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Studies on Hypolipidemic Agents. III.[1] Comparison of the Hypolipidemic Properties of 5-Tridecylpyrazole-3-carboxylic Acid and Clofibrate[2]

KUNIO SEKI,* MINORU FUKUDA, JUNICHI ISEGAWA and MASAHIKO OHKI

Research Laboratories, Morishita Pharmaceutical Co., Ltd., 1658, Ohshinohara, Yasu-cho, Yasu-gun, Shiga 520–23, Japan

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In the hyperlipidemia induced by fructose in rats, 5-tridecylpyrazole-3-carboxylic acid (TDPC) reduced serum triglyceride (TG) and cholesterol (CH) levels dose-dependently. The hypolipidemic profile of this compound was quite similar to that of clofibrate. In a time-course experiment, TDPC lowered serum lipid levels shortly after the treatment, and the maximum effect was observed at 4—8 h, which is quite similar to the action of clofibrate. However, there were some differences between TDPC and clofibrate in the effects on lipoprotein patterns; TDPC increased the ratio of high density lipoprotein (HDL) to very low density lipoprotein + lower density lipoprotein (VLDL + LDL)-CH in a dose-dependent manner but clofibrate did not. The hypolipidemic effect of TDPC was due to a decrease in serum lower density lipoprotein rather than high density lipoprotein. In addition, TDPC remarkably increased the rate of disappearance of exogenous triglyceride. No adverse effects of TDPC were found on parameters such as relative liver weight and hepatic lipid content.

Keywords——5-tridecylpyrazole-3-carboxylic acid; clofibrate; hypertriglyceridemic rat; hypolipidemic activity; lipoprotein cholesterol; triglyceride catabolism; triglyceride secretion

In the previous paper,[1] we reported the synthesis and hypolipidemic activity of various alkylpyrazole derivatives. Of these derivatives, 5-tridecylpyrazole-3-carboxylic acid (TDPC) was found to have a potent hypolipidemic activity in hypertriglyceridemic rats, with low toxicity. This paper reports the results of detailed experiments on the hypolipidemic actions of TDPC in the hypertriglyceridemic model in comparison with those of clofibrate, which is the most widely used hypolipidemic drug.

Results and Discussion

Hyperlipidemia induced by fructose in rats was characterized by an increase in serum triglyceride (TG) or very low density lipoprotein (VLDL), on the basis of lipoprotein analysis by disc electrophoresis (not shown) and ultracentrifugal methods (Table II). It is widely known that excessive intake of carbohydrate induces in humans, a certain type of hyperlipidemia (type IV).[3] Fructose-induced hyperlipidemia is relatively similar to human type IV hyperlipidemia in some respects, and in addition this model is useful because of its high reproducibility and the short-time required. Thus the fructose model is considered to be very useful for evaluation of hypolipidemic activities of test compounds. Another advantage of this model is that it seems more physiological than other systems[4,5] (*e.g.* with triton WR-1339 and glycerol), which have been widely used as assay systems for hypolipidemic agents.

In the hyperlipidemia, TDPC (10—300 mg/kg) reduced serum TG and cholesterol (CH) levels dose-dependently as shown in Table I. There was no statistically significant difference in body weight or intake of drinking fluid, which exerts a marked influence on the serum lipid

TABLE I. Hypolipidemic Activities of TDPC and Clofibrate in Hypertriglyceridemic Rats

