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Ameliorating effects of D-47, a newly developed compound, on lipid metabolism in an animal model of familial hypercholesterolemia (WHHLMI rabbits)

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ABSTRACT

Improvements induced in lipid metabolism in the liver by D-47, a newly developed compound, were examined herein.

WHHLMI rabbits, an animal model of hypercholesterolemia and coronary atherosclerosis, was fed D-47-supplemented chow for 5 weeks at a dose of 30 mg/kg. Lipid concentration were assayed using enzymatic methods. Plasma lipoproteins were fractionated with an ultracentrifuge. mRNA expression was analyzed with real-time PCR. Lipidome analyses of lipoproteins were performed using supercritical fluid chromatography mass spectrometry. In the D-47-treated group, serum lipid levels decreased by 23% for total cholesterol and by 40% for triglycerides. These reductions were mainly attributed to decreases in the VLDL fraction. Compared with the control, in the D-47 group, lipid contents in the liver were decreased by 22% in cholesterol and by 69% in triglycerides, and fat accumulation was decreased by 57% in pericardial fat and by 17% in mesenteric fat. In lipidome analyses of VLDL fraction, lysophosphatidylcholine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylethanolamine plasmalogen, sphingomyelin, and ceramide were decreased by the D-47

treatment. mRNA expression in the liver was 51% lower for FAS and 24% lower for MTP, but 5.9- and 5.1-fold higher for CYP7A1 and CPT-1, respectively, in the D-47 group than in the control. mRNA expression was 72%, 64%, and 36% higher for LPL, CTP-1, and PPARγ, respectively, in mesenteric fat in the D-47 group.

D-47 is a potent lipid-lowering compound that uses a different mechanism of action from that of statins. It has potential as a compound in the treatment of steatohepatitis and metabolic syndrome.

Keywords: adipose tissue accumulation, gene expression, D-47, lipid-lowering effects, lipidome analyses, liver lipid contents

1. Introduction

Hyperlipidemia is associated with cardiovascular disease, metabolic syndrome, obesity, and nonalcoholic fatty liver disease (NAFLD) / nonalcoholic steatohepatitis (NASH). These diseases represent an important public health issue worldwide. A variety of powerful lipid-lowering agents have been developed. However, cardiovascular disease remains an important disease to be overcome, and the number of obese individuals and patients with metabolic syndrome and/or NAFLD/NASH has increased worldwide due to the ingestion of a Western diet and the lack of exercise (Hannah and Harrison, 2016). Therefore, it seems necessary to develop widely available novel compounds with different mechanisms to improve lipid metabolism. In addition, therapeutic drugs for NAFLD/NASH were not available until recently (Verbeek et al., 2017), although several lipid-lowering drugs are known to ameliorate NAFLD/NASH (Liss et al., 2017; Nakade et al., 2017; Park et al., 2016; Scorlettii et al., 2014; Kelley, 2016: Orime et al., 2016: Bae et al., 2017).

In developing novel lipid metabolism improving drugs, it is important to develop compounds showing a mechanism different from that of

commercially available drugs. Although a potent lipid lowering compound, S-2E, was previously developed (Ohmori et al., 2003; Ohmori et al., 2004), the absorption in intestine was very low. In order to improve the intestine absorption of S-2E, S-2E-arginine salt (a derivative of S-2E), was developed (Ogawa et al., 2016; Nakaya et al., 2017), and a solid dispersion of S-2E-arginine salt (D-47) was developed. In the present study, we examined the effects of D-47 on serum lipid levels, lipid contents in the liver, fat accumulation in tissues, and body weight using WHHLMI rabbits. WHHLMI rabbits are an animal model that spontaneously develop hypercholesterolemia even fed standard chow due to abnormality of the LDL receptor gene, and spontaneously develop myocardial infarction caused by the progression of coronary artery atherosclerosis. (Shiomi et al., 2003; Shiomi and Ito, 2009). Lipoprotein metabolism in humans more closely resembles that in rabbits than in mice and rats (Shiomi et al., 2012). The present results indicate that D-47 not only exhibits a potent lipid lowering effect but also suppresses lipid accumulation in the liver and adipose tissue.

2. Materials and methods

2.1. Materials

D-47 was produced in Tokushima-Bunri University (Tokushima, Japan), and is a solid dispersion of the S-2E-arginine salt ($C_{28}H_{38}N_5O_6$, MW: 540, PubChem CID: 9864020) (Nakaya et al., 2017). The solubility and plasma concentration of the S-2E-arginine salt were markedly improved by making it a solid dispersion (Supplemental Table 1). Pitavastatin (PubChem CID: 5282451, Kowa Pharmaceutical Co., Ltd., Tokyo, Japan), epadel (EPA, PubChem CID: 3298, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), and bezafibrate (PubChem CID: 39042, Kissei Pharmaceutical Co., Ltd., Tokyo, Japan) are commercially available products.

2.2. Animals

WHHLMI rabbits bred at the Institute for Experimental Animals, Kobe University Graduate School of Medicine (Kobe, Japan) were used. Rabbits were housed individually in metal cages (550 mm x 600 mm x 450 mm; in width, depth, and height, respectively) with a flat metal floor. Animals were

maintained under SPF conditions with a constant temperature (22 ± 2 °C), relative humidity (50–60%), ventilation rate (15 cycles/h), air supply (through a HEPA filter), and lighting cycle (12 h light/dark). This study was approved by the Kobe University Animal Care and Use Committee (approval number: P140612), and all animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe University, the Act on Welfare and Management of Animals (Law No. 105, 1973, revised in 2006), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88, 2006), and Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the Jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (Notice No. 71, 2006).

2.3. Administration of lipid-lowering agents

The design of the D-47 study was shown in Supplemental Fig. 1. Twelve female WHHLMI rabbits aged 12 months were divided into two groups

(control and D-47-treated groups) to ensure uniform plasma lipid levels and body weights. The D-47 group was fed D-47-supplemented chow at a dose of 30 mg/kg according to a previous study using the drug substance, S-2E (Ohmori et al., 2004), followed by standard chow (CR-3, CLEA Japan, Inc., Tokyo, Japan) for 5 weeks. Total daily feeding was 120 g/day. Rabbits in the control group were fed standard chow at 120 g/day. Water was given *ad libitum*. In comparisons of commercially available lipid-lowering drugs, 24 female WHHLMI rabbits aged 10-12 months were divided into four groups (control-2, pitavastatin, EPA, and bezafibrate groups) to ensure uniform plasma cholesterol levels. The dose of each commercially available drug was selected based on previous studies. Pitavastatin, EPA, and bezafibrate were suspended in a 0.5% carboxymethylcellulose (PubChem CID: 24748, Iwai Chemicals Company Ltd., Tokyo, Japan) suspension and administered to rabbits for 4 weeks at doses of 0.5 mg/kg, 300 mg/kg, and 50 mg/kg, respectively. Control rabbits were given 0.5% carboxymethylcellulose suspension as a placebo.

2.4. Fractionation of plasma lipoproteins and measurement of lipid

concentration

After overnight fasting, blood samples were collected every week from the marginal ear vein, and serum lipid levels were measured by enzymatic methods. At the start and after 4 weeks of the treatment, lipoproteins were fractionated by ultracentrifugation to yield very low-density lipoproteins (VLDL, d<1.006 g/ml), LDL (1.006<d<1.063 g/ml), and high-density lipoproteins (HDL, d>1.063 g/ml). Lipoprotein lipid concentration were assayed using enzymatic methods.

2.5. Lipidome analyses of lipoprotein fractions in the D-47 study

Lipidome analyses of plasma VLDL and LDL fractions were performed at the start and after 5 weeks of the treatment. VLDL (d<1.006 g/ml) and LDL (1.019<d<1.063 g/ml) were fractionated with an ultracentrifuge using a stepwise method. Lipids were extracted from lipoprotein fractions using Bligh and Dyer's method (Bligh and Dyer, 1959) with minor modifications (Takeda et al., 2015). Prior to lipid extraction, a dodecanoyl or

heptadecanoyl-based synthetic internal standard mixture (Supplemental Table 2) was spiked to lipoprotein fractions for the quantification of each lipid molecular species. Lipoprotein lipids were quantified using supercritical fluid chromatography triple quadrupole mass spectrometry (SFC/MS/MS) in the multiple reaction monitoring mode. The SFC/MS/MS system is composed of an ACQUITY UltraPerformance Convergence Chromatography (UPC2) system (Waters Co., Milford, MA, USA) and Xevo TQ−S micro tandem mass spectrometer (Waters Co.), which are controlled by MassLynx software version 4.1 (Waters Co.).

2.6. Real-time PCR analyses for mRNA expression in the D-47 study

After 5 weeks of the D-47 treatment, rabbits were euthanized by exsanguination under anesthesia using an intravenous injection of ketamine hydrochloride (15 mg/kg, Daiichi-Sankyo Co., Ltd., Tokyo, Japan) and midazolam (1 mg/kg, Dormicum, Astellas Pharma Inc., Tokyo, Japan), and were perfused with saline. The liver, mesenteric fat, intestines, and epicardial fat were removed. Total RNA from each tissue was isolated using

Trizol reagent (Thermo Scientific) (Liang et al., 2006). RNA expression levels were evaluated using One Step SYBR PrimeScript RT PCR Kit II（Takara Bio Inc., Tokyo, Japan) according to the manufacturer's instructions. Primers used in these analyses are shown in Supplemental Table 3. cDNAs were prepared from extracted total RNA and the PrimeScript RT reagent kit with the gDNA Eraser (Takara Bio Inc., Tokyo, Japan). Real-time PCR analyses were performed with the Thermal Cycler Dice (Takara Bio Inc., Kusatsu, Japan) with the intercalator method (Wang et al., 2004). Rabbit GAPDH was used as the reference gene.

