

## Effects of Compound-326, a Selective Delta-5 Desaturase Inhibitor, in ApoE Knockout Mice with Two Different Protocols for Atherosclerosis Development

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**ABSTRACT -- Purpose:** We previously confirmed its anti-atherosclerotic effects by pre-treatment with compound-326, a selective delta-5 desaturase (D5D) inhibitor, in Western diet-fed ApoE knockout mice. In the present study, we evaluated effects of compound-326 in ApoE knockout mice with two different protocols for atherosclerosis development. **Methods:** In a post-treatment protocol, where the compound treatment started after 10 weeks pre-feeding of Western diet, compound-326 (1 and 3 mg/kg/day, *p.o.* for 12 weeks) significantly reduced the atherosclerotic lesion area in the aorta (24% reduction at 3 mg/kg/day). In another protocol using Paigen diet (containing 12.5% cholesterol and 5% sodium cholate), compound-326 (3 and 10 mg/kg/day, *p.o.* for 7 weeks) also significantly reduced the lesion area (36% reduction at 3 mg/kg/day). **Results:** In both protocols, Compound-326 significantly reduced the hepatic ratio of arachidonic acid to dihomo- $\gamma$ -linolenic acid, blood inflammatory eicosanoid production and plasma soluble intercellular adhesion molecule 1 (sICAM-1) levels, similarly to the previous pre-treatment study. **Conclusions:** Compound-326 exerted anti-atherosclerotic effects in ApoE knockout mice with the two different protocols for atherosclerosis development further supporting D5D inhibition as a promising strategy in treating atherosclerosis.

### INTRODUCTION

New drugs that can treat atherosclerosis and prevent subsequent cardiovascular events are urgently needed in clinical practice (1,2). One of the important characteristics of atherosclerosis is chronic inflammation in the vascular wall (3). Recently, CANTOS trial revealed that canakinumab, an interleukin 1 $\beta$  antibody, showed significantly lower rate of recurrent cardiovascular events independent of plasma lipid lowering (4). Anti-inflammatory strategy for blood vessels is thus considered to be an effective measure for atherosclerotic plaque stabilization and rupture prevention (5,6).

Delta-5 desaturase (D5D) is considered an attractive target molecule for treating atherosclerosis (7,8). D5D is an enzyme mainly expressed in the liver (9,10), which metabolizes dihomo- $\gamma$ -linolenic acid (DGLA) into arachidonic acid (11). Arachidonic acid is considered to play a role in the progression of atherosclerosis because of its pro-inflammatory properties (12). Several reports have suggested that D5D gene polymorphism in humans is associated

with coronary artery diseases (13,14). D5D-deficient apolipoprotein E (ApoE) knockout mice were resistant to diet-induced development of atherosclerosis (7,8). We recently discovered that an orally active and selective D5D inhibitor, compound-326, that exhibited *in vitro* D5D inhibition (IC<sub>50</sub>: 22 nM) in human cells (15), reduced liver and blood arachidonic acid levels in mice, accompanied by an increase in DGLA levels (8). In ApoE knockout mice fed a Western diet for 14 weeks, the atherosclerotic lesion area was significantly reduced by a 15-week treatment with compound-326 (1-week pre-treatment plus 14-week concurrent treatment with Western diet). These results suggest that D5D inhibition could be a promising strategy for the treatment of atherosclerosis.

Diet composition and duration are known to affect the characteristics of atherosclerotic plaques (16). ApoE knockout mice fed a Western diet for a long duration showed an atherogenesis process strikingly similar to that seen in humans (17); Paigen diet (containing high cholesterol and cholate) induced severe dysfunction in the reverse cholesterol transport system (18), resulting in high plasma

cholesterol levels and accelerated plaque development in ApoE knockout mice. These different diets are known to induce different pathologies and states of inflammation (19,20). Since the inflammatory properties of atherosclerosis are known to vary in clinical practice according to stages of the pathology and to complications in patients (21-24), it is important to evaluate effects of D5D inhibition in several animal models of atherosclerosis with different states of inflammation and different pathophysiology.

In the present study, we investigated the anti-atherosclerotic and anti-inflammatory effects of the D5D inhibitor compound-326 in ApoE knockout mice with two different protocols for atherosclerosis development.

## MATERIALS AND METHODS

### Animals

Male ApoE knockout mice (6 weeks old) were supplied by Takeda Rabics, Ltd (Fukuchiyama, Japan). All animals were maintained on a laboratory chow diet (CE-2, Clea Japan, Suita, Osaka) before experiments. Mice were allowed free access to water and food before and during experiments. The care and use of the animals and the experimental protocols used in this research were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Co., Ltd., and were in accordance with the Guide to the Care and Use of Experimental Animal Care.

### Chemicals

A D5D selective inhibitor, 2-(2,2,3,3,3-pentafluoropropoxy)-3-[4-(2,2,2-trifluoroethoxy)phenyl]-5,7-dihydro-3H-pyrrolo[2,3-d]pyrimidine-4,6-dione, compound-326 (15), was synthesized. Compound-326 was suspended in 0.5% (w/v) methylcellulose (Wako, Osaka, Japan).

### Experimental design for atherosclerosis intervention studies: post-treatment protocol

From 11 weeks of age, mice were fed a Western diet (D12079B, Research Diets, Inc., New Brunswick, NJ, USA) for 10 weeks to develop atherosclerotic lesions. Mice were then divided into four groups (n = 10–14) based on body weight and plasma biochemical parameters (alanine aminotransferase [ALT], total cholesterol [TC], triglycerides [TG], and glucose [GLU]), and 10 mice were dissected to obtain data for this group (Pre group). Compound-326 was administered to the rest of the mice at gravimetric doses of either 1 or 3 mg/kg of body

weight per day for 12 weeks. The vehicle group received 0.5% (w/v) methylcellulose without compound-326. Food intake was measured every two weeks. Blood samples for interim measurements of plasma TC and the cell adhesion molecule sICAM-1 were obtained every four weeks from the orbital veins. After 12 weeks of treatment, mice were anesthetized by pentobarbital sodium (50–100 mg/kg, *i.p.*), and blood was collected from the abdominal aorta for eicosanoid analysis and from the caudal vena cava for other analyses. Whole aortas were dissected for quantification of atherosclerotic lesions and livers were dissected for fatty composition analysis and TG content measurement.

### Experimental design for atherosclerosis intervention studies: Paigen diet protocol

From 13 weeks of age, mice were fed a control diet (D12337, Research Diets, Inc.) for one week and were divided into five groups (n = 6–12) based on body weight and plasma biochemical parameters (ALT, TC, TG, GLU). Compound-326 was administered at gravimetric doses of either 0.3, 3, or 10 mg/kg of body weight per day for 7 weeks. Control and vehicle groups received 0.5% (w/v) methylcellulose without compound-326. In the latter 4 weeks, the mice were fed a Paigen diet (D12336, Research Diets, Inc., containing 12.5% cholesterol and 5% sodium cholate) except for the control group, which received the control diet throughout the study period. Food intake was measured on week 1, 4, and 6 from the onset of the experiment three days. Blood samples for interim measurements of plasma TC and sICAM-1 were obtained from the orbital veins. After 7 weeks of treatment, mice were anesthetized by pentobarbital sodium (50–100 mg/kg, *i.p.*), and blood was collected from the abdominal aorta for eicosanoid analysis and from the caudal vena cava for other analyses. Whole aortas were dissected for quantification of atherosclerotic lesions and the livers were dissected for fatty composition analysis and TG content measurement.

