects. Use of the R → Q substitutions did however increase the hydrophobicity of peptides (Table 1), which may have facilitated lipid binding.

The α-helical content of the R → Q variants was largely unaffected by removal of positively charged residues (Tables 1 and 2), i.e. at relatively high concentrations (>0.1 mg/ml, i.e. 31 μM) of peptide that far exceed those required for mediating cholesterol efflux [23–25]. Thus, peptides with single or multiple

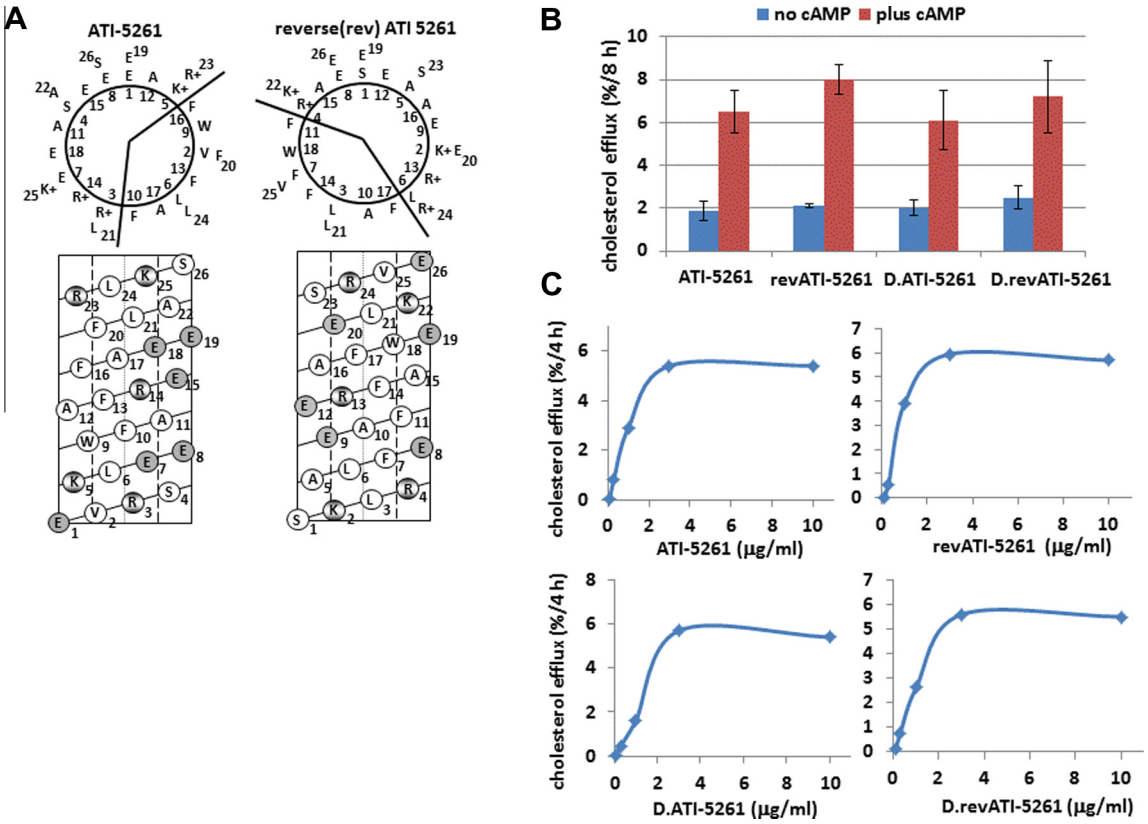


Fig. 1. Cholesterol efflux activity of ATI-5261, its reverse sequence analog and D-amino form. Panel A – the sequences of ATI-5261 and its reverse (rev) analog are shown enface projected as helical wheel diagrams (top). Both peptides conform to the structure of a class A α-helix with positively charged amino acids (arginine and lysine, i.e., R and K) located at the lipid–water interface and negatively charged residues (glutamate, E) positioned toward the center of the polar face. Helical net diagrams (bottom) generated by cutting α-helices down the long axis (starting at position 1) and flattening. The topographical arrangement of amino acids is shown. Note the differences in the relative positions of positively charged residues (partially shaded