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MTP inhibitor decreases plasma cholesterol levels in LDL receptor-deficient WHHL rabbits by lowering the VLDL secretion

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Abstract

To examine whether a microsomal triglyceride transfer protein (MTP)-inhibitor is effective in patients with homozygous familial hypercholesterolemia, we administered (2*S*)-2-cyclopentyl-2-{4-[(2,4-dimethyl-9*H*-pyrido[2,3-*b*]indol-9-yl)methyl]phenyl}-*N*-[(1*S*)-2-hydroxy-1-phenylethyl]ethanamide (Implitapide), a new MTP inhibitor, to low-density lipoprotein (LDL)-receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits at doses of 3, 6, and 12 mg/kg for 4 weeks. In the 12 mg/kg group, the plasma cholesterol and triglyceride levels were decreased by 70% and 45%, respectively, and the very low-density lipoprotein (VLDL) secretion rate was decreased by 80%. The composition of newly secreted VLDL was similar in each group. This suggests that Implitapide diminished the number of VLDL particles secreted from the liver. Although the ratio of vitamin E/LDL was not altered by Implitapide, triglyceride accumulation and a decrease in vitamin E were observed in the liver. In conclusion, an inhibition of VLDL secretion led to a decrease of plasma LDL in WHHL rabbits, and MTP inhibitors should have hypolipidemic effects against homozygous familial hypercholesterolemia.

Keywords: MTP inhibitor; Implitapide; VLDL (very low-density lipoprotein) secretion; Hypolipidemic effect; WHHL, rabbit

1. Introduction

In patients with homozygous familial hypercholesterolemia, especially low-density lipoprotein (LDL)-receptor null type, therapy using inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statin), which competitively inhibits cholesterol biosynthesis and increase the LDL-receptor function, has almost no effect. To reduce the plasma cholesterol levels of homozygous familial hypercholesterolemia, reduction in the secretion of very low-density lipoprotein (VLDL)-cholesterol from the liver may be one of the important approaches.

Microsomal triglyceride transfer protein (MTP) plays an important role in the assembly of VLDL particles in the liver and of chylomicron particles in the intestine (Wetterau et al., 1992; Sharp et al., 1993; Sorbera et al., 2000). In vitro studies have demonstrated that if the assembly of VLDL is suppressed, secretion of the lipoproteins is reduced (Jamil et al., 1996; Gruetzmann et al., 2000). The main mechanisms of hypolipidemic effects of MTP inhibitors are largely different from inhibitors for HMG-CoA reductase. Therefore, MTP inhibitors have the possibility of lowering the plasma cholesterol levels of patients with homozygous familial hypercholesterolemia.

Recently, Wetterau et al. (1998) reported the effect of an MTP inhibitor on lipoprotein lipid levels and the triglyceride secretion rate using rats and hamsters. They also showed an MTP inhibitor that normalized the plasma lipid levels of homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, which is an LDL receptor-deficient animal model (Tanzawa et al., 1980; Kita et al., 1981). There are no original studies reporting whether MTP inhibitors suppress secretion of VLDL particles from the liver in vivo. Therefore, we attempted to examine the effects of an MTP inhibitor on the VLDL secretion rate, lipoprotein levels, and plasma vitamin E levels in homozygous WHHL rabbits, using another MTP inhibitor (2*S*)-2-cyclopentyl-2-{4-[(2,4-dimethyl-9*H*-pyrido[2,3-*b*]indol-9-yl)methyl]phenyl}-*N*-[(1*S*)-2-hydroxy-1-phenylethyl]ethanamide (Implitapide) (Sorbera et al., 2000).

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2. Materials and methods

2.1. Materials

Implitapide was provided by Bayer Yakuhin (Osaka, Japan). Triton WR-1339 (4-(1,1,3,3-tetramethylbutyl)phenol polymer with formaldehyde and oxirane) was purchased from Nakarai Tesque (Tokyo, Japan).

