JPET Fast Forward. Published on September 5, 2019 as DOI: 10.1124/jpet.119.259846
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A novel orally available delta-5 desaturase inhibitor prevents atherosclerotic lesions accompanied with changes of fatty acid composition and eicosanoid production in *ApoE* knockout mice

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Running Title: D5D inhibitor prevents atherosclerosis in *ApoE* KO mice

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Text Pages: 20

Figures: 6

References: 29

Words in Abstract: 192

Words in Introduction: 618

Words in Discussion: 1231

Abbreviations

AA, arachidonic acid; ACS, acute coronary syndrome; D5D, delta-5 desaturase; D6D, delta-6 desaturase; DGLA, dihomo-γ-linolenic acid; ELISA, enzyme-linked immunosorbent assay; 15-HETrE, 15-hydroxy eicosatrienoic acid; KO, knockout; LA, linoleic acid; LTs, leukotrienes; MRM, multiple reaction monitoring; PGs, prostaglandins; TC, total cholesterol; TXs, thromboxanes; WT, wild-type.

Recommended section assignment: Cardiovascular

Abstract

Delta-5 desaturase (D5D), encoded by fatty acid desaturase 1 (*Fads1*), is the rate-limiting enzyme for the conversion from dihomo-γ-linolenic acid (DGLA) to arachidonic acid (AA) in the ω-6 polyunsaturated fatty acid pathway. Several AA-derived eicosanoids (e.g., prostaglandins, thromboxanes, leukotrienes) and DGLA-derived eicosanoids are reported to promote and/or prevent atherosclerosis progression through, at least in part, its pro-inflammatory or anti-inflammatory effects. To elucidate effects of D5D inhibition by a D5D inhibitor on atherosclerosis, we generated a potent, orally available and selective D5D inhibitor, compound-326, and examined its effects on Western-diet fed *ApoE* knockout (KO) mice. Oral administration of compound-326 (3–10 mg/kg/day for 15 weeks) significantly inhibited the progression of atherosclerotic lesions in the aorta without affecting plasma total cholesterol and triglyceride levels. Compound-326 significantly decreased AA levels while increased DGLA levels in the liver and the blood accompanied with decreases in AA-derived eicosanoid production and increases in DGLA-derived eicosanoid production from the blood cells. We conclude that compound-326 prevented the progression of atherosclerosis in Western-diet fed *ApoE* KO mice by modulating a profile of eicosanoid production, suggesting that D5D inhibitors can be a novel remedy for preventing atherosclerosis and subsequent cardiovascular events.

Significance statement: This study shows a D5D specific and orally available potent inhibitor provided the first evidence to support the concept that D5D inhibitors will be a novel remedy for preventing the

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atherosclerosis progression.

Introduction

Atherosclerosis and subsequent cardiovascular (CV) complications (including death, acute myocardial infarction, unstable angina, and stroke) are the leading causes of morbidity and mortality worldwide, in spite of major therapeutic progress for controlling classical risk factors, such as hypertension, dyslipidemia, and diabetes (Arbab-Zadeh et al., 2012; Galkina and Ley, 2009). Even when treated with powerful statins, some patients still have a high risk of CV complications after an acute coronary syndrome (ACS) and stroke, suggesting an unmet need for new medications. One possibility that has strong potential is anti-inflammatory therapy. Several anti-inflammatory drugs, such as darapladib (LP-PLA2 inhibitor), varespladib (sPLA2 inhibitor), canakinumab (IL-1β mAb), and low-dose methotrexate have been evaluated. Although some of the trials reported negative results (O'Donoghue *et al*., 2014; White *et al*., 2014; Nicholls *et al*., 2014; Mukherjee *et al*., 2001), anti-inflammatory therapy is still considered a promising strategy, since many clinical and pre-clinical studies suggest that inflammation plays a significant role in the development of atherosclerosis and subsequent complications (Ross, 1999; Duchatelle *et al*., 2012). In fact, it was recently reported that targeting the interleukin-1β pathway with canakinumab lead to a significant prevention of CV events independent of lipid-level lowering in CANTOS trial (Ridker *et al*., 2017), suggesting that anti-inflammatory therapy could be a new remedy for atherosclerosis and subsequent CV complications (Hansson, 2017).

The enzyme delta-5 desaturase (D5D) is encoded by the gene fatty acid desaturase 1 (*Fads1*). This

desaturase is the rate-limiting enzymes for polyunsaturated fatty acids (PUFA) conversion and determines systemic PUFA levels (Tosi *et al*., 2014). In the long chain PUFA synthesis pathway, D5D catalyzes the conversion of dihomo-γ-linolenic acid (DGLA) into arachidonic acid (AA, ω-6 PUFA pathway) and the conversion of ω-3 AA (20:4 ω-3) into eicosapentaenoic acid (EPA, ω-3 pathway). Several AA-derived eicosanoids including prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) function as pro-inflammatory mediators (Capra *et al*., 2013) while some AA- and DGLA-derived eicosanoids including PGI2, PGE1 and 15-hydroxy eicosatrienoic acid (15-HETrE) have anti-inflammatory effects (Wang *et al*., 2012; Vang and Ziboh, 2005). These pro-inflammatory eicosanoids are reported to promote atherosclerosis, while inhibition of their synthesis showed anti-atherosclerotic effects in rodents, rabbits, and pigs (Wang *et al*., 2006; Subbarao *et al*., 2004; Kobayashi *et al*., 2004; Cyrus *et al*., 2002; Shen *et al*., 1996; Braun *et al*., 1993; Merhi *et al*., 1997; Takai *et al*., 2009). These results suggest that modulation of a profile of AA- and DGLA-derived eicosanoids could affect atherosclerosis progression. In fact, Powell *et al* and Fan *et al* recently reported that *Fads1* knockout (KO) mice showed systemic decreases in AA and AA-derived prostaglandin amounts and increases in DGLA and DGLA-derived prostaglandin amounts, and decreased atherosclerotic lesion formation in the aorta. These results suggest that D5D inhibition could be an attractive target for the treatment of atherosclerosis through its novel mechanism, despite the potential to induce developmental adaptations as one limitation of the genetic loss-of-function approach. To obtain proof-of-concept that D5D inhibition by a selective D5D inhibitor has promise as a translatable

therapy for atherosclerosis, it is important to develop a selective D5D inhibitor and to characterize its therapeutic efficacy in a pre-clinical animal model of atherosclerosis.

