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Studies on Hypolipidemic Agents. IV.[1] Influence of a New Hypolipidemic Agent, 5-Tridecylpyrazole-3-carboxylic Acid, on Cholesterol Metabolism in Rats

KUNIO SEKI,* MINORU FUKUDA and MASAHIKO OHKI

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

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The comparative effects of 5-tridecylpyrazole-3-carboxylic acid (TDPC) and clofibrate or β-sitosterol on cholesterol (CH) metabolism were investigated in normal and hyperlipidemic rats. The present study demonstrated that TDPC possesses a potent hypocholesterolemic activity in both types of rats, and also improves the α/β-lipoprotein ratio and anti-atherogenic index in hyperlipidemic rats. Although TDPC at 0.3% caused an increase of liver weight in both types of rats, the adverse effect of TDPC was less than that of clofibrate in normal rats. TDPC, like clofibrate, remarkably suppressed sterol biosynthesis from [^{14}C]acetate. Dual-isotope experiments suggested that TDPC (300 mg/kg) and β-sitosterol (100 mg/kg) inhibit the intestinal absorption of CH, though the effect was not statistically significant.

It is apparent from the data presented that TDPC, unlike clofibrate, exerts its hypocholesterolemic effect by multiple modes of action, including the inhibition of both synthesis and absorption of CH.

Keywords——5-tridecylpyrazole-3-carboxylic acid; clofibrate; hypocholesterolemic activity; lipoprotein; normal rat; hyperlipidemic rat; sterol biosynthesis; cholesterol absorption

Introduction

5-Tridecylpyrazole-3-carboxylic acid (TDPC) was selected from among a series of analogues on the basis of the results of pharmacological and acute toxicological tests in rats. The compounds can be classified as a member of a new type of hypolipidemic agents structurally unrelated to clofibrate, which is the most widely used hypolipidemic drug. In the previous paper,[1,2] we demonstrated that TDPC has hypolipidemic activity comparable to that of clofibrate, and that there is a major difference between these two agents in terms of effect on cholesterol (CH) distribution in lipoproteins; TDPC enhanced the ratio of high density lipoprotein (HDL)-CH to lower density lipoprotein (VLDL+LDL)-CH but clofibrate did not.

In assessment of the hypolipidemic properties of an active compounds, it is considered to be important to employ more than one animal model. In this work, we investigated the effects of TDPC on serum CH and lipoprotein levels in normal and hyperlipidemic rats. The effects of TDPC on sterol biosynthesis and intestinal absorption of CH were also investigated to clarify the mechanism of hypocholesterolemic action.

Results

1) Effect on Normal Rats

TDPC as well as clofibrate reduced serum total CH in a dose-dependent manner. Addtion of TDPC to the diet at levels of 0.1% and 0.3% significantly reduced serum CH levels

TABLE I. Effect of TDPC on the Serum CH and Liver Weight in Normal Rats

Treatment		Serum CH	Liver weight
Agent	% in diet	(mg/dl)	(% of body wt.)
Control	—	47.8±2.7	4.5±0.2
TDPC	0.03	44.7±2.2	4.5±0.1
	0.1	30.0±1.4[b]	4.5±0.1
	0.3	10.1±1.2[b]	5.4±0.1[a]
Clofibrate	0.03	41.7±2.2	4.5±0.1
	0.1	29.5±1.2[b]	4.9±0.2
	0.3	26.7±0.9[b]	6.5±0.1[a]

The rats were allowed free access to the powdered CE-II diet containing test agent for 10 d. The animals were sacrified at 4 h after withdrawing the diets. For details concerning the experimental methods, see the experimental section. Each value represents the mean±S.E. of 6 rats. Significantly different from the control by Student's *t*-test. *a*) $p<0.01$. *b*) $p<0.001$.

TABLE II. Effect of TDPC on the Serum CH Level, Anti-atherogenic Index, α/β-Lipoprotein and Liver Weight in Hyperlipidemic Rats