Treatment	Dose (mg/kg)	No. of rats	Serum lipids (mg/dl) TG	Serum lipids (mg/dl) CH
Normal	—	30	85.4±3.8[a]	53.4±1.3
Control	—	60	170.5±5.5	56.7±1.3
TDPC	10	30	153.5±2.1 (10.0)[b]	54.4±2.4 (4.0)
	20	30	131.5±1.9[d] (22.9)	50.5±2.7[c] (11.0)
	40	30	101.4±1.6[e] (40.5)	47.5±2.1[d] (16.2)
	75	30	84.7±2.3[e] (50.3)	41.8±1.1[e] (26.2)
	150	30	73.7±2.1[e] (56.8)	36.5±1.3[e] (35.7)
	300	30	41.8±1.5[e] (75.5)	26.5±0.6[e] (53.2)
	ED_{50} (mg/kg)[f]		81.7	291.1
Clofibrate	10	24	160.3±3.0 (6.0)	56.5±2.5
	20	24	130.9±2.4[d] (23.2)	51.3±2.0[c] (9.6)
	40	24	114.1±2.0[e] (33.1)	44.8±1.9[d] (21.0)
	75	24	87.6±2.2[e] (48.6)	39.0±1.7[e] (31.3)
	150	24	75.5±1.9[e] (55.7)	31.6±1.1[e] (44.3)
	300	24	63.6±1.7[e] (62.7)	29.3±0.7[e] (48.3)
	ED_{50} (mg/kg)[f]		112.9	255.5

Rats were allowed free access to the standard diet and 10% fructose solution for 2 d. The agents were administered orally to the rats at 0 (immediately), 24 and 42 h after provision of fructose solution, respectively. Six h after the last dose, blood samples were obtained after decapitation of the animals.

a) Each value represents the mean±S.E. of 5—6 experiments.

b) Values in parenthesis represent the percent reduction from the control.

Significantly different from the control by Student's *t*-test (*c*) $p<0.05$, *d*) $p<0.01$, *e*) $p<0.001$).

f) This is the dose of agent required to produce 50% reduction of the serum lipid levels.

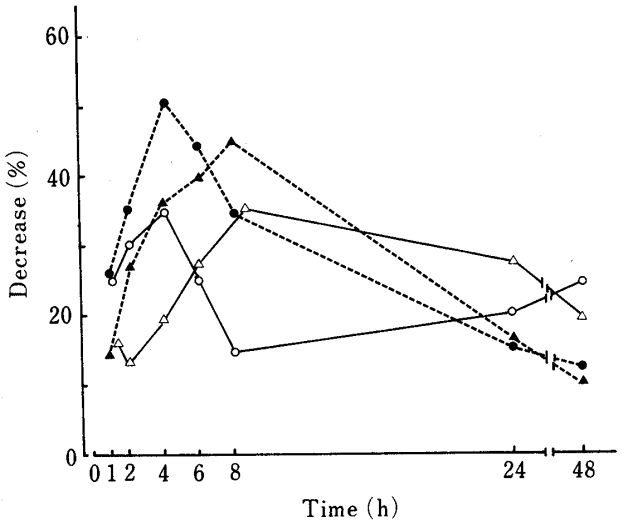

Fig. 1. Time Course of Changes in Hypolipidemic Activity after Single Administration (150 mg/kg) of TDPC or Clofibrate in Hypertriglyceridemic Rats

Rats were allowed free access to the standard diet and 10% fructose solution for 2 d. Blood samples were obtained after decapitation at the times indicated on the graph after single administration. Each point indicates the percent reduction calculated from mean values of 6 rats.

(---●---) and (---▲---); TG and CH reduction (%) in TDPC-treated rats.

(—○—) and (—△—); TG and CH reduction (%) in clofibrate-treated rats.

levels, between treated and untreated groups (not shown). The minimal effective doses of TDPC and clofibrate which produced a statistically significant decrease were 20 mg/kg for both lipids. When compared on the basis of 50% effective dose (ED_{50}, mg/kg), the hypotriglyceridemic effect of TDPC (81.7 mg/kg) was 1.4-fold higher than that of clofibrate (112.9 mg/kg). On the other hand, the hypocholesterolemic effect of TDPC (291.1 mg/kg) was slightly lower than that of clofibrate (255.5 mg/kg). Thus, the hypotriglyceridemic effects of the two agents were greater than the effects on serum CH. In an overall comparison of the

activities of both agents, TDPC appears to be as effective as clofibrate in hypertriglyceridemic rats.

