Atherosclerosis 222 (2012) 99–105

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Effect of an oral astaxanthin prodrug (CDX-085) on lipoprotein levels and progression of atherosclerosis in LDLR$^{-/-}$ and ApoE$^{-/-}$ mice

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ARTICLE INFO

Article history:
Received 30 September 2011
Received in revised form 23 January 2012
Accepted 2 February 2012
Available online 8 February 2012

Keywords:
Antioxidants
Lipoproteins
Atherosclerosis
Carotenoids
Inflammation

ABSTRACT

Oxidative stress and inflammation are key promoters of atherosclerosis and myocardial damage. When orally administered, the novel astaxanthin prodrug CDX-085 delivers high levels of the xanthophyll antioxidant astaxanthin that protects LDL from oxidation and reduces primary thrombosis. In this study, we analyzed whether delivery of astaxanthin from administration of the CDX-085 prodrug reduces plasma lipoprotein levels and the progression of atherosclerosis in low-density lipoprotein receptor negative (LDLR$^{-/-}$) and apolipoprotein E deficient (ApoE$^{-/-}$) mice.

Methods: Relative circulating levels of astaxanthin derived from CDX-085 administration compared to administration of pure astaxanthin was initially evaluated in a canine model. In mouse Study #1, 16 wild-type and 16 LDLR$^{-/-}$ mice on 0.5% cholesterol diet supplemented with either 0.0%, 0.08%, 0.2% and 0.4% CDX-085 were used to assess plasma levels and lipoprotein biodistribution measured by FPLC after 4 weeks treatment. In Study #2, 36 male LDLR$^{-/-}$ mice were randomized to a 0.5% cholesterol chow diet (CHOW group, $n = 12$) or 0.5% cholesterol chow fortified with 0.08% CDX-085 ($n = 12$) or 0.5% cholesterol chow with 0.4% CDX-085 ($n = 12$) for 12 weeks. In Study #3, 34 male ApoE$^{-/-}$ mice were randomized in the same fashion as the Study #2 and fed similar diets for 9 weeks.

Results: CDX-085 administration was shown to result in significantly higher levels of circulating astaxanthin ($p < 0.001$ ANOVA) over a 72 h period compared to pure, non-esterified astaxanthin in a single-dose pharmacokinetic study in beagles. In Study #1, plasma astaxanthin levels were 5–9-fold higher in LDLR$^{-/-}$ mice compared to wild-type mice. Astaxanthin was highly distributed among all lipoprotein fractions, generally reflecting cholesterol content of lipoproteins. In Study #2, administration of CDX-085 resulted in significantly lower total cholesterol levels (528 ± 68 mg/dL vs. 550 ± 67 mg/dL vs. 602 ± 80 mg/dL, $p = 0.047$) and aortic arch atherosclerosis ($9.0 \pm 4.2\%$ vs. $9.8 \pm 3.5\%$ vs. $13.2 \pm 3.6\%$, $p = 0.023$) in the 0.4% CDX-085 group compared to the 0.08% CDX-085 and CHOW groups, respectively. In ApoE$^{-/-}$ mice, a 72% reduction in triglycerides in the 0.4% CDX-085 group and 50% reduction in the 0.08% CDX-085 groups was noted compared to CHOW group (final levels 17 ± 11 mg/dL vs. 30 ± 15 mg/dL vs. 60 ± 32 mg/dL, respectively, $p = 0.001$).

Conclusion: Oral administration of the novel astaxanthin prodrug CDX-085 shows that it distributes among lipoproteins. CDX-085 lowers total cholesterol and aortic arch atherosclerosis in LDLR$^{-/-}$ mice and triglyceride levels in ApoE$^{-/-}$ mice and shows promise for further evaluation in human studies.

1. Introduction

While active lipid lowering agents such as statins and antiplatelet therapy reduce cardiovascular risk significantly, residual risk still remains [1,2]. Mechanistically, oxidative stress induces a variety of detrimental effects on the vessel wall, including endothelial dysfunction, vasoconstriction, thrombosis, inflammation, vessel remodeling, and plaque instability [3]. Various antioxidants, including vitamin E, have been evaluated clinically to reduce cardiovascular events but have shown reduced events in only high oxidative stress situations, such as dialysis patients [4–6] or diabetics with pro-inflammatory haptoglobin genotypes [7,8].

Astaxanthin is a xanthophyll carotenoid pigment found in various living organisms, including crustaceans and marine animals,

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doi:10.1016/j.atherosclerosis.2012.02.002

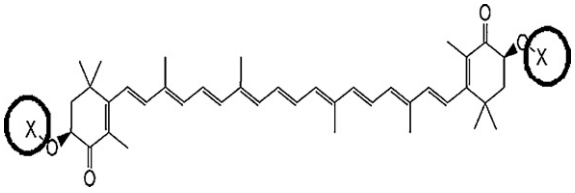

Fig. 1. Structure of astaxanthin prodrug CDX-085 (X = proprietary esterified moiety).

and is acquired by humans at low levels through diet [9]. Astaxanthin is a powerful lipophilic antioxidant effective against both aqueous and lipid oxidants and has been previously studied in both animal models and humans [9–14]. However, a major limitation of natural astaxanthin as a therapeutic agent in humans is its limited bioavailability due to poor oral absorption. Despite the low oral bioavailability, natural astaxanthin has been shown in recent human clinical trials to modestly reduce triglyceride levels, increase adiponectin and reduce inflammatory markers associated with metabolic syndrome such as tumor necrosis factor alpha (TNF-α) [12,14]. Additionally, astaxanthin significantly delayed formation of oxidized LDL ex vivo when compared to vitamin E and lutein in humans [15]. Beside anti-oxidant effects, astaxanthin has been shown to have anti-cancer, anti-diabetic and anti-inflammatory effects [9].