2.2. Animals

Twenty male homozygous WHHL rabbits (Shiomi et al., 1992) aged 6 months were divided into four groups, i.e., a placebo group and Implitapide-treated groups administered daily doses of 3, 6, and 12 mg/kg, respectively. Implitapide was suspended in 0.5% methylcellulose every day and was administered to WHHL rabbits orally for 4 weeks. All animal experimentation and care were conducted according to the Guidelines of Animal Experimentation of Kobe University.

2.3. Fractionation of plasma lipoprotein and measurement of lipid levels

After overnight fasting, blood samples were taken every week from the marginal ear vein. The plasma lipid levels were measured every week by enzymatic methods. Before and at the end of the treatment, lipoprotein was fractionated by ultracentrifugation to yield the following fractions: VLDL ($d < 1.006$ g/ml), intermediate-density lipoprotein (IDL, $1.006 < d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$ g/ml), and high-density lipoprotein (HDL, $d > 1.063$ g/ml).

2.4. Determination of VLDL secretion rate

At the end of Implitapide treatment, we determined the VLDL secretion rate (Shiomi et al., 1994; Shiomi and Ito, 1994). The VLDL secretion rate was determined by intravenous injection of Triton WR-1339 after overnight fasting to eliminate the influence of chylomicrons. It was reported that Triton WR-1339 blocks degradation of VLDL (Shotz et al., 1957; Borenztajn et al., 1976) and that the VLDL secretion rate determined by a Triton injection reflects well the secretion rate of VLDL in vivo (Guettet et al., 1989; Maeda et al., 1993). In addition, the apolipoprotein B of liver perfusates was contained almost exclusively in VLDL in WHHL rabbits (Yamada et al., 1987). Prior to Triton injection, Implitapide was administered to rabbits in the treated group. Triton WR-1339 at 200 mg/ml in 0.15 M NaCl solution was injected into an ear vein at a dose of 400 mg/kg body weight. Before and at 6 h after Triton injection, blood samples were obtained and the VLDL fraction was prepared by ultracentrifugation. The lipid concentration was measured enzymatically and the protein concentration was determined by the method of Lowry et al. (1951). The increased rate of VLDL was calculated by dividing the difference between the VLDLs obtained before and at 6 h after the Triton injection by 6 h.

2.5. Measurement of plasma vitamin E levels

Before and at the end of the Implitapide administration, the vitamin E levels were measured in the plasma samples by analysis in a commercial laboratory (SRL, Tokyo, Japan). Vitamin E was extracted with hexane, and α-, β-, and γ-tocopherol were separated and measured by high-performance liquid chromatography employing a column (Unisil Q NH_2, GL Science, Tokyo, Japan) and a spectrofluorometer (FP-821, JASCO, Tokyo, Japan).

2.6. Measurement of lipid contents in the liver

After the Triton experiment, rabbits were anesthetized by an intravenous injection of sodium pentobarbital (25 mg/kg) and perfused with saline solution. After the perfusion, the liver was excised. Using 5 g of the tissue, the lipid accumulated in the liver was extracted according to the method of Folch et al. (1957). The concentration of extracted lipid and vitamin E were determined as described above.

2.7. Statistical analysis

Values are presented as the means ± SEM. Statistical analysis was carried out by Williams–Shirley multiple comparison test. (Shirley, 1977).

3. Results

3.1. Effect of Implitapide on the plasma and lipoprotein lipid levels

All plasma lipid levels were decreased dose-dependently by Implitapide treatment. Comparing the highest dose group to the placebo group, the decrease in plasma lipid levels was 70% ($P < 0.01$) for cholesterol (20.4 ± 1.0 vs. 6.2 ± 0.8 mM) and 45% ($P < 0.01$) for triglyceride (2.0 ± 0.4 vs. 1.1 ± 0.1 mM). In Fig. 1, each lipoprotein containing apolipoprotein-B100 was decreased dose-dependently by Implitapide treatment. In the highest dose group, the VLDL fraction was markedly decreased: the decrease was 79% ($P < 0.01$) for cholesterol and 65% ($P < 0.05$) for triglyceride, and the decrease in the LDL fraction was similar to that of the plasma lipid levels. However, the HDL-lipid levels did not show significant changes.