### Assessment of atherosclerotic lesions

Aortas were carefully cleared of all connective tissues under a dissecting microscope and were cut open, washed with saline, and then fixed in 10% neutral buffered formalin for over 7 days. Aortas were stained with Oil Red O to detect the lipid-accumulated area. The stained areas were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) and expressed as a percentage of the surface area of the entire aorta.

### Hepatic fatty acid composition analysis

Liver samples were homogenized in 4-fold liver wet weight of saline. The homogenized liver samples were added to a 4.5-fold amount of hexane-isopropanol-butylated hydroxytoluene (60:40:0.01, v/v/w) containing 800  $\mu$ M margaric acid as an internal standard, and were vortexed for 30 min. The hexane phase was removed to a plastic tube and evaporated to dryness under a nitrogen stream and vacuum. The phospholipid fraction was isolated from the residue using Sep-Pak PSA columns (Waters, Milford, MA, USA), based on previously described procedures (25), and was derivatized using a YMC long- and short-chain fatty acid labeling kit (YMC, Kyoto, Japan). Samples were redissolved in methanol and were analyzed by reverse-phase HPLC using an LC column (6.0 $\phi$   $\times$  250 mm, Shim-pack XR-ODS, Shimadzu, Kyoto, Japan) and by UV absorbance detection at 230 nm. HPLC solvent conditions were as follows: A mobile phase of acetonitrile-water-trifluoro acetic acid (1,660:340:0.004, v/v/w), a flow rate of 1.4 mL/min, and a temperature of 40°C.

### Measurement of hepatic TG contents

Homogenized liver samples were added to an equal amount of 0.4% CaCl<sub>2</sub> (w/v) solution and a 4.5-fold amount of hexane-isopropanol-butylated hydroxytoluene (60:40:0.01, v/v/w), and were vortexed for 30 min. The hexane phase was removed to a plastic tube and evaporated to dryness under a nitrogen stream and vacuum. The residues were suspended in nonaethylene glycol monododecyl ether-1,4-dioxane (1:1, v/v). TG contents were determined using a Triglyceride E-test (Wako).

### Measurements of plasma ALT, TC, sICAM-1, and MCP-1

Blood samples were centrifuged (20,000  $\times$  g) at 4°C for five min to isolate plasma and were 6-fold, 200-fold, and 2-fold diluted with saline for quantification of TC, sICAM-1, and monocyte chemoattractant protein 1 (MCP-1), respectively. ALT and TC levels were determined using a 9000 Series Automatic Analyzer (Hitachi, Tokyo, Japan). sICAM and MCP-1 were assessed by a Mouse Soluble ICAM-1 (CD54) ELISA kit (EMICAM1, Thermo Fisher Scientific, Yokohama, Japan) and a Quantikine Mouse CCL2/JE/MCP-1 kit (MJE00, R&D Systems, Inc., Minneapolis, MN, USA), respectively.

### Blood eicosanoid production assay

Blood eicosanoid production assay was performed as

described previously (8). Blood samples were stimulated with 30  $\mu$ M A23187 and 10  $\mu$ M fMLP (N-Formylmethionyl-leucyl-phenylalanine) and were incubated at 37°C for 10 min. Samples were centrifuged (20,000 $\times$ g) at room temperature for 5 min to isolate plasma. Blood eicosanoid composition was analyzed by LC/MS/MS.

### STATISTICAL ANALYSIS

Results are expressed as the mean  $\pm$  SEM. Statistical significance between two groups was determined by Student's *t*-test (for homogenous data) or Welch's test (for non-homogenous data). Statistical significance between groups of multiple doses of test compounds was determined by a two-tailed Williams' test (for homogenous data) or a two-tailed Shirley-Williams test (for non-homogenous data). Differences were considered significant at  $p < 0.05$ .

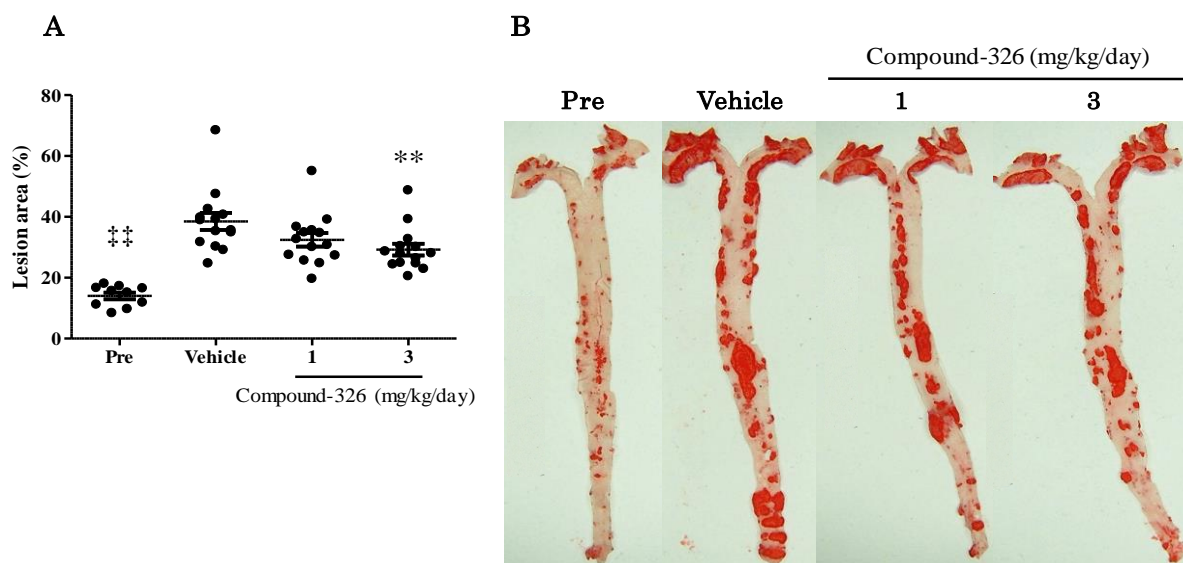
### RESULTS

#### Post-treatment protocol

##### *Anti-atherosclerotic effects of compound-326*

To evaluate the effects of D5D inhibition on atherogenic progression in a post-treatment protocol, atherosclerotic lesions were developed in advance by a 10-week administration of a Western diet to ApoE knockout mice (Pre group). The group developed a 14% surface lesion area for the entire aorta, indicating sufficient formation of the atherosclerotic lesion area before the administration of compound-326. The vehicle group developed a 38% atherosclerotic lesion area over additional 12 weeks of the Western diet, indicating continuous atherogenic progression during the drug treatment phase. A 12-week post-treatment with compound-326 (1–3 mg/kg/day, *p.o.*) reduced the atherosclerotic lesion area of the aorta in a dose-dependent manner (16 and 24% reduction at 1 and 3 mg/kg/day, Figure 1), statistically significant at 3 mg/kg/day. The lesion area reduction was 38% at 3 mg/kg when the Pre group was set as the basal line, so the efficacy of compound-326 was comparable to that seen previously in the previous pre-treatment study (8).