CDX-085 is a novel prodrug of the naturally occurring xanthophyll carotenoid astaxanthin that has been modified to have markedly increased water solubility and when administered orally to result in high circulating levels of astaxanthin (~10-fold compared to pure astaxanthin) (Fig. 1) [9,16]. Following conversion of the prodrug to solubilized astaxanthin in the gastrointestinal tract, absorbed astaxanthin transits to the liver in chylomicrons where it is stored and incorporated into LDL, HDL and VLDL particles. As these lipoproteins circulate, astaxanthin is distributed in various tissues including heart and vasculature, ultimately localizing within cellular, nuclear and mitochondrial membranes [9]. The molecular structure of astaxanthin demonstrates an optimal transverse-membrane spanning orientation resulting in stabilization of biological membrane integrity and prevention of lipid oxidation all in the absence of membrane disruption [17]. CDX-085 and similar compounds have decreased myocardial damage following ischemia in several animal species [18] and reduced platelet aggregation, decreased thrombus weights and attenuated recurrent thrombosis in a canine model [19]. Recently, Khan et al. [20] showed that CDX-085 reduced thrombosis and increased arterial patency in a mouse model of oxidative stress induced thrombosis, which appeared to be partially mediated by increased nitric oxide levels and decreased peroxynitrite levels in endothelial cells and platelets. Diminished nitric oxide formation and endothelial dysfunction are strongly implicated in atherothrombosis [21,22].

In the studies presented here, we tested the capacity of astaxanthin delivered from orally administered CDX-085 to reduce plasma lipoprotein levels and alter the progression of atherosclerosis in two mouse models of hyperlipidemia and atherosclerosis.

2. Methods

2.1. Quantification of astaxanthin levels in canine plasma

We evaluated the resulting circulating levels of astaxanthin following administration of non-esterified astaxanthin in comparison to CDX-085 administration in a single-dose pharmacokinetic study in beagles (MPI Research, Inc., Mattawan, Michigan). Male beagle dogs ($N = 3$/group, 10–12 kg wt.) were fasted overnight and then administered a single oral gavage dose of pure astaxanthin (36 mg/kg at a concentration of 4.8 mg/ml in 3:2:1 water:olive oil emulsion:20 mg/ml soy lecithin) or CDX-085 (50 mg/kg at a concentration of 10 mg/ml in 3:2:1 water:olive oil emulsion:20 mg/ml soy lecithin). Pure astaxanthin was administered at a slightly lower dose (36 mg/kg) compared to CDX-085 (50 mg/kg) due to the limited capacity for pure, non-esterified astaxanthin to be solubilized even in organic mixtures. However, as roughly 2/3 of CDX-085 molecular weight is astaxanthin, this does equate to approximately the same molar amount of astaxanthin delivered between the two groups. Blood samples (2 ml) were collected from the jugular vein at various time points (predose, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168 h) into tubes containing potassium-EDTA and quenching solution consisting of 0.1% (v/v) paraoxon-ethyl in absolute (200 proof) ethanol. Quantification of astaxanthin levels was conducted similarly to that described below for mouse plasma analysis.

2.2. Mouse feeding and treatment protocols

Study #1: 16 LDL receptor negative ($LDLR^{-/-}$) and 16 wild type mice (C57BL6 background) were fed a high cholesterol chow (0.5%) for 2 weeks. Animals were then divided into 4 groups (8 mice/group) and fed chow containing 0.5% cholesterol, 0.5% cholesterol + 0.08% CDX-085, 0.5% cholesterol + 0.2% CDX-085, or 0.5% cholesterol + 0.4% CDX-085 for 4 additional weeks. CDX-085 was supplied by Cardax Pharmaceuticals (Honolulu, Hawaii) and all diets were prepared by Harlan Teklad as previously described [20].

Study #2: In the second study, 11–16-week-old $LDLR^{-/-}$ male mice were assigned to 3 groups (12 mice/group, 36 total). All mice were initially fed high cholesterol chow (0.5%) for two weeks and then subsequently switched to either 0.5% cholesterol alone chow (CHOW), 0.5% cholesterol + 0.08% CDX-085 chow, or 0.5% cholesterol + 0.4% CDX-085 chow for an additional 12 weeks. Body weight check and blood sampling was performed at baseline, initial 2 weeks 0.5% cholesterol chow, and 4, 8 and 12 week treatment periods. The study was terminated following 12 weeks of diet.