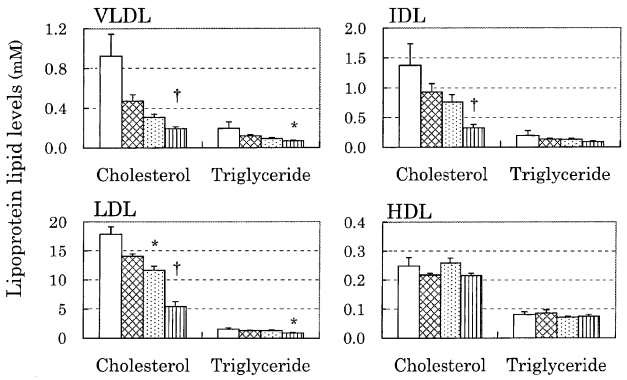

Fig. 1. Effects of Implitapide on the lipoprotein lipid levels of homozygous WHHL rabbits. □, placebo ($n = 5$); ▨, 3 mg/kg of Implitapide ($n = 5$); ▩, 6 mg/kg of Implitapide ($n = 5$); and ▥, 12 mg/kg of Implitapide ($n = 5$). Values are presented as means ± SEM. Statistical analyses were carried out with the Williams–Shirley multiple comparison test ($^{*}P < 0.05$; and $\dagger P < 0.01$ vs. placebo group). The plasma cholesterol levels were 20 ± 1 mM in the placebo group, 16 ± 1 mM in the 3 mg/kg group, 13 ± 1 mM in the 6 mg/kg group, and 6 ± 1 mM in the 12 mg/kg group.

3.2. Effect of Implitapide on VLDL secretion

The VLDL secretion rate was decreased dose-dependently (Fig. 2). Using all parameters, the VLDL secretion rate of the highest dose group was decreased significantly by about 80% ($P < 0.05$ or $P < 0.01$) compared with the placebo group. Fig. 3 shows the composition of newly secreted VLDL at 6 h after Triton injection. Guettet et al. (1989) and Maeda et al. (1993) reported that VLDL obtained after injection of Triton WR-1339 more closely resembled nascent VLDL rather than circulating VLDL. Therefore, VLDL obtained after Triton injection is considered newly secreted VLDL. The VLDL composition of

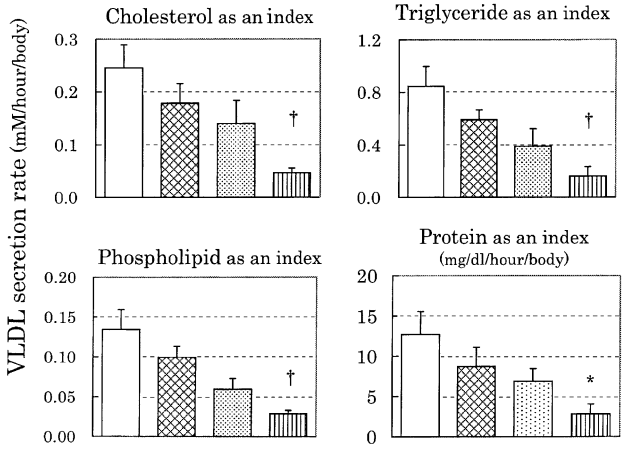

Fig. 2. Effects of Implitapide on the VLDL secretion rate of homozygous WHHL rabbits. □, placebo ($n = 5$); ▨, 3 mg/kg of Implitapide ($n = 5$); ▩, 6 mg/kg of Implitapide ($n = 5$); and ▥, 12 mg/kg of Implitapide ($n = 5$). Values are presented as means ± SEM. Statistical analyses were carried out with the Williams–Shirley multiple comparison test ($^{*}P < 0.05$ and $\dagger P < 0.01$ versus placebo group).