In addition, Smirnov-Grubbs' outlier test revealed that the ones with the largest lesion areas in vehicle, 1 mg/kg and 3 mg/kg groups were outliers ( $p < 0.05$ ). When excluded these three outliers, two-tailed Williams' test indicated the statistically significant reduction of the lesion area in the 1 and 3



**Figure 1.** Effects of compound-326 on atherosclerotic lesion area in ApoE knockout mice with the post-treatment protocol. Percentage of surface lesion area over the entire aorta were assessed by Oil Red O staining. From 11 weeks of age, mice were fed a Western diet for 10 weeks to develop atherosclerotic lesions. Ten mice were dissected at this point to obtain data for Pre group. Compound-326 was administered to the rest of the mice at gravimetric doses of either 1 or 3 mg/kg of body weight per day. Vehicle group received 0.5% (w/v) MC without compound-326. After 12 weeks of administration, whole aortas were dissected for quantification of atherosclerotic lesions. (A) Percentage of surface lesion area for the entire aorta. (B) Representative images of oil red-O staining of en-face preparations of aortas in each group. Results are expressed as mean  $\pm$  SEM (Pre: n = 10, others: n = 14).  $^{\ddagger\ddagger}p < 0.01$  vs. vehicle (Welch's test).  $^{**}p < 0.01$  vs. vehicle (Williams' test).

mg/kg groups ( $p < 0.01$ ). This analysis suggest that the outliers of the data may affect the statistics.

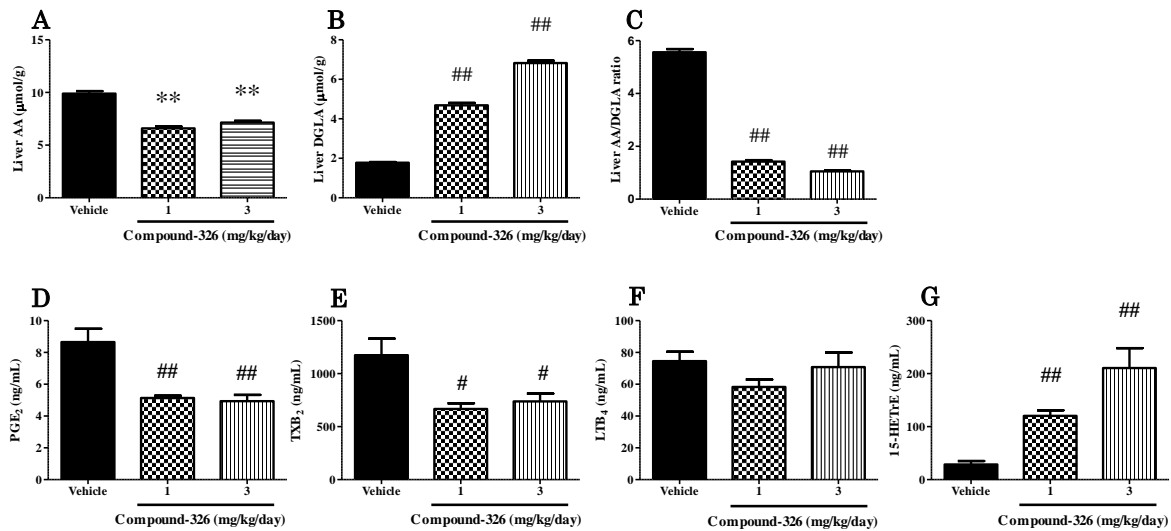
#### ***Effects of compound-326 on fatty acid composition and blood eicosanoid production***

To confirm the mechanism of action of compound-326, hepatic fatty acid composition and blood eicosanoid production (stimulated by calcium ionophore A23187 and fMLP) were measured at the end of the treatment period. Compound-326 significantly reduced the reaction product, arachidonic acid contents (33% and 28% reduction at 1 and 3 mg/kg/day, respectively; Figure 2A), and increased the substrate, DGLA contents (163% and 284% increase at 1 and 3 mg/kg/day, respectively; Figure 2B) in the liver in a dose-dependent manner. Compound-326 therefore significantly reduced the arachidonic acid/DGLA ratio in the liver (74% and 81% reduction at 1 and 3 mg/kg/day, respectively; Figure 2C). Similar changes in fatty acid composition were also observed in the blood (data not shown). Corresponding to the composition change in hepatic fatty acids, blood eicosanoid

production, a biomarker for D5D inhibitors, was also altered. Arachidonic acid-derived eicosanoids PGE<sub>2</sub> (41% and 43% reduction at 1 and 3 mg/kg/day, respectively; Figure 2D) and TXB<sub>2</sub> (43% and 37% reduction at 1 and 3 mg/kg/day, respectively; Figure 2E) were significantly reduced. Production of LTB<sub>4</sub>, an arachidonic acid-derived eicosanoid, in blood was not altered (Figure 2F). A DGLA-derived eicosanoid, 15-HETrE, increased significantly following compound-326 administration (321% and 636% increase at 1 and 3 mg/kg/day, respectively; Figure 2G).

#### ***Effects of compound-326 on inflammatory markers***

Compound-326 suppressed plasma sICAM-1 levels during treatment (Figure 3A), resulting in a significant reduction in area under the curve (AUC) (8.4% reduction at 3 mg/kg/day; Figure 3B). In addition, compound-326 did not alter plasma cholesterol levels (Table 1), suggesting that the anti-atherosclerotic effects were independent of plasma cholesterol levels and were due to anti-inflammatory effects.