Study #3: In the third study, 22–24-week-old apolipoprotein E negative mice ($ApoE^{-/-}$, 18 male, 16 female) were initially fed a high cholesterol chow (0.5%) for two weeks and then subsequently divided into 3 groups each receiving either 0.5% cholesterol chow alone (11 mice, CHOW), 0.5% cholesterol + 0.08% CDX-085 chow (11 mice) or 0.5% cholesterol + 0.4% CDX-085 chow (12 mice) for an additional 9 weeks. Body weight check and blood sampling was performed at baseline, initial 2 weeks 0.5% cholesterol chow, and 4, 9 week treatment periods.

2.3. Determination of total cholesterol and triglyceride levels in mice

Total cholesterol and triglycerides were determined by commercial enzymatic assay using the Roche Cobas Mira Plus Analyzer.

2.4. Quantification of astaxanthin levels in mouse plasma and FPLC fractions

Study #1 and #2: Lipoproteins were fractionized by size using fast protein liquid chromatography (FPLC) equipped with a Superose 6 column, and cholesterol and triglyceride levels were determined in each fraction (250 μl), as previously described [23].

Astaxanthin levels were determined using API-4000 with reversed phase C30 column separation. Plasma or FPLC fractions (50 μl) and internal standard solution (retinyl acetate, 100 ng/ml, 50 μl) were vortex-mixed with methyl *tert*-butyl ether (2.5 ml) for 10 min. After centrifugation, supernatant was dried with NitroVap evaporator and residual extract was reconstituted with mobile phase (100 μl) and quantified by LC–MS/MS. Mobile phases: A, water; B, methanol; containing 0.1% formic acid (v/v). Flow rate:

0.5 ml/min. Gradient: 90% B, 0–2 min; 95% B, 6–10 min. Electrospray ionization source was operated in the positive multiple reaction monitoring mode at 650 °C with the following optimized voltages: ion spray, 5500 V; declustering potential, 16 V; entrance potential, 10 V; collision energy, 39 V; collision cell exit potential, 14 V. Nitrogen was the collision gas. Q1/Q3 masses: 593.7/147.2 for astaxanthin; 269.3/93.0 for retinyl acetate. Retention times: 5.0 min for astaxanthin; 3.5 min for retinyl acetate. Linearity was satisfactory from 0.3 to 3000 ng/ml. A signal-to-noise ratio of ten is used for estimating quantification limit and quantification limit was determined to be 1 ng/ml.

2.5. Preparation of aortas

Following euthanasia, the aorta was extracted after a 20 min perfusion with phosphate buffered saline (PBS) containing 20 μM butylated hydroxytoluene (BHT) and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, via a cannula inserted into the left ventricle, and the aorta was fixed by perfusion with formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 μM EDTA, pH 7.4). The aorta was exposed, minor branching arteries (e.g., the intercostal arteries) were cut off, and the adventitia was removed in situ as far as possible. The aorta was then opened longitudinally, from the heart to the iliac arteries, while still attached to the heart and major branching arteries. The primary incision followed the ventral side of the aorta and the inner curvature of the arch. To obtain a flat preparation for imaging, a second incision was made along the outer curvature of the arch. The remaining branches were then cut off, and the aorta (from the heart to approximately 3 mm distal to the iliac bifurcation) was removed and pinned out on a black wax surface in a dissecting pan, using 0.2 mm diameter stainless steel pins (Fine Science Tools, Foster City, CA). Each pin was placed either well within a lesion or well away from lesions, so that it could easily be edited out in the processed image. The aortas were then subjected to an additional fixation with formal-sucrose for 12 h and stained with Sudan IV.

The upper half of the heart was dissected, fixed overnight in formal-sucrose, and paraffin-embedded. Sequential 7 μm thick sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 21–30 sections were collected and stained with hematoxylin and eosin.

2.6. Quantification of atherosclerosis

After fixation of aorta on paraffin plate, images of Sudan IV-stained aorta were captured by a Sony DXC-960MD three-chip color video camera. Total area and Sudan IV-stained area (atherosclerosis area) was measured by Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) and percent atherosclerosis area was calculated. In the aortic arch, quantification of atherosclerosis was done as a separate analysis (Fig. 2). The extent of atherosclerosis in the cross-sections of the aortic origin was determined with the same image analysis system. Seven images were captured of each hematoxylin-eosin stained cross-section in every 3rd slice since the first slice with aortic valve cusp, using the same video camera mounted on a Leica microscope. Area of atherosclerosis was measured using Image-pro Plus with total and mean atherosclerotic area measured. Preparation of the aortas and quantification of atherosclerosis were performed using previously validated protocols [24–26].

2.7. Statistical analysis

All continuous variables are described as mean ± standard deviation (SD). Comparison between three treatment groups was made with the use of one-way ANOVA test for quantitative variables with the post test Tukey test to assess differences between groups. Chi-square analysis was used to compare sex distribution of mice.