Since a definite effect of TDPC on serum lipid levels could be observed by using this model, we made a detailed comparison of the duration of hypolipidemic actions of both agents in order to investigate the bioavailability and mechanism of action of TDPC. Figure 1 shows the time course of the changes in hypolipidemic activity after a single administration of TDPC or clofibrate (150 mg/kg). Decreases in serum TG and CH were observed immediately after the oral administration of TDPC. A clear hypolipidemic effect of TDPC was observed during 24 h with the maximal effects on TG and CH at 4 and 8 h after the treatment, respectively. Although the overall change of activity of TDPC was quite similar to that of clofibrate, its hypocholesterolemic effect was sustained better than that of clofibrate.

TABLE II. Effects of TDPC and Clofibrate on CH Distribution in Lipoproteins in Hypertriglyceridemic Rats

Treatment	Dose (mg/kg)	No. of rats	CH (mg/dl)			B/A−B
			Total (A)	HDL (B)	VLDL+LDL[a] (A−B)	
Normal	—	12	52±1.8[c]	32±1.2	20±1.7[d]	1.8±0.1[d]
Control	—	24	59±1.5	30±0.6	29±1.5	1.1±0.1
TDPC	75	12	49±2.7[c]	27±0.9[b]	22±2.3[c]	1.4±0.1[b]
	150	12	35±1.5[d]	26±0.6[d]	9±1.4[d]	3.6±0.6[d]
	300	12	24±1.0[d]	23±0.5[d]	2±0.5[d]	18.9±2.6[d]
Clofibrate	75	6	39±2.8[d]	20±1.2[d]	19±1.9[c]	1.1±0.1
	150	6	32±1.9[d]	15±0.6[d]	16±2.2[c]	1.0±0.2
	300	6	26±2.2[d]	12±1.0[d]	14±1.8[d]	0.9±0.1

For details concerning the experimental conditions see the footnote to Table I or the experimental section.
a) (VLDL+LDL)-CH level estimated as the difference of total CH and HDL-CH levels.
Significantly different from the control by Student's *t*-test (*b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$).

TABLE III. Effects of TDPC and Clofibrate on TG and CH Levels in Lipoproteins of Hypertriglyceridemic Rats

Lipids in lipoproteins[d]		Treatment			
		Normal	Control	TDPC (300 mg/kg)	Clofibrate (300 mg/kg)
Serum	TG	86.5±2.9[c]	159.2±6.2	27.3±3.3[c]	76.1±10.5[c]
	CH	48.7±0.8	50.0±1.6	25.8±2.5[c]	27.0±2.5[c]
VLDL	TG	72.8±0.9[c]	144.5±9.5	20.9±3.5[c]	50.8±8.0[c]
	CH	8.8±1.3[b]	14.3±1.3	1.5±0.4[c]	9.2±1.6
LDL	TG	6.8±1.7	6.3±1.5	4.4±1.2	10.5±3.0
	CH	9.4±0.2[a]	13.0±1.0	1.2±0.5[c]	8.0±1.0[b]
HDL	TG	5.1±0.4	4.6±1.2	4.4±1.7	4.8±1.3
	CH	33.7±1.0	29.4±2.0	20.1±2.2[b]	9.7±0.7[c]

For details concerning the experimental conditions see the footnote to Table I or the experimental section. Portions of the samples in Table II were employed for ultracentrifugal analysis of lipoprotein.
a) Values of lipid in lipoproteins are based on determinations of 3 pools of 2 rats/pool.
Significantly different from the control by Student's *t*-test (*b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$).

The finding that TDPC produced a very rapid reduction of serum lipid may imply that this compound is absorbed rapidly through the intestinal tract.

Many hypolipidemic agents are reported to alter lipoprotein CH distribution and/or lipoprotein pattern.[6] Therefore, the influence of TDPC on the lipoprotein CH distribution in this model was examined by two methods as described in the experimental section. Table II shows the results of analysis of lipoprotein CH by the precipitation method in rats treated with 75, 150 and 300 mg/kg of TDPC or clofibrate. TDPC and clofibrate decreased in a dose-dependent manner the total CH, high density lipoprotein (HDL)-CH and lower density lipoprotein (VLDL+LDL)-CH levels; the last of these was determined as the difference between total CH and HDL-CH levels. There was no marked difference in the total CH and HDL-CH lowering effects of clofibrate, whereas TDPC decreased total CH more effectively than HDL-CH. Consequently, the ratio of HDL-CH to (VLDL+LDL)-CH increased remarkably in a dose-dependent manner after treatment with TDPC, but did not with clofibrate (control, 1.1 ± 0.1; 150 mg/kg TDPC, 3.6 ± 0.6, $p < 0.001$; 300 mg/kg TDPC, 18.9 ± 2.6, $p < 0.001$; 300 mg/kg clofibrate, 0.9 ± 0.1).