We previously reported that compound-326 (Suzuki *et al*., 2010), a newly discovered orally available, potent, and selective D5D inhibitor, ameliorated insulin resistance and obesity in a high-fat diet-induced obese mouse model accompanied with alterations of the balance between DGLA and AA in the blood (Yashiro *et al*., 2016). In the present study, we evaluated anti-atherosclerotic effects of compound-326 in Western-diet fed *ApoE* KO mice. We examined effects of the D5D inhibitor on the development of atherosclerosis, PUFA levels, eicosanoid production, and plasma levels of soluble ICAM-1 (sICAM-1), a systemic inflammatory marker, in this well-described animal model of atherosclerosis.

Material and methods

Compound. Compound-326 potently and selectively inhibits D5D activity whereas it has almost no inhibitory effects of D6D and D9D activity (Yashiro *et al*., 2016). Its selectivity was investigated using a Cerep/PanLab selectivity profiling panel (Eurofins discovery, St Charles, MO) for 94 targets at 10 μM of compound-326. The results are presented in Supplemental table S1 as the percent of inhibition of specific binding or activity.

Animal studies. All laboratory animal care and protocols were approved by the Institutional Animal Care and Use Committee in Takeda Pharmaceutical Company, and were conducted in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals. Supplemental table S2 shows fatty acid composition in the diets used in the study.

(1) D5D inhibitory efficacy evaluation study using normal mice.

Male C57BL/6 mice (CLEA, Japan) were fed standard rodent chow (CE2, CLEA). Compound-326 (0.1–30 mg/kg/day, p.o., suspended in 0.5% [w/v] methylcellulose) was administered once daily to the mice via gavage for 1 to 4 days. Twenty-four hours after the final dosing, the mice were killed and the livers were isolated. Levels of fatty acids in the liver were analyzed as mentioned below.

(2) Anti-atherosclerotic study using *Fads1* x *ApoE* double KO mice.

Fads1 KO mice were obtained from Lexicon Pharmaceuticals, Inc. [Powell *et al*., 2016] and *Fads1* x

ApoE double KO mice were generated by the Takeda KO animal generation group. Eight- to 9-week-old male *Fads1* x *ApoE* double KO mice were fed a Western diet (D12079B; Research Diets, New Brunswick, NJ) containing 21% fat and 0.15% cholesterol for 14 weeks. After 2, 6, 10, and 14 weeks from the start of the Western diet, a blood sample was obtained via retro-orbital bleed, and assayed for biochemical parameters (fatty acid, total cholesterol, triglyceride, and glucose) analysis. At the end of the study period, mice were anesthetized by intraperitoneal administration of pentobarbital sodium, and the liver and the aorta were removed. Levels of fatty acids in the liver were analyzed as mentioned below. The isolated aortae, after removing extra tissues, were fixed with formalin and then stained with Oil red O to identify atherosclerotic lesions. The percentage of the lesion area was quantified with Image-Pro 6.0 software.

(3) Anti-atherosclerosis study using compound-326 (Studies 1 and 2).

Study 1: Male *ApoE* KO mice were used in this study. At 10 weeks of age, the mice were subjected to a control diet (98121701, Research Diets) for a week. At 11 weeks of age, the mice were then divided into 5 groups based on the baseline values of their body weight and plasma biochemical parameters (total cholesterol, triglyceride, and glucose in the non-fasting condition), followed by once-daily administration of compound-326 (0.3, 3, 10 mg/kg/day, p.o.) or vehicle (0.5% [w/v] methylcellulose) every morning via gavage. One week after the start of treatment, the mice were subjected to a Western diet for 14 weeks. One group of mice (administered with the same vehicle) as a control group was maintained on the control diet. After 3, 7, 11 and 15 weeks of compound-326 once-daily administration, blood samples were

obtained by retro-orbital bleed and were assayed for fatty acid composition and biochemical parameters (total cholesterol, triglyceride and soluble ICAM1) analysis. At the end of the study period, the mice were killed, and the blood, liver, and aorta were harvested. The atherosclerotic lesion area of the aorta was quantified as described above in a blind manner.

Study 2: At 11 weeks of age, the mice were divided into 6 groups based on the baseline values of their body weight and plasma parameters (total cholesterol, triglyceride, glucose and soluble ICAM1 levels in the non-fasting condition), followed by once-daily administration of compound-326 (0.3, 1, 3, 10 mg/kg/day, p.o.) or vehicle as described above. Other procedures were conducted as described in study 1.

Plasma biochemical parameter analysis. Plasma total cholesterol, triglyceride, glucose levels were analyzed using an automatic chemistry analyzer (7180 Clinical Analyzer; Hitachi, Tokyo, Japan). Plasma soluble-ICAM1 levels were assayed using Mouse Soluble ICAM-1 (CD54) enzyme-linked immunosorbent assay (ELISA) kits (Pierce Biotechnology, Rockford, lL, USA).

Eicosanoid production assay. At the end of the study period, blood collected from the abdominal aorta was stimulated using 30 μM A23187 and 10 μM N-formylmethionyl-leucyl-phenylalanine (fMLP) and was incubated at 37°C for 10 min. The blood samples were then centrifuged at room temperature for 5 min to isolate the plasma. The plasma eicosanoid fraction was extracted using a solid phase extraction column (Oasis HLB 30 mg/cc, Waters, japan). An aliquot (5 μL) of each sample was injected onto a UK-C18 reverse phase column (150×2 mm, Imtakt, Japan), then chromatographic separation was

performed by gradient elution of mobile phase A (0.1% acetic acid in MilliQ) and mobile phase B (0.1% acetic acid in acetonitrile). Eicosanoids (PGE2, TXB2, 11-dehydro TXB2, LTB4, 6-keto PGF1α, PGF2α, PGD2, 15-HETrE and 13, 14-dihydro, 15-keto PGE1) were measured by multiple reaction monitoring (MRM) with a TSQ Vantage Triple Stage Quadrupole LC/MS Mass Spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA). Since we could not directly detect PGE1 and PGI2 production in blood cells by our method, we measured 13, 14-dihydro, 15-keto PGE1 and as 6-keto PGF1α production as a surrogate marker of unstable PGE1 and PGI2, respectively.