circles) between the two peptides. Numbers refer to the primary sequence of amino acids. Panel B – cholesterol efflux (8 h) from [^{3}H] cholesterol-labeled J774 cells to D- and L-amino acid forms of ATI-5261 (10 μg/ml, lipid-free peptides). Cells were treated (18 h) with cAMP to up-regulate ABCA1 or with no cAMP (control) prior to assessment of cholesterol efflux to peptides. Values are means ± SD, $n = 3$. Panel C – dependence of cholesterol efflux from cAMP-treated J774 cells on the concentration of lipid-free peptides. Values are representative of two independent experiments from which similar results were obtained. In all cases, maximal levels of cholesterol efflux were observed at 3 μg/ml peptides.

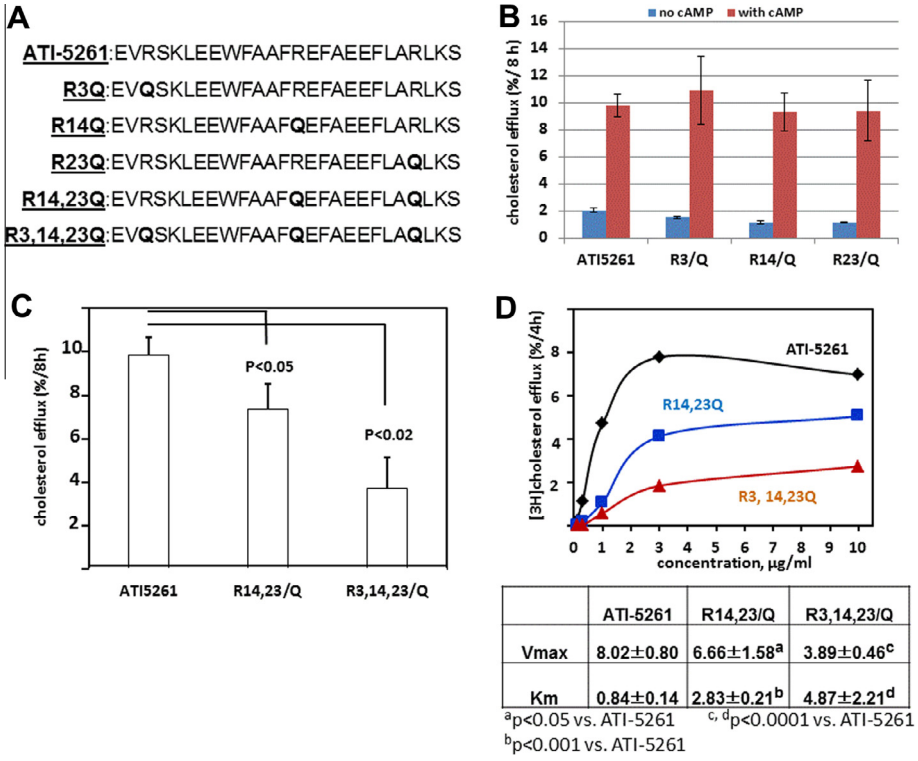
R → Q substitutions were capable of adopting an α-helical configuration under conditions that favor self-association [24]. Moreover, treatment of the R3, 14, 23 → Q peptide (20 μg/ml) with 50% TFE increased the α-helical content by 31.8 ± 1.9% ($n = 3$). An increase (22 ± 1.3%) in α-helicity was also observed upon formulation of this triple R → Q peptide with POPC. Thus the ability of R → Q peptides to form an α-helix and bind phospholipid was largely unaffected by the loss of multiple (>2) cationic residues.