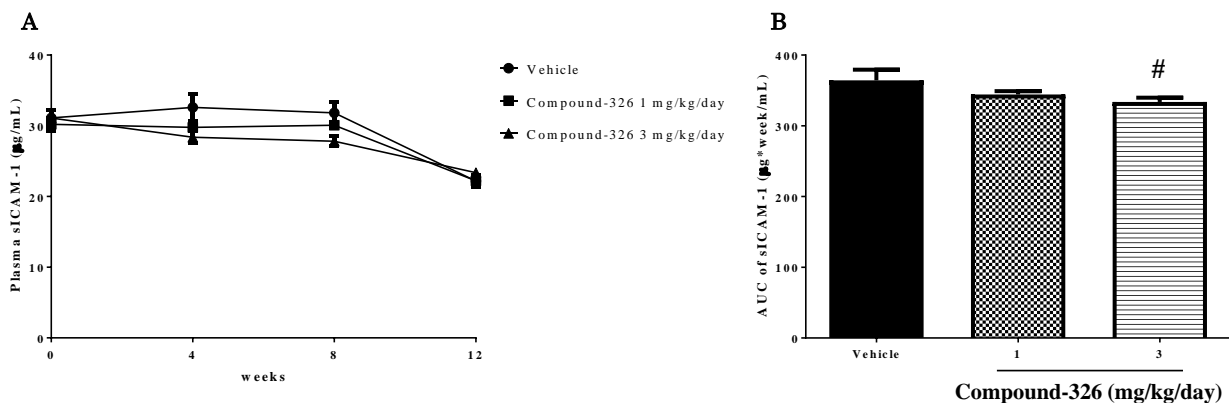


**Figure 2.** Effects of compound-326 on hepatic fatty acid composition (A-C) and blood eicosanoid production (D-G) in ApoE knockout mice with the post-treatment protocol. Livers and bloods were obtained from animals shown in Figure 1 at the end of the study. (A) Arachidonic acid (AA) contents. (B) Dihomo- $\gamma$ -linolenic acid (DGLA) contents. (C) AA/DGLA ratio. Results are expressed as mean  $\pm$  SEM (n = 14). (D) PGE<sub>2</sub>, an arachidonic acid (AA) derivative. (E) TXB<sub>2</sub>, an AA derivative. (F) LTB<sub>4</sub>, an AA derivative. (G) 15-HETE, a dihomogamma-linolenic acid (DGLA) derivative. Results are expressed as mean  $\pm$  SEM (n = 7). \*\**p* < 0.01 vs. vehicle (Williams' test). #*p* < 0.05, ##*p* < 0.01 vs. vehicle (Shirley-Williams test).

**Table 1.** Effects of compound-326 on plasma total cholesterol (mg/dL) in the post-treatment protocol.

Group	Weeks after commencing of compound-326 treatment			
	0	4	8	12
Vehicle	1,459 $\pm$ 65	1,451 $\pm$ 70	1,349 $\pm$ 98	1,313 $\pm$ 67
1 mg/kg/day	1,494 $\pm$ 55	1,456 $\pm$ 59	1,490 $\pm$ 51	1,288 $\pm$ 56
3 mg/kg/day	1,500 $\pm$ 53	1,406 $\pm$ 54	1,392 $\pm$ 52	1,298 $\pm$ 38

Results are expressed as mean  $\pm$  SEM (n = 14).



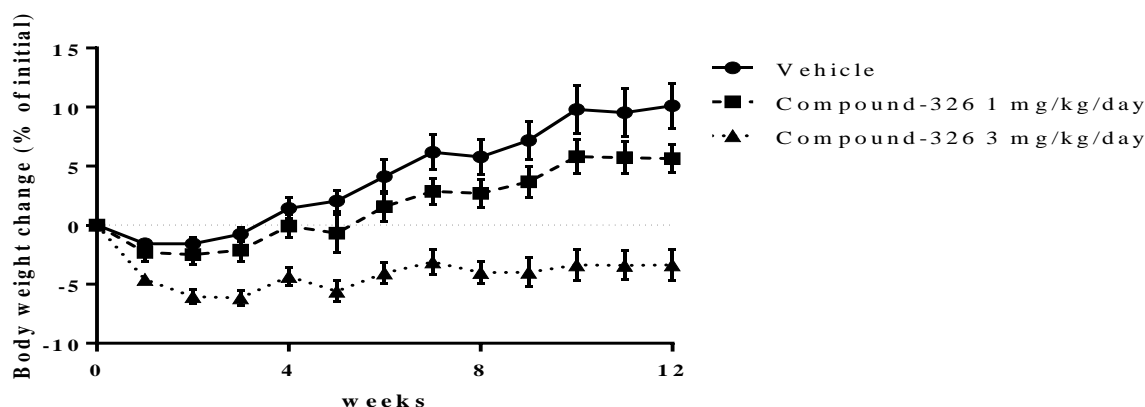
**Figure 3.** Effects of compound-326 on plasma sICAM-1 levels in ApoE knockout mice with the post-treatment protocol. Plasma samples were collected every four weeks from the onset of administration from animals shown in Figure 1. (A) Time-dependent alteration of plasma sICAM-1 levels. (B) Area under the curve (AUC) of plasma sICAM-1 concentrations. Plasma sICAM-1 levels were measured at baseline and on weeks 4, 8, and 12. Results are expressed as mean  $\pm$  SEM (n = 14). #*p* < 0.05 vs. vehicle (Shirley-Williams test).

### Other findings related to compound-326 administration

Compound-326 suppressed Western diet-induced body weight gain (Figure 4). Food intake was not altered (Vehicle:  $2.81 \pm 0.14$  g/day, 1 mg/kg/day:  $2.92 \pm 0.18$  g/day, 3 mg/kg/day:  $2.82 \pm 0.10$  g/day, mean  $\pm$  SEM,  $n = 14$ ), indicating that the suppression of body weight gain by compound-326 was

independent of food intake.

Liver weight (Vehicle:  $1.88 \pm 0.14$  g, 1 mg/kg/day:  $1.87 \pm 0.07$  g, 3 mg/kg/day:  $1.67 \pm 0.06$  g/day) and hepatic TG (Vehicle:  $137 \pm 24$   $\mu$ g/mg liver, 1 mg/kg/day:  $136 \pm 12$   $\mu$ g/mg liver, 3 mg/kg/day:  $89 \pm 8.3$   $\mu$ g/mg liver) were not altered by compound-326 (mean  $\pm$  SEM,  $n=14$ ). Plasma ALT were not altered (Vehicle:  $80.3 \pm 30.5$  U/L, 1 mg/kg/day:  $51.0 \pm 5.36$  U/L, 3 mg/kg/day:  $31.3 \pm 3.22$  U/L, mean  $\pm$  SEM,  $n=14$ ) by compound-326.



**Figure 4.** Effects of compound-326 on body weight changes in ApoE knockout mice with the post-treatment protocol. Results are expressed as mean  $\pm$  SEM ( $n = 14$ ).

### Paigen diet protocol

#### Anti-atherosclerotic effects of compound-326

To evaluate the effects of D5D inhibition on atherogenic progression induced by Paigen diet (containing 12.5% cholesterol and 5% sodium cholate), the atherosclerotic lesion area in the aorta was determined after 7 weeks of compound-326 administration (0.3–10 mg/kg/day, *p.o.*, with a 3-week pre-treatment before commencing the Paigen diet). Compound-326 significantly reduced the atherosclerotic lesion area of the aorta in a dose-dependent manner (36% and 38% reduction at 3 and 10 mg/kg/day, respectively; Figure 5).