2.7. Other assays in the D-47 study

Lipid contents in the liver were assayed by Skylight Biotech Inc. (Akita, Japan) after the extraction of lipids by Folch's method (Folch et al., 1957). Serum AST, ALT, and CK levels were measured by enzymatic methods at the start and after 4 weeks of the treatment.

2.8. Statistical analysis

Data are represented as the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using the Student's *t*-test, Welch's *t*-test, Mann-Whitney U-test, or paired *t*-test. P-values less than 0.05 were considered to be significant.

3. Results

3.1. Baseline data of WHHLMI rabbits

Baseline data of WHHLMI rabbits used in the D-47 study are shown in Supplemental Table 4. No significant differences were observed in any parameters between the control and D-47 groups. However, one rabbit in the control group had extremely high serum AST (121 IU/l) and ALT (111 IU/l) levels (Supplemental Fig. 2). Therefore, this rabbit was excluded from the study. No abnormality was found in other rabbits during the experiment. Every rabbit consumed 120 g of chow every day.

3.2. Lipid-lowering effects of D-47 and commercially available lipid-lowering

drugs

Fig. 1 shows the lipid-lowering effects of D-47. Serum cholesterol concentration was 16% lower (1149±63 mg/dl vs. 967±40 mg/dl, P=0.034, Panel A) and serum triglyceride concentration was 46% lower (467±61 mg/dl vs. 252±20 mg/dl, P=0.022, Panel B) in the D-47-treated group than in the control. In the VLDL fraction (Panel C), compared to 0 week, D-47 decreased total cholesterol concentration by 35% (119±8 mg/dl vs. 78±5 mg/dl, P=0.001) and triglyceride concentration by 73% (111±20 mg/dl vs. 31±3 mg/dl, P=0.015). In the LDL fraction (Panel D), D-47 decreased triglyceride concentration by 45% (309±26 mg/dl vs. 170±9 mg/dl, P<0.001). No significant differences were observed in HDL lipid concentration. Among commercially available lipid-lowering drugs (Fig. 2), pitavastatin and EPA showed cholesterol-lowering effects in whole plasma and the VLDL and LDL fractions. However, a decrease in triglyceride concentration was only observed in the whole plasma and VLDL fraction in the EPA group.

3.3. Effects of D-47 on fat accumulation in tissues

Fig. 3 shows the suppressive effects of D-47 on lipid accumulation in tissues. In the liver (Panel-A), D-47 decreased the cholesterol content by 22% (3.2±0.2 mg/g vs. 2.5±0.1 mg/g, P=0.008) and triglyceride content by 69% (13.7±3.5 mg/g vs. 4.2±0.4 mg/g, P=0.011). The weight of fat was 57% lower (1.9±0.4 g vs. 0.8±0.1 g, P=0.008) for epicardial fat and 17% lower (126±9 g vs. 105±5 g, P=0.053) for mesenteric fat in the D-47 group than in the control (Panel-B). Furthermore, the percentage of the initial body weight was significantly lower in the D-47 group than in the control at 5 weeks of treatment despite ingesting the total amount of chow given (120 g/day) during this study (Panel-C).

3.4. Results of lipidome analyses of lipoprotein fractions of the D-47 treatment.

In lipidome analyses of the VLDL fraction (Fig. 4A), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine plasmalogen (PE-pln), phosphatidylinositol (PI),

sphingomyelin (SM), and ceramide (Cer) were significantly lower in the D-47 group than in the control. In lipidome analyses of the LDL fraction (Fig. 4B), a significant decrease was only observed in Cer.

3.5. Effects of D-47 on mRNA expression associated with lipid metabolism

Fig. 5 shows real-time PCR results for mRNA expression. The mRNA expression of CYP7A1, the gene of cholesterol-7α-hydroxylase, which is a rate-limiting enzyme in the synthesis of bile acids from cholesterol, and CPT-1, the gene of carnitine palmitoyltransferase-I, which plays an important role in the β-oxidation of fatty acids, was significantly higher (5.95- and 2.07-fold, respectively), while that of MTP, the gene of the mitochondrial triglyceride transfer protein, which plays an important role in VLDL formation in the liver, and FAS, the gene of fatty acid synthase, was significantly lower (by 24% and 51%, respectively) in livers in the D-47 group (Panel-A). In mesenteric adipose tissue in the D-47 group (Panel-B), the expression of LPL, the gene of lipoprotein lipase, was significantly increased by 1.72-fold (P=0.028). The expression of CPT-1 was showed tendency to

increase by 1.64-fold (P=0.055). Although there were no statistical significance, the expression of ADPOQ, the gene of adiponectin, and PPAR-γ, the gene of peroxisome proliferator-activated receptor-γ was high, while that of VLDLR, Leptin, and FAS was low in the D-47 group. In the intestines, the expression of NPC1L1, the gene of Niemann-pick C1-Like 1 Protein, which is involved in cholesterol absorption in the intestines, was showed tendency to increase by 20% (P=0.068) in the D-47 group (Panel-C).

4. Discussion

The present results demonstrated that the new compound, D-47, exerts potent improving effects on not only lipid concentration in whole serum, VLDL, and LDL, but also lipid contents in the liver and fat accumulation in adipose tissues.

The results of PCR analyses for gene expression suggest a mechanism for the lipid-lowering effects of D-47 (Supplemental Fig. 3). The D-47-induced suppression of FAS expression in the liver decreased triglyceride synthesis, while an increase in CPT-1 expression enhanced fatty acid degradation. A remarkable decrease in triglyceride accumulation in the

liver may be due to suppression of FAS expression and/or upregulation of CPT-1 expression. Regarding cholesterol metabolism in the liver, increased CYP7A1 expression may enhance the conversion of cholesterol to bile acids. Reduction of cholesterol pool in the liver may be an increase in CYP7A1 expression. Decreases in NPC1L1 expression may also be related to reductions in the cholesterol pool. In order to prove these hypotheses, it is necessary to confirm the change in activity of the relevant enzymes and change in protein expression. Although Ohmori et al. (2003) reported that lipid lowering effect of S-2E, bulk drug of D-47, was due to non-competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase and acetyl-CoA carboxylase (ACC), expression of mRNA of HMG-CoA reductase and ACC did not increase by D-47 treatment in the present assay. Therefore, the detail mechanism is unclear. Assay of enzyme activity in D-47 treatment is necessary to elucidate the mechanism of the lipid lowering effects. In the present study using WHHLMI rabbits with abnormality in LDL receptor gene, D-47 did not up-regulate the expression of LDL receptor gene. To clarify the effect of D-47 on LDL receptor, it is necessary to use other animal models or normal cells with normal LDL receptor function.

These results suggest that D-47 has potential as a therapeutic agent for NAFLD/NASH. Recent studies demonstrated that simple steatosis progressed to NASH following exposure to lipotoxicity and glucotoxicity (Polyzos et al., 2017; Singh et al., 2015). Furthermore, decreases in plasma concentration of LPC and ceramides in the D-47 group in lipidome analyses may reflect less fat accumulation in the liver because LPC and ceramides are associated with hepatic toxicity (Mota et al., 2016). Since therapeutic drugs have not yet been developed for NAFLD/NASH (Verbeek et al., 2017), future studies on the effect of D-47 on NAFLD / NASH using an animal model of NAFLD / NASH is necessary.

In the present study, MTP expression was decreased by 24% in D-47 treated group. This result is consistent with the observation about decrease in VLDL secretion in S-2E treated rats (Ohmori et al., 2003). Decreases in MTP expression in the liver reduced VLDL formation, which, in turn, decreased VLDL secreted from the liver. Previous studies demonstrated that the suppression of MTP activity by MTP inhibitors increased lipid accumulation in the liver (Tep et al., 2012; Pereira et al., 2011). In these cases, synthesized lipids may have accumulated in the liver through the

inhibition of MTP. Since D-47 reduced lipid accumulation in liver, a combination treatment with D-47 and MTP inhibitors may have potent lipid metabolism improving effects without increasing lipid accumulation in liver.

In mesenteric adipose tissue, D-47-induced increases in CPT-1 expression may be associated with less fat accumulation. Decreased lipid contents in the liver and the accumulation of epicardial fat and mesenteric fat may be associated with decreased body weight. The suppression of visceral fat accumulation by D-47 may ameliorate metabolic syndrome, and high expression of PPARγ in mesenteric adipose tissue may relate to ameliorating metabolic syndrome. Furthermore, since epicardial fat accumulation correlates with coronary artery disease (Shimabukuro et al., 2013), the suppression of epicardial fat accumulation by D-47 may be associated with the prevention of coronary events. Therefore, future studies to investigate the effect of D-47 on pericardial fat is important.