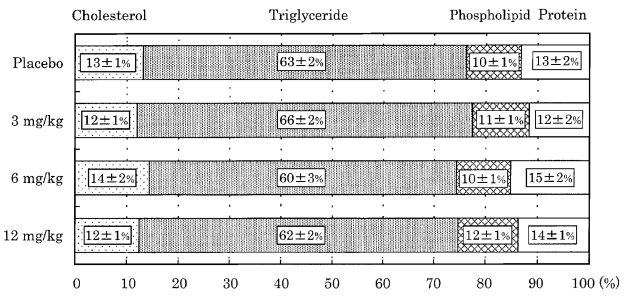

Fig. 3. Effects of Implitapide on the lipid composition of the newly secreted VLDL of homozygous WHHL rabbits. We analyzed the VLDL fraction obtained at 6 h after Triton injection as newly secreted VLDL. Values are presented as means ± SEM. There were no significant differences among the groups ($n = 5$) by the Williams–Shirley multiple comparison test.

newly secreted VLDL showed almost no changes in all the groups.

3.3. Plasma vitamin E levels

Before the experiment, the plasma α-tocopherol level was 64 ± 4 μM ($n = 20$) and the β- or γ-tocopherol levels were below 1 μM. At the end of the treatment (Fig. 4), the plasma α-tocopherol level in the highest dosage group was decreased by 76% ($P < 0.01$) compared with the placebo group. The decrease was dose-dependent. However, the plasma α-tocopherol level was closely correlated with the LDL-cholesterol level ($r = 0.8842$, $P < 0.001$). In addition, the ratio of plasma α-tocopherol/plasma LDL-cholesterol was 2.9 ± 0.3 ($n = 5$) in the highest dose group at the end of the treatment, and this was similar to the ratio

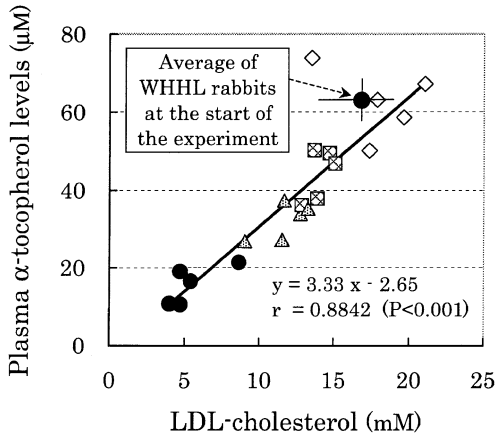

Fig. 4. Relation between the plasma α-tocopherol levels and the LDL cholesterol levels of homozygous WHHL rabbits treated with Implitapide, an MTP inhibitor. ◇, placebo ($n = 5$); ▨, 3 mg/kg of Implitapide ($n = 5$); △, 6 mg/kg of Implitapide ($n = 5$); and ●, 12 mg/kg of Implitapide ($n = 5$). The plasma α-tocopherol levels were 62 ± 4 μM in the placebo group, 44 ± 3 μM in the 3 mg/kg group, 31 ± 2 μM in the 6 mg/kg group, and 15 ± 2 μM in the 12 mg/kg group.