Treatment		Serum CH (mg/dl)		Anti-atherogenic index[a] (%)	α/β-LP[b] (%)	Liver weight (% of body wt.)
Agent	% in diet	Total	HDL			
Normal	—	54± 2.7[e]	20±1.0[e]	71±11[e]	406±47[e]	4.3±0.1[e]
Control	—	205± 8.8	11±0.7	6± 1	20± 1	5.8±0.1
TDPC	0.03	180± 6.2[c]	8±0.7[c]	5± 1	19± 1	5.7±0.1
	0.1	135± 9.2[e]	10±0.5	9± 1[c]	30± 3[d]	5.8±0.1
	0.3	26± 1.2[e]	7±0.5[e]	33± 2[e]	250±18[e]	6.4±0.2[d]
β-Sitosterol						
	0.3	145±11.2[d]	15±0.8[c]	12± 1[e]	36± 3[e]	5.4±0.1[c]
	1.0	108± 7.0[e]	17±1.7[e]	18± 3[d]	47± 4[e]	5.3±0.1[e]
	3.0	77± 4.6[e]	16±1.1[e]	28± 2[e]	74± 6[e]	4.9±0.2[e]

The rats were allowed free access to the experimental diet containing cholesterol and test agent for 10 d. Details concerning the experimental methods are given in the experimental section. *a*) Anti-atherogenic index = ratio of HDL-CH to total CH minus HDL-CH ×100. *b*) Ratio of intensities of α- to β-lipoprotein bands ×100. Each value represents the mean±S.E. of 8 rats. Significantly different from the control by Student's *t*-test. *c*) $p<0.05$. *d*) $p<0.01$. *e*) $p<0.001$.

by 37% and 79%, respectively. TDPC was more active than clofibrate at the 0.3% level, though its activity was identical with that of clofibrate at 0.1%. TDPC and clofibrate significantly increased the relative liver weight at high dose, but did not do so at the 0.1% level.

2) Effect on Cholesterol-Induced Hyperlipidemia

In rats fed the cholesterol-cholate diet, serum total CH increased to four times that in normal rats, and this change was accompanied with marked changes in serum α/β-lipoprotein ratio and CH distribution (anti-atherogenic index), as can be seen in Table II. In this model of hyperlipidemia, the elevation of serum total CH was inhibited in a dose-dependent manner by treatment with TDPC or β-sitosterol. The minimal effective dose of TDPC was 0.03% in the diet; this corresponds approximately to a daily oral intake of 30 mg/kg. In particular, the serum total CH level (26 mg/dl) or rats treated with the highest dose (0.3% in diet) was much lower than that of normal rats (54 mg/dl). As a whole, the hypolipidemic activity of TDPC was much higher than that of β-sitosterol, which is a typical inhibitor of CH absorption. The results of analysis of serum lipoprotein in hyperlipidemic rats are shown in the right column in

Table II. As can be seen from the normal and control values, this animal model is characterized by a decrease in HDL-CH (11 mg/dl); the ratio of HDL- to (VLDL+LDL)-CH levels (anti-atherogenic index) was 6% and the ratio of α- to β-lipoprotein band intensities (α/β-LP) was 20%. The corresponding values for normal rats were 20 mg/dl, 71% and 406%, respectively.

Treatment with TDPC significantly improved these parameters toward normal levels, though the HDL-CH level declined. These parameters were also improved by β-sitosterol in a dose-dependent manner.

TDPC showed a hepatomegalic effect at a dose of 0.3%, which is about 10 times the effective dose in this model. In contrast, β-sitosterol caused a significant decrease in the relative liver weight even at a fairly high dose.

3) Effect on Incorporation of [1-^{14}C]Acetate into Sterol

Table III shows the effects of TDPC and clofibrate on [1-^{14}C]acetate incorporation into sterol. The data indicate that reversal of the lighting cycle (light period; 19:00—07:00) caused a two- or three-fold increase in the sterol synthetic rates in comparison with a daylight-dependent lighting cycle (light period; 07:00—19:00), normal group. In this case, TDPC and clofibrate showed a dose-dependent inhibitory effect on the incorporation of labelled acetate into sterol in serum. TDPC at doses of 30, 100 and 300 mg/kg significantly decreased the radioactivity in serum (dpm/ml) by 44% ($p<0.01$), 68% ($p<0.001$) and 81% ($p<0.001$), respectively. Similarly, the incorporation of labelled acetate into sterol in the liver was strongly inhibited by these two agents. The inhibitory activity of TDPC was, however, more potent than that of clofibrate in both the serum and liver.