#### Effects of compound-326 on fatty acid composition and blood eicosanoid production

Hepatic fatty acid composition and blood eicosanoid production (stimulated by calcium ionophore A23187 and fMLP) were measured at the end of the treatment period. Compound-326 significantly reduced the product arachidonic acid contents (36% and 56% reduction at 3 and 10 mg/kg/day, respectively; Figure 6A) and increased the substrate DGLA contents (56%, 347%, and 676% increase at

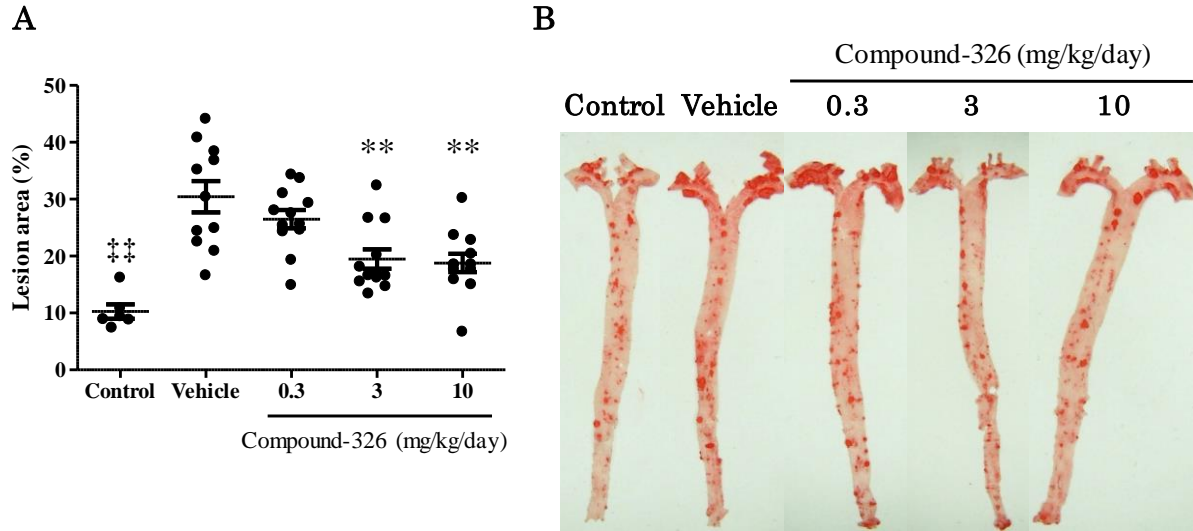
0.3, 3, and 10 mg/kg/day, respectively; Figure 6B) in the liver in a dose-dependent manner. Compound-326 therefore significantly reduced the arachidonic acid/DGLA ratio in the liver (39%, 84%, and 94% reduction at 0.3, 3, and 10 mg/kg/day, respectively; Figure 6C), in a dose-dependent manner. Similar changes in fatty acid composition were also observed in the blood (data not shown). Corresponding to the composition change in hepatic fatty acids, blood eicosanoid production was also altered. Arachidonic acid-derived eicosanoids PGE<sub>2</sub> (56% and 87% reduction at 3 and 10 mg/kg/day, respectively; Figure 6D), TXB<sub>2</sub> (46% and 82% reduction at 3 and 10 mg/kg/day, respectively; Figure 6E), and LTB<sub>4</sub> (53% and 83% reduction at 3 and 10 mg/kg/day, respectively; Figure 6F) were significantly reduced. The DGLA-derived eicosanoid 15-HETrE increased significantly (104%, 626%, and 754% increase at 0.3, 3, and 10 mg/kg/day, respectively; Figure 6G) following compound-326 administration.

#### Effects of compound-326 on inflammatory markers

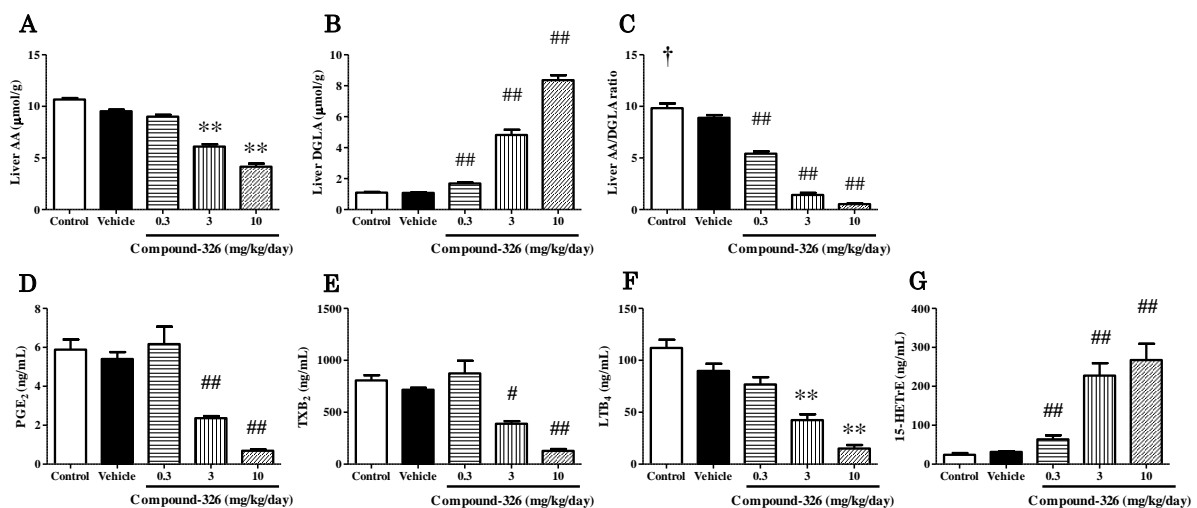
Compound-326 suppressed plasma sICAM-1 levels

during treatment (Figure 7A), resulting in a significant reduction in area under the curve (AUC) (11% reduction at 10 mg/kg/day; Figure 7B). Compound-326 also significantly reduced plasma MCP-1 levels (30% reduction at 10 mg/kg/day;

Figure 7C) in a dose-dependent manner. In addition, compound-326 did not alter plasma TC levels (Table 2), suggesting that the anti-atherosclerotic effects were independent of plasma cholesterol levels and were due to anti-inflammatory effects.