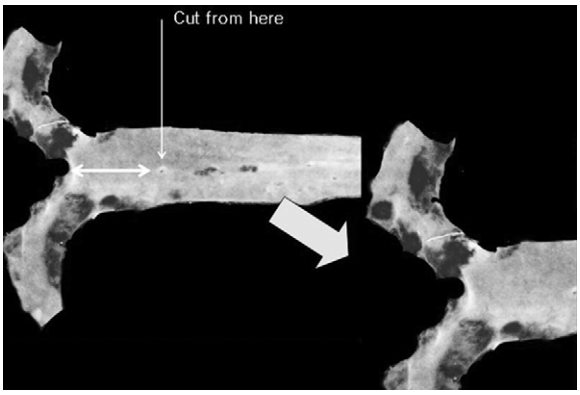

Fig. 2. Demonstration of quantitation of atherosclerosis in the aortic arch. The upper part of aorta was defined as starting from the ascending aorta to origin of the 1st intercostal artery.

3. Results

3.1. Circulating levels of astaxanthin resulting from oral administration of CDX-085

We evaluated the resulting circulating levels of astaxanthin following administration of non-esterified astaxanthin in comparison to CDX-085 administration in a single-dose pharmacokinetic study in beagles. As shown in Fig. 3, administration of CDX-085 resulted in rapid and dramatic levels of astaxanthin in plasma compared to pure, non-esterified astaxanthin administration which was undetectable in plasma at all time-points (below 1 ng/ml) ($p < 0.0001$ ANOVA). CDX-085 administration resulted in an AUC of 4.711 μg/ml h, a Tmax of 4hr and a T1/2 of 15 h with no detectable astaxanthin remaining after the 72 h point. These data support the in vivo superiority of orally administered CDX-085 to deliver astaxanthin systemically compared to pure astaxanthin.

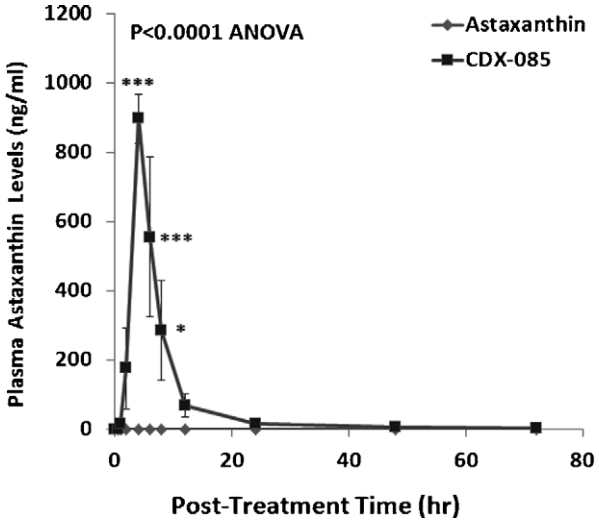

Fig. 3. Oral bioavailability of pure, non-esterified astaxanthin in comparison to astaxanthin derived from administration of CDX-085 in a single-dose pharmacokinetic study in beagles. The data was analyzed by ANOVA for the trend over time. ***$p < 0.001$ and *$p < 0.05$ for astaxanthin concentrations compared to the zero time-point using Bonferroni post test analysis.

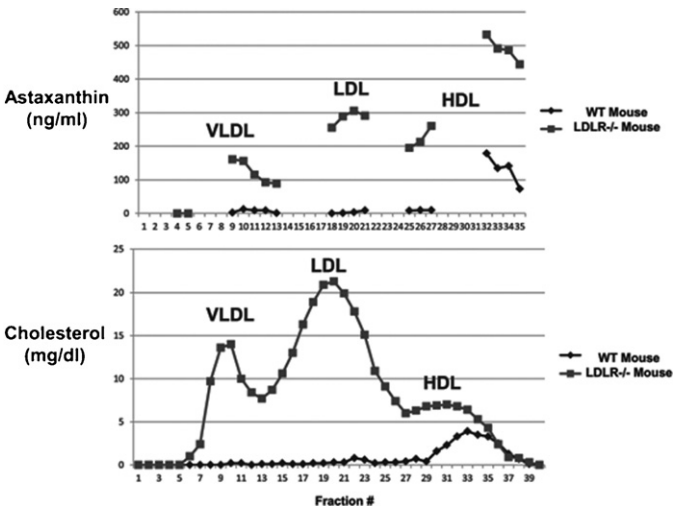

Fig. 4. Determination of astaxanthin levels (A), derived from pro-drug CDX-085, and total cholesterol (B) levels in FPLC fractions from LDLR$^{-/-}$ mouse plasma. The mice were treated with 0.5% cholesterol supplemented with 0.4% CDX-085 for 4 weeks.