4) Effect on Cholesterol Absorption

Table IV shows the effects of TDPC and β-sitosterol on CH absorption measured by a dual-isotope ratio method in normal rats. Treatment with TDPC (300 mg/kg) or β-sitosterol (100 mg/kg) moderately inhibited the CH absorption, but the effects did not prove to be

Table III. Effect of TDPC on Sterol Biosynthesis from [1-^{14}C]Acetate

Treatment		Radioactivity (dpm)			
		Serum		Liver	
Agent	Dose (mg/kg)	dpm/ml	dpm/mg sterol	dpm/g	dpm/mg sterol
Normal[a]	—	348± 17[c]	1799±197[c]	3790± 411[b]	3177±393[b]
Control	—	1189±100 (100)	3564±442 (100)	7073±1219 (100)	5376±864 (100)
TDPC	30	663±102[c] (55.8)	3039±494 (85.3)	4754± 568 (67.2)	4065±644 (75.6)
	100	376± 86[d] (31.6)	1696±331[c] (47.6)	2035± 578[c] (28.8)	1324±592[c] (24.6)
	300	222± 36[d] (18.7)	1516±273[c] (42.5)	2550± 852[b] (36.1)	1860±580[c] (34.6)
Clofibrate	30	762±112[b] (64.1)	3427±620 (96.2)	6201± 759 (87.7)	4873±644 (90.6)
	100	371± 61[d] (31.2)	2323±373 (65.2)	3744± 646[b] (52.9)	4100±801 (76.3)

The rats were maintained on a reversed-lighting cycle (light period; 19:00—07:00) for 10 d with oral administration of test agent once a day (10:00—11:00). Three hours after the last administration, they were given an intraperitoneal injection of [1-^{14}C]acetate (10 μCi/100 g body weight) and decapitated 60 min later. *a*) This group alone was maintained under a daylight-dependent lighting cycle (light period; 07:00—19:00). For details concerning the experimental methods, see the experimental section. Each value represents the mean±S.E. of 6 rats. Values in parenthesis represent the relative values with respect to the control. Significantly different from the control by Student's *t*-test. *b*) $p<0.05$. *c*) $p<0.01$. *d*) $p<0.001$.

TABLE IV. Effect of TDPC on Cholesterol Absorption in Normal Rats

Treatment		Radioactivities in serum (dpm/ml)		Cholesterol absorption[a] (%)
Agent	Dose (mg/kg)	[^{3}H]-CH (*p.o.*)	[^{14}C]-CH (*i.v.*)	
Control	—	5873±421 (100)	2794±146 (100)	47.0±2.7 (100)
TDPC	30	5249±303 (89.4)	2377± 69[b] (85.1)	49.6±3.7 (106)
	100	4073±264[c] (69.4)	1974± 57[d] (70.7)	43.2±2.0 (91.9)
	300	2205±125[d] (37.5)	1370±100[d] (49.0)	39.0±3.2 (83.0)
β-Sitosterol	100	3793±355[c] (64.6)	2408±211 (86.2)	36.6±4.3 (77.9)

The rats were maintained on laboratory chow (CE-II) for 10 d with oral administration of test agent once a day. On the 8th day, [4-^{14}C]-CH (0.381 μCi/100 g body weight) was injected into the tail vein followed immediately by [1α,2α-^{3}H]-CH (1.712 μCi/100 g body weight), given through a stomach tube. The rats were decapitate 72 h after the isotope doses and 4 h after the last administration of agents. For details concerning the experimental methods, see the experimental section. *a*) Cholesterol absorption was calculated from the ^{3}H/^{14}C ratio in the digitonin-precipitable sterol and the administration doses. Each value represents the mean±S.E. of 5—6 rats. Values in parenthesis represent the relative values with respect to the control. Significantly different from the control by Student's *t*-test. *b*) $p<0.05$. *c*) $p<0.01$. *d*) $p<0.001$.

statistically significant.

In addition, the serum [^{3}H] derived from an oral dose of [1α,2α-^{3}H]-CH and serum [^{14}C] derived from an intravenous dose of [4-^{14}C]-CH were significantly decreased in TDPC-treated rats.