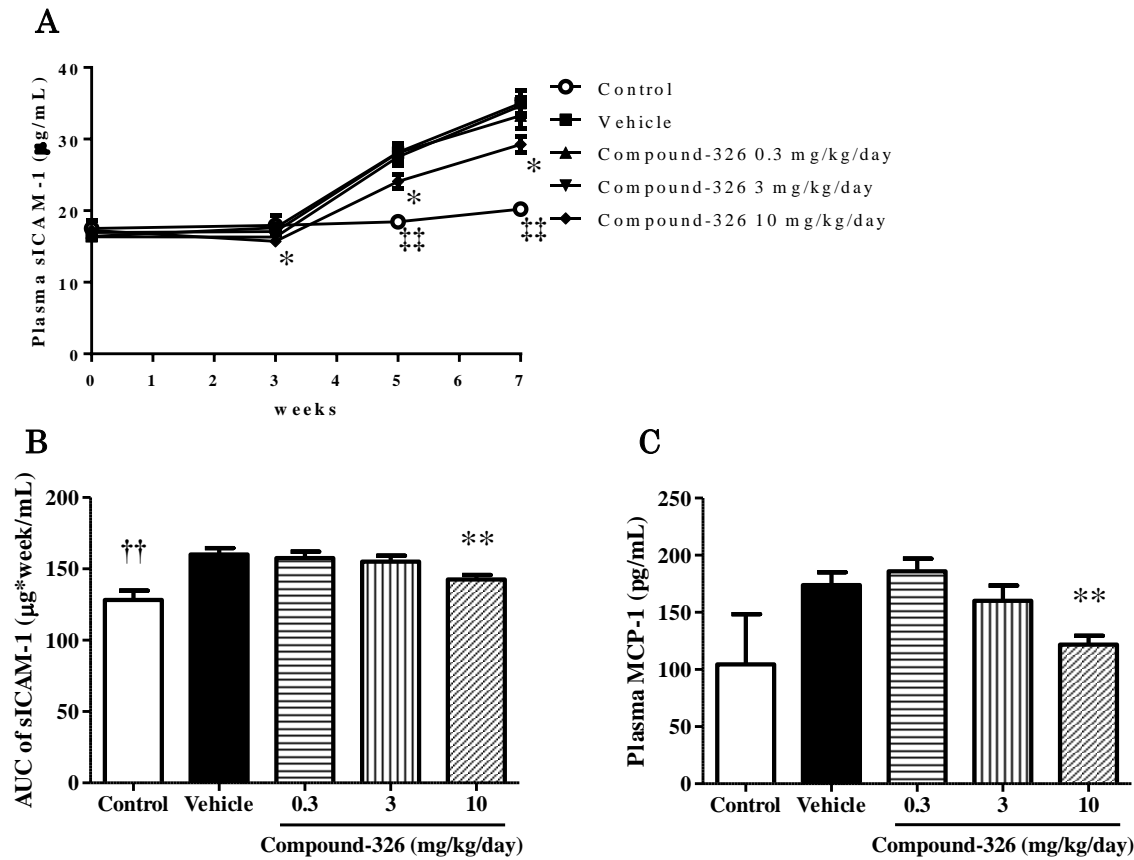


**Figure 5.** Effects of compound-326 on atherosclerotic lesion area in ApoE knockout mice with the Paigen diet protocol. From 13 weeks of age, mice were fed a control diet for one week and were divided into five groups (control: n = 6, others: n = 12). Compound-326 was administered at gravimetric doses of either 0.3, 3, or 10 mg/kg of body weight per day for 7 weeks. Control and vehicle groups received 0.5% (w/v) methylcellulose without compound-326. In the latter 4 weeks, the mice were fed a Paigen diet except for the control group, which received the control diet throughout the study period. After 7 weeks of administration, whole aortas were dissected for quantification of atherosclerotic lesions. (A) Percentage of surface lesion area for the entire aorta. (B) Representative images of oil red-O staining of en-face preparations of aortas in each group. Results are expressed as mean ± SEM (control: n = 6, others: n = 12). ††*p* < 0.01 vs. vehicle (Welch's test). \*\**p* < 0.01 vs. vehicle (Williams' test).



**Figure 6.** Effects of compound-326 on hepatic fatty acid composition (A-C) and blood eicosanoid production (D-G) in ApoE knockout mice with the Paigen diet protocol. Livers and bloods were obtained from animals shown in Figure 5 at the end of the study. (A) arachidonic acid (AA) contents. (B) dihomo-γ-linolenic acid (DGLA) contents. (C) AA/DGLA ratio. Results are expressed as mean ± SEM (control: n = 6, others: n = 12). (D) Prostaglandin E2 (PGE<sub>2</sub>), an arachidonic

acid (AA) derivative. (E) TXB<sub>2</sub>, an AA derivative. (F) LTB<sub>4</sub>, an AA derivative. (G) 15-HETrE, a dihomog- $\gamma$ -linolenic acid (DGLA) derivative. †*p* < 0.05 vs. vehicle (*t*-test). \*\**p* < 0.01 vs. vehicle (Williams' test). #*p* < 0.05, ##*p* < 0.01 vs. vehicle (Shirley-Williams test).



**Figure 7.** Effects of compound-326 on inflammatory markers in ApoE knockout mice with the Paigen diet protocol. Plasma samples were collected on weeks 3, 5, 7 from the onset of administration from animals shown in Figure 5. (A) Time-dependent alteration of plasma sICAM-1 levels. (B) Area under the curve (AUC) of plasma sICAM-1 concentrations. Plasma sICAM-1 levels were measured at baseline and on weeks 3, 5, and 7. (C) Plasma monocyte chemoattractant protein (MCP)-1 concentrations after 7 weeks of treatment. Results are expressed as mean  $\pm$  SEM (control: n = 6, others: n = 12). ††*p* < 0.01 vs. vehicle (*t*-test). \*\**p* < 0.01 vs. vehicle (Williams' test).

**Table 2.** Effects of compound-326 on plasma total cholesterol (mg/dL) in the Paigen diet protocol.

Group	Weeks after commencing of compound-326 treatment			
	0	3	5	7
Control	783 $\pm$ 58	803 $\pm$ 84	873 $\pm$ 88	938 $\pm$ 89††
Vehicle	787 $\pm$ 30	826 $\pm$ 34	4,022 $\pm$ 131	4,162 $\pm$ 112
0.3 mg/kg/day	798 $\pm$ 40	820 $\pm$ 32	4,275 $\pm$ 112	4,219 $\pm$ 123
3 mg/kg/day	790 $\pm$ 32	856 $\pm$ 40	4,297 $\pm$ 142	4,260 $\pm$ 141
10 mg/kg/day	795 $\pm$ 31	801 $\pm$ 29	4,271 $\pm$ 177	4,084 $\pm$ 237

Results are expressed as mean  $\pm$  SEM (control: n = 6, others: n = 12). ††*p* < 0.01 vs. vehicle (*t*-test).