Since ATI-5261 is active at mediating cholesterol efflux at low concentrations, we evaluated the secondary structure of the R → Q peptides following extensive dilution. Both the double and triple R → Q variants displayed decreased α-helicity below 0.1 mg/ml concentrations (Table 3). The loss of α-helical structure was consistently more severe for the triple R → Q variant compared to the double R → Q variant (Table 3), paralleling the loss of cholesterol efflux activity (Fig. 2C and D). Consequently, the ability of ATI-5261 to mediate cellular cholesterol efflux was directly related to the α-helical content of the peptide at low concentrations (Fig. 4). No difference in α-helical content was observed between the single R → Q variants (i.e. R3Q, R14Q or R23Q) and ATI-5261 at any of the concentrations tested (data not shown), consistent with the peptides retaining cholesterol efflux activity (Fig. 2B). Therefore, reductions in cholesterol efflux activity seen with the double and triple R → Q variants were associated with loss of secondary structure upon extensive dilution.

4. Discussion

ATI-5261 constitutes a unique α-helical peptide. It possesses high α-helical content over a broad-range of concentrations in solution and in the absence of lipid [23,24]. This behavior contrasts that of previous apolipoprotein mimetic peptides that require lipid or high concentrations to induce α-helical formation [29–34]. The latter favors peptide self-association, suggesting intermolecular interactions are necessary to drive the formation of α-helical structure. Indeed, it is generally considered difficult to create small peptides having substantial secondary structure, i.e. without the use of chemical staples and/or end-group capping to stabilize and/or promote intra-helical interactions [35,36]. This apparently is not the case for ATI-5261, which retains high α-helical content upon extensive dilution in physiological buffers [24].

The ability of ATI-5261 to maintain an α-helical structure upon dilution may account for the potent cholesterol efflux activity of the peptide. The α-helical content of apolipoproteins is thought to be critical for mediating cellular cholesterol efflux via ABCA1 [26,37]. The results of the present study support this idea, wherein peptide analogs of ATI-5261 with double and triple R → Q substitutions failed to maintain α-helical structure and activity upon dilution. These peptides were weak mediators of cellular cholesterol efflux, despite having increased hydrophobicity compared to the parent ATI-5261 peptide and being able to bind phospholipid effectively and form an amphipathic α-helix. These observations



	ATI-5261	R14,23/Q	R3,14,23/Q
Vmax	8.02±0.80	6.66±1.58[a]	3.89±0.46[c]
Km	0.84±0.14	2.83±0.21[b]	4.87±2.21[d]

[a]p<0.05 vs. ATI-5261 [c, d]p<0.0001 vs. ATI-5261
[b]p<0.001 vs. ATI-5261

Fig. 2. Cholesterol efflux activity of ATI-5261 variants with R → Q substitutions. J774 macrophages were labeled with [^{3}H]cholesterol and incubated with and without cAMP as described in Fig. 1. Panel A – sequences of peptides showing location (bold) of single or multiple R → Q substitutions down the length of ATI-5261. Panel B – cholesterol efflux activity (8 h) determined using 3 µg/ml of lipid-free peptides. Values are means ± SD, $n = 3$. Panel C – ABCA1-dependent cholesterol efflux activity of the double and triple R → Q variants of ATI-5261. Peptides were used in lipid-free form at 3 µg/ml concentrations. Panel D – dependence of cholesterol efflux on peptide concentration. Efflux data were obtained using cAMP-treated J774 cells incubated 4 h with increasing concentrations of lipid-free peptides. Values are means ± SD, $n = 3$. Km and Vmax were calculated using the Michaelis Menten equation.

Table 1
Biophysical properties and lipid-binding activity of ATI-5261 analogs.

