EFFECTS OF N-(4-METHYLBENZYLTHIOCARBONYL)-L-PHENYLALANINE (KF 1492), A NEW PHENYLALANINE DERIVATIVE, ON LIPID SYNTHESIS *IN VIVO* INHIBITION OF HEPATIC CHOLESTEROL SYNTHESIS AND 3-HYDROXY-3-METHYLGLUTARYL-Co A REDUCTASE

AKIO ISHII, TAKASHI DEGUCHI, HIROFUTO MARUMO AND MASAO TANAKA

Pharmaceuticals Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.,* Nagaizumi-cho, Sunto-gun, Shizuoka 411, Japan

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KF 1492, a new phenylalanine derivative, was administered at a dose of 100 mg/kg or 0.25% (w/w) diet to Wistar rats. After different periods of drug administration, incorporation of ^{14}C -acetate or ^{14}C -mevalonic acid into digitonin-precipitable sterols and hepatic 3-hydroxy-3-methylglutaryl-Co A (HMG-CoA) reductase activity were measured. KF 1492 feeding was associated with depressed incorporation of ^{14}C -acetate into sterols in liver slices or homogenate. There was no effect on incorporation of ^{14}C -mevalonic acid into sterols. Thus, KF 1492 administration induced inhibition of hepatic HMG-CoA reductase correlated with reduction of incorporation of ^{14}C -acetate into digitonin-precipitable sterols. KF 1492 feeding also inhibited *in vivo* sterol synthesis in rats, which was intraperitoneally injected with ^{14}C -acetate. Stereoisomers, metabolites and chemically similar compounds of KF 1492 reduced HMG-CoA reductase activity, and this was correlated with lowering the activity of plasma cholesterol. These findings indicate that a major site of the action of KF 1492 in the inhibition of sterol synthesis may be at the level of HMG-CoA reductase.

Keywords—KF 1492; phenylalanine; hypolipidemic drug; cholesterol; 3-hydroxy-3-methylglutaryl-Co A reductase; rat liver

INTRODUCTION

Among various N-protected amino acid analogs, derivatives of L-phenylalanine (such as N-acylated derivatives) have been reported to possess hypocholesterolemic activity in rats¹⁾ and phenylalanine has been shown to interfere with the formation of sterols from mevalonic acid.^{2,3)} KF 1492, which is the abbreviation of N-(4-methylbenzylthiocarbonyl)-L-phenylalanine, has been shown to decrease plasma cholesterol concentration in various experimental hyperlipidemic rats⁴⁾ and monkeys.⁴⁾

In this paper investigations regarding the effects of clofibrate, ethylester of α -*p*-chlorophenoxyisobutyrate, and a chemically unrelated hypolipidemic agent, KF 1492, on rat hepatic, 3-hydroxy-3-methylglutaryl-Co A (HMG-CoA) reductase activity and sterol bio-synthesis after *in*

in vivo administration, are reported.

In view of the important role of HMG-CoA reductase in controlling cholesterol bio-synthesis and to obtain some insight into the mode of action, a series of structurally-related derivatives and rat metabolites of KF 1492 were also studied, regarding the effect of HMG-CoA reductase activity.

MATERIALS AND METHODS

Materials—KF 1492 was synthesized in Kyowa Hakko Kogyo Co., Ltd.¹⁾ and the structure of KF 1492 is shown in Fig. 1.

[1- ^{14}C]Acetate (sodium salt, 59.1 mCi/mmol), [2- ^{14}C]acetate (sodium salt, 58 mCi/mmol) and DL-[2- ^{14}C]mevalonic acid lactone (10.9 mCi/mmol, ^{14}C -MVA) were purchased from the Radio Chemical Centre and DL-[3- ^{14}C]-3-

hydroxy-3-methylglutaryl-CoA (49.5 mCi/mmol) was purchased from New England Nuclear Co. Dithiothreitol and cholesterol were obtained from Nakarai Chemicals, 3-hydroxy-3-methylglutaryl-CoA was obtained from Calbiochem, glucose 6-phosphate dehydrogenase (E.C.1.1.4.9) was obtained from Sigma Chemical Co., glucose 6-phosphate was obtained from Boehringer-Mannheim and nicotinamide and digitonin were obtained from Wako Pure Chemical Industries. Clofibrate was obtained from Sumitomo Kagaku Kogyo.

Animals—Male rats of the Wistar strain, weighing 200–240 g, were maintained for one week on commercial chow (Funabashi Nojo) according to a three-hour feeding schedule from 8:30 A.M. to 11:30 A.M. daily, and were provided with free access to water. The animals were adapted to a lighting schedule where the lights were turned on from 7:00 A.M. until 7:00 P.M.

