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# Amphipathic polyproline peptides stimulate cholesterol efflux by the ABCA1 transporter

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ABSTRACT

ApoA-I mimetics are short synthetic peptides that contain an amphipathic α-helix and stimulate cholesterol efflux by the ABCA1 transporter in a detergent-like extraction mechanism. We investigated the use of amphipathic peptides with a polypro helix for stimulating cholesterol efflux by ABCA1. Polypro peptides were synthesized with modified prolines, containing either a hydrophobic phenyl group ($Pro_p$) or a polar N-acetylgalactosamine ($Pro_g$) attached to the pyrrolidine ring and were designated as either PP-2, 3, 4, or 5, depending on the number of 3 amino acid repeat units ($Pro_p$-$Pro_g$-$Pro_p$). Based on molecular modeling, these peptides were predicted to be relatively rigid and to bind to a phospholipid bilayer. By CD spectroscopy, PP peptides formed a Type-II polypro helix in an aqueous solution. PP-2 was inactive in promoting cholesterol efflux, but peptides with more than 2 repeat units were active. PP-4 showed a similar $V_{max}$ as a much longer amphipathic α-helical peptide, containing 37 amino acids, but had a $K_m$ that was approximately 20-fold lower. PP peptides were specific in that they did not stimulate cholesterol efflux from cells not expressing ABCA1 and were also non-cytotoxic. Addition of PP-3, 4 and 5 to serum promoted the formation of smaller size HDL species (7 nM) and increased its capacity for ABCA1-dependent cholesterol efflux by approximately 20–35% ($p < 0.05$). Because of their relatively small size and increased potency, amphipathic peptides with a polypro helix may represent an alternative structural motif for the development of apoA-I mimetic peptides.



## 1. Introduction

There is great interest in the development of synthetic High Density Lipoprotein (HDL) particles as a potential therapy for cardiovascular diseases [1–3]. Either full length ApoA-I [4], which contains a tandem array of amphipathic α-helices, or short ApoA-I mimetic peptides [5,6], are used as the protein component of synthetic HDL particles. ApoA-I mimetic peptides are typically designed to form an amphipathic α-helix, so that one side of the helix contains hydrophobic amino acids and faces the lipid core of HDL, whereas the other side contains amino acids with hydrophilic side chains that face toward the aqueous environment. It has been shown in numerous studies that apoA-I mimetic peptides, like the full-length apoA-I protein, can efflux excess cellular cholesterol by the ABCA1 transporter in a detergent-like extraction process [7,8]. These peptides reduce atherosclerosis in various animal models [5,6,9], and also show benefit in several other disease models, particularly inflammatory diseases [10].

Peptides that are enriched in proline can also form helices, namely type I and type II polypro helices [11]. The polypro type II helix, with its peptide bond in the *trans*-configuration, is more common than the type I polypro helix and is structurally quite different than the α-helix. It forms a left-handed helix with 3 amino acids per turn and has an overall shape of a triangular prism, with a rise per residue of approximately 3.1 Å. In contrast, the α-helix is right-handed, contains 3.6 amino acids per turn and has a rise per residue of only 1.5 Å, which means that for the same number of amino acids a polypro type II helix will be about twice as long an α-helix. Even though the polypro type II helix has no backbone hydrogen bonds to stabilize its secondary structure like the α-helix, it is, nevertheless, very rigid. In fact, polypro peptides are used as "molecular rulers" in FRET type analysis [12]. The reason that polypro peptides are so rigid is because the pyrrolidine side chain ring

*Abbreviations:* ABCA1, ATP-binding cassette transporter A1; BHK, baby hamster kidney cell line; HDL, high density lipoproteins; LDL, low density lipoproteins; PEG, polyethylene glycol.

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of proline forms a covalent bond with the amino group on the peptide backbone, which limits the permissible dihedral angles of the peptide bond to a conformation that favors helix formation [11]. As a consequence, polypro helices can be much shorter than α-helices, because they do not require stabilization by backbone hydrogen bonding. A cross section of polypro peptides shows almost a perfect 3-fold symmetry, because their side chains are arranged every 120° unlike α-helices, which instead occur about every 100°. Polypro helices, containing modified prolines with attached side chain groups [13], can potentially form amphipathic helices much longer than the typical limit of 18–20 residues for α-helices, because the relative orientation of their side chains will be maintained throughout the length of the helix.

In this study, we used modified prolines to synthesize novel amphipathic polypro peptides. To form the hydrophobic base of the triangular prism shape of the polypro peptide, we used two modified proline residues ($Pro_p$), containing a hydrophobic phenyl group covalently attached to the pyrrolidine ring. The hydrophilic "apex" of the peptide was designed to contain a modified proline residue with a polar N-Acetylgalactosamine (GalNAc) sugar attached to its side chain ($Pro_g$). We hypothesized that short peptides based on a repeating unit of these 3 modified amino acids ($Pro_p$-$Pro_g$-$Pro_p$) would form polypro helices and be effective in promoting cholesterol efflux by the ABCA1 transporter, because of their relative rigidity and amphipathic structure.