Increased LPL expression in mesenteric adipose tissue may be associated with decreased LDL lipid concentration. The Epick-Norfolk study demonstrated that serum LPL concentrations negatively correlated not only with serum triglyceride concentration, but also the risk of future CAD (Rip et

al., 2006). A similar concept was reported by Kobayashi (2007). Furthermore, VLDL-LPC was decreased by the D-47 treatment in the lipidome analysis. Since LPC is an important component of oxidized LDL (Itabe et al., 1999), reductions in LPC in the VLDL fraction indicate the suppression of oxidative stress in this fraction. Atherogenicity is greater in oxidized VLDL than oxidized LDL (Argmann et al., 2001). Furthermore, human and rabbit, but not mouse or rat macrophages have VLDL receptors, and VLDL and VLDL remnants cause atherosclerosis in humans and rabbits (Takahashi et al., 2017). Therefore, the reductions observed in VLDL-LPC in the D-47 group suggest the anti-atherosclerotic effects of D-47. Further studies are needed.

In studies using commercially available lipid-lowering drugs, statins (Park et al., 2016; Orime et al., 2016), fibrate (Liss and Finck, 2017; Park et al., 2016; Orime et al., 2016), EPA (Scorletti et al., 2014; Kelley et al., 2016; Bae et al., 2017), and ezetimibe (Nakade et al., 2017; Orime et al., 2016) also suppressed lipid accumulation in tissues. Serum triglyceride concentration were reduced in all these studies. However, the mechanisms responsible for inhibiting lipid accumulation in the liver remain unclear.

5. Conclusions

The present study demonstrated that D-47, which has a different mechanism from statins and other commercially available lipid lowering agents, is a potent lipid lowering compound. It also has potential as a compound in the treatment of steatohepatitis and metabolic syndrome. Further studies about the improving effects of D-47 on lipid metabolism will advance the development of therapeutic drugs for these diseases.

Author contributions

MS designed, executed, and supervised the study and drafted the manuscript. ST, YY, RN, and TK contributed to animal experimentation and the analysis of data. HT, YI, and TB contributed to lipidome analyses. TT, MT, and KO contributed to the production of compounds and evaluation of the characteristics of compounds.

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Disclosure of conflicts of interest

The authors declare they do not have anything to disclose regarding conflicts of interest with respect to this manuscript.

Appendix

Supplementary data associated with this article can be found in the online version.

References

Argmann, C.A., Van Den Diepstraten, C.H., Sawyez, C.G., Edwards, J.Y., Hegele, R.A., Wolfe, B.M., Huff, M.W., 2001. Transforming growth factor-β1 inhibits macrophage cholesterol ester accumulation induced by native and oxidized VLDL remnants. Arterioscler. Thromb. Vasc. Biol. 21, 2011-2018.

Bae, J.S., Park, J.M., Lee, J., Oh, B.C., Jang, A.H., Lee, Y.B., Han, Y.M., Ock, C.Y., Cha, J.Y., Hahm, K.B., 2017. Amelioration of non-alcoholic fatty

liver disease with NPC1L1-targeted IgY or n-3 polyunsaturated fatty acids in mice. Metabolism 66, 32-44.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37, 911-917.

Folch, J., Less, M., Stanley, G.M., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-507.

Hannah, W.N. Jr., Harrison, S.A., 2016. Lifestyle and dietary interventions in the management of nonalcoholic fatty liver disease. Dig. Dis, Sci. 61, 1365-1374.

Itabe, H., Hosoya, R., Karasawa, K., Jimi, S., Saku, K., Takebayashi, S., Imanaka, T., Takano, T., 1999. Metabolism of oxidized phosphatidylcholines formed in oxidized low density lipoprotein by lecithin-cholesterol acyltransferase. J. Biochem. 126, 153-161.

Kelley, N.S., 2016. Treatment of nonalcoholic fatty liver disease with long-chain n-3 polyunsaturated fatty acids in humans. Metab. Syndr. Relat. Disord. 14, 417-430.

Kobayashi, J., Nohara, A., Kawashiri, M., Inazu, A., Koizumi, J., Nakajima, K., Mabuchi, H., 2007. Serum lipoprotein lipase mass: clinical significance of its measurement. Clin. Chim. Acta. 378, 7-12.

Liang, J., Liu, E., Yu, Y., Kitajima, S., Koike, T., Jin, Y., Morimoto, M., Hatakeyama, K., Asada, Y., Watanabe, T., Sasaguri, Y., Watanabe, S., Fan, J., 2006. Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits. Circulation 113, 1993-2001.

Liss, K.H.H., Finck, B.N., 2017. PPARs and nonalcoholic fatty liver disease. Biocim. 136, 65-74.

Mota, M., Banini, B.A., Cazanave, S.C., Sanyal, A.J., 2016. Molecular mechanisms of lipotoxicity and glucotoxicity in nonalcoholic fatty liver disease. Metabolism 65,1049-1061.

Nakade, Y., Murotani, K., Inoue, T., Kobayashi, Y., Yamamoto, T., Ishii, N., Ohashi, T., Ito, K., Fukuzawa, Y., Yoneda, M., 2017. Ezetimibe for the treatment of nonalcoholic fatty liver disease: a meta-analysis. Hepatol. Res. doi: 10.1111/hepr.12887.

Nakaya, Y., Fukuda, D., Oyamada, T., Ogawa, K., Harada, N., Nakagami, H., Morishita, R., Sata, M., Sakaue, H., 2017. A novel lipoprotein (a)

lowering drug, D-47, decreases neointima thickening after vascular injury. J. Med. Invest. 64, 64-67.

Ogawa, K., Okamoto, I., Akagi, M., Toori, M., Tsuonoda, T., Sata, M., Nakaya, Y., 2016. Research development of D-47 as new anti-arteriosclerosis. Abstract Book of the 136th Annual Meeting of the Pharmaceutical Society of Japan (March 26-29, 2016). 2, 168.

Ohmori, K., Yamada H., Yasuda, A., Yamamoto, A., Matsuura, N., Kiniwa, M., 2003. Anti-hyperlipidemic action of a newly synthesized benzoic acid derivative, S-2E. Eur. J. Pharmacol. 471, 69-76.

Ohmori, K., Yamada, H., Yasuda, A., Yamamoto, A., Matsuura, N., Kiniwa, M., 2004. Effects of a novel antihyperlipidemic agent, S-2E, on the blood lipid abnormalities in homozygous WHHL rabbits. Metabolism 53, 680-685.

Orime, K., Shirakawa, J., Togashi, Y., Tajima, K., Inoue, H., Nagashima, Y., Terauchi, Y., 2016. Lipid-lowering agents inhibit hepatic steatosis in a non-alcoholic steatohepatitis-derived hepatocellular carcinoma mouse model. Eur. J. Pharmacol. 772, 22-32.

Park, H.S., Jang, J.E., Ko, M.S., Woo, S.H., Kim, B.J., Kim, H.S., Park, H.S., Park, I.S., Koh, E.H., Lee, K.U., 2016. Statins increase mitochondrial and peroxisomal fatty acid oxidation in the liver and prevent non-alcoholic steatohepatitis in mice. Diabetes Metab. J. 40, 376-385.

Pereira, I.V., Stefano, J.T., Oliveira, C.P., 2011. Microsomal triglyceride transfer protein and nonalcoholic fatty liver disease. Expert. Rev. Gastroenterol. Hepatol. 5, 245–251.

Polyzos, S.A., Kountouras, J., Mantzoros, C.S., 2017. Endocrine and metabolic disorders interplaying with non-alcoholic fatty liver disease. Minerva. Endocrinol. 42, 92-108.

Rip, J., Nierman, M.C., Wareham, N.J., Luben, R., Bingham, S.A., Day, N.E., Van Miert, J.N., Hutten, B.A., Kastelein, J.J., Kuivenhoven, J.A., Khaw, K.T., Boekholdt, S.M., 2006. Serum lipoprotein lipase concentration and risk for future coronary artery disease: the EPIC-Norfolk prospective population study. Arterioscler. Thromb. Vasc. Biol. 26, 637-642.

Scorletti, E., Bhatia, L., McCormick, K.G., Clough, G.F., Nash, K., Hodson, L., Moyses, H.E., Calder, P.C., Byrne, C.D.; WELCOME Study Collaborators (15), 2014. Effects of purified eicosapentaenoic and

docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology 60,1211-1221.

Shimabukuro, M., Hirata, Y., Tabata, M., Dagvasumberel, M., Sato, H., Kurobe, H., Fukuda, D., Soeki, T., Kitagawa, T., Takanashi, S., Sata, M., 2013. Epicardial adipose tissue volume and adipocytokine imbalance are strongly linked to human coronary atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 33, 1077-1084.

Shiomi, M., Ito, T., Yamada, S., Kawashima, S., Fan, J., 2003. Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). Arterioscler. Thromb. Vasc. Biol. 23, 1239-1244.

Shiomi, M., Ito, T., 2009. The Watanabe heritable hyperlipidemic (WHHL) rabbit, its characteristics and history of development: A tribute to the late Dr. Yoshio Watanabe. Atherosclerosis 207, 1-7.

Shiomi, M., Koike, T., Ishida, T., 2012. Genetically Modified Animal Models for Lipoprotein Research. In Frank S, Kostner G eds. Lipoproteins - Role in Health and Diseases. InTechopen.com. (University Campus STeP Ri, Rijeka, Croatia), Chapter 22, 533-560.

Singh, S., Allen, A.M., Wang, Z., Prokop, L.J., Murad, M.H., Loomba, R., 2015. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: A systematic review and meta-analysis of paired-biopsy studies. Clin. Gastroenterol. Hepatol. 13, 643–654.