Studies in Vitro with Rat Liver Homogenate and Slice—Rats were fed with commercial chow or the same diet to which 0.25% (w/w) KF 1492 was added. After drug treatment for 3 to 22 days, non-fasted rats were killed by decapitation and the livers were rapidly removed into ice-cold beakers and washed with chilled saline. They were homogenized in 2.5 vols of cold 100 mM potassium phosphate buffer pH 7.4, containing 37.5 mM nicotinamide and 6 mM MgCl_2 in a Potter-Elvehjem type homogenizer with a loose-fitting teflon pestle. After homogenization, the mixture was centrifuged at $500 \times g$ for 10 min, and the supernatant solution was obtained.

The activity of cholesterol synthesis in the liver supernatant was assayed by the method of Ozawa *et al.*⁵⁾ using ^{14}C -acetate as the substrate. Two ml

of liver supernatant were incubated with ^{14}C -acetate (13 mM, 50 μCi) and NAD (0.8 mM) for 100 min at 37°C . At the end of the incubation period, 1 ml of 20% alcoholic KOH was added to the incubation tubes. Sterols were extracted from the saponification mixture and isolated as the digitonide. The cholesterol digitonide thus obtained was dissolved in Hyamine, and the radioactivity was measured with a liquid scintillation spectrometer (Aloka LSC 653).

In the rat liver slice experiment, liver slices (100 mg, approximately 0.5 mm thick) were placed in a test tube containing 1 ml of Krebs Ringer bicarbonate buffer and 4.23 μmol ^{14}C -acetate (50 μCi) or 0.183 μmol ^{14}C -MVA (2 μCi) for sterol synthesis. After incubation for 120 min at 37°C , 1 ml of 20% alcoholic KOH was added to each incubation mixture. An aliquot of the saponified mixture was assayed for protein concentration by the method of Lowry *et al.*⁶⁾ using bovine serum albumin as a standard. The non-saponifiable lipids were extracted with petroleum ether and precipitated as the digitonide following the method of Endo.⁷⁾

Studies in Vivo with Rats—The rats were housed in stainless steel cages in a temperature-humidity controlled room. Food (commercial rat pellets) and water were always available. The rats were not fasted during the entire experiment. The drugs were suspended in a 1% carboxymethyl cellulose (CMC) and administered (100 mg/kg) daily by gavage (0.1 ml/g body weight) for 11 days. Control animals received 1% CMC alone. Four hours after the last administration, the rats received intra-peritoneal injections of $[1-^{14}\text{C}]$ -acetate (approximately 80 $\mu\text{Ci/kg}$), and after 1 hour, the animals were killed by cardiac puncture. Blood was collected and the organs to be studied were rapidly removed, washed thoroughly with chilled saline and then minced. The synthesized non-saponifiable products and digitonin-precipitable sterols were measured.⁷⁾

Preparation of Microsomes and Assay for HMG-CoA Reductase Activity—Rats were fed with commercial chow or the same diet to which 0.25% of the drug was added. After a period of 3

FIG. 1. Chemical Structure of KF 1492

to 23 days of drug treatments, the non-fasted rats were sacrificed three hours after termination of the last feeding schedule.

The rats were decapitated and the livers were excised. Homogenization of the liver was performed with a motor-driven teflon pestle in a Porter-Elvehjem type homogenizer in 3 vols of the cold buffer containing 50 mM potassium phosphate, pH 7.4, 250 mM NaCl, 30 mM EDTA and 1 mM dithiothreitol. The homogenate were centrifuged for 15 min at $16000 \times g$, and the supernatant was decanted and re-centrifuged at $105000 \times g$ for 45 min. The microsomal pellet was re-suspended in the same buffer. The HMG-CoA reductase assay was carried out according to the method of Shapiro *et al.*^{8,9)} The co-factor substrate solution (150 μ l) containing NADP 1 μ mol, glucose-6-phosphate 5 μ mol, glucose-6-phosphate dehydrogenase 1 unit, dithiothreitol 4 μ mol, HMG-CoA 0.15 μ mol, [$3\text{-}^{14}\text{C}$]HMG-CoA 0.33 μ Ci and potassium phosphate buffer, pH 7.0, 100 μ mol, was pre-incubated for 10 min at 37°C. After the addition of 50 μ l of microsomal solution, the mixture was incubated for 30 min at 37°C. After the termination of the reaction by the addition of 20 μ l of conc. HCl, the mixture was further incubated for 30 min at 37°C and then centrifuged at $200 \times g$ for 10 min. The supernatant was applied to a silica gel F₂₅₄ plate (Merck). The chromatograms were developed in benzene/acetone (1:1). A section of the thin layer plates where the mevalonolactone was located was scraped and assayed for radioactivity. Plasma

cholesterol was determined by Determiner TC (Kyowa Medex Co.).