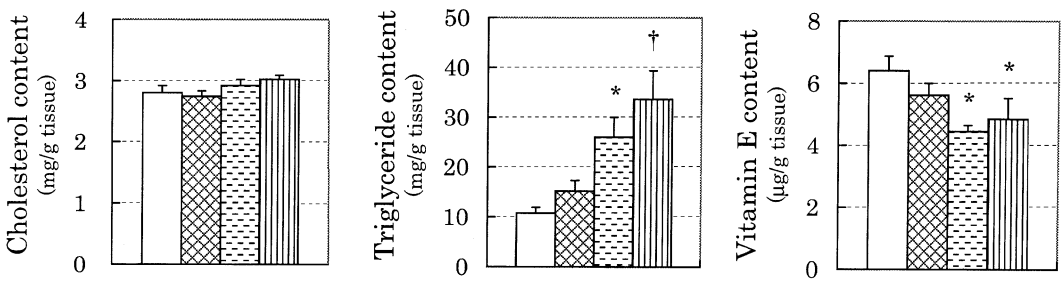

Fig. 5. Accumulation of lipids and α-tocopherol in the livers of homozygous WHHL rabbits treated with Implitapide, an MTP inhibitor. □, placebo ($n = 5$); ▨, 3 mg/kg of Implitapide ($n = 5$); ▩, 6 mg/kg of Implitapide ($n = 5$); and ▥, 12 mg/kg of Implitapide ($n = 5$). Values are presented as means ± SEM. Statistical analyses were carried out with the Williams–Shirley multiple comparison test ($^{*}P < 0.05$ and $\dagger P < 0.01$ vs. placebo group).

in each group. This suggests that reduction in the plasma α-tocopherol levels by Implitapide was due to the reduction in the plasma LDL levels.

3.4. Lipid accumulation in the liver

Fig. 5 shows accumulation of lipids and α-tocopherol in the liver after 4 weeks administration of Implitapide. Although the cholesterol content was similar in each group, the triglyceride accumulation was increased dose-dependently. In the highest dose group, the triglyceride content was about three-fold greater than that of the placebo group. In addition, the α-tocopherol content was decreased dose-dependently.

4. Discussion

In this study, we examined whether inhibition of VLDL secretion by MTP inhibitor administration could reduce the plasma cholesterol levels even in the LDL receptor-deficient state. We found that 12 mg/kg of Implitapide markedly decreased the level of atherogenic apolipoprotein-B100-containing lipoproteins and the VLDL secretion rate in homozygous WHHL rabbits. This suggests that a potent inhibition of VLDL secretion led to a marked decrease in LDL in the plasma even in the LDL-receptor deficient state.

A previous study by Wetterau et al. (1998) reported the effect of another MTP inhibitor on the triglyceride secretion rate but not on the VLDL secretion rate. Implitapide reduced the VLDL secretion rate by about 80%, using not only cholesterol as an index but also triglyceride or protein as an index, and did not affect the VLDL composition. These results suggest that Implitapide decreased the number of VLDL particles secreted from the liver. This is a novel observation in vivo, although in vitro studies demonstrated that MTP inhibitors reduced apolipoprotein-B secretion from HepG2 cells by an inhibition in the assembly of VLDL particles (Jamil et al., 1996; Wetterau et al., 1998). The present findings suggest that the potent hypolipidemic effect of Implitapide was mainly due to suppression of VLDL particle secretion from the liver.

In the statin treatment, the hypolipidemic effects of statins are mainly the induction of the LDL-receptor function in patients with normal LDL-receptor function. In WHHL rabbit studies using statins, the hypolipidemic effects were due to an induction of LDL-receptor mRNA (Kuroda et al., 1992) and a reduction of cholesterol content in the VLDL secreted from the liver (Shiomi et al., 1994; Shiomi and Ito, 1994). In this latter case, the secretion of VLDL-triglyceride was not affected despite the reduction in the secretion of VLDL-cholesterol. Although the reduction in VLDL-cholesterol secretion was about 20% in the statin-treated WHHL rabbits (Shiomi et al., 1994; Shiomi and Ito, 1994), Implitapide (12 mg/kg) deceased the VLDL secretion by about 80% in the present study. Implitapide was very efficient in the suppression of VLDL secretion compared with statin treatments. Therefore, this compound has the possibility of being an effective agent for patients with homozygous familial hypercholesterolemia, although statins have almost no effects.