**Other findings related to compound-326 administration**

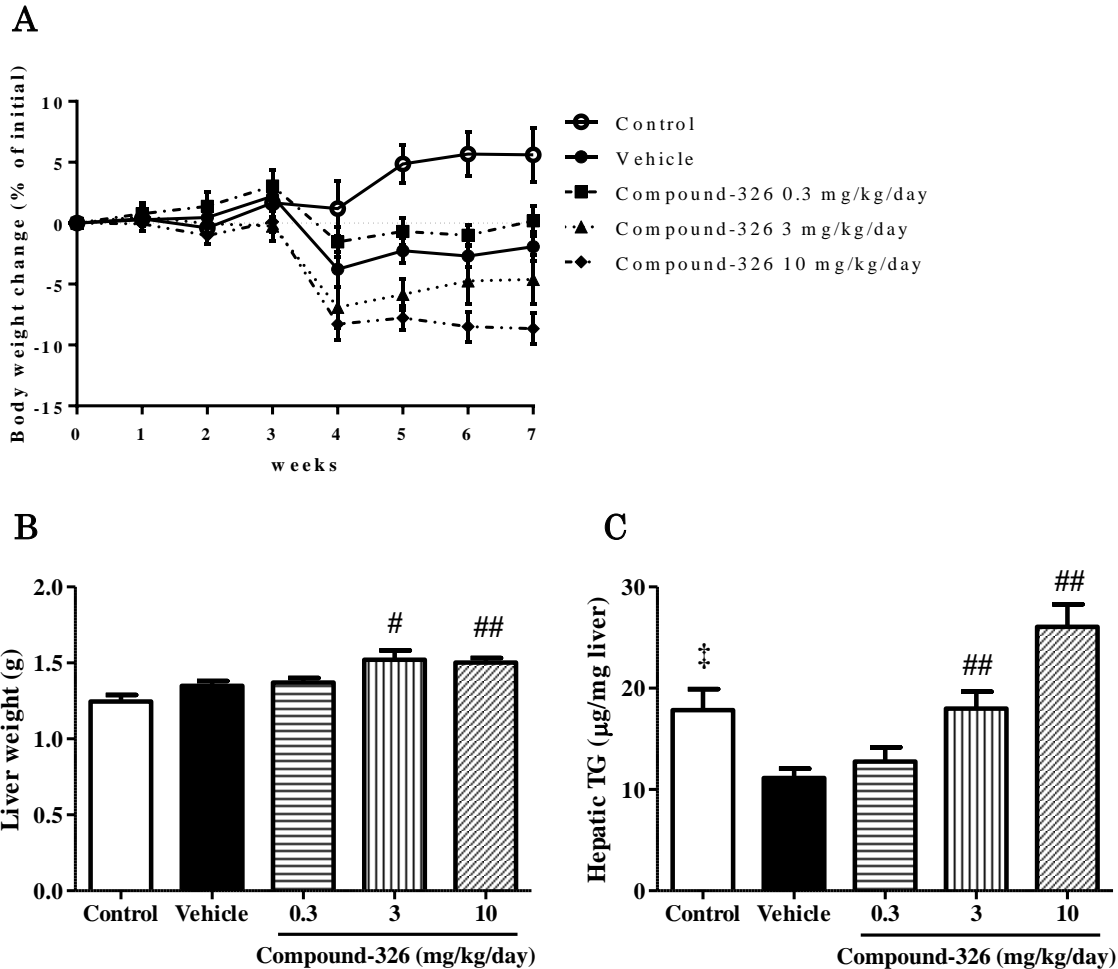
Compound-326 enhanced Paigen diet-induced body

weight reduction (Figure 8A). Food intake was not altered (Vehicle: 2.88  $\pm$  0.12 g/day, 0.3 mg/kg/day: 2.83  $\pm$  0.13 g/day, 3 mg/kg/day: 2.87  $\pm$  0.11 g/day, 10



mg/kg/day:  $2.83 \pm 0.14$  g/day, mean  $\pm$  SEM, n = 12), suggesting that the suppression of body weight by compound-326 was not caused by the reduction of food intake. Compound-326 significantly increased liver weight and hepatic TG contents in a dose-

dependent manner (Figure 8B,8C). Plasma ALT were not altered (Vehicle:  $75.8 \pm 13.3$  U/L, 0.3 mg/kg/day:  $53.5 \pm 6.00$  U/L, 3 mg/kg/day:  $114 \pm 46.1$  U/L, 10 mg/kg/day:  $54.7 \pm 3.88$  U/L, mean  $\pm$  SEM, n = 12) by compound-326.



**Figure 8.** Effects of compound-326 on body weight changes (A), liver weight (B) and hepatic triglyceride (TG) contents (C) in ApoE knockout mice with the Paigen diet protocol. (A) Body weight change. Results are expressed as mean  $\pm$  SEM (control: n = 6, others: n = 12). (B) Liver weight. (C) Hepatic TG contents. Results are expressed as mean  $\pm$  SEM (control: n = 6, others: n = 12). ‡*p* < 0.05 vs. vehicle (Welch's test). #*p* < 0.05, ##*p* < 0.01 vs. vehicle (Shirley-Williams test).

**DISCUSSION**

In the present study, we demonstrated that a D5D inhibitor, compound-326, exerts anti-atherosclerotic effects in ApoE knockout mice with two different protocols for atherosclerosis development. The main findings in this study were as follows: 1) Compound-326 exhibited anti-atherosclerotic effects in a dose-dependent manner with similar effective dose ranges in the two protocols with different states of inflammation; 2) compound-326 decreased hepatic arachidonic acid levels and increased hepatic DGLA levels in both protocols; 3) compound-326 decreased

blood production of arachidonic acid-derived eicosanoids and increased blood production of a DGLA-derived eicosanoid in both protocols; 4) compound-326 reduced plasma sICAM-1 levels without altering plasma cholesterol levels in both protocols; 5) compound-326 exhibited suppressive effects on body weight gain in both protocols; 6) compound-326 increased hepatic TG in ApoE knockout mice fed Paigen diet.

This D5D inhibitor exerted anti-atherosclerotic effects in a dose-dependent manner in ApoE knockout mice with both protocols. The effective dose ranges in both protocols were similar

to those reported in previous pre-treatment study (8). In the vehicle-treated groups of this study, Western diet induced a 38.5% atherosclerotic lesion area and a stable sICAM-1 level of approximately 30  $\mu\text{g/mL}$  at 10–22 weeks, while Paigen diet developed a 30.5% atherosclerotic lesion area and a 35  $\mu\text{g/mL}$  sICAM-1 level at 4 weeks. This suggests a clear difference in the relationship between atherosclerotic lesion formation and inflammatory status in these two protocols. Several reports have also indicated that the two diets display different inflammation states. The Paigen diet triggered greater MCP-1 and interleukin 6 gene expression in the mouse aorta compared to that reported with a Western diet. Mice fed the two diets exhibit different expression patterns of M1 and M2 macrophages, suggesting that the macrophages that accumulate in atherosclerotic lesions vary in polarity. Several other genes are differentially expressed in the two models (17), suggesting a difference in atherogenic progression. Paigen diet-fed mice have been reported not to develop lesions beyond the early foam cells and fatty streaks, while Western diet-fed mice form monocytic adhesions and advanced lesions at earlier stages, displaying a histological similarity to tissues from humans (19). The Paigen diet itself is inflammatory because of its cholic acid levels, leading to liver toxicity by inducing hepatic NF- $\kappa$ B activation, which is not seen in Western diet-fed mice (19). These reports outline the differences between the two diet-induced atherosclerosis, supporting the importance of testing drug efficacy in different protocols for atherosclerosis development.