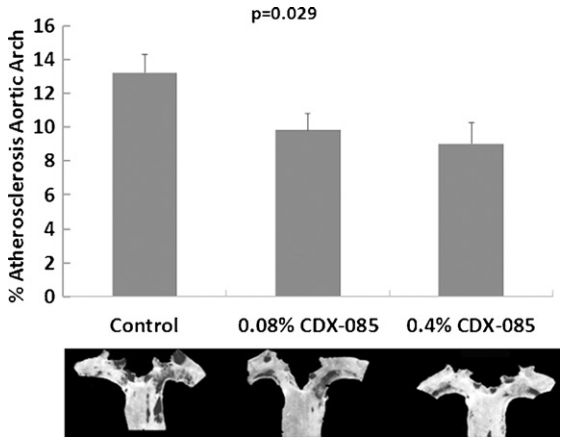

Fig. 5. Comparison of percent en face atherosclerotic lesion area in the ascending aorta plus the aortic arch (as described in Fig. 2) between treatment groups in LDLR$^{-/-}$ mice. Under each group are representative images of Sudan stained (stains neutral lipid) aortic arch lesions in the 3 groups corresponding to the atherosclerosis quantitation.

3.2. Distribution of astaxanthin in plasma and lipoprotein particles

In Study #1, oral administration of CDX-085 resulted in high levels of astaxanthin in plasma (1.19 ± 0.51 μg/ml in WT mice and 10.59 ± 5.29 μg/ml in LDLR$^{-/-}$ mice at 0.4% CDX-085). Interestingly, LDLR$^{-/-}$ mice exhibited much higher circulating levels of astaxanthin (5–9-fold) at all doses compared to WT mice (0.17 μg/ml in WT vs. 0.96 μg/ml in LDLR$^{-/-}$ mice at 0.08% CDX-085; 0.55 μg/ml in WT mice vs. 3.24 μg/ml in LDLR$^{-/-}$ mice at 0.2% CDX-085; 1.19 μg/ml in WT mice vs. 10.59 μg/ml in LDLR$^{-/-}$ mice at 0.4% CDX-085). In Study #2, administration of CDX-085 to LDLR$^{-/-}$ mice achieved astaxanthin levels of 0.76 ± 0.22 μg/ml in the 0.08% CDX-085 dose and 3.80 ± 1.36 μg/ml in the 0.4% CDX-085 dose. No astaxanthin was detected in plasma from mice administered control chow lacking CDX-085 in either Study #1 or Study #2.

FLPC fraction analysis of Study #1 samples revealed higher cholesterol levels in VLDL, LDL, and HDL fractions in LDLR$^{-/-}$ mice compared to wild type mice. This was associated with increased astaxanthin levels in LDLR$^{-/-}$ mice in VLDL, LDL, and HDL fractions (Fig. 4).

3.3. Effect CDX-085 on lipoprotein profiles

3.3.1. LDLR$^{-/-}$ mice

No significant changes were noted in body weights over the time course of the study. Oral CDX-085 administration significantly reduced total cholesterol levels significantly in LDLR$^{-/-}$ mice in a dose dependent manner (Table 1). This observed lipid lowering effect was noted early in the treatment after only a month of CDX-085 treatment (total cholesterol: CHOW: 759 ± 86 mg/dL, 0.08% CDX-085: 640 ± 70 mg/dL, 0.4% CDX-085; 590 ± 59 mg/dL, $p < 0.001$). No significant changes were noted in triglyceride levels.

3.3.2. ApoE$^{-/-}$ mice

In ApoE$^{-/-}$ mice, total cholesterol levels diminished slightly over time after CDX-085 treatment regardless of dose, which is typical when first starting a high cholesterol diet in mice [23]; however, none of these changes were significant (Table 2). Triglyceride levels were similar in all groups at baseline and 2 weeks after 0.5% cholesterol diet; however, after 4 weeks of 0.08% CDX-085 treatment there was a strong trend toward triglyceride reduction compared to control. After 9 weeks of treatment, triglyceride levels were significantly lower in both the 0.08% CDX-085 and 0.4% CDX-085 treatment groups compared to the control group and were most dramatic in the high CDX-085 group (control: 60.7 ± 31.9, 0.08% CDX-085 dose: 30.4 ± 15.3, 0.4% CDX-085 dose: 17.4 ± 11.0 mg/dL, $p = 0.001$) (Table 2).

3.4. Effect CDX-085 on atherosclerosis

3.4.1. LDLR$^{-/-}$ mice

In en-face analysis, there was no significant difference in % lesion area in whole aorta between CDX-085 treatment groups (2.66 ± 1.04%, 2.37 ± 0.76%, and 2.34 ± 1.22% in control, LOW CDX-085 and 0.4% CDX-085 dose groups respectively, $p = 0.70$) (Table 3). Mean aortic valve root atherosclerotic area measurement in 7 sections was: 0.08% CDX-085: 0.10197 ± 0.04302, 0.4% CDX-085: 0.10204 ± 0.05770 mm^2 vs. control: 0.12507 ± 0.04302 mm^2, $p = 0.41$, (Table 3). However, in the aortic arch, there was significantly lower extent of atherosclerosis in the CDX-085 treatment groups (0.08% CDX-085: 9.821 ± 3.48, 0.4% CDX-085: 9.02 ± 4.23% vs. control: 13.23 ± 3.56%, $p = 0.023$) (Table 3, Fig. 5), suggesting site-specific effects.