Wetterau et al. (1998) and Sorbera et al. (2000) reported MTP inhibitors decreased HDL levels. In the present study, the HDL cholesterol level did not show any significant decrease. Although we have no findings to explain this disagreement, this difference may be due to the very low HDL levels in WHHL rabbits compared with normal rabbits.

The plasma vitamin E levels were decreased by the MTP inhibitor treatment. This decrease was due to a reduction in LDL. It is well known that vitamin E binds to lipoproteins (Cohn et al., 1992). The ratio of vitamin E/LDL-cholesterol did not decrease in the present study. In addition, the plasma vitamin E level of age-matched normal rabbits was about one-third of the highest dose group of the present study (data not shown). This suggests that MTP inhibitors do not interfere with antioxidative effects on LDL particle.

In the present study, we did not examine any side effects of Implitapide because the purpose of this study was to examine the effects on the VLDL secretion and lipoprotein levels in the LDL-receptor deficient state. In the experimental period, no rabbits show steatorrhoea or other clinical findings, and there were no significant differ-

ences in food consumption or body weight changes in each group. However, in examination of lipid accumulation in the liver, Implitapide showed a dose-dependent increase in the triglyceride levels and a dose-dependent decrease in the α-tocopherol levels despite the normal external appearance. Since the liver triglyceride content of hamsters treated with another MTP inhibitor was increased temporally (Wetterau et al., 1998), it may be inappropriate to conclude that the present 4 weeks treatment demonstrates that Implitapide treatment causes fatty liver. At the dose of 3 mg/kg, Implitapide decreased plasma cholesterol levels by 23% (20.4 ± 1.0 vs. 15.7 ± 0.5 mM, $P = 0.003$ by Student's *t*-test). At this dose, the liver triglyceride and α-tocopherol levels did not show any significant changes compared with the placebo group. Although Implitapide showed potent hypolipidemic effects, it is important to select a safe dose and to measure activities of plasma enzymes, which reflect liver damage.

It is well known that there are no effective compounds to reduce the plasma cholesterol levels of patients with homozygous familial hypercholesterolemia, especially LDL-receptor null type. However, the present results suggest that potent inhibition of VLDL secretion can reduce the plasma cholesterol levels of homozygous WHHL rabbits, and MTP inhibitors are thus considered candidates as compounds effective for treatment of homozygous familial hypercholesterolemia patients.

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References

Borenztajn, J., Roune, M.S., Kotlar, T.J., 1976. The inhibition in vivo of lipoprotein lipase (clearing-factor lipase) activity by Triton WR-1339. Biochem. J. 156, 539–543.

Cohn, W., Goss-Sampson, M.A., Grun, H., Muller, D.P.R., 1992. Plasma clearance and net uptake of α-tocopherol and low-density lipoprotein by tissues in WHHL and control rabbits. Biochem. J. 287, 247–254.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Gruetzmann, R., Beuck, M., Mueller, U., Nielsch, U., 2000. Bay 13-9952 (Implitapide), an inhibitor of the microsomal triglyceride transfer protein (MTP), blocks secretion of apo-B-lipoproteins. Atherosclerosis 151 (Suppl.) 91–92 (Abstract).

Guettet, C., Mathe, D., Navarro, N., Lecuyer, B., 1989. Effects of chronic glucagons administration on rat lipoprotein composition. Biochim. Biophys. Acta 1005, 233–238.

Jamil, H., Gordon, D.A., Eustice, D.C., Brooks, C.M., Dickson Jr., J.K., Chen, Y., Ricci, B., Chu, C.H., Harrity, T.W., Ciosek Jr., C.P., Biller, S.A., Gregg, R.E., Wetterau, J.R., 1996. An inhibitor of the microsomal triglyceride transfer protein inhibits apoB secretion from HepG2 cells. Proc. Natl. Acad. Sci. U. S. A. 93, 11991–11995.

Kita, T., Brown, M.S., Watanabe, Y., Goldstein, J.L., 1981. Deficiency of low density lipoprotein receptors in liver and adrenal gland of the WHHL rabbit, an animal model of familial hypercholesterolemia. Proc. Natl. Acad. Sci. U. S. A. 78, 2268–2272.