Quantitative measurement of fatty acid levels. Total lipids in blood and liver samples were extracted with hexane/2-propanol/butylated hydroxytoluene (60:40:0.01, v/v). A liver phospholipid fraction was extracted using a solid phase extraction column (Sep-Pak Vac NH2, Waters). Blood lipids and liver phospholipids were de-esterified and labeled with an esterified long-chain and short-chain fatty acid labeling reagent (YMC). The labeled fatty acid contents were measured via high performance liquid chromatography (Agilent 1200, Agilent Technologies). In the anti-atherosclerotic study using *Fads1* KO mice, peak areas of AA to linoleic acid (LA) ratio (AA/LA) and DGLA to LA ratio (DGLA/LA) were quantified. In the D5D inhibitory efficacy evaluation study and the anti-atherosclerosis study, each peak area of fatty acids were quantified and absolute amounts of fatty acids were quantified using margaric acid as an internal standard. The ratio of AA/DGLA was used for estimating D5D activity.

Data analysis. Data were expressed as mean ± standard deviation (S.D.) or mean ± standard error of

the mean (S.E.M.). Statistical analysis was conducted in a step-by-step approach. A significant difference between control and vehicle-treated groups was assessed by Student's *t*-test or *Aspin-Welch* test depending on equal or unequal variances and a *p*-value less than 0.05 was considered significant. The dose-dependent effect of compound-326 vs. vehicle was then evaluated by the one-tailed *Williams* test or *Shirley-Williams* test depending on equal or unequal variances and a *p*-value less than 0.025 was considered significant.

Results

D5D inhibitory efficacy evaluation study. Repeated administration of the inhibitor compound-326 (at 0.1-30 mg/kg/day, p.o. for 4 days) markedly and dose-dependently decreased the AA/DGLA ratio and AA levels and increased DGLA levels in the liver of normal mice. The effect of single administration showed moderate changes in AA and DGLA levels in the liver compared with repeated administration (Figure 1). It showed only moderate or no effects on other fatty acids including EPA and DHA in normal mice put on the normal diet (CE2), which richly contains ω-3 PUFA (Supplemental table S2).

Anti-atherosclerotic study using *Fads1* KO mice. To determine the anti-atherosclerotic effects of *Fads1* gene deficiency, *Fads1* homo KO (Homo), *Fads1* hetero KO (Hetero) and wild-type (WT) mice with an *ApoE* KO background were subjected to a Western diet for 14 weeks. Likewise Powell *et al* reported, Homo mice showed significant prevention of aortic atherosclerosis lesion formation and body weight gaining, and changes of fatty acid compositions in tissues with no significant changes of plasma total cholesterol (TC) and triglyceride (TG) levels (Supplemental figure S1-S2)..

Anti-atherosclerosis study using the D5D inhibitor, compound-326 (Studies 1 and 2). In order to elucidate whether a D5D inhibitor could cause anti-atherosclerotic effects as observed in *Fads1* mutant mice, we administered compound-326 to *ApoE* KO mice fed a Western diet. We conducted two studies using two independent cohorts of mice to confirm the reproducibility of our results and a minimum effective dose. In both studies (Studies 1 and 2), compound-326 (at 3–10 mg/kg/day, p.o.) showed

statistically significant and reproducible prevention of aortic atherosclerosis lesion formation (Figure 2).

Fatty acid levels were quantified in the liver and the blood in test mice. Compound-326 dose-dependently decreased AA and DHA levels and increased DGLA levels in the liver, leading to a decreased AA/DGLA ratio (Figure 3A). EPA levels in the liver were undetectable (the lower limit of the detection: 0.2 μmol/g tissue) in mice who received compound-326 (3-10 mg/kg/day). In the blood, dose-dependent decreases in AA, EPA, and DHA levels and increases in DGLA levels were observed throughout the study period (Figure 3B). In addition, similar fatty acid changes were also observed in Study 1 (Supplemental figure S3A-B).

In order to elucidate the anti-atherosclerotic mechanism, eicosanoid production was measured in the blood of vehicle- and compound-326-treated *ApoE* KO mice. Figure 4 shows the results of blood samples taken from Study 2. Compound-326 dose-dependently decreased the A23187 and fMLP-stimulated production of AA-derived eicosanoids (PGE2, TXB2, LTB4, and 6-keto PGF1α), while the DGLA-derived eicosanoid production (15-HETrE and 13, 14-dihydro, 15-keto PGE1) was dose-dependently increased by the treatment of compound-326. Other eicosanoids (11-dehydro TXB2, PGF2α, PGD2) were shown in Supplemental figure S4. The same results were observed in samples from Study 1 (Supplemental figure S5). Accompanied with anti-atherosclerotic effects, plasma levels of sICAM-1 modestly but statistically significantly decreased by compound-326 administration in some points during the study period (Supplemental figure S6).

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During the study period, body weight and the time course of plasma TC and TG levels were monitored. Plasma TC and TG levels were not altered by compound-326 administration (Figure 5A-B). As we previously reported (Yashiro *et al*., 2016), compound-326-treated mice were dose-dependently leaner than the vehicle-treated mice (Figure 6).