3.4.2. ApoE$^{-/-}$ mice

No differences were present in % en-face atherosclerosis in both 0.08% CDX-085 and 0.4% CDX-085 treatment (control: 12.98 ± 6.88%, 0.08% CDX-085: 9.44 ± 4.78%, 0.4% CDX-085: 9.44 ± 4.78%, $p = 0.34$) or in the aortic arch (control: 0.41252 ± 0.15493, 0.08% CDX-085 dose: 0.37428 ± 0.11504, 0.4% CDX-085: 0.33603 ± 0.12793 mm^2, $p = 0.45$) (Table 3) (Table 4).

4. Discussion

In this study, oral administration of the novel astaxanthin pro-drug CDX-085 resulted in a dose-dependent increase in plasma astaxanthin levels that were effectively distributed in individual lipoproteins. CDX-085 administration dose-dependently reduced total cholesterol levels and aortic arch atherosclerosis in LDLR$^{-/-}$ mice and triglyceride levels in ApoE$^{-/-}$ mice. These findings suggest that the ability of CDX-085 administration to provide high circulating astaxanthin levels compared to unesterified pure astaxanthin may allow optimal dosing to further assess its cardiovascular properties in future human studies.

While oxidative stress plays a key role in all stages of atherosclerosis from plaque initiation to plaque rupture and resulting

Table 1
Changes in body weight, total cholesterol and triglycerides in LDLR$^{-/-}$ mice according to CDX-085 dose.

LDLR$^{-/-}$ Mice	No treatment	0.08% CDX-085	0.4% CDX-085	*p*-Value*
Male% (*n*)	100(12)	100(12)	100(12)	NA
Weight (g)				
Baseline	29.4 ± 2.3	29.1 ± 1.9	27.6 ± 2.0	0.088
2 wk 0.5% cholesterol	29.8 ± 2.1	29.4 ± 1.5	28.8 ± 1.6	0.399
1 month treatment	31.2 ± 2.7	31.1 ± 1.9	29.9 ± 1.8	0.313
2 months treatment	33.2 ± 2.7	33.1 ± 2.6	31.9 ± 1.8	0.368
3 months treatment	33.7 ± 2.8	33.9 ± 2.7	33.5 ± 2.6	0.570
Total cholesterol (mg/dL)				
Baseline	269 ± 26	254 ± 36	244 ± 37	0.219
2 wk 0.5% cholesterol	823 ± 94	838 ± 121	842 ± 182	0.934
1 month treatment	759 ± 86	640 ± 70§	590 ± 59§	<0.001
2 months treatment	674 ± 125	623 ± 63	586 ± 72	0.070
3 months treatment	602 ± 80	550 ± 67	528 ± 68¶	0.047
Triglycerides (mg/dL)				
Baseline	159 ± 40	151 ± 32	178 ± 42	0.278
2 wk 0.5% cholesterol	132 ± 30	125 ± 30	111 ± 24	0.218
1 month treatment	154 ± 34	135 ± 31	139 ± 28	0.297
2 months treatment	168 ± 44	161 ± 37	155 ± 27	0.682
3 months treatment	145 ± 28	131 ± 22	124 ± 16	0.082

* One-way ANOVA with Tukey post test.
§ $p < 0.001$ compared to no treatment group.
¶ $p < 0.05$ compared to no treatment group.

Table 2
Baseline characteristics of ApoE$^{-/-}$ mice and changes of body weight and lipid profiles according to CDX-085 dose.

	No treatment	0.08% CDX-085	0.4% CDX-085	*p*-Value*
Male% (*n*)	72.7(8)	36.4(4)	58.3(7)	0.224
Weight (g)				
Baseline	28.15 ± 3.18	27.42 ± 4.12	28.33 ± 3.70	0.824
0.5% cholesterol	28.75 ± 3.27	27.62 ± 4.55	28.64 ± 3.90	0.757
4 weeks treatment	30.42 ± 3.27	29.25 ± 5.10	30.18 ± 3.00	0.755
9 weeks treatment	30.71 ± 3.14	27.49 ± 3.27¶	30.16 ± 2.72	0.032
Total cholesterol (mg/dL)				
Baseline				
0.5% cholesterol	1116.7 ± 193.5	1170.1 ± 190.0	1143.8 ± 219.9	0.827
4 weeks treatment	994.7 ± 114.3	997.1 ± 170.1	967.4 ± 212.3	0.899
9 weeks treatment	728.1 ± 96.2	829.3 ± 216.6	844.1 ± 199.6	0.270
Triglycerides (mg/dL)				
Baseline	138.4 ± 51.7	128.5 ± 84.3	134.8 ± 106.3	0.963
0.5% cholesterol	73.7 ± 34.6	51.5 ± 33.9	77.2 ± 50.0	0.281
4 weeks treatment	81.4 ± 58.4	34.8 ± 27.1	49.2 ± 47.9	0.053
9 weeks treatment	60.7 ± 31.9	30.4 ± 15.3	17.4 ± 11.0¶	0.001

* One-way ANOVA with Tukey post test.
¶ $p < 0.05$ compared to no treatment group.

thrombosis, disappointingly, antioxidants such as vitamin E have only been effective in high risk subjects such as dialysis and diabetics. One may have expected improved clinical outcomes in prospective studies as expected from theoretical background, successful animal studies and epidemiologic human studies. The reasons for this are complex and previously discussed in detail [9,27–31]. Collectively, they may reflect treatment starting too late in life when disease is quite advanced, poor efficacy of vitamin E derivatives in humans, potential pro-oxidant effects of some anti-oxidants, lack of measurement of oxidative stress, treatment

Table 3
Quantification of en face atherosclerosis in LDLR$^{-/-}$ mice and direct measurement of atherosclerotic area in aortic root.