D5D inhibition by compound-326 was confirmed in both protocols by a decrease in liver levels of arachidonic acid and an increase in DGLA, a consistent fatty acid compositional alteration in blood, reduced blood production of arachidonic acid-derived inflammatory eicosanoids such as PGE<sub>2</sub>, TXB<sub>2</sub>, and LTB<sub>4</sub>, and increased blood production of DGLA-derived anti-inflammatory eicosanoids such as 15-HETrE. However, the decrease in hepatic arachidonic acid and the reduction in blood production of arachidonic acid-derived inflammatory eicosanoids did not show a clear dose-dependency in the post-treatment protocol, displaying a maximum effect on arachidonic acid reduction at a dose of 1 mg/kg/day. In contrast, the increase in hepatic DGLA and blood production of DGLA-derived anti-inflammatory eicosanoids showed a clear dose-dependency. Since a 3-mg/kg/day compound-326 treatment showed more potent anti-atherosclerotic effects than the 1-mg/kg/day dose did, these results may suggest the

importance of DGLA elevation and DGLA-derived anti-inflammatory eicosanoid increase for D5D inhibition in showing anti-atherosclerotic effects. Several studies have indicated the involvement of eicosanoids in inflammation and atherosclerosis. Studies on mice lacking EP2 and EP4, receptors for PGE<sub>2</sub>, described pro-inflammatory and pro-atherogenic properties of PGE<sub>2</sub> (26). TXA<sub>2</sub> is involved in atherogenesis and ICAM-1 expression in vascular endothelial cells (27). LTB<sub>4</sub> is reported to promote atherogenesis by inducing MCP-1 production (28,29). 15-HETrE has anti-inflammatory properties and has been suggested to exert further anti-inflammatory effects by inhibiting 5-lipoxygenase, an enzyme that converts arachidonic acid into inflammatory eicosanoids (30). The anti-atherosclerotic effects of DGLA have been previously reported (31), and a DGLA-derived eicosanoid, PGE<sub>1</sub>, is thought to be the key to anti-atherosclerotic effects. PGE<sub>1</sub> has been reported to reduce ICAM-1 expression in monocytes, resulting in repression of T-cell infiltration under vascular endothelial cells (32,33). Confirming the effects of D5D inhibition on atherosclerotic plaque stabilization should be done in future work by measuring the pathophysiological changes in plaque composition. Taken together, the present results suggest that reduced levels of pro-inflammatory eicosanoids and increased levels of anti-inflammatory eicosanoids exert anti-inflammatory impact, resulting in anti-atherosclerotic effects by D5D inhibition.

In the post-treatment protocol study, treatment of compound-326 did not reduce the LTB<sub>4</sub> production despite of the significant reduction in the liver AA content and in the production of PGE<sub>2</sub> and TXB<sub>2</sub> (Figure 2). This result shows a clear difference from our previous pre-treatment protocol study (8) and current Paigen diet-study (Figure 6), which indicated concurrent decreases in the liver AA content and in the production of PGE<sub>2</sub>, TXB<sub>2</sub> and LTB<sub>4</sub>. Interestingly, several studies reported that long-term high fat diet feeding enhances the expression of 5-lipoxygenase (5-LO) and 5-LO-activating protein (34,35). In the future, it may be interesting to investigate whether the long-term high fat diet feeding may change the balance between LTB<sub>4</sub>-generating 5-LO pathway and PGE<sub>2</sub>/TXB<sub>2</sub>-generating cyclooxygenase-2 (COX-2) pathways in ApoE knockout mice, where the 5-LO pathway is known to be activated (36).

Compound-326 reduced plasma sICAM-1 levels without altering plasma cholesterol levels in ApoE knockout mice with both protocols, indicating

that D5D inhibition can potentially exert anti-inflammatory action. Plasma sICAM-1 levels are a marker of vascular inflammation and a risk factor for cardiovascular diseases (37,38). Plasma sICAM-1 levels also can predict future cardiovascular events (39). One report suggested that high levels of plasma sICAM-1 correlate with a poor prognosis (40). Regarding the plasma MCP-1 level reduction observed in the Paigen diet protocol, several reports have suggested a relationship between plasma MCP-1 concentrations and atherosclerosis (41,42). The anti-inflammatory properties of D5D inhibitors, which are independent of plasma cholesterol levels, can realize further benefits in treating atherosclerosis in addition to statins, the standard treatment (2).

Suppressive effects of D5D inhibition on body weight gain independent of food intake were also confirmed in both protocols in this study, indicating a beneficial and advantageous aspect of D5D inhibitors. Since D5D inhibition does not alter body weight in normal chow-fed conditions, it is likely that D5D inhibition results in fat mass gain reduction (8). Several reports refer to the contribution of chronic inflammation to insulin resistance and related metabolic disorders (43,44). Previous studies have suggested that D5D inhibition can exert anti-obesity and anti-diabetic effects, accompanied by fat reduction (7,8,15). The study of Yashiro et. al. (15) reported that treatment of compound-326 in diet-induced obese C57BL/6J mice decreased expression of macrophage-related inflammatory genes in adipose tissues and increased the whole-body energy expenditure, which effects could account for the anti-obesity effects of compound-326. Further analysis should, however, be needed to clarify the underlying mechanisms.

An increase in hepatic TG was observed in the Paigen diet protocol, although it was within the normal range (under 50 µg/mg). Plasma ALT were not concurrently increased, suggesting that the increase in the hepatic TG is a physiological but not a pathological change. Our preliminary studies indicate that this hepatic TG increase is observed under specific conditions and depends on diet composition and duration. In fact, the hepatic TG increase was not observed in the present post-treatment protocol. Some studies have suggested that increased lipogenesis related to polyunsaturated fatty acid-mediated regulation of SREBP-1c may be a potential mechanism underlying this phenomenon. Moon et al. (2009) reported that SREBP-1c positively regulated the expression of genes involved in the synthesis of free fatty acid from citrate, and that polyunsaturated fatty acid negatively regulated

SREBP-1c expression. Since D5D catalyzes the formation of arachidonic acid, eicosapentaenoic acid, and the downstream product docosahexaenoic acid, which are all polyunsaturated fatty acids (45), inhibiting D5D may attenuate the suppression of SREBP-1c by these polyunsaturated fatty acids, resulting in increased fatty acid and eventual TG synthesis in hepatocytes. This may be a limited effect only in animal experiments since diets we used in this study contain poor polyunsaturated fatty acids, unlike clinical situation with exogenous polyunsaturated fatty acid consumption. Future work should elucidate the mechanism underlying this effect, including whether a similar phenomenon occurs in humans.

## CONCLUSION

In conclusion, the findings of the present study clearly indicate that compound-326 exerts anti-atherosclerotic effects accompanied by anti-inflammatory action in ApoE knockout mice with two different protocols for atherosclerosis development, supporting D5D inhibition as a promising strategy in treating atherosclerosis.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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