Takahashi, S., 2017. Triglyceride rich lipoprotein-LPL-VLDL receptor and Lp(a)-VLDL receptor pathways for macrophage foam cell formation. J. Atheroscler. Thromb. 24, 552-559.

Takeda, H., Koike, T., Izumi, Y., Yamada, T., Yoshida, M., Shiomi, M., Fukusaki, E., Bamba, T., 2015. Lipidomic analysis of plasma lipoprotein fractions in myocardial infarction-prone rabbits. J. Biosci. Bioeng. 120, 4764-82.

Tep, S., Mihaila, R., Freeman, A., Pickering, V., Huynh, F., Tadin-Strapps, M., Stracks, A., Hubbard, B., Caldwell, J., Flanagan, W.M., Kuklin, N.A., Ason, B., 2012. Rescue of Mtp siRNA-induced hepatic steatosis by DGAT2 siRNA silencing. J. Lipid Res. 53, 859-867.

Verbeek, J., Spincemaille, P., Vanhorebeek, I., Van den Berghe, G., Elst, I.V., Windmolders, P., Van Pelt, J., Van der Merwe, S., Bedossa, P., Nevens, F., Cammue, B., Thevissen, K., Cassiman, D., 2017. Dietary intervention,

but not losartan, completely reverses non-alcoholic steatohepatitis in obese and insulin resistant mice. Lipids Health Dis. 16, 46-55.

Wang, X., Liang, J., Koike, T., Sun, H., Ichikawa, T., Kitajima, S., Morimoto, M., Shikama, H., Watanabe, T., Sasaguri, Y., Fan, J., 2004. Overexpression of human matrix metalloproteinase-12 enhances the development of inflammatory arthritis in transgenic rabbits. Am. J. Pathol. 165, 1375–1383.

Figure Legends

Fig. 1

Lipid-lowering effects of D-47. (A) Changes in serum total cholesterol concentration. (B) Changes in serum triglyceride concentration. (C) Changes in VLDL lipid concentration. (D) Changes in LDL lipid concentration. (E) Changes in HDL lipid concentration. Data are represented as the mean ± S.E.M. Statistical analyses were performed with the Student's *t*-test for serum lipid concentration. Open and solid symbols indicate the control and D-47-treated groups.

Fig. 2

Lipid-lowering effects of eicosapentaenoic acid (EPA), pitavastatin (Statin), and bezafibrate (Fibrate) in WHHLMI rabbits. (A) Plasma cholesterol concentration, (B) VLDL-cholesterol concentration, (C) LDL-cholesterol concentration, (D) plasma triglyceride concentration, (E) VLDL-triglyceride concentration, and (F) LDL-triglyceride concentration. Data are represented as the mean ± S.E.M. Statistics analyses were performed with the paired *t*-test. Open bars indicate 0 weeks, and solid bars indicate 4 weeks of the

treatment.

Fig. 3

Effects of D-47 on lipid accumulation in various tissues and body weight. (A) Lipid content in the liver. (B) Weight of pericardial fat and mesenteric fat. (C) Changes in body weight during the treatment. Data are represented as the mean ± S.E.M. Statistical analyses were performed with the Student's *t*-test or Welch's *t*-test. Italic numbers indicate P-values. Open symbols indicate the control group, and solid symbols indicate the D-47-treated group.

Fig. 4

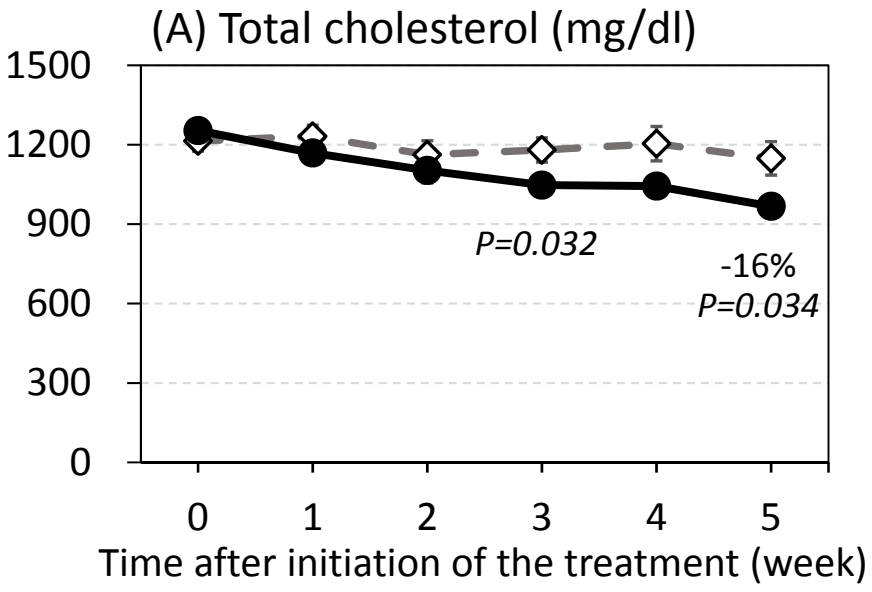
Results of lipidome analyses of VLDL (A) and LDL (B) fractions at the end of the D-47 treatment. Data are represented as the mean ± S.E.M. Statistical analyses were performed with the Student's *t*-test or Welch's *t*-test. Upper row values indicate changes from the control group, and lower row italic values indicate P-values. Open bars indicate the control group, and solid bars indicate the D-47-treated group. LPC, Lysophosphatidylcholine; LPE,

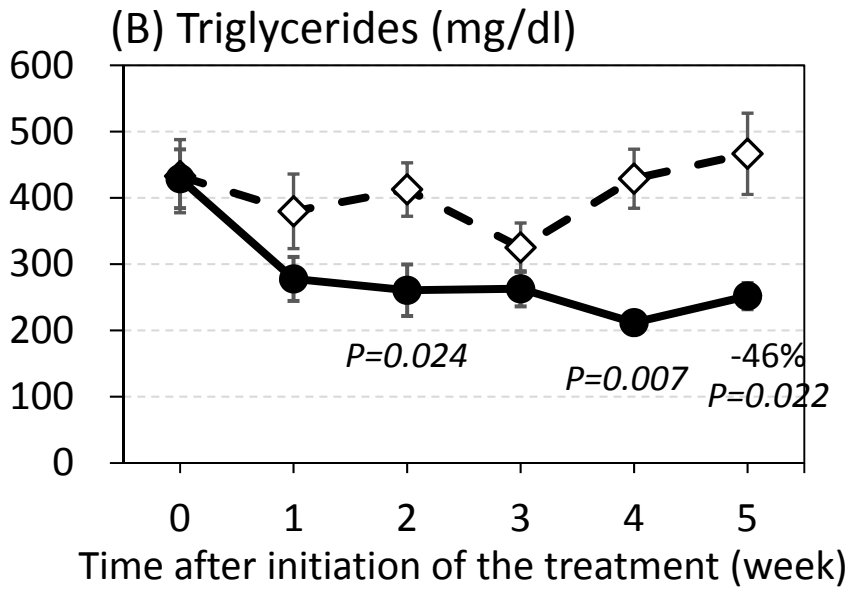
Lysophosphatidylethanolamine; PC, phosphatidylcholine; E-PC, Ether PC; PE, Phosphatidylethanolamine; PEpln, PE plasmalogen; PI, phosphatidylinositol; SM, Sphingomyelin; Cer, Ceramide.

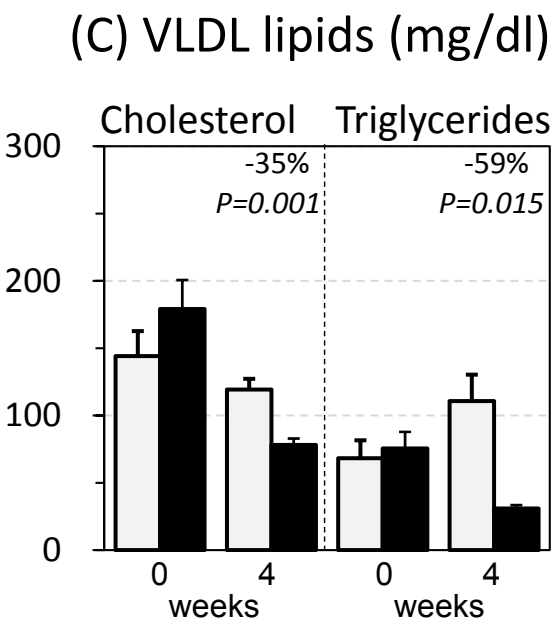
Fig. 5

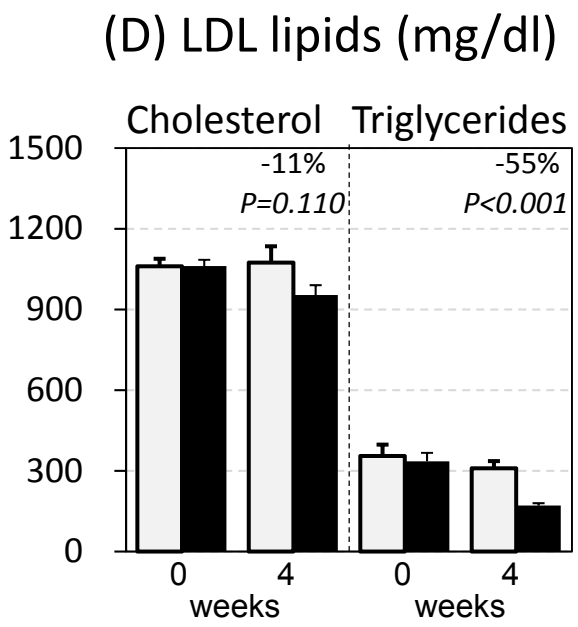
mRNA expression in the liver (A), mesenteric adipose tissues (B), and intestines (C) at the end of the D-47 treatment. Data are represented as the mean ± S.E.M. Statistical analyses were performed with the Mann-Whitney *U*-test. Upper row values indicate changes from the control group, and lower row italic values indicate P-values. Open symbols indicate the control group, and solid symbols indicate the D-47-treated group.