Discussion

The present study shows that the hypocholesterolemic profile of TDPC differs somewhat from that of clofibrate in rats: the former produced a significant effect in both normal and hyperlipidemic rats whereas the latter did not exert any significant effect in hyperlipidemic rats in a preliminary study (data not shown). This suggests that the hypocholesterolemic action of TDPC in rats may be mediated by a different mechanism from that of clofibrate, which inhibits sterol biosynthesis. TDPC as well as clofibrate significantly decreased the serum CH level in a dose-dependent manner in normal rats. In view of this result, it is suggested that the inhibition of sterol biosynthesis may account for the reduction of serum CH in TDPC-treated rats. This possibility was further explored in an experiment using a radioactive precursor. TDPC and clofibrate strongly inhibited the incorporation of [^{14}C]acetate into sterol in the serum and liver (Table III). These results suggest that a possible mechanism for the hypocholesterolemic effect produced by TDPC, at least partly, is an inhibition of sterol biosynthesis in the liver, and that the activity of TDPC is greater than that of clofibrate. In the present experiment using rats which were maintained under a reversed-lighting cycle, hepatic sterol synthesis from labelled acetate was twice as high as that in a daylight-dependent lighting cycle (normal group). This coincides well with the report of Fears and Morgan[3] that the peak activity in the diurnal cycle of sterol synthesis could be observed at midnight. Therefore, this model is considered to be very useful for the evaluation of the effect of hypolipidemic agents on sterol biosynthesis, since the sterol synthetic rate is markedly enhanced in the liver, which is the major organ that supplies serum CH.

Hyperlipidemia in rats fed a high CH diet has been widely used for the exploration of the hypolipidemic profiles of test agents. It is known that the amount of CH absorbed through the

intestinal tract increases remarkably in the presence of bile acids and fat, resulting in increased serum and liver CH level. To clarify the mechanism of action of TDPC in our hyperlipidemic model, the effect of TDPC on CH absorption in normal rats was examined using a dual-isotope ratio method in comparison with that of β-sitosterol, which is a typical inhibitor of CH absorption. In this model, TDPC (300 mg/kg) moderately inhibited CH absorption; the magnitude of inhibiton was similar to that of β-sitosterol (100 mg/kg); the former inhibited it by 17% and the latter by 22%. However, the moderate inhibition of CH absorption by TDPC was relatively remote from expectation based on the activity of TDPC in the hyperlipidemic model. The reason for this discrepancy is not clear at present, but may be related to differences in the animals used or in the experimental methods. Further investigation is required to determine why different results were obtained in the two experiments.

From the present results, it is apparent that the hypocholesterolemic activity of TDPC is due to inhibition of CH absorption from the intestinal tract. In addition, TDPC remarkably promoted the rate of disappearance of labelled CH from serum. It is therefore suggested that TDPC may stimulate the excretion and/or catabolism of CH. Further investigations are in progress.

TDPC increased the anti-atherogenic index and α/β-lipoprotein ratio concomitantly with its remarkable activity in the hyperlipidemic model. It appears that these changes are due to a decrease in VLDL + LDL and its CH content rather than to an increase in HDL and its CH content. Hence TDPC is considered to improve the abnormal pattern of lipoprotein toward a normal level pattern.

Hepatomegaly is a well-known side effect of hypolipidemic drugs such as clofibrate and its derivatives.[4-7] Although TDPC caused hepatomegaly at high dosage (0.3% in diet) in both types of rats, its hepatomegalic effect was less than that of clofibrate at the same dosage level in normal rats.

In conclusion, the present study demonstrated that the inhibitory effect on sterol synthesis, in addition to the inhibition of CH absorption, accounts for at least part of the hypocholesterolemic activity of TDPC, and showed that there are substantial differences between TDPC and clofibrate with respect to their effects on hyperlipidemia.

Experimental

Materials——Animals: Male Sprague-Dawley rats (CD, Charles River Japan, Inc.,) weighing 140—150 g were maintained on commercial laboratory chow (CE-II, Nippon Clea). They were maintained under a daylight-dependent lighting cycle (light period; 07:00—19:00), unless otherwise specified.

Chemicals: TDPC was prepared by the method described in our previous paper.[2] Clofibrate (ethyl *p*-chlorophenoxyisobutyrate), used as a reference agent, was synthesized by us. β-Sitosterol (80% purity) and other chemicals (cholesterol, sodium cholate and olive oil) were purchased from Nakarai Chemicals Ltd. The labelled compounds were obtained from Amersham, and had the following specific activities: [1-^{14}C]sodium acetate, 57 mCi/mmol; [4-^{14}C]-CH, 56 mCi/mmol; [1α,2α-^{3}H]-CH, 44 Ci/mmol.