To confirm this effect of TDPC the lipoprotein fractions obtained at high dosages of both agents were also analyzed by the combination of ultracentrifugal fractionation and lipid analysis. As shown in Table III, the effects of the two agents on CH distribution in lipoprotein were found to be similar to those described above.

The results suggest that the hypocholesterolemic effect of TDPC is due to a decrease in CH level in VLDL and LDL rather than in HDL. It is especially noteworthy that these two agents are different with regard to the magnitude of their effects on the HDL-CH level. This indicates that the hypocholesterolemic activity of TDPC in rats may be mediated by a different mechanism from that of clofibrate. However, the detailed mechanism of TDPC action is not clear at present.

Moreover, lipoprotein analysis revealed that the decrease in serum TG by TDPC was mainly due to the decrease of TG in VLDL. The mechanism of TG reduction by TDPC is not known, but a preliminary study showed that TDPC remarkably promoted the disappearance rate of TG which was intravenously administered as a fat emulsion in rats, as shown in Fig. 2. That is to say, the half-time ($t_{1/2}$) for the disappearance of exogenous TG was 24.9 ± 0.7 min in TDPC-treated rats and 46.8 ± 4.2 min in the control. The results suggest that the acceleration of TG catabolism may account for a part of the hypolipidemic action of TDPC.

We next attempted to determine whether the hypolipidemic effect of TDPC results in

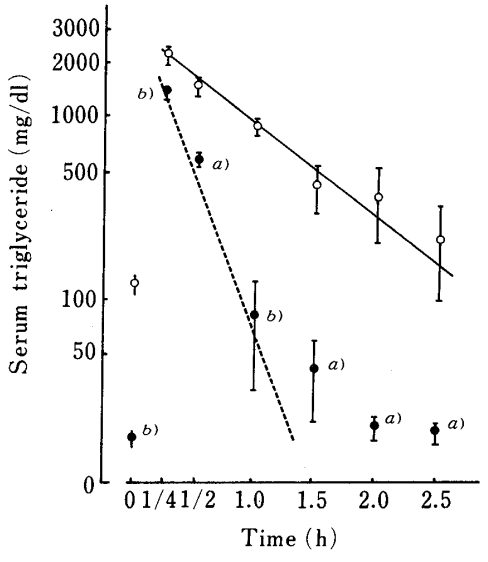

Fig. 2. Disappearance Rate ($t_{1/2}, K_2$) of Exogenous Triglyceride in Normal (—○—) and TDPC-treated (---●---) Rats

The half-times ($t_{1/2}$) for normal and TDPC-treated rats were 46.8 ± 4.2 and 24.9 ± 0.7 min, respectively ($p < 0.01$). The disappearance rates (K_2) for normal and TDPC-treated rats were 1.53 ± 0.14 and 2.80 ± 0.08%/min, respectively ($p < 0.01$). Significantly different from the control by Student's t-test. (*a*) $p < 0.01$, (*b*) $p < 0.001$).

TABLE IV. Effects of TDPC and Clofibrate on the Rate of TG Secretion from the Liver in Hypertriglyceridemic Rats

Treatment	Dose (mg/kg)	No. of rats	TG concentration (mg/ml)			Estimated rate of TG secretion (mg/min)
			Before Triton	2 h after Triton	Increment in 2 h	
Normal	—	6	$0.79 \pm 0.14^{a)}$	$5.88 \pm 0.19^{b)}$	$7.75 \pm 0.21^{b)}$	$0.48 \pm 0.03^{b)}$
Control	—	12	1.25 ± 0.07	9.02 ± 0.22	5.09 ± 0.25	0.73 ± 0.22
TDPC	300	6	$0.32 \pm 0.03^{b)}$	$7.89 \pm 0.22^{a)}$	7.20 ± 0.20	0.72 ± 0.02
Clofibrate	300	6	$0.53 \pm 0.07^{b)}$	$4.39 \pm 0.57^{b)}$	$3.86 \pm 0.51^{b)}$	$0.35 \pm 0.05^{b)}$