Figure 1


(A) Total cholesterol (mg/dl)
1500
1200
900
600
300
0
P=0.032
-16%
P=0.034
0 1 2 3 4 5
Time after initiation of the treatment (week)


(B) Triglycerides (mg/dl)
600
500
400
300
200
100
0
P=0.024
P=0.007
-46%
P=0.022
0 1 2 3 4 5
Time after initiation of the treatment (week)


(C) VLDL lipids (mg/dl)
Cholesterol
Triglycerides
300
200
100
0
-35%
P=0.001
-59%
P=0.015
0 4 0 4
weeks weeks


(D) LDL lipids (mg/dl)
Cholesterol
Triglycerides
1500
1200
900
600
300
0
-11%
P=0.110
-55%
P<0.001
0 4 0 4
weeks weeks

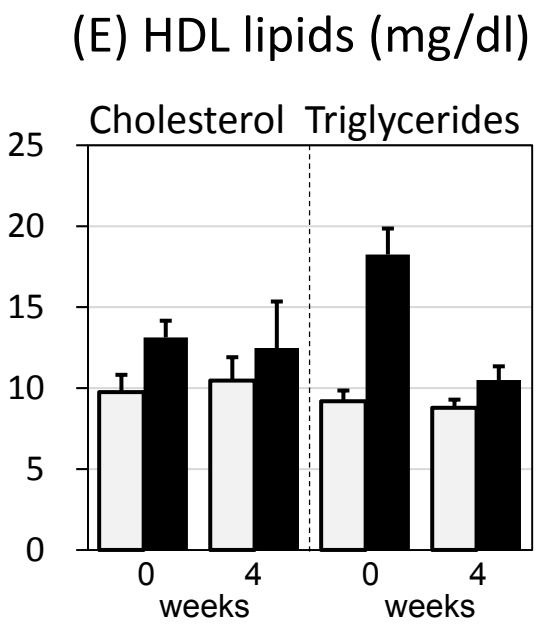

(E) HDL lipids (mg/dl)
Cholesterol
Triglycerides
25
20
15
10
5
0
0 4 0 4
weeks weeks

Figure 2

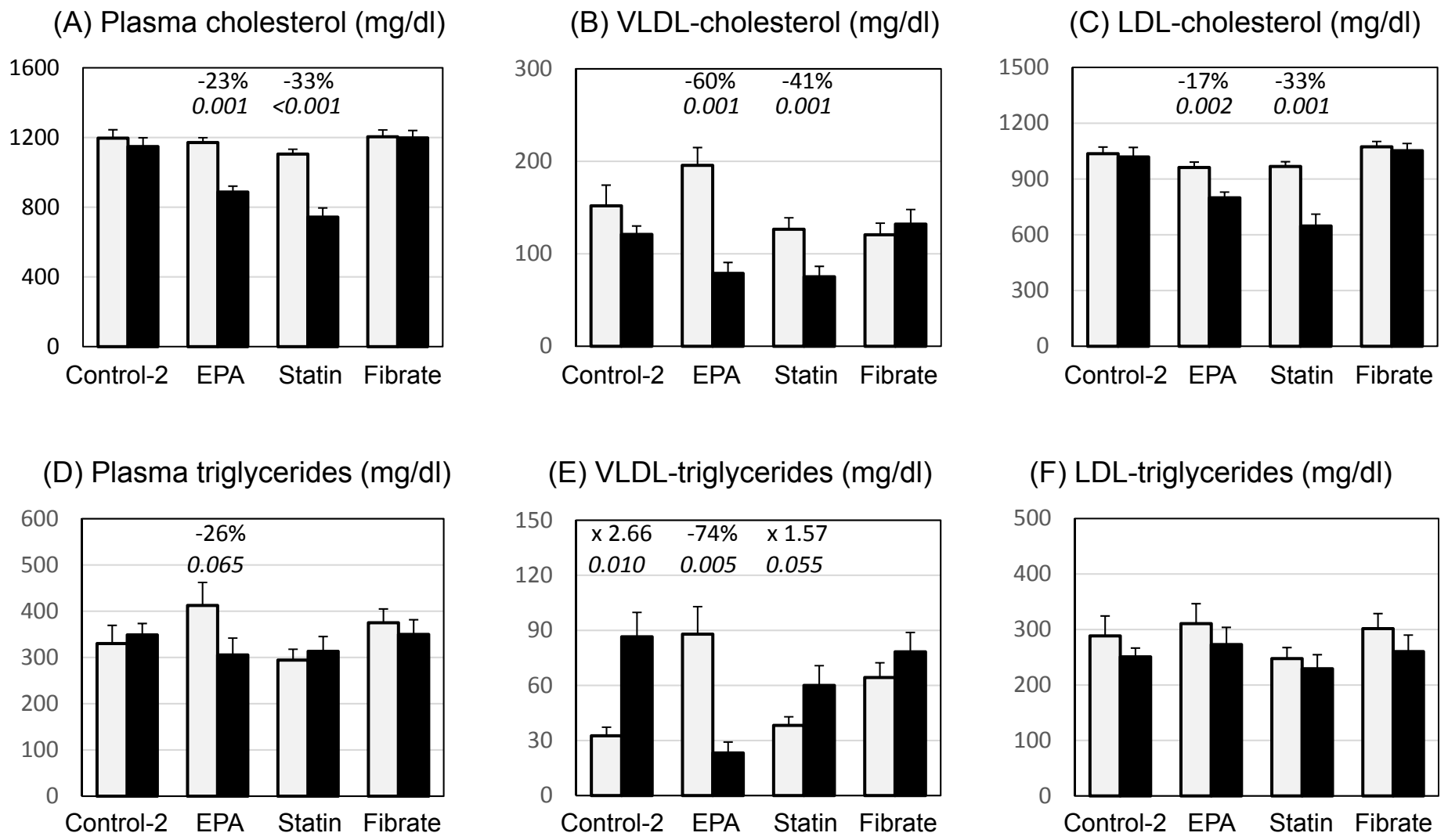

(A) Plasma cholesterol (mg/dl)
1600
1200
800
400
0
-23%
0.001
-33%
<0.001
Control-2
EPA
Statin
Fibrate
(B) VLDL-cholesterol (mg/dl)
300
200
100
0
-60%
0.001
-41%
0.001
Control-2
EPA
Statin
Fibrate
(C) LDL-cholesterol (mg/dl)
1500
1200
900
600
300
0
-17%
0.002
-33%
0.001
Control-2
EPA
Statin
Fibrate
(D) Plasma triglycerides (mg/dl)
600
500
400
300
200
100
0
-26%
0.065
Control-2
EPA
Statin
Fibrate
(E) VLDL-triglycerides (mg/dl)
150
120
90
60
30
0
x 2.66
0.010
-74%
0.005
x 1.57
0.055
Control-2
EPA
Statin
Fibrate
(F) LDL-triglycerides (mg/dl)
500
400
300
200
100
0
Control-2
EPA
Statin
Fibrate