Discussion

In the present study, we provide proof-of-concept that D5D inhibition by a selective D5D inhibitor has promise as a translatable therapy for the treatment of atherosclerosis. Fan *et al* and Powell *et al* reported that *Fads1* KO mice showed a decrease in AA levels and an increase in DGLA levels in colon mucosa, liver, splenocytes, the brain and serum accompanied with a decrease in PGE2 levels and an increase in PGE1 levels in tissues, and a decrease of atherosclerotic lesion formation in the aorta. (Fan *et al*., 2012, Powell *et al*., 2016). Although these results clearly suggest the value of D5D as a promising target for atherosclerosis, it is important to obtain proof-of concept data with a specific D5D inhibitor, in order to show the clinical feasibility and translatability. We provide three critical lines of evidence utilizing a potent, orally available and selective D5D inhibitor, compound-326. First, 4 day oral treatment of compound-326 was enough to induce significant changes in AA and DGLA levels in normal mice in a dose-dependent manner. Second, the administration of compound-326 also showed significant alterations of this fatty acid composition in Western-diet fed *ApoE* KO mice accompanied with modulating profiles of eicosanoid production in blood cells. Finally, compound-326 significantly prevented aortic atherosclerosis lesion formation in the two independent cohorts of mice. These results provide proof-of-concept that D5D inhibition by a selective D5D inhibitor represents a promising potential therapy for atherosclerosis.

Several lines of previous work suggests that mechanism of the anti-atherosclerosis efficacy of D5D inhibition may be related to the change in a profile of eicosanoid production and inflammatory pathway

modulation through alterations of AA and DGLA levels. Several reports suggest the unique roles of AA-derived eicosanoids in the progression of atherosclerosis by utilizing low-dose aspirin administration, PGE2 synthase enzyme (*mPGES-1*) deletion, LTB4 receptor (*BLT-1*) deletion. thromboxane receptor (*TP*) deletion, PGI receptor (*IP*) deletion in mice (Cyrus *et al*., 2002, Wang *et al*., 2006, Subbarao *et al*., 2004, Kobayashi *et al*., 2004, Kobayashi *et al*., 2004). Based on the crucial role of inflammation in the pathogenesis of atherosclerosis, and the well-described inflammatory signaling pathways of these eicosanoids, the mechanism of anti-atherosclerosis effects observed in the present mouse studies is considered to be, at least in part, related to its inflammatory modulation. In the present study, we found compound-326 showed a weak but statistically significant decrease of a systemic inflammation marker sICAM-1 in plasma in some points during the study period, in agreement with the relationship between its anti-atherosclerosis effects.

As Powell *et al* reported, we confirmed that genetic D5D deficiency could prevent the progression of atherosclerotic lesions in Western-diet fed *Fads1* Homo KO x *ApoE* KO mice. As Hetero mice did not show a decrease in atherosclerotic lesion in the aorta, it is likely that 50% inhibition of the enzyme accompanied with a slight change of AA and DGLA levels (less than 10 % of decrease of AA/LA ratio and less than 40 % of increase of DGLA/LA ratio in blood cells) was not enough to show anti-atherosclerotic effects. The anti-atherosclerotic effects of compound-326 were observed with more than 30 % of decreases of AA levels and more than 120 % of increases of DGLA levels in blood cells,

suggesting there is a threshold of arachinodante for showing anti-atherosclerotic effects. D5D inhibition showed a significant decrease among AA-derived eicosanoids both pro-inflammatory eicosanoids and a metabolite of anti-inflammatory eicosanoid PGI2, 6-keto PGF1α, and ω-3 fatty acid (EPA and DHA) levels, which have been reported to show anti-atherosclerotic effects through anti-inflammatory mechanisms in mouse models (Matsumoto *et al*., 2008; Wan *et al*., 2010). The details of its mechanism should be examined, but we speculate that an increase in DGLA-derived anti-inflammatory eicosanoids (e.g. PGE1 and 15-HETrE) production and a decrease of several AA-derived inflammatory eicosanoids (e.g. PGE2, LTB4 and TXB2) simultaneously could overcome the negative effects of a decrease in PGI2 and ω-3 fatty acid levels (Wang *et al*., 2012; Vang *et al.*, 2005).

Compound-326 showed minimum effects on plasma TC and TG levels in *ApoE* KO mice fed a Western diet. It suggests that its anti-atherosclerotic effects are independent of cholesterol-lowering effects. Hypercholesterolemia is an important factor for the progression of atherosclerotic lesions, and drugs that decrease cholesterol levels reduce cardiovascular (CV) events in the clinical setting. The present results may suggest the usefulness of combination therapy with a D5D inhibitor and cholesterol-lowering drugs for the prevention of CV events.

We observed body weight loss in both *Fads1* x *ApoE* double KO mice and compound-326 treated *ApoE* KO mice fed a Western diet fed. Since a difference in body weight between Wild mice and Homo mice was not observed under normal chow fed conditions, it is likely that D5D inhibition leads to reduction of

fat mass gain. Powell *et al* reported reduction of fat mass in *Fads1* x *ApoE* double KO mice fed both a high fat diet and a Western diet with minimum effects on lean body mass (Powell *et al*., 2016). We also reported that compound-326 lowered insulin resistance and body weight gain accompanied with a decrease of macrophage infiltration into adipose tissue in a DIO mouse model (Yashiro *et al*., 2016). These results are consistent with the present results. Although one might be interested in the interaction between D5D inhibition-induced anti-obesity and anti-atherosclerotic effects, its detailed mechanism of action remains to be elucidated.

Our study has some limitations. First, characteristics of atherosclerotic lesions in *ApoE* KO mice we used can differ considerably from those of human unstable atherosclerotic plaques. Although inflammation plays an important role in pathogenesis of both settings, human unstable plaques are much more severe and can progress to plaque rupture, unlike those of mice. It is, therefore, important to clarify whether D5D inhibition is effective in preventing rupture in unstable atherosclerosis or an atherosclerosis pre-existing model. If so, D5D inhibition is expected to show benefits in the secondary prevention of myocardial infarction. Second, more research is needed to compare effects of D5D inhibition and other anti-atherosclerosis strategies, such as lipid-lowering drugs or anti-inflammatory drugs. Although several inflammatory enzymes (Lp-PLA2, sPLA2, COX-2, *etc*.), highly expressed in atherosclerotic lesions, produce pro-inflammatory and pro-apoptotic mediators, these enzyme inhibitors have not shown promising results in clinical studies (O'Donoghue *et al*., 2014; White *et al*., 2014; Nicholls *et al*., 2014;

Mukherjee *et al*., 2001). Further studies are needed for a better understanding of the detailed mechanism of action that can relate D5D-inhibition to its anti-atherosclerotic effects. We think it is interesting to conduct *in vivo* and *in vitro* studies such as analysis of inflammatory cell infiltrations and macrophage activity to test the hypothesis that D5D inhibition has anti-inflammatory effects. Finally, we need to clarify potential adverse effects by D5D inhibition especially in the central nervous systems. We identified that D5D inhibition caused a reduction in AA levels in the brain (Supplemental figure S7). AA is the substrate for the synthesis of endocannabinoids which mediate their endocannabinoid receptor, CB1, signaling. Signal alterations by CB1 antagonist treatments have been reported to lead to psychiatric adverse effects including depression (Leite *et al*., 2009). It would be critical to clarify if D5D inhibition can cause neuropsychiatric effects.