RESULTS

Incorporation of Acetate into Digitonin-precipitable Sterols in Vitro

The effects of KF 1492 and clofibrate on incorporation of ^{14}C -substrates into sterols are shown in Table I and Table II.

Clofibrate or KF 1492-treated rats showed an increase in liver weight: however, this activity of KF 1492 was less potent than clofibrate. After KF 1492 administration (with diet), incorporation of [$1\text{-}^{14}\text{C}$] or [$2\text{-}^{14}\text{C}$]acetate into digitonin-precipitable sterols was significantly depressed in rat liver homogenate (approximately 69.5% inhibition against control), and slices (72.3–82.9% inhibition). After 3 days, 72.3% of the reduction was attained in slices while after 7 days, the reduction reached 82.9%.

When ^{14}C -mevalonic acid was used as a substrate, incorporation of the radioactivity into digitonin-precipitable sterols was not influenced by the control level in liver slices. These data indicate that like clofibrate, KF 1492 has no effect on steps between mevalonate and cholesterol, and influences the bio-synthetic pathway prior to MVA formation.

Incorporation of Acetate into Digitonin-precipitable Sterols in Vivo

Inhibition of sterol bio-synthesis by KF 1492 was examined after 11 days of treatment in normolipidemic rats *in vivo*.

TABLE I. Effects of KF 1492 or Clofibrate Treatment on Sterol Synthesis in Liver Homogenate

Treatment	n	Period	Liver weight ^{a)}	[$2\text{-}^{14}\text{C}$]Acetate	→ Digitonin precipitable sterols
		(d)	(g/100 g body weight)	(pmol/mg protein/100 min) ^{a)}	
Control	4	22	2.877 ± 0.030	5.43 ± 3.51	
KF 1492	4	22	3.647 ± 0.095 $p < 0.01$	1.57 ± 0.37 (69.5) ^{b)}	
Clofibrate	4	22	4.708 ± 0.211 $p < 0.01$	2.48 ± 0.64 (60.2)	

a) Values represent mean $\pm$ S.D. b) Values in parenthesis represent inhibitory percent.

As shown in Table III, incorporation of radioactivity into digitonin-precipitable sterols widely varied from tissue to tissue. KF 1492 effectively inhibited *in vivo* sterol synthesis in the plasma and liver, but in other tested organs (adrenal, kidney, lung, spleen, testis and ileum),

TABLE II. *Effects of KF 1492 or Clofibrate Treatment on Sterol Synthesis in Liver Slices*

Treatment	n	Period (d)	Liver weight (g/100g body weight)	[1- ¹⁴ C]Acetate → Digitonin precipitable sterols (pmol/mg protein/120 min) ^{a)}	[2- ¹⁴ C]MVA → Digitonin precipitable sterols (pmol/mg protein/120 min) ^{a)}
Control	5	7	3.549	7.31 ± 4.12	22.08 ± 8.17
KF 1492	5	3	4.482*	2.02 ± 0.74* (72.3) ^{b)}	25.70 ± 4.85 (−16.4) ^{b)}
	5	7	4.844**	1.25 ± 0.56* (82.9)	21.06 ± 6.61 (6.4)
Clofibrate	5	7	5.267**	1.30 ± 0.44* (82.3)	17.32 ± 6.78 (21.6)

* $p < 0.05$, ** $p < 0.01$. a) Values represent mean ± S.D. b) Values in parenthesis represent inhibitory percent.

TABLE III. *Effects of KF 1492 in Vivo on the Incorporation of [1-¹⁴C]Acetate into Digitonin-Precipitable Sterols in Various Tissues of Rats*

Tissue	Incorporation (dpm/g tissue) ^{a)}			% of control	
	Control group		Treated group		
Plasma	700 ± 122		228 ± 49	32.6	$p < 0.01$
Liver	2893 ± 463		1307 ± 170	45.2	$p < 0.05$
	(4154 ± 879)		1719 ± 248	41.4	$p < 0.05$) ^{b)}
Ileum	48876 ± 13707		33750 ± 8912	69.1	
Adrenal	4837 ± 540		3815 ± 468	78.9	
Kidney	1499 ± 232		2219 ± 280	148.3	
Lung	1477 ± 187		1657 ± 217	112.2	
Spleen	2580 ± 210		2309 ± 205	89.5	
Testis	770 ± 135		908 ± 153	117.9	

a) Values represent means of 6 rats ± S.E. b) Data in parenthesis represent the incorporation of radioactivity into non-saponifiable lipids.