For details concerning the experimental conditions see the footnote to Table I or the experimental section. Triton WR-1339 (800 mg/kg) was injected into the tail vein at 4 h after the last dose of agents. Blood samples were obtained from the carotid by the method described in the experimental section prior to the injection of Triton and 2 h later.
Significantly different from the control by Student's t-test (a) $p<0.01$, b) $p<0.001$).

TABLE V. Influence of TDPC and Clofibrate on Liver Weight and Lipid Content in Hypertriglyceridemic Rats

Treatment	Dose (mg/kg)	Liver weight (% of b.w.)	Lipids (mg)	
			TG	CH
Normal	—	4.4 ± 0.1	114 ± 8	21.4 ± 0.8
Control	—	4.6 ± 0.1	170 ± 6	24.2 ± 0.4
TDPC	150	4.5 ± 0.2	152 ± 8	23.6 ± 1.1
	300	4.5 ± 0.1	175 ± 23	22.4 ± 1.1
Clofibrate	150	$5.1 \pm 0.1^{a)}$	176 ± 12	$29.2 \pm 0.9^{b)}$
	300	$5.3 \pm 0.1^{b)}$	182 ± 16	$29.2 \pm 2.5^{a)}$

For details concerning the experimental conditions see the footnote to Table I or the experimental section. After decapitation of rats, the liver was immediately removed, washed, blotted, weighed and kept at $-20\,^{\circ}C$ until used for analysis of lipid.
Significantly different from the control by Student's t-test (a) $p<0.05$, b) $p<0.01$).

suppression of the rate of liver TG secretion, which might be the rate-limiting step in determining the serum lipid levels. Table IV shows the changes in the rate of TG secretion in rats treated with 300 mg/kg of TDPC or clofibrate. The rate of TG secretion under conditions of lipoprotein lipase inhibition with Triton WR-1339 decreased remarkably in clofibrate-treated rats, but did not in TDPC-treated rats. Therefore, the serum lipid-lowering action exerted by TDPC may not be due to an altered distribution of lipid between the liver and serum.

Hepatomegaly is a well-known side effect of hypolipidemic drugs such as clofibrate and its derivatives.[7] Table V shows the changes in liver weight and lipid content of rats treated with test agents, which were administered orally to the rats three times over 2 d. As expected, clofibrate at dose levels of 150 and 300 mg/kg increased the relative liver weight by 10 and 15%, respectively. In contrast, TDPC caused no hepatomegaly at a dose of 300 mg/kg, which is about 15 times the effective dose. Thus, a noticeable difference was observed between the effects of TDPC and clofibrate on the liver weight.

In addition, the hepatic lipid content was not significantly affected by TDPC treatment, while clofibrate significantly increased the CH content.

In conclusion, the present study demonstrated that TDPC can exert a hypolipidemic effect comparable to that of clofibrate, and that there are substantial differences between these

two agents with respect to their effects on CH distribution in lipoprotein, rate of liver TG secretion, liver weight and hepatic lipid content.

Further work is in progress to clarify the mechanism of action of TDPC and to compare it with other pharmacologically active agents in various experimental models. These results will be reported elsewhere.

Experimental

Materials——Animals: Male Sprague-Dawley rats weighing 175—185 g were purchased from Charles River Japan, Inc., and maintained on commercial laboratory chow (CE-II, Nipon Clea) for at least 1 week before use.

Chemicals: TDPC was synthesized in this laboratory as a colorless crystalline powder. Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) was also synthesized by us. Triton WR-1339 was purchased from Ruger Chemicals Co., Inc. (U.S.A.). Fructose was purchased from Nakarai Chemicals, Ltd.