In summary, in the present study we identified a D5D specific and orally available potent inhibitor and provided the first evidence to support the concept that D5D inhibitors will be a novel remedy for preventing atherosclerosis and subsequent CV events through its novel mechanism.

Acknowledgments

We thank Makiko Itasaka and Masatoshi Yoshimura for excellent technical assistance. We also thank Hidenori Kamiguchi for helpful discussions.

Authorship contributions

Participated in research design: Takagahara, Itokawa, Kubo, Ikeda

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Performed data analysis: Takagahara, Ikeda

Wrote or contributed to the writing of the manuscript: Takagahara, Ikeda

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Footnotes

This work was not supported by any grants.

Figure legends

Figure 1. ω-6 fatty acid levels from the liver of C57BL/6 mice fed a normal diet.

(A) Daily administration for four consecutive days, (B) Single administration. Data are presented mean ± standard deviation (S.D., n=5). #: P < 0.025, ##: P < 0.005 vs. Vehicle by *Williams*-test, ††: P < 0.01 vs. Vehicle by *Aspin-Welch* test. Abbreviations: LA, linoleic acid; AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 2. Compound-326 administration inhibited the progression of atherosclerotic lesion in Western-diet fed ApoE KO mice (Studies 1 and 2).

After 15 weeks of compound-326 administration, aorta was harvested. The percentage of stained aortic area was quantified. Control mice were fed normal chow. The upper left panel shows the result of Study 1. The number of mice in each group was as follows: Control (n=6), vehicle (n=11), 0.3 mg/kg (n=12), 3 mg/kg (n=11), 10 mg/kg (n=11). The upper right panel shows the result of Study 2. The number of mice in each group was as follows: Control (n=6), Vehicle (n=12), 0.3 mg/kg (n=12), 1 mg/kg (n=11), 3 mg/kg (n=12), 10 mg/kg (n=12). Data are presented mean ± standard deviation (S.D.). Representative images of each group were presented. The values of the percentage of the lesion were described below the images. ††: P < 0.01 vs. Control by *Aspin-Welch* test, ‡ ‡: P < 0.01 vs. Control by *Student's t*-test, #: P < 0.025, ##: P < 0.005 vs. Vehicle by *Williams*-test.

Figure 3. ω-6 and ω-3 fatty acid levels from ApoE KO mouse liver and the blood administered with compound-326 (Study 2).

Fatty acid analysis of (A) mouse liver and (B) mouse blood. After 15 weeks of compound-326 administration, livers were harvested. The number of mice in each group is described in the legend of Figure 2. The EPA levels in the blood from the control were undetectable. Data are presented mean ± standard deviation (S.D.). **: $p<0.01$ vs. Vehicle by *Student's t*-test, ##: $P < 0.005$ vs. Vehicle by *Williams*-test, †: $p<0.05$, ††: $p<0.01$ vs. Vehicle by *Aspin-Welch*-test, ‡: $P < 0.025$, ‡‡: $P < 0.005$ vs. Vehicle by *Shirley-Williams* test. Abbreviations: AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 4. Eicosanoid production from ApoE KO mouse blood stimulated with A23187 and fMLP (Study 2). ApoE KO mouse blood was stimulated with A23187 (30 μM) and fMLP (10 μM). Both reagents were added to blood samples at the same time. The number of mice in each group is described in the legend of Figure 2. Some samples (control, n=6; vehicle, n=6; 0.3mg/kg, n=5) were removed from the data because the levels of 13, 14-dh, and 15-keto PGE1 were not detectable. Data are presented as mean ± standard deviation (S.D.). ‡ ‡: $P < 0.01$ vs. Control by *Student's t*-test, #: $P < 0.025$, ##: $P < 0.005$ vs. Vehicle by *Shirley-Williams* test, †: $P < 0.025$, ††: $P < 0.005$ vs. Vehicle by *Williams* test. Abbreviations:

PG, prostaglandin; TXB2, thromboxane B2; LTB4, leukotriene B4; 15-HETrE, 15-hydroxyeicosatrienoic acid.

Figure 5. Effects of compound-326 on plasma total cholesterol and triglyceride levels (Studies 1 and 2). Time course of plasma total cholesterol and triglyceride levels. Plasma was obtained at 0, 3, 7, 11 and 15 weeks of the study period; Study 1 (A) and Study 2 (B). The number of mice in each group is described in the legend of Figure 2. Data are presented as mean ± standard deviation (S.D.). There is no statistical significance between vehicle and compound-326 treated group.

Figure 6. Effects of compound-326 on body weight (Studies 1 and 2). The left panel and the right panel show the result of Studies 1 and 2, respectively. The number of mice in each group is described in the legend of Figure 1. Data are presented as mean ± standard error of the mean (S.E.M.). *: $p<0.05$, **: $p<0.01$ vs. Control by *Aspin-Welch*-test, #: $p<0.025$, ##: $p<0.005$ vs. Vehicle by *Shirley-Williams* test, †: $p<0.025$, ††: $p<0.005$ vs. Vehicle by *Williams* test.