## 2. Materials and methods

### 2.1. Peptide synthesis and modification

Peptides were synthesized by a solid-phase procedure, using FMOC–protected amino acids on a Biosearch 9600 peptide synthesizer (Bioresearch, Japan), as described in Fig. 1. Trans-Fmoc-4-azido-L-proline ($Pro_a$) from IRIS BIOTECH, GMBH (Germany) and Fmoc-(2S, 4R)-4-benzyl-pyrrolidine-2-carboxylic acid ($Pro_p$) from AnaSpec, Inc. (Fremont, CA) were used to form polypro peptides with a variable number of the ($Pro_p$-$Pro_a$-$Pro_p$)$_n$ repeat units and were abbreviated as PP-n, with n designating the number of repeat units (Fig. 1A). After cleavage from resin with trifluoroacetic acid, the peptides were lyophilized and α-GalNAc was attached via a PEG trimer linkage to the azido group of $Pro_a$ residues by the following click chemistry reaction: 3 mM peptides (PP-2, PP-3, PP-4, PP-5) and α-GalNAc-TEG-Alkyne (IRIS BIOTECH, GMBH) at a concentration of 9 mM, 13.5 mM, 18 mM and 22.5 mM respectively (alkyne/azide molar ratio was 1.5) were solubilized in dimethylformamide (Sigma–Aldrich, Saint-Louis, MO) and then added to the reaction mixture of 70% dimethylformamide with 4.5 mM CuSO4 and 9 mM sodium ascorbate. The reaction was heated in a microwave (CEM Corp., USA) at 50 °C with 25 W power for 2 h. The reaction was monitored by taking 2ul of the final peptide product and placing it on an Anchorchip target and allowed to dry at 45 °C. It was overlaid with 2ul of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% ACN, 2.5% TFA and analyzed in a MALDI AutoFlex III (Bruker Daltonic, Billerica, MA), using the linear detector in positive mode between 2000 and 20,000 Da.

### 2.2. Circular Dichroism (CD) spectroscopy

Peptides at a concentration of 0.1 mg/ml in 0.015 M sodium phosphate buffer, pH 7.4, were loaded into a quartz cuvette ($d = 0.2$ cm path length) and the CD spectra from 185 to 240 nm were recorded on a Jasco J715 spectropolarimeter at 24 °C. The polypro type II helix was identified by its characteristic CD spectrum, which has a nadir at 198 nm and a peak at 218 nm [14].

### 2.3. In vitro cholesterol efflux assay

Cholesterol efflux studies were performed as previously described [6]. Briefly, BHK-mock (control) and ABCA1-transfected BHK cells were incubated with 1 mCi/ml of [$^3$H]-cholesterol in DMEM. After 24 h, the media was replaced with DMEM containing the peptides or PBS vehicle control. After 18 h of incubation, radioactive counts in media and cell lysates were measured by liquid scintillation counting on a Perkin Elmer MicroBeta 1450 scintillation counter. Results are expressed as the percentage of total counts appearing in the media.

Cholesterol efflux to LDL-depleted plasma spiked with the polypro peptides was performed as described above but only with 4 h of incubation. LDL-depleted plasma was prepared by PEG precipitation [15] and tested at a final concentration of 1% (vol:vol).

### 2.4. Plasma HDL remodeling

Remodeling of plasma HDL was assessed by adding 50 uL of 5 mg/ml of peptides or 50 uL of PBS as a vehicle control to 450 uL of pooled human plasma. Samples were incubated at 37 °C for 1 h in an orbital shaker at 300 rpm. HDL subclasses were separated by size by native PAGE, using 10-well Tris-Borate-EDTA gradient (3–25%) acrylamide gels (Jule, Inc.,USA) [16]. Proteins were transferred onto PVDF membrane and incubated overnight with anti-human apoA-I-HRP conjugate antibody (Meridian Life Science,USA). Images were acquired on an Alpha Innotech Chemi Imager 5500.

### 2.5. Molecular dynamic modeling

PP-5 structure was modelled using UCSF Chimera software (v.1.10.2, Regents of the University of California, USA) [17], with φ, ψ torsional angles of −75°, 145° for the peptide's secondary structure [11]. The peptide was relaxed in an aqueous environment by all atom simulation, using Desmond Molecular Dynamics System (version 4.3, DE Shaw Research, USA, 2015) [18]. The finalized structure was oriented against a POPC lipid bilayer and energy minimization was performed for 25 nanosec, using the same software application. The results were visualized using Maestro software (v.10.2.011, Schrödinger, New York, NY, 2015.) Initial GSG-10 peptide structure was modeled as an α-helix (φ, ψ = −60°, −45°), using UCSF Chimera software followed by relaxation in an aqueous environment as described above.

## 3. Results

### 3.1. Molecular dynamic modeling of the polyproline peptides

Before peptide synthesis, we first performed molecular dynamic modeling to predict the possible behavior of the polypro peptide PP-5, containing 5 trimeric repeat units ($Pro_p$-$Pro_g$-$Pro_p$)$_5$ in an aqueous solvent and when associated with a phospholipid bilayer. A structural model for one turn of the PP-5 peptide is shown in Fig. 2A. Each side chain radiates off the central peptide backbone at 120°, so that the two hydrophobic phenyl rings attached to the modified $Pro_p$ residues form a hydrophobic base and the polar Pro residue with the attached polar GalNac side chain ($Pro_g$) is at the top. The stability of a hypothetical PP-5 peptide forced into a polypro Type-II configuration was compared to a theoretical GSG-10 peptide, containing 10 repeat units of Gly-Ser-Gly, which were initially arranged in an α-helical conformation. The distance between α-carbon atoms in the first and the last peptide bonds were plotted against the simulation time (Fig. 2B). The starting distance for a "perfect" helix for both peptides was calculated to be approximately 42 Å. After a 12 nanosec stabilization period, the PP-