Figure 3

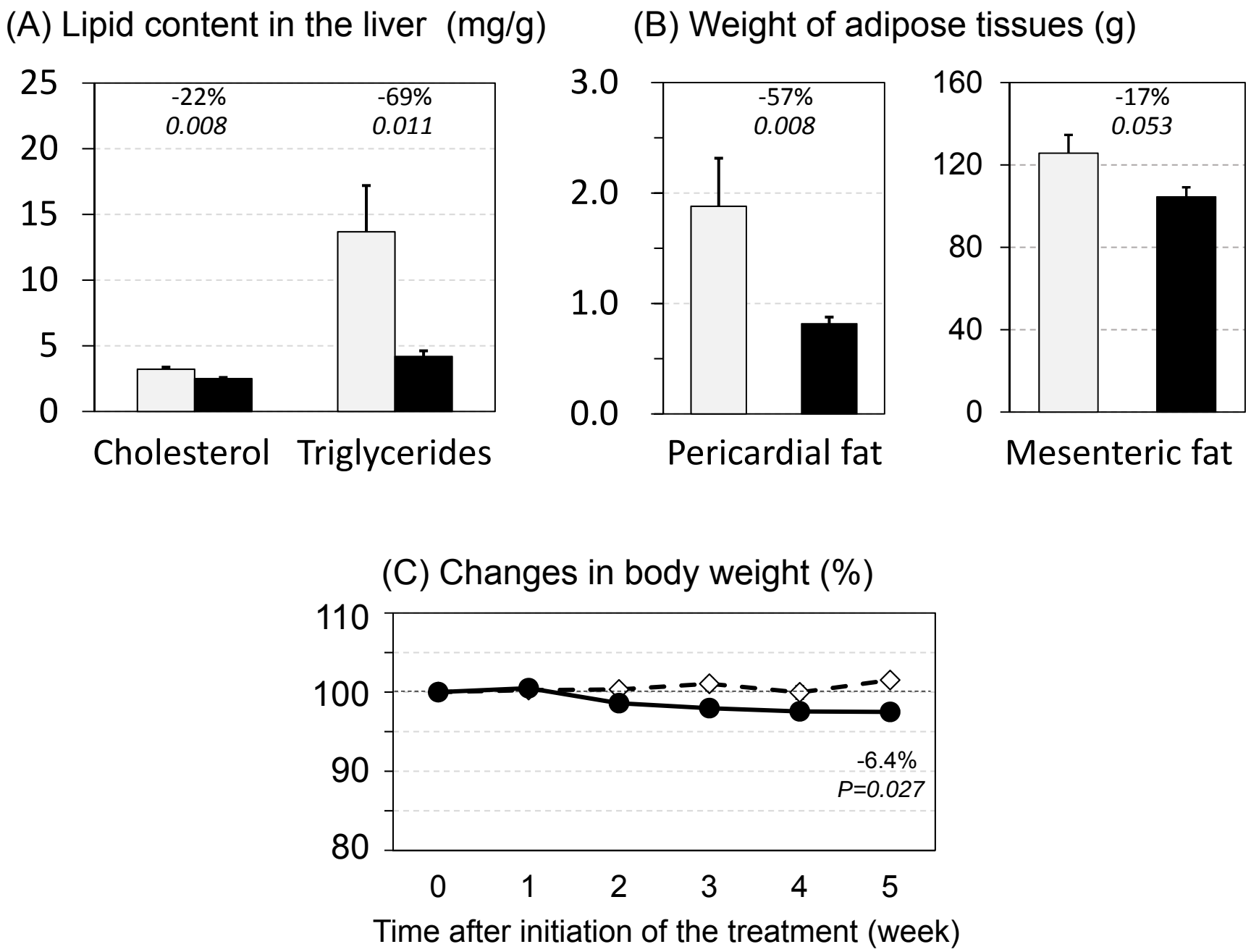

(A) Lipid content in the liver (mg/g)
25
20
15
10
5
0
-22%
0.008
-69%
0.011
Cholesterol
Triglycerides
(B) Weight of adipose tissues (g)
3.0
2.0
1.0
0.0
-57%
0.008
Pericardial fat
160
120
80
40
0
-17%
0.053
Mesenteric fat
(C) Changes in body weight (%)
110
100
90
80
-6.4%
P=0.027
0
1
2
3
4
5
Time after initiation of the treatment (week)

Figure 4

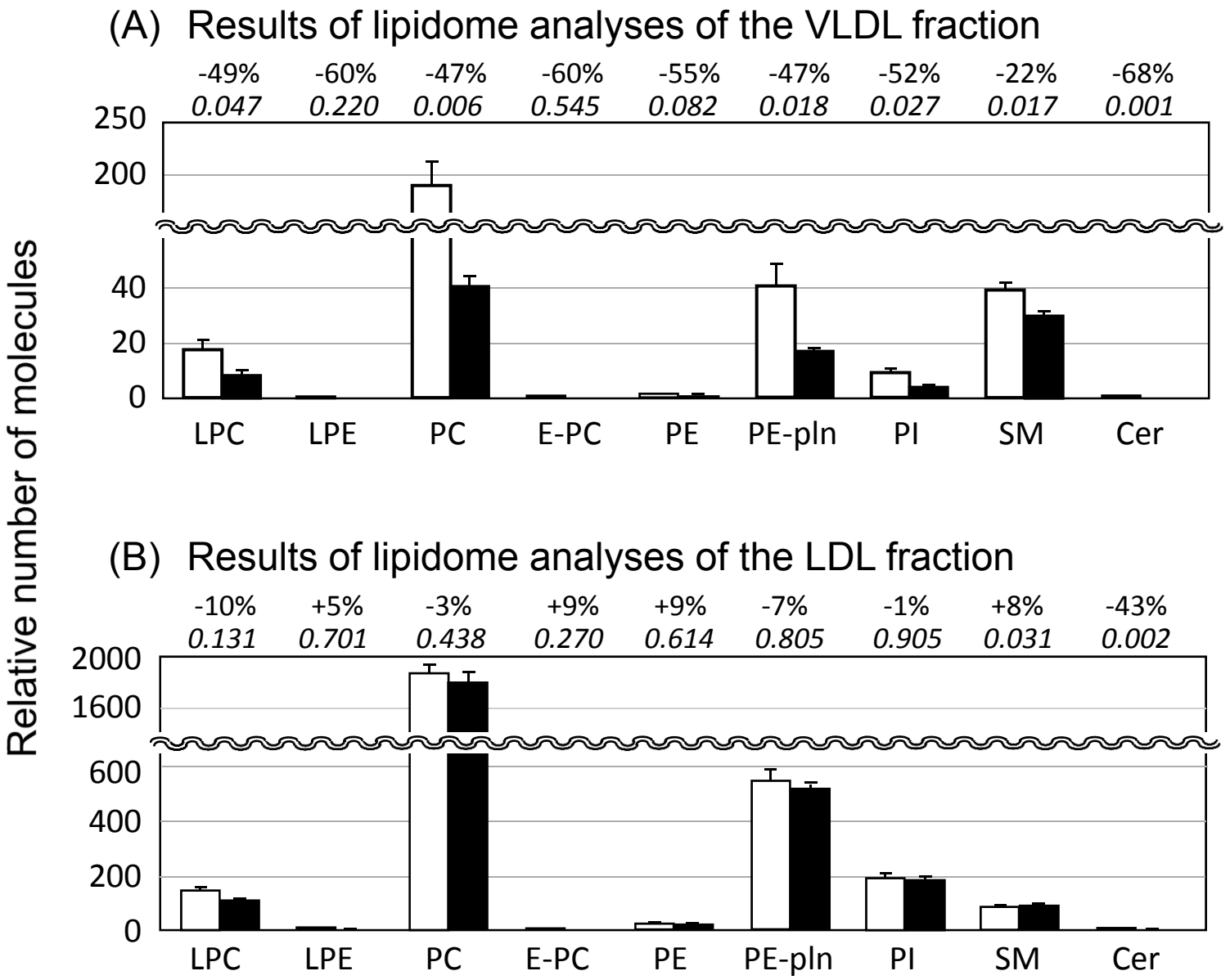
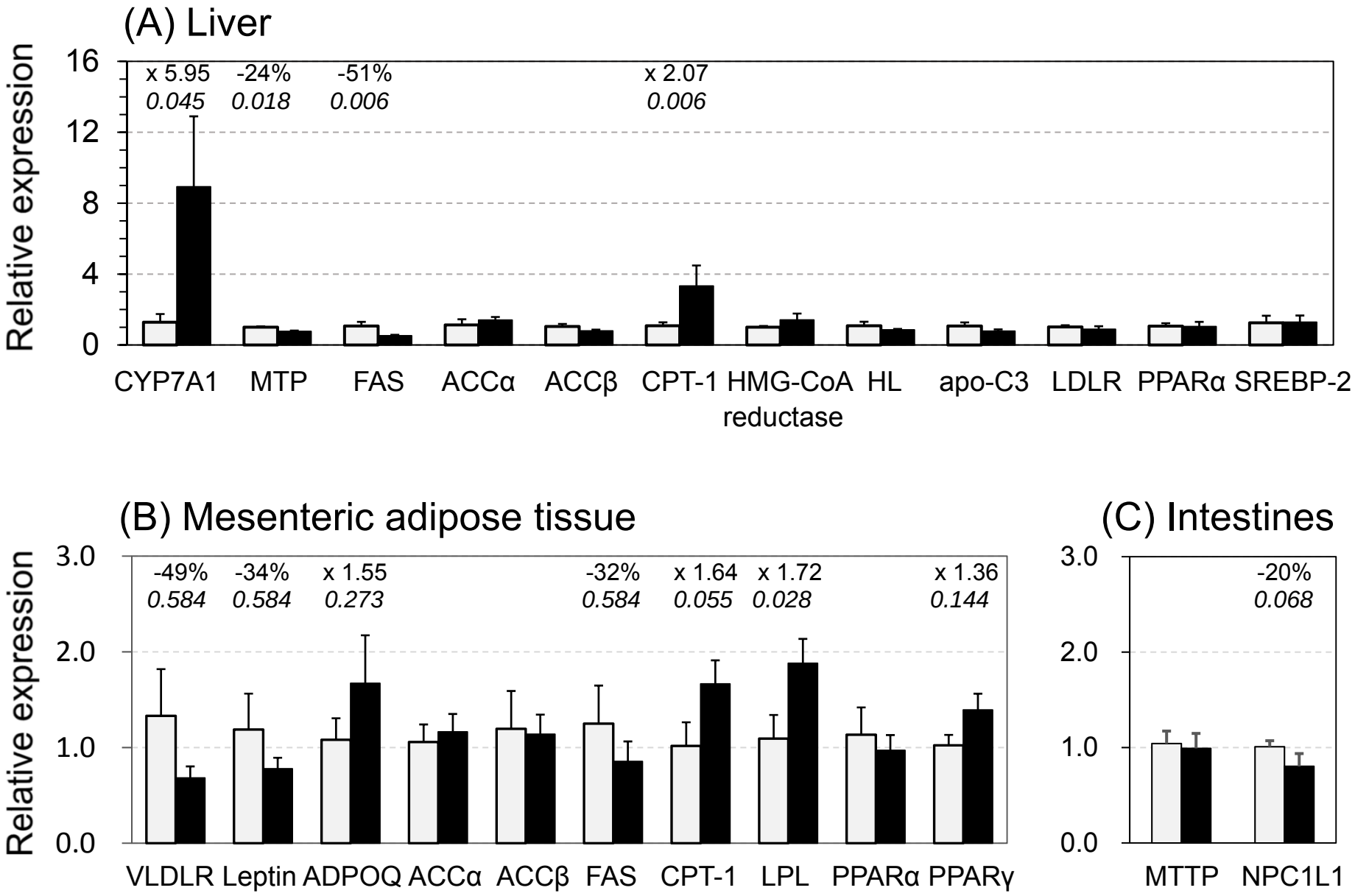