Figures

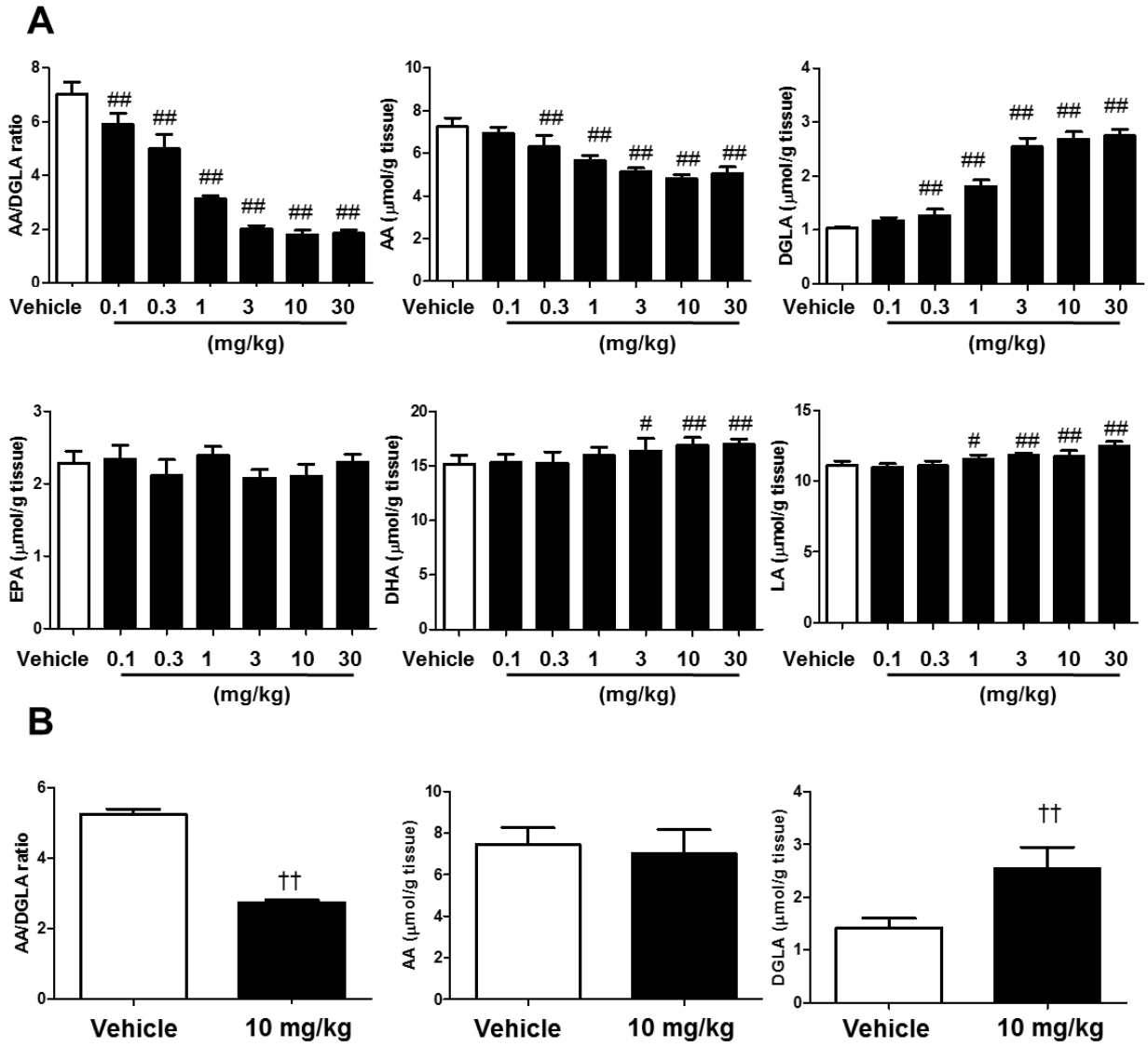

Figure 1.

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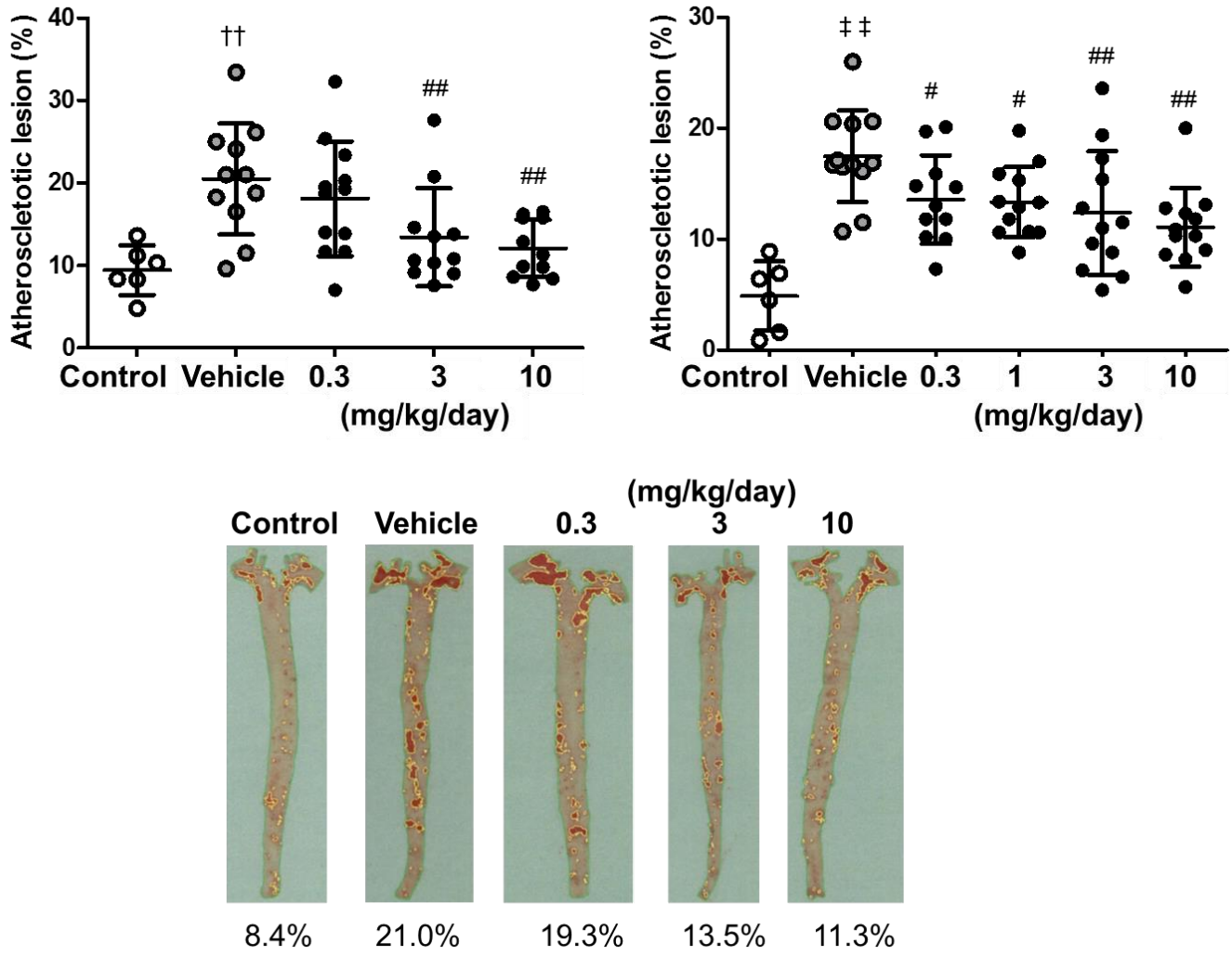