	No treatment	0.08% CDX-085	0.4% CDX-085	*p*-Value*
Total aorta (pixel)				
Lesion area	6832 ± 2770	63857 ± 2199	6213 ± 3011	0.84
Aorta area	254471 ± 3787	267553 ± 4024¶	269988 ± 3742¶	0.001
Lesion %	2.66 ± 1.04	2.37 ± 0.76	2.34 ± 1.22	0.70
Aortic root + aortic arch (pixel)[a]				
Lesion area	6798 ± 1726	5545 ± 2043	5258 ± 2460	0.18
Aorta area	51736 ± 5666	56123 ± 4477	58176 ± 3847§	0.007
Lesion %	13.23 ± 3.56	9.821 ± 3.48	9.02 ± 4.23¶	0.023
Aortic root extent of atherosclerosis (mm^2)				
Average	0.12507 ± 0.04302	0.10197 ± 0.04302	0.10204 ± 0.05770	0.41

* One-way ANOVA with Tukey post test.
¶ $p < 0.05$ compared to no treatment group.
[a] Aortic arch analysis; 1 specimen of no treatment group was excluded because of loss of root and ascending aorta.
§ $p < 0.01$ compared to no treatment group.

Table 4
Quantification of en face atherosclerosis in ApoE$^{-/-}$ mice and direct measurement of atherosclerotic area in aortic root.

	No treatment	0.08% CDX-085	0.4% CDX-085	*p*-Value*
Total aorta (pixel)				
Lesion area	21715 ± 11814	20593 ± 10735	16773 ± 8434	0.48
Aorta area	168770 ± 15702	172943 ± 11105	177804 ± 10572	0.23
Lesion %	12.98 ± 6.88	11.86 ± 5.99	9.44 ± 4.78	0.34
Aortic root extent of atherosclerosis (mm^2)				
Average	0.41252 ± 0.15493	0.37428 ± 0.11504	0.33603 ± 0.12793	0.45

* One-way ANOVA with Tukey post test.

of subjects without evidence of increased oxidative stress rather than subjects with documented increased oxidative stress where one may predict enhanced efficacy, and diminished local bioavailability and low doses of antioxidants leading to poor local tissue effects where these antioxidants cannot overcome the pro-oxidant kinetics of oxidizing species. Clearly, a more precise understanding regarding the role of oxidative stress in this complex pathophysiology is required to infer predicted antioxidant therapy efficacy [32].

The capacity of orally administered CDX-085 to result in high circulating levels of astaxanthin addresses some of the limitations of prior anti-oxidants. For example, McNulty et al. [17] showed that the unique transmembrane alignment of astaxanthin is important to protect cellular membranes from oxidation and that the antioxidant effects of carotenoids are associated with specific "physio-chemical" interactions with the membrane. Additionally, McNulty et al. compared various polar carotenoids (astaxanthin) and apolar carotenoids (beta-carotene) and showed the apolar carotenoids to be pro-oxidants and associated with a disarrangement of the membrane bilayer. In contrast, astaxanthin did not result in any disruption of the cell membrane structure and also showed a strong antioxidant effect, as measured by reduction in LOOH levels. Iwamoto et al. [15] also showed that astaxanthin delayed ex vivo LDL oxidation in a human study, whereas other antioxidants such as vitamin E (α-tocopherol) and lutein (another carotenoid) did not delay LDL oxidation.

This presented series of studies used CDX-085 that is an orally administered prodrug of astaxanthin that when de-esterified in vivo delivers high levels of astaxanthin to plasma and tissues. The canine study and murine Study #1 presented here showed that oral administration of this prodrug increased plasma astaxanthin levels in support of previously observed findings that orally administered CDX-085 resulted in increased astaxanthin levels in plasma, heart and liver [20]. This oral administration of CDX-085 resulted in high levels of astaxanthin in plasma and was proportionately distributed between lipoproteins. Based on results from these previous studies and the observed plasma astaxanthin levels in the present studies, we can hypothesize that in these experiments astaxanthin has been stored in the liver and incorporated into lipoproteins for distribution to various tissues including heart and vasculature.