Peptide	Molecular weight (Da)	Amphiphilicity[a]	Mean hydrophobicity[a]	α-Helicity (%)[b]	$t_{1/2}$ DMPC[c] (s)	Net charge
ATI-5261	3230	0.25	−0.28	70.0	56	−1
R3Q	3204	0.26	−0.23	75.4	57	−2
R14Q	3202	0.24	−0.23	61.4	26	−2
R23Q	3202	0.26	−0.23	72.2	22	−2
R14, 23Q	3174	0.25	−0.19	61.3	23	−3
R3, 14, 23Q	3146	0.26	−0.15	61.9	34	−4

[a] Amphiphilicity and mean hydrophobicity were calculated described [17,18].
[b] α-Helicity content was determined using 62 µM lipid-free peptides (~0.2 mg/ml) in 10 nM phosphate buffer, pH = 7.4.
[c] Initial rates of DMPC clearence determined using peptide: DMC ratio of 1:4 at 25 °C.

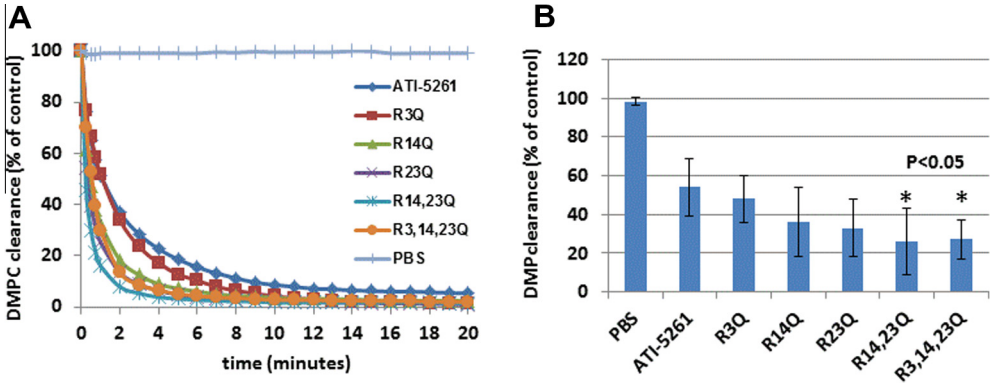


Fig. 3. DMPC clearance activity of ATI-5261 and its analogs. Turbid solutions of DMPC (160 µg/ml) were prepared in PBS as described in Section 2. Panel A – kinetics of DMPC clearance obtained at 25 °C with using 1:4 mol ratios of peptides relative to phospholipid. Panel B – extent of DMPC clearance (% of PBS control) obtained 2 min after adding peptide to phospholipid (i.e., 1:4 mol ratios). Results are means ± SD, $n = 4$. The asterisk denotes statistical significance ($p < 0.05$) compared to ATI-5261.

Table 2
α-Helical content of ATI-5261 analogs at different concentrations.

Peptide variant	% α-Helical content			
	31 μM	62 μM	155 μM	310 μM
R3/Q	71.8	75.4	78.2	77.4
R14/Q	59.2	61.4	65.2	64.8
R23/Q	68.0	72.2	73.5	72.2
R14, 23/Q	68.0	61.3	64.6	67.1
R3, 14, 23/Q	66.1	61.9	64.8	75.3

CD spectroscopy was performed using lipid-free peptides (62 μM ≈ 0.2 mg/ml) in 10 mM phosphate buffer, pH = 7.4. Values are representative of two independent experiments with different batches of peptides and mutiple readings at each concentration. The α-helical content of control ATI-5261 was 69%, 73%, 78%, and 76% at 31, 62, 155 and 310 μM concentrations, respectively.

Table 3
α-Helical content of ATI-5261 analogs upon extensive dilution.

Concentration (μM)	ATI-5261 % α-helical content	R14, 23/Q % α-helical content	R3, 14,2 3/Q % α-helical content
155	68.3	52.2	59.2
62	78.3	64.8	62.4
31	69.5	60.1	57.0
15	61.6	55.1	44.2
7.5	76.8	61.1	40.6
3	66.4	38.8	17.2

CD spectroscopy was performed using lipid-free peptides (62 μM ≈ 0.2 mg/ml) in 10 mM phosphate buffer, pH = 7.4. Values are representative of two independent experiments with different batches of peptides and multiple readings at each concentration.