TABLE IV. *Effects of KF 1492 Treatment on HMG-CoA Reductase*

Treatment	n	Period (d)	Liver weight ^{a)} (g/100 g body weight)	HMG-CoA Reductase Activity ^{a)} (nmol/mg protein/30 min)
Control	4	7	3.116 ± 0.060	12.01 ± 2.12
KF 1492	4	3	3.499 ± 0.096 $p < 0.01$	3.89 ± 0.59 (67.6) ^{b)} $p < 0.001$
	4	7	3.820 ± 0.062 $p < 0.01$	2.59 ± 0.25 (78.4) $p < 0.001$
Control	4	23	2.960 ± 0.187	14.83 ± 6.14
KF 1492	4	23	4.020 ± 0.069 $p < 0.01$	2.74 ± 0.86 (81.5) $p < 0.02$
Clofibrate	4	23	4.628 ± 0.130 $p < 0.01$	7.02 ± 1.95 (52.7) $p < 0.1$

a) Values represent mean ± S.D. b) Values in parenthesis represent inhibitory percent.

the effects of the drug were not observed. In the liver, incorporation of non-saponifiable lipids was also depressed (58.6%) as well as digitonin-precipitable sterols (54.8%). These findings confirmed the above *in vitro* results. The fraction of cholesterol in the non-saponifiable lipids

TABLE V. Effects of Stereoisomers of KF 1492 on HMG-CoA Reductase Activity

Treatment	n	Period (d)	Liver weight ^{a)} (g/100 g body weight)	HMG-CoA Reductase ^{a)} (nmol/mg protein/30 min)
Control	4	7	3.028±0.073	10.97±2.45
D form	4	7	3.120±0.127	8.77±1.17 (20) ^{b)}
DL form	4	7	3.516±0.108 <i>p</i> < 0.01	5.37±1.03 (51) <i>p</i> < 0.02

a) Values represent mean ± S.D. b) Values in parenthesis represent inhibitory percent.

TABLE VI. Effects of KF 1492 Analogs on HMG-CoA Reductase Activity and Plasma Cholesterol Level

Treatment	R ₁	Structure	R ₂	R ₃	R ₄	HMG-CoA Reductase ^{a)} (nmol/mg protein/30 min)	Plasma cholesterol (% of decrease)
Control	—	—	—	—	—	9.03±1.26	
Compound 1		O	H	H		4.75±1.36 <i>p</i> < 0.05	36.9 <i>p</i> < 0.05
2	Cl	O	H	CH ₃		3.22±0.10 <i>p</i> < 0.01	64.7 <i>p</i> < 0.01
3	CH ₃	S	H	CH ₃		5.01±2.44	38.6 <i>p</i> < 0.01
4	HOOC	O	H	H		11.08±1.01	13.0
5	HOCH ₂	O	H	H		6.55±0.66 <i>p</i> < 0.05	20.0 <i>p</i> < 0.05
6		O	H	H		7.61±1.28	12.2
7		O	H	H		8.59±0.98	28.9 <i>p</i> < 0.01
8	NO ₂	O	H	H		11.20±2.14	28.1 <i>p</i> < 0.01
9	CH ₃	O	OH	H		16.54±5.23	11.3

a) Results are means of 3 rats ± S.D.

(cholesterol, squalene and lanosterol, *etc.*) in the group treated with KF 1492 was at the same level as in the control group (77.5% and 72.6%, respectively).

Effects of Liver HMG-CoA Reductase Activity

Since KF 1492 significantly inhibited incorporation of ^{14}C -acetate into digitonin-precipitable sterols, it was of interest to investigate which pathway on hepatic cholesterologenesis was inhibited by KF 1492 feeding. Rats were given a diet containing 0.25% of drug for 3, 7 or 23 days. The reductase activity was measured 3 hours after administration, and the results are shown in Table IV. After consecutive administration of KF 1492 or clofibrate, this enzyme activity was significantly depressed. A KF 1492 diet produced a 68% decrease in reductase activity after 3 days. The prolonged treatment for 7 days produced approximately 78% reduction.

Furthermore, when the stereoisomers of KF 1492 were administered to rats for 3 days, the HMG-CoA reductase activity was also decreased to 20 (D form) and 51 (DL form) percent, and these inhibitory activities were weaker than the L form (KF 1492) [refer to Table IV and Table V]. Similar to the inhibitory activity, the increment of the liver weight was in the following order: L, DL and D forms.