(A) Results of lipidome analyses of the VLDL fraction
-49% -60% -47% -60% -55% -47% -52% -22% -68%
0.047 0.220 0.006 0.545 0.082 0.018 0.027 0.017 0.001
250
200
40
20
0
Relative number of molecules
LPC LPE PC E-PC PE PE-pln PI SM Cer
(B) Results of lipidome analyses of the LDL fraction
-10% +5% -3% +9% +9% -7% -1% +8% -43%
0.131 0.701 0.438 0.270 0.614 0.805 0.905 0.031 0.002
2000
1600
600
400
200
0
LPC LPE PC E-PC PE PE-pln PI SM Cer

Figure 5


(A) Liver
Relative expression
16
12
8
4
0
x 5.95
0.045
-24%
0.018
-51%
0.006
x 2.07
0.006
CYP7A1
MTP
FAS
ACCα
ACCβ
CPT-1
HMG-CoA reductase
HL
apo-C3
LDLR
PPARα
SREBP-2
(B) Mesenteric adipose tissue
Relative expression
3.0
2.0
1.0
0.0
-49%
0.584
-34%
0.584
x 1.55
0.273
-32%
0.584
x 1.64
0.055
x 1.72
0.028
x 1.36
0.144
VLDLR
Leptin
ADPOQ
ACCα
ACCβ
FAS
CPT-1
LPL
PPARα
PPARγ
(C) Intestines
3.0
2.0
1.0
0.0
-20%
0.068
MTTP
NPC1L1

Supplemental Table 1. Solubility and plasma drug concentrations

Structural formula of the S-2E-Arg salt (drug substance of D-47)

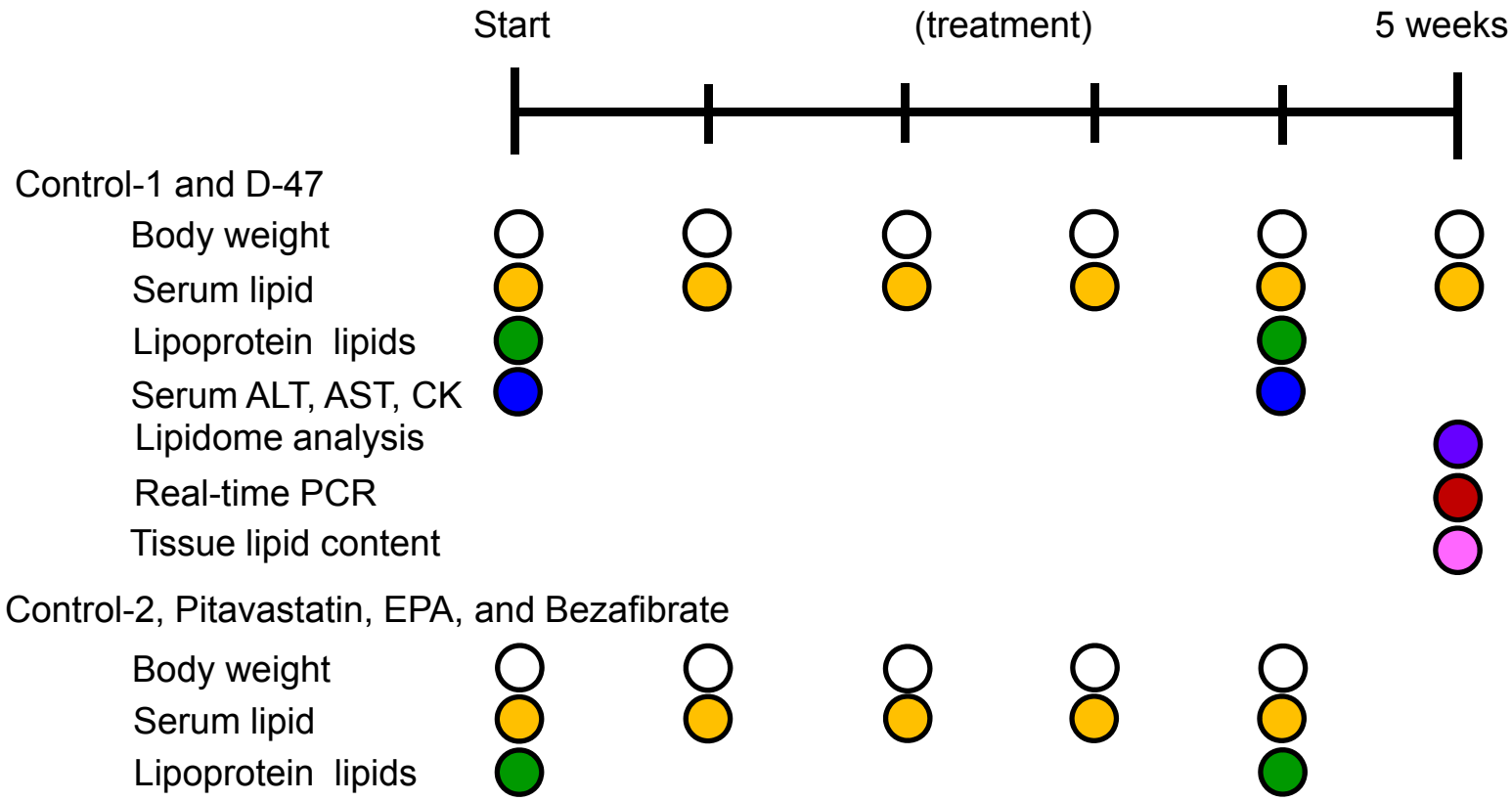
Solubility of S-2E, the S-2E-Arg-salt, and the solid dispersion (D-47)

	In JP-1 fluid (pH 1.2)	In JP-2 fluid (pH 6.8)	In JP-2 fluid treated with JP-1 fluid
S-2E	0.0%	2.4%	n.d.
The S-2E-Arg salt	0.1 %	19 %	n.d.
D-47	**n.d.**	**98 %**	**92 %**

Drug concentrations in plasma after oral administration

	Beagle dog			WHHLMI rabbits
	Tmax (h)	T-half (h)	Cmax (μg/ml)	Cmax (μg/ml)
S-2E	4	------	3.5	2.6±1.3
D-47	**2**	**3.7**	**13.2**	**10.0±0.6**

(Ogawa K et al. 136th Meeting of the Pharmaceutical Society of Japan, Mar 26-29, 2016)



Supplemental Fig. 1. Study design of D-47

In this study, we used WHHLMI rabbits, an animal model for human familial hypercholesterolemia.

Rabbits in D-47 treated group (n=6) were given drug supplemented chow at a dose of 30 mg/kg.
Control rabbits (n=6) were given standard chow. Chow was given to rabbits 120 g/day.

Supplemental Table 2. Internal standard substances used lipidome analyses

Lipid class	ISD conc. (μM)	Final conc. (nM)	Injection (fmol)	Total extraction volume (pmol)	Serum concentration (nmol/ml)
LPC 17:0	25	112	112	250	12. 5
LPE 17:1	500	2,243	2,244	4,999	250
PC 17:0-17:0	500	2,243	2,244	4,999	250
PE 17:0-17:0	200	1,794	1,795	3,999	200
PS 17:0-17:0	2,000	17,948	17,948	399,887	1,999
SM d18:1-17:0	5	22.4	22.4	50.0	2.50
Cer d18:1-17:0	5	22.4	22.4	50.0	2.50

ISD, internal standard; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, Phosphatidylethanolamine; PS, phosphatidylserine; SM, Sphingomyelin; Cer, Ceramide