Animal Experiments——Hypolipidemic Activity: Male rats were employed in this study. Fructose induction of hypertriglyceridemia were carried out by the method described previously.[1] TDPC was administered by gastric intubation with 1% carboxymethylcellulose as vehicle. Except in examination of the duration of hypolipidemic activity, TDPC was administered immediately after the provision of fructose solution, second and third doses were administered 24 and 42 h after the first dose, respectively. Six hours after the last dose, blood samples for serum lipids and lipoprotein analysis were taken after decapitation of the animals. The liver was immediately removed, washed, blotted, weighed and kept at −20 °C until used for analysis of lipid.

Measurement of Disappearance Rate of Exogenous TG——Male rats were allocated to experimental groups of 6 animals. The commercial diet containing 0.3% TDPC was given to rats for 10 d *ad libitum*. Animals were fasted for 4 h prior to the experiment. Base-line blood samples (0.1 ml) were drawn with a syringe from the carotid of unanesthetized rats in a CFK restraining apparatus (CFK Technology Institute, Tokyo) and then Intralipos® (Green Cross, Co.) was injected into the tail vein (1 ml/100 g body weight). Blood samples (0.1 ml) were obtained from the carotid by the above-mentioned method at 1/4, 1/2, 1, 1.5, 2.0 and 2.5 h later. Serum TG levels at each point were plotted on semilogarithmic graph paper to estimate the half-time ($t_{1/2}$) for the disappearance of TG. The rate of disappearance (K_2) was calculated according to the following formula.

$$K_2(\%/\text{min}) = 0.693/t_{1/2} \times 100$$

Measurement of Secretion Rate of TG[8]——TDPC-treated hypertriglyceridemic rats were employed in this study. TG secretion rates were estimated by injecting Triton WR-1339 into rats and measuring TG accumulation in the serum during the following 2 h. Base-line blood samples were obtained from the carotid by the above-mentioned method and then Triton WR-1339 was injected intravenously (800 mg/4 ml/kg). Further blood samples were also obtained by the above-mentioned method 2 h later. The increment in serum TG during the 2 h period provides an estimate of the rate of TG secretion.

$$\text{secretion rate of TG (mg/min)} = \text{TG increment (mg/ml)} \times \text{plasma volume (ml)}/120\,\text{min}$$

Plasma volumes of six additional animals under the same conditions were estimated by the standard dye dilution technique after the intravenous injection of Evans blue; the average volume obtained by this method was found to be 5.34 ml/100 g body weight, and this figure was used in the above formula to calculate the secretion rate of TG.

Analytical Procedures——Serum CH and TG were measured by an enzymatic method using commercial reagent sets (Determiner TC, Kyowa Medex Co., Ltd.; Triglyceride G-test Wako, Wako Pure Chemicals). Lipoprotein fractionation was performed by an isoelectric method (Isopol, International Reagent Co.) and by ultracentrifugation in a discontinuous sucrose gradient.[9] In the former method, the lower density lipoprotein CH levels were estimated as the difference between total CH and HDL-CH levels.

The ultracentrifugal separation of lipoprotein was achieved by the method of Kudo *et al.*[9] Sucrose (0.5 g), KBr (0.4 g) and NaCl (0.1 g) were added to 2.0 ml of serum to make a final density (d) = 1.21, and 1.0 ml of d = 1.21 solution [sucrose (20 g), KBr (15 g) and NaCl (5 g)/100 ml (H_2O)] was stirred gently, then centrifuged for 20 h at 50000 rpm (Hitachi 65 P). Next, 2.0 ml of d = 1.063 solution (6 w/v% in sucrose) and 2.5 ml of d = 1.006 solution (2.5 w/v% in sucrose) were overlayered and the gradient was centrifuged for 4 h. The lipoprotein fractions were collected by using a tube slicer (model TSU 2, Hitachi Koki Co., Ltd.), and samples were taken up with a capillary pipette. CH and TG in lipoproteins were determined by the above-mentioned methods.

For the determination of hepatic lipids, 0.5 g of wet tissue was extracted with 10 ml of isopropanol using a Polytron (Kinematica GmBH, Switzerland), and CH and TG in the extracts were determined by the above-mentioned methods.

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