Kuroda, M., Matsumoto, A., Itakura, H., Watanabe, Y., Ito, T., Shiomi, M., Fukushige, J., Nara, F., Fukami, M., Tsujita, Y., 1992. Effects of pravastatin sodium alone and in combination with cholestyramine on hepatic, intestinal and adrenal low density lipoprotein receptors in homozygous Watanabe heritable hyperlipidemic rabbits. Jpn. J. Pharmacol. 59, 65–70.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maeda, E., Yoshino, G., Matsuba, M., Nagata, K., Morita, M., Murata, Y., Naka, Y., Kazumi, T., 1993. Effect of a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor on triglyceride kinetics in chronically streptozotocin-diabetic rats. Metabolism 42, 1–8.

Sharp, D., Blinderman, L., Combs, K.A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C.M., Turck, C.W., Bouma, M.E., Rader, D.J., Aggerbeck, L.P., Gregg, R.E., Gordon, D.A., Wetterau, J.R., 1993. Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. Nature 365, 65–69.

Shiomi, M., Ito, T., 1994. Pravastatin sodium, a competitive inhibitor of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase, decreased the cholesterol content of newly secreted very-low-density lipoprotein in Watanabe heritable hyperlipidemic rabbits. Metabolism 43, 559–564.

Shiomi, M., Ito, T., Shiraishi, M., Watanabe, Y., 1992. Inheritability of atherosclerosis and the role of lipoproteins as risk factors in the development of atherosclerosis in WHHL rabbits: risk factors related to coronary atherosclerosis are different from those related to aortic atherosclerosis. Atherosclerosis 96, 43–52.

Shiomi, M., Shiraishi, M., Yata, T., Ito, T., 1994. Effect of fluvastatin sodium on secretion of very low density lipoprotein and serum cholesterol levels. Arzneim.-Forsch. 44, 1154–1156.

Shirley, E., 1977. A non-parametric equivalent of Williams' test for containing increasing dose level of a treatment. Biometrics 33, 386–389.

Shotz, M.C., Scanu, A.M., Page, I.H., 1957. Effect of Triton on lipoprotein lipase of rat plasma. Am. J. Physiol. 188, 399–402.

Sorbera, L.A., Martin, L., Silvestre, J., Castaner, J., 2000. Implitapide. Drugs Future 25, 1138–1144.

Tanzawa, K., Shimada, Y., Kuroda, M., Tsujita, Y., Arai, M., Watanabe, Y., 1980. WHHL-rabbit: a low density lipoprotein receptor-deficient animal model for familial hypercholesterolemia. FEBS Lett. 118, 81–84.

Wetterau, J.R., Aggerbeck, L.P., Bouma, M.E., Eisenberg, C., Munck, A., Hermier, M., Schmitz, J., Gay, G., Rader, D.J., Gregg, R.E., 1992. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 258, 999–1001.

Wetterau, J.R., Gregg, R.E., Harrity, T.W., Arbeeny, C., Cap, M., Connoly, F., Chu, C.H., George, R.J., Gordon, D.A., Jamil, H., Jolibois, K.G., Kunselman, L.K., Lan, S.J., Maccagnan, T.J., Ricci, B., Yan, M., Young, D., Chen, Y., Fryszman, O.M., Logan, J.V.H., Musial, C.L., Poss, M.A., Robl, J.A., Simpkins, L.M., Slusarchyk, W.A., Sulsky, R., Taunk, P., Magnin, D.R., Tino, J.A., Lawrence, R.M., Dickson, J.K., Biller, S.A., 1998. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. Science 282, 751–754.

Yamada, N., Shames, D.M., Havel, R.J., 1987. Effect of low density lipoprotein receptor deficiency on the metabolism of apolipoprotein B-100 in blood plasma. J. Clin. Invest. 80, 507–515.