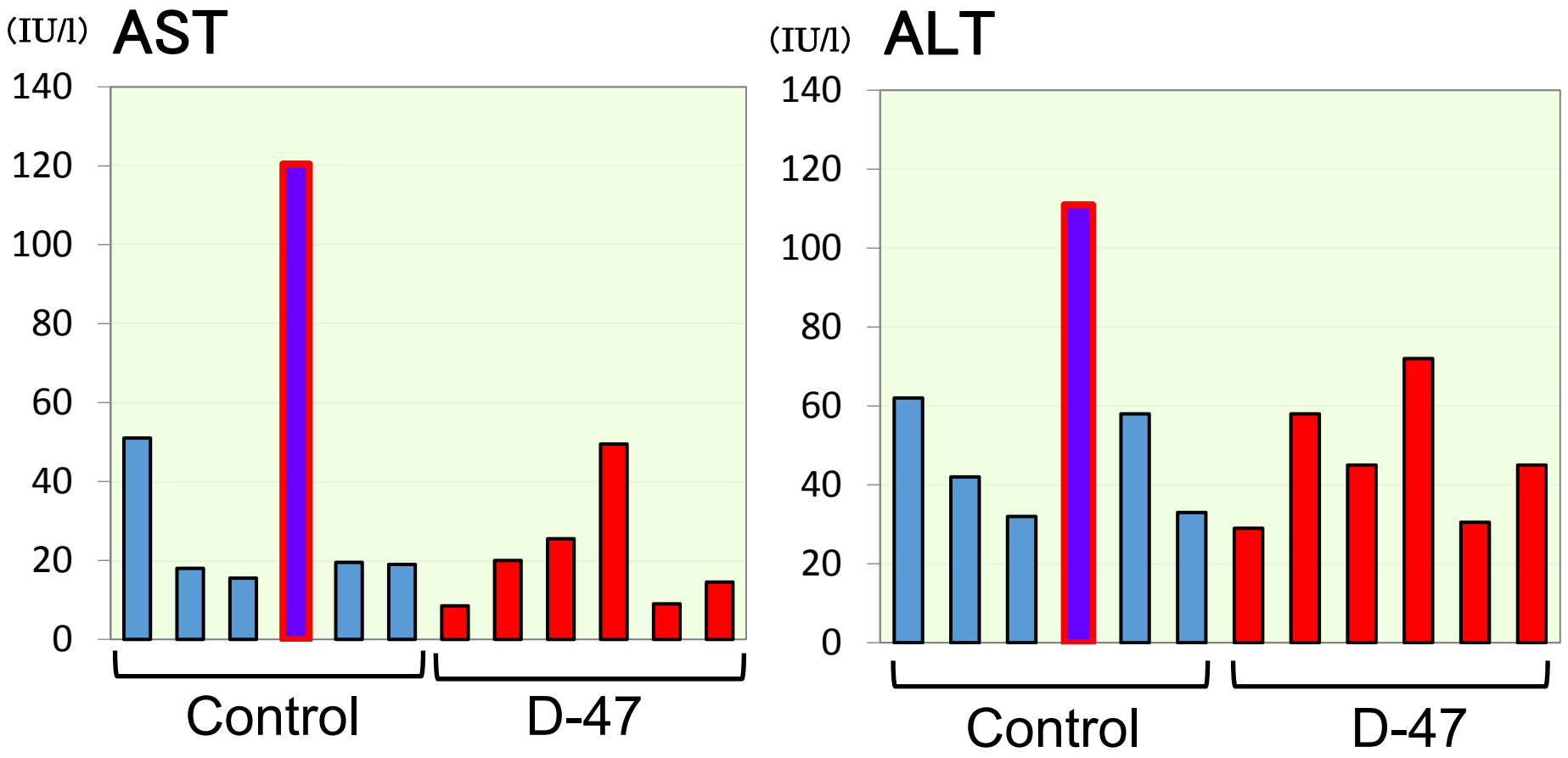
Supplemental Table 3. Primers for Real-time PCR

Rabbit mRNA	Former Primer	Revers Primer
MTP	aagcagagaagaaggaggacac	ctctgcagggtggtggtc
HMG-CoA reductase	ttgagccagaacgcagtttc	tggatccatcaagtgctcag
Acetyl-CoA carboxylase (ACC) α	ttggtgcttacatcgtggac	tcggcatacatctccatgtg
Acetyl-CoA carboxylase (ACC) β	tgttcgagttcatggagcag	acttgcaagcgaggattcag
FAS	aagacacatccggagaccag	tacccagatcccggtcatag
Carnitine Palmitoyltransferase (CPT)1	tctttctcttccgccaaacc	tcttgccgtgcatctcaaac
CYP7A1	tgagacgtcctgaaccaaagag	aatgctcgttgtgcgtcttg
ApoC3	atgcagggctatgtgcaac	aagtagcctttcagggagctg
LDLR	tcatgtactggaccgactgg	tggaaagatccagggtgatg
VLDLR	gatgaatccctggagcagtg	ttgacctcatcgctaccatc
NPC1L1	tgaaccgctactttgaggtg	tggcgtattggatcttctgg
Hepatic Lipase (HL)	tcttcattgactccctgctg	atgtggtagcccagtgtgttg
LPL	gggccacgttgacatttatc	cgatgaagaggtggatggag
Leptin	cgatgaagaggtggatggag	accacctccgtggagtagag
ADPOQ	gagatgcaggtcttgttggtc	tgctgagcggtagacatagg
SREBP-2	aagtgcccattaagcaggtg	accgcttctcgatgatgttg
PPAR-α	atgcatgtgaaggctgcaag	ttgtgaaagcggcagtactg
PPAR-γ	tgctgaatgtgaagcccatc	tcatcttctgcagcagcttg

Supplemental Table 4. Baseline data of WHHLMI rabbits in D-47 study.

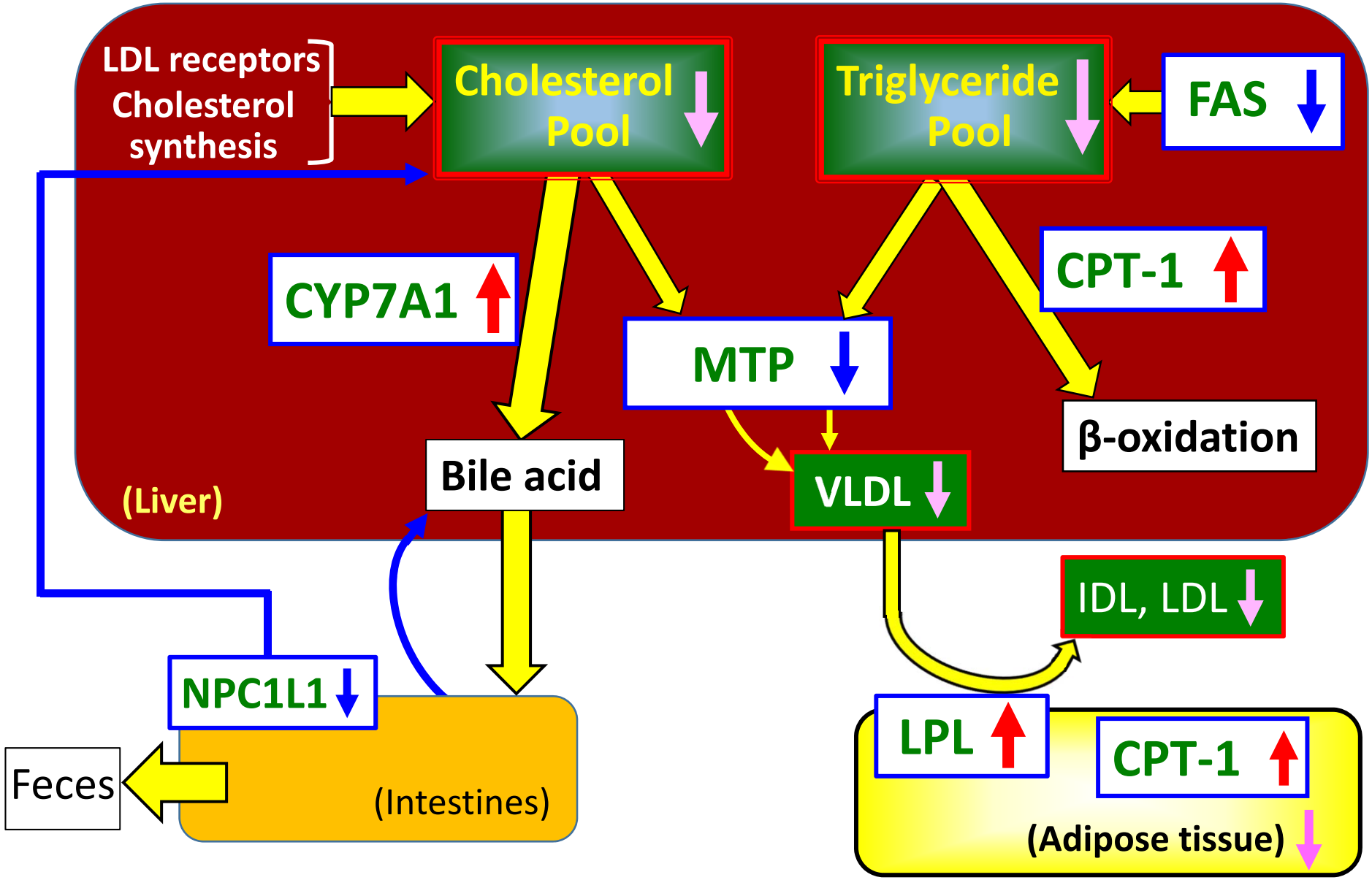
	Control	D-47	P-value
Examined rabbits	6	6	
Gender	female	female	
Age (months)	12	12	
Body weight (kg)	3.32±0.11	3.43±0.10	*0.491*
Serum lipid concentration (mg/dl)			
Total cholesterol	1,165±39	1,225±56	*0.299*
Triglyceride	414±49	429±44	*0.828*
Serum concentration of AST (IU/l)	40.6±16.9	21.2±6.3	*0.320*
Serum concentration of ALT (IU/l)	56.3±12.1	46.6±6.7	*0.496*
Serum concentration of CK (IU/l)	210±33	283±27	*0.116*

Data are represented as mean ± S.E.M. Statistical analyses were performed by Student t-test or Welch's t-test.



Supplemental Fig. 2. Serum AST and ALT levels at initiation of the treatment.

Data from a rabbit showing extremely high concentration of AST and ALT were deleted in analyses of this experiment.



Supplemental Fig. 3
Potential mechanism underlying lipid-lowering effects of D-47.