When 10 groups of rats were fed for 3 days with a diet containing 0.25% each of compounds, chemical structures of which are shown in Table VI, plasma cholesterol was lowered by 11.3%–64.7%. Compounds **1**–**3** and **5** caused a significant reduction of hepatic HMG-CoA reductase, and the activities were in good agreement with the reduction in plasma cholesterol levels in rats. Incorporation of *p*-hydroxy (compound **9**), nitro (compound **8**) and carboxyl (compound **4**) groups gave no *in vivo* reduction of HMG-CoA reductase.

DISCUSSION

To prevent atherosclerosis and related complications, hypocholesterolemic drugs and modification of diet have been investigated with respect to lowering the cholesterol level. Plasma

cholesterol levels are thought to be affected by cholesterol synthesis, its metabolic degradation to bile acids, intestinal absorption of dietary or biliary cholesterol and re-distribution of cholesterol between plasma and tissue compartments.

In this paper, a new phenylalanine derivative, KF 1492, has been investigated in regard to the effect on rat hepatic HMG-CoA reductase activity and sterol synthesis after *in vivo* administration, and the effect compared with clofibrate. Clofibrate used as a reference compound is a well-known drug to lower the levels of plasma lipids; however, the mechanism of action is not yet completely known. White¹⁰⁾ reported that clofibrate regulated hepatic cholesterol biosynthesis by inhibiting the microsomal reduction of HMG-CoA to mevalonate. The same observations were obtained in the present experiment; namely, a 0.25% clofibrate-treated rat produced a 52.7% decrease in reductase activity after 23 days of treatment.

A wide variety of N-acylated derivatives of phenylalanine has been reported. Many are of special interest in peptide synthesis, and some N-acylated amino acids including phenylalanine have been shown to exhibit hypolipidemic action in hypercholesterolemic rats,¹¹⁾ and another approach has been performed to exhibit anti-tumor activity against the growth of Ehrlich ascites carcinoma in CF₁ male mice.¹¹⁾ As shown in the present investigation, the structurally-similar compounds and stereoisomers of KF 1492 possess the inhibitory action of HMG-CoA reductase, and the inhibitory activity was in good agreement with the cholesterol-lowering activity. Therefore, the inhibition *in vivo* may partly participate with the hypocholesterolemic action of KF 1492.

In this experiment, the *p*-methyl group of KF 1492 is metabolized to hydroxymethyl (compound **5**) and the carboxy group (compound **4**) in rats.¹²⁾ It was investigated as to whether these metabolites participate in the hypolipidemic action *in vivo* or whether there is any inhibitory action on HMG-CoA reductase activity. However, the inhibitory effects on HMG-CoA reductase and hypocholesterolemic activity of

normolipidemic rats were weaker than the parent drug. Phenylalanine and related metabolites (phenylpyruvate, phenylacetate) have been shown to inhibit the incorporation of ^{14}C -mevalonate into non-saponifiable lipids and digitonin-precipitable sterols.^{2,3,13} Since KF 1492 has no effect on the incorporation of mevalonic acid into sterols and inhibited the HMG-CoA reductase *in vivo*, the inhibitory activity of KF 1492 on sterol synthesis *in vivo* seemed to differ with the mode of these phenylalanine metabolites.

To investigate whether KF 1492 or related metabolites (compound 4 and compound 5) have any inhibitory effects on HMG-CoA reductase, inhibitory experiments have been performed *in vitro*. However, neither of these compounds displayed inhibitory activities on the reductase at 10^{-3} – 10^{-5} M. Since clofibrate was shown to strongly suppress HMG-CoA reductase activity in cultured mouse L cells at concentrations of 20–50 $\mu\text{g/ml}$,¹⁴ There might be some differences between clofibrate and KF 1492.

In conclusion, KF 1492 induced inhibition of hepatic HMG-CoA reductase correlated with reduction of incorporation of acetate into digitonin-precipitable sterols. A major site of action of KF 1492 in the inhibition of sterol synthesis may be at the level of HMG-CoA reductase. The decrease in the activity of HMG-CoA reductase in the liver may be due either to changes in the synthesis (or degradation) of the enzyme or to a modification of the enzyme, or both.

Yamada has shown that KF 1492 was weaker in hepatomegalic activity than clofibrate, and also had different responses in hypocholesterolemic activity with clofibrate in various hyperlipidemic rats.⁴ It will be required to further investigate the inhibitory mode of HMG-CoA reductase *in vivo*, and also to clarify other hypolipidemic factors; namely, cholesterol absorption or excretion.

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