Figure 2.

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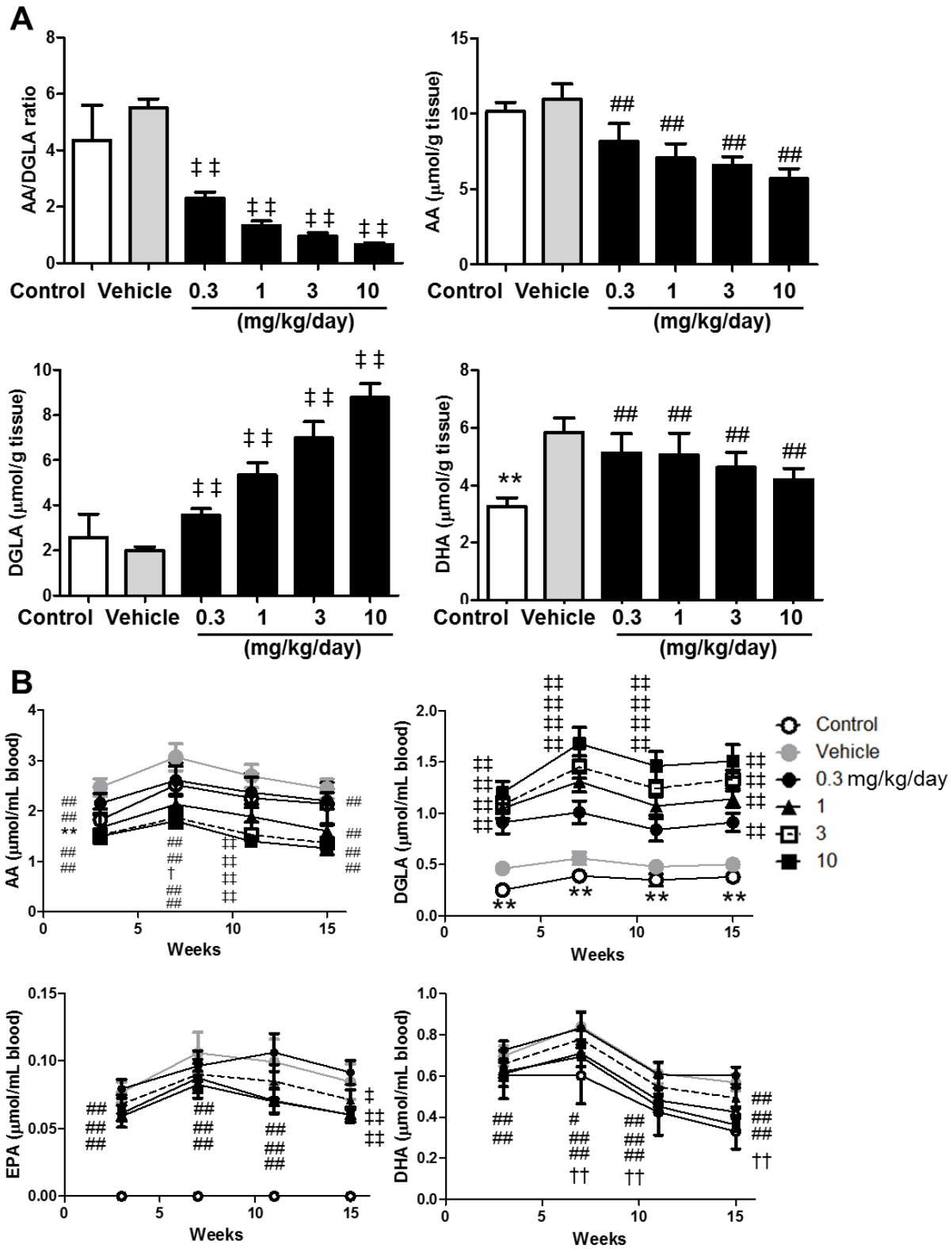

Figure 3.