In the murine Study #2, we tested the lipid lowering and anti-atherosclerotic effects of CDX-085 administration for the first time. Total cholesterol was significantly lowered by CDX-085 administration in a dose dependent manner. This lipid lowering effect was apparent following only a month of CDX-085 treatment and was mainly seen in lowering total cholesterol levels. The mechanism of cholesterol reduction was not addressed in this study. However, recent studies have suggested that carotenoid intestinal and cellular uptake may be in part a facilitated process dependent on SR-BI receptors and have shown that ezetimibe reduces carotenoid cellular uptake [33]. Additionally, other groups have published reductions in total cholesterol following astaxanthin administration in rodents in congruence with our observations with CDX-085 [13,34]. Furthermore, although not a definitive mechanistic explanation, Yang et al. [34] suggest upregulation of SREBP2 resulted in increased LDL receptor upregulation and decreased circulating LDL-C. However, as we observed cholesterol reduction in an LDL receptor deficient mouse strain, receptor upregulation clearly cannot be the mechanism for cholesterol reduction in our study. Astaxanthin may also alter the functionality of LDL and HDL leading to increased reverse cholesterol transport and elevated cholesterol efflux but this hypothesis has yet to be tested.

In the atherosclerosis assessment, while analysis of whole aorta showed only a tendency toward atherosclerosis reduction without statistical significance, the LDLR$^{-/-}$ mice were young (11–16 weeks) and total cholesterol levels never reached the high levels usually required to induce extensive progression of atherosclerosis in whole aorta (±2.5%). Additionally, the relatively short treatment duration of these studies may also be a contributing factor in the lack of CDX-085 anti-atherosclerotic effect. However, a statistically significant anti-atherosclerotic effect of CDX-085 was observed in aortic arch in LDLR$^{-/-}$ mice. The upper part of the aorta (aortic root, ascending aorta, and aortic arch) is directly exposed to sheer stress from blood flow and is associated with vital arteries such as coronary and carotid arteries. We analyzed the upper part of the aorta apart from the other portions due to the fact that atherosclerosis often evolves earlier in this area in these models. These data are consistent with a study in Watanabe heritable hyperlipidemic rabbits showing natural astaxanthin, although it did not reduce atherogenesis, reduced macrophage infiltration, apoptosis and MMP3 expression, which are markers of plaque vulnerability, following 24 weeks of diet [10,11]. Additional studies are needed to assess whether this effect is mediated though a cholesterol lowering effect, through an independent pathway or both.

In murine Study #3 we used older ApoE$^{-/-}$ mice that showed higher total cholesterol levels compared to levels achieved in the LDLR$^{-/-}$ mice in Study #2. Additionally, the ApoE$^{-/-}$ mice showed a greater atherosclerotic burden compared to the LDLR$^{-/-}$ mice in Study #2. Importantly, CDX-085 treatment significantly reduced triglyceride levels in a dose-dependent manner. Although the mechanisms underlying this observation were not tested here, in human studies, natural astaxanthin reduced triglyceride levels and increased adiponectin, suggesting that this may be through increasing VLDL catabolism and increasing insulin resistance [14,35,36].

In Study #3, although numerically lower amounts of atherosclerosis were seen in the HIGH CDX-085 group and to a similar extent as there LDLR$^{-/-}$ mice of ~25%, we found no statistically significant reduction atherosclerosis in the CDX-085 treatment groups, which appeared to be due to the higher mouse-to-mouse variability of the extent off atherosclerosis in this model. A higher dose and/or longer duration of CDX-085 treatment may be postulated to result in a significant effect on atherosclerosis in this model.

Interestingly, similar lipid lowering effects of astaxanthin have been previously reported in animals [13,35] and human studies with nutraceutical astaxanthin [14]. Specifically, an experiment using obese mice fed a high fat diet showed that astaxanthin significantly reduced plasma total cholesterol, triglyceride, liver triglyceride, fat mass, and body weight, suggesting it may have

effects on fatty liver in humans [13]. The first randomized human study reported daily administration of 12 and 18 mg of astaxanthin increased HDL-C up to 15.4% and reduced triglycerides 25.2% [14]. Additionally, triglyceride levels were lowered almost to one fourth of the control group with the high dose treatment for 9 weeks (60.7 ± 31.9 mg/dL vs. 17.4 ± 11.0 mg/dL). This suggests that a more pure prodrug, such as CDX-085, that is capable of being orally administered in a dose-regulated manner and that can result in high levels of circulating astaxanthin may have an even stronger effect and therefore can be more definitively tested in future human studies.

5. Limitations

Due to the expense of CDX-085 at these early stages of synthesis, we were not able to carry out longer-term studies in the mice, which may have potentiated both the lipid lowering and anti-atherosclerosis effects. Additionally, to minimize wide variations in plasma cholesterol levels, we used a 0.5% cholesterol diet [23] which does not seem to generate as much inflammation as western diets which are additionally supplemented with fat, and may be associated with worse lipid profile, particularly in triglycerides and a stronger atherosclerosis stimulus. These diets may more closely reflect the metabolic syndrome where potential clinical use may be optimal. Future studies are needed to establish the proper dose and duration associated with efficacy.

6. Conclusion

Oral administration of the astaxanthin prodrug CDX-085 resulted in high plasma levels of astaxanthin that were effectively distributed in lipoproteins, associated with reduced total cholesterol and triglycerides and diminished atherosclerosis in the root–arch. These results support the continued pre-clinical and ultimately clinical development of CDX-085 in lipid lowering and prevention of atherosclerosis and its complications.

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