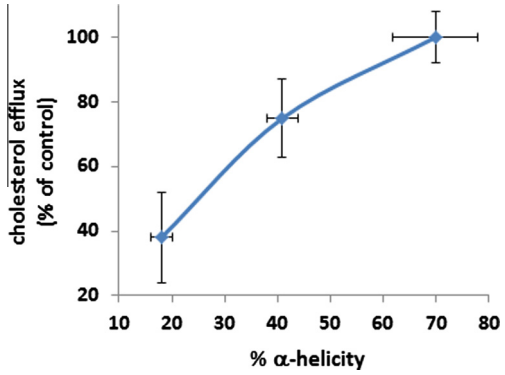


Fig. 4. Relationship between cholesterol efflux activity and α-helical content of ATI-5261. Cholesterol efflux activity (4 h) was obtained using [3H]cholesterol-labeled J774 macrophages treated with cAMP and peptides (R14, 23 → Q and R3, 14, 23 → Q) with different α-helicity values, i.e., both determined at 10 μg/ml concentrations. Values are means ± SD, $n = 3$.

indicate that stimulation of cholesterol efflux by ATI-5261 requires a preformed α-helical structure being presented to cells to function effectively.

ATI-5261 does not require end-group capping or special buffers to induce α-helical formation [23,24]. These observations indicate that the α-helical structure and activity of ATI-5261 are largely governed by intra-molecular forces, contrasting previous apo mimetics. Indeed the ability of ATI-5261 to retain high α-helical content and activity upon dilution appears to be dependent on two or more positively charged residues, involving R3, R14 and/or R23. Neither of these individual residues nor their position within ATI-5261 seems necessary for activity, as reverse sequence analogs and peptides with single R → Q substitutions retained the ability to stimulate cholesterol efflux like the parent peptide. These observations are consistent with previous results, where analogs of ATI-5261 with glutamine (Q) in place of lysine (i.e. K25Q) had little impact on cholesterol efflux activity [25]. As discussed, ATI-5261 analogs with glutamine substitutions were able to bind phospholipid, indicating the double and triple R → Q substitution did not disrupt the overall amphipathic nature and potential of the peptide to adopt an α-helix configuration. These results are consistent with a role of cationic residues maintaining critical interactions that stabilize the α-helix structure of ATI-5261. This could involve ionic interactions with negatively charged residues located 4 residues from each the aforementioned cationic residues (i.e. R3, R14 and R23), consistent with a $i + 4$ arrangement of putative salt-bridges in native apoE sequence from which ATI-5261 was designed [23].

Our data suggest that studies optimizing the cholesterol efflux activity of apo mimetic peptides should take into account the α-helical structure over a broad-range of peptide concentrations. For example, we have found that the α-helical content of peptides when assessed at high concentrations does not always correlate positively with the ability to stimulate cellular cholesterol efflux. This no doubt relates to peptide self-association that can induce α-helical formation of amphipathic peptides and confound interpretation of experimental results, particularly when extrapolating results to low concentrations where the peptides mediate cellular cholesterol efflux. Such measurements should take into account the secondary structure at low concentrations of peptide, which reflect a dynamic state and interactions of peptide with ABCA1 expressing cells. Similarly, lipid-binding affinity may not always predict efficiency for mediating cholesterol efflux, as revealed in studies of R → Q mutants of ATI-5261 that show increased hydrophobicity/lipid binding but lower levels of cholesterol efflux activity than ATI-5261. These factors may also be important *in vivo* for optimizing ABCA1 targeting and efficacy, as apo mimetic peptides are likely to be extensively diluted and/or distribute between lipoprotein particles in blood and extravascular spaces in mediating cellular cholesterol efflux.

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