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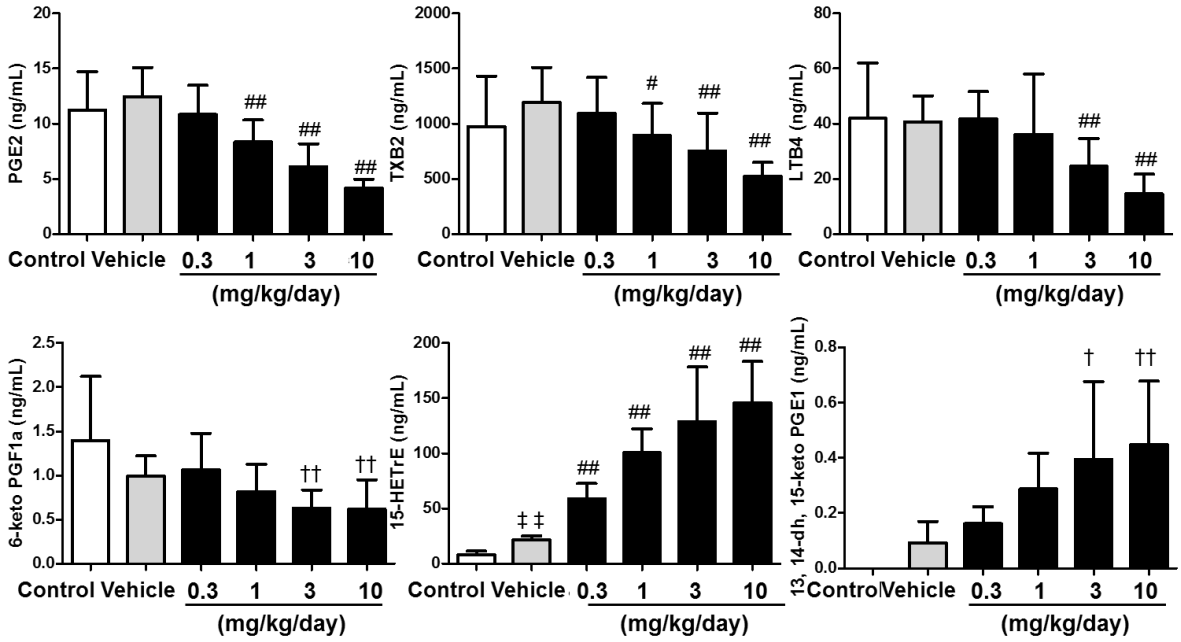

Figure 4.

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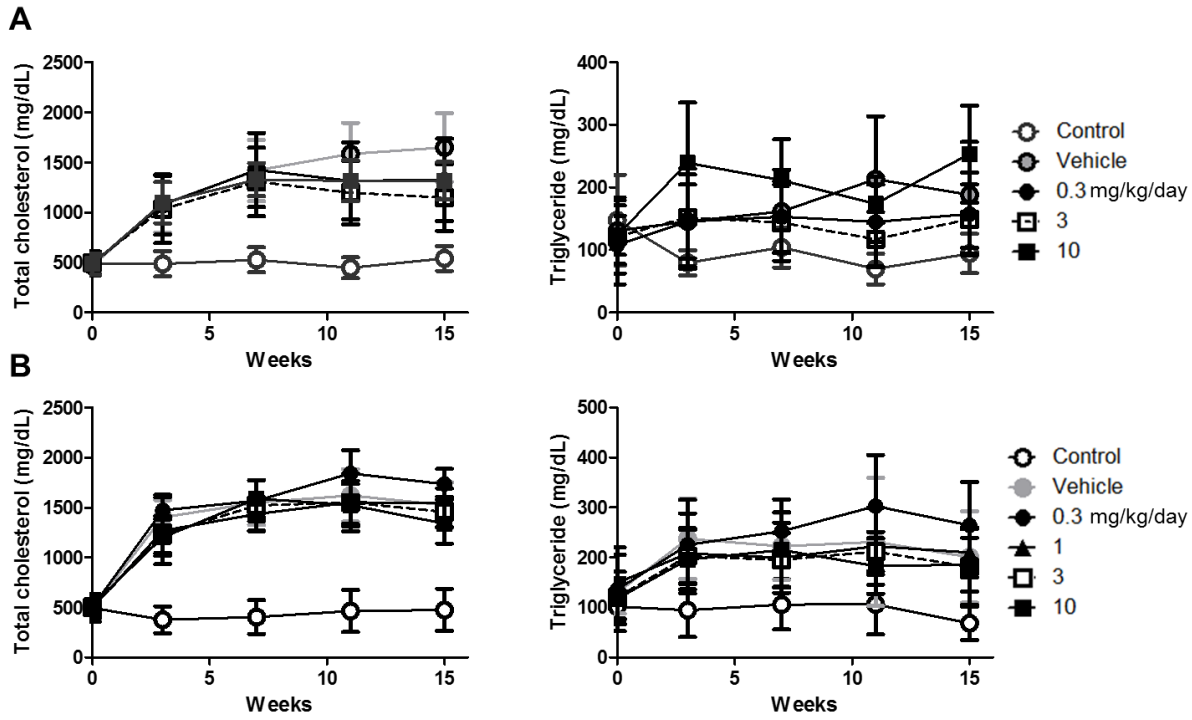

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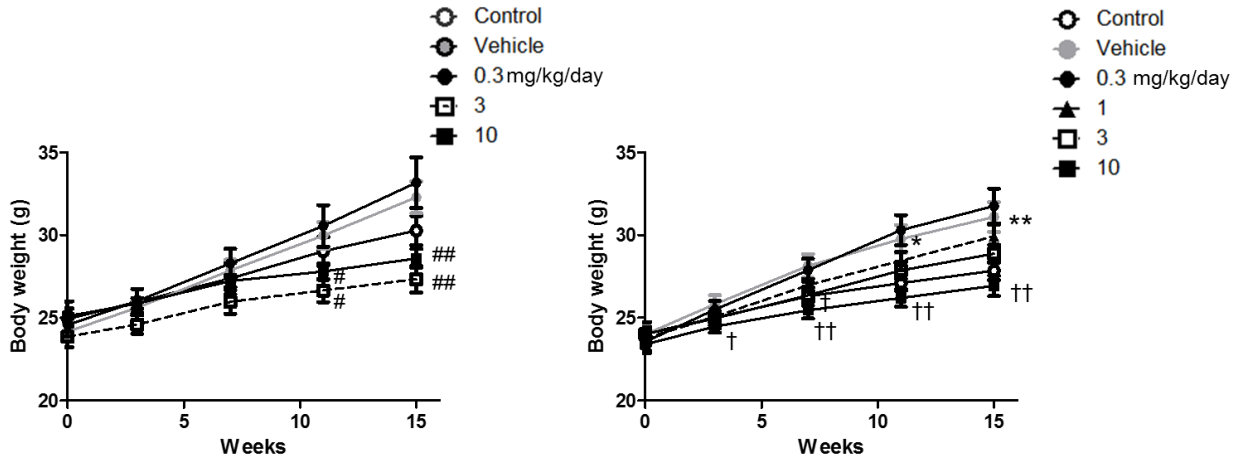

Figure 6.