Animal Experiments——Hypocholesterolemic Activity: Male rats were employed in this experiment. Whenever test agents were supplemented in powdered basal diet (CE-II, Nipon Clea) or high-cholesterol diet containing 1% CH, 0.2% sodium cholate and 5% olive oil in powdered CE-II diet, an equivalent amount of the powdered CE-II diet was subtracted. The test agents were mixed thoroughly with the experimental diets as described above. The animals were allowed free access to the experimental diets and tap water *ad libitum* for 10 d, and then they were sacrified at 4 h after withdrawing the diet. The blood samples for lipid analysis were obtained from the abdominal aorta under anesthesia with pentobarbital (50 mg/kg, *i.p.*). The liver was immediately removed, washed, blotted on filter paper and weighed. The determination of CH in serum was performed by an enzymatic method using a commercial reagent kit as described previously.[1]

Food consumption and body weight were routinely recorded every other day throughout the experimental period, and these data were used to calculate the average daily dose of the test agent. Though the data are not shown, there was no difference in weight gain or food consumption between TDPC-treated and corresponding control groups.

Incorporation of [1-^{14}C]Acetate into Sterol——Male rats were maintained under a reversed-lighting cycle (light period; 19:00—07:00). However, the normal rats alone were maintained under a daylight-dependent lighting cycle. Suspension of a test agent in 1% carboxymethylcellulose was given daily to the rats (10:00—11:00) for 10 d by oral administration. The rats were fasted for 4 h after the last dose of agent. Three hours after the last dose, they were given an intraperitoneal injection of [1-^{14}C]acetate (10 μCi/100 g body weight), and decapitated 60 min later. The liver was quickly removed, blotted on filter paper and weighed. To 0.8 ml of serum was added 2 ml of 15% alcoholic KOH. The mixture was saponified at 85 °C for 2 h and the nonsaponifiable fraction was extracted three times, each with 4 ml of petroleum ether. The minced liver (1 g) was transferred to glass-stoppered extraction tubes. After adding 1 ml of water and 2 ml of 15% alcoholic KOH, the mixture was saponified at 85 °C for 6 h. The nonsaponifiable fraction was extracted by the above-mentioned method. The extracts were evaporated to dryness, then the digitonin-precipitable sterol was isolated as described by Matteis.[8] The materials were dissolved in 1.0 ml of methanol, and an aliquot (0.7 ml) was transferred to a scintillation vial for counting. The radioactivity was determined in toluene scintillator (Liquiflor®, New England Nuclear) with a liquid scintillation counter (Tri-Carb 4640, Parckard). For quantitative determination of sterol, an aliquot (0.2 ml) was transferred to another tube, dried under a stream of nitrogen and assayed by the method of Zak.[9] The data were expressed as dpm per mg of sterol, dpm per ml of serum and dpm per g of liver.

Measurement of CH Absorption——CH absorption was measured by a dual-isotope ratio method as described by Zilversmit and Hughes.[10] The intravenous dose of [4-^{14}C]-CH (13.7 μCi) was prepared by dissolving the compound in 0.45 ml of 95% ethanol and by adding 8.54 ml of 0.9% NaCl; the rats received 0.381 μCi of [4-^{14}C]-CH per 100 g of body weight. The oral dose of [^{3}H]-CH (59.92 μCi) was prepared by dissolving 105 mg of CH in 2730 mg of triolein. The oil phase was then suspended by sonification in 30 ml of water containing 131.25 mg of sodium taurocholate, and the final volume was adjusted with water to 35 ml. Rats received 1.5 mg of CH plus 1.712 μCi of [^{3}H]-CH per 100 g of body weight.

The rats were maintained on commercial laboratory chow for 10 d. The agents were suspended in 1% carboxymethylcellulose and orally given to animals through stomach tube once a day during the experimental period. On the 8th day, [4-^{14}C]-CH was injected into the tail vein of rats under ether anesthesia. This was immediately followed by the [^{3}H]-CH, which was given to the animals through a stomach tube. The rats were fasted for 18 h before and for 6 h after treatment with the isotopes. The rats were decapitated 72 h after the isotope doses and 4 h after the last administration of agents. The digitonin-precipitable sterol in serum was isolated by the above-mentioned method. CH absorption was calculated from the ^{3}H/^{14}C ratio in the digitonin-precipitable sterol and the administration doses.

Analysis of Lipoprotein——Lipoprotein fractionation was performed by using a commercial reagent as described previously.[1] The lower density lipoprotein-CH was estimated as the difference between total CH and HDL-CH levels. The data are presented as HDL-CH level and the ratio of HDL-CH to lower density lipoprotein-CH (anti-atherogenic index). The distribution of α- to β-lipoproteins were determined by densitometric measurement (Autoscanner, quick Quant II, filter 595 nm, Helena Laboratories) of patterns obtained by polyacrylamide gel electrophoresis of serum.[11] The data are presented as the ratio of intensities of α- and β-lipoprotein bands (α/β-LP).

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