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EFFECTS OF S-8527 (1,1-BIS[4'-(1''-CARBOXY-1''-METHYLPROPOXY)PHENYL] CYCLOHEXANE), A NEW HYPOLIPIDEMIC COMPOUND, ON CHOLESTEROL AND LIPOPROTEIN METABOLISM IN RATS

KOTARO SUZUKI

Research Department, Pharmaceuticals Division, Sumitomo Chemical Co. Ltd., Takarazuka, Japan

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Abstract—The effects of S-8527 (1,1-bis[4'-(1''-carboxy-1''-methylpropoxy)phenyl]cyclohexane on cholesterol and lipoprotein metabolism were examined and compared with those of clofibrate in rats under various experimental conditions. When rats were given a daily oral dose of S-8527 for 8 days, the incorporation of [^{14}C]acetate into liver cholesterol was not inhibited at the dose of 30 mg/kg of S-8527, which was reported to decrease the serum cholesterol significantly, but the higher dose (300 mg/kg) of S-8527 decreased the incorporation of [^{14}C]acetate into liver cholesterol. Under these experimental conditions, clofibrate (300 mg/kg) caused a decrease in labeled cholesterol in the liver. Oral doses of S-8527 or clofibrate for 8 days did not affect the incorporation of [^{14}C]mevalonate into liver cholesterol. Also, when the drugs were added to normal rat liver slices, the effects of S-8527 were not so marked as those seen with clofibrate. Oral doses of S-8527 (30 mg/kg) or clofibrate (300 mg/kg) for 8 days decreased the incorporation of [^{14}C]leucine into the protein of serum lipoproteins. S-8527 and clofibrate did not affect the body retention of radioactivity after the injection of labeled cholesterol into rats. In view of the above results, it is conceivable that S-8527 primarily inhibits either the secretion of lipoproteins containing cholesterol into plasma or the formation of lipoproteins containing cholesterol in the liver, or both, and secondarily interferes with the biosynthesis of liver cholesterol.

S-8527 (1,1-bis[4'-(1''-carboxy-1''-methylpropoxy)-phenyl]cyclohexane) has been reported to possess pronounced hypolipidemic properties in experimental animals and is considered to be about ten times more potent in hypolipidemic activity and less potent in its hepatomegalic effect than clofibrate [1, 2]. It was also reported that one of the possible mechanisms for the decrease in serum and liver triglyceride levels produced by S-8527 was an inhibition of triglyceride synthesis in the liver [3].

The present study was designed to investigate the nature of the hypocholesterolemic effect exerted by S-8527. S-8527 and clofibrate are different with regard to their effects on the concentration of liver cholesterol [2]. We have, therefore, studied the effects of S-8527 on incorporation of [^{14}C]acetate or [^{14}C]mevalonate into liver and intestinal cholesterol in rats, incorporation of [^{14}C]leucine into serum lipoproteins in rats, and body retention of radioactivity after the injection of [^{14}C]cholesterol into rats.

METHODS

Animals. Male Wistar rats weighing 150–200 g were used throughout the study. Drugs were suspended in 5% gum arabic solution and given to rats every morning for 8 or 21 days by oral intubation. During the experimental period, the animals were fed on a commercial chow pellet (NIPPON CLEA, CE-2) and water *ad lib.*

Incorporation of [^{14}C]acetate or [^{14}C]mevalonate into cholesterol. In experiments *in vivo*, the rats were injected with [1-^{14}C]sodium acetate (10 μCi/100 g of body weight; 46·1 μCi/μmole) or [2-^{14}C]sodium mevalonate (5 μCi/100 g of body weight; 6·3 μCi/μmole) intraperitoneally 24 hr after the last dose of S-8527 or clofibrate; 4 hr later blood samples were obtained from the inferior vena cava under ether anesthesia. After the animals were sacrificed, the liver and small intestine were removed and washed with ice-cold physiological saline, blotted on a filter paper and weighed. The samples of liver and small intestine were homogenized with 20 vol. of chloroform methanol (2:1, v/v), and total lipid extracts were obtained as described previously [2]. An aliquot of the extracts was saponified and cholesterol was extracted from the saponification mixture. Cholesterol digitonide was synthesized and isolated as described by Avoy *et al.* [4]. The digitonide was dissolved in ethanol [5] and radioactivity was measured by a liquid scintillation spectrometer. In experiments *in vitro*, the rats were sacrificed by decapitation and their livers were removed immediately, washed in chilled saline and sliced with a tissue slicer (Natsume Seisakusho, Co., Ltd., Japan). Samples weighing 1·0 g were incubated in 10 ml Krebs-Ringer phosphate buffer (pH 7·4) containing [1-^{14}C]sodium acetate (3 μCi/30 μmole) at 37° for 3 hr at atmospheric pressure. S-8527 was added as the Na salt dissolved in saline. Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) is not water soluble. The Na salt of chlorophenoxyisobutyric acid (CPIB), the nonesterified derivative of the drug, was dissolved in saline and added. The reaction was stopped by adding 15 ml of alcoholic KOH solution (20%), and the radioactivity of cholesterol was assayed as described above.

Incorporation of [^{14}C]leucine into serum lipoproteins. Twenty-four hr after the last dose, uniformly labeled L-[^{14}C]leucine (7 μCi/animal; 216 μC/μmole), dissolved in physiological saline, was injected into the femoral vein, and 2 hr later blood samples were ob-

tained from the inferior vena cava under ether anesthesia. Serum samples from two rats were pooled for separation of lipoproteins. The lipoprotein fraction was separated into VLDL ($d < 1.019$), LDL ($1.019 < d < 1.063$) and HDL ($d > 1.063$) according to the method of Havel *et al.* [6]. Ultracentrifugation was carried out in a Hitachi model 55-2 ultracentrifuge using an R. P. 55A rotor (Hitachi, Japan). The density of the pooled serum for each of two rats was adjusted to 1.019 and centrifuged at 105,000 *g* for 22 hr at 12°; after centrifugation, the VLDL fraction was obtained. The density of the infranatant solution from the first ultracentrifugation was then adjusted to 1.063 and again centrifuged at 105,000 *g* for 22 hr. After centrifugation, the LDL fraction was obtained. The final infranatant solution represented the HDL fraction. The isolated lipoprotein fractions were precipitated with trichloroacetic acid and the precipitates were washed and delipidized with ethanol–acetone (1:1, v/v) and ether as described by Radding and Steinberg [7]. The dried protein precipitate was dissolved in 1 N NaOH solution. An aliquot of the alkaline solution was dissolved in Bray's scintillator [8] and radioactivity was counted as described above.

Measurement of whole body retention of [^{14}C]*cholesterol.* The rats were injected with [4-^{14}C]cholesterol (1·7 μCi/animal; 49·4 μCi/μmole) dissolved in 10% HCO-60(NIKKOL) [9] intravenously. Immediately after the injection of radioactive cholesterol, S-8527 or clofibrate was given by oral intubation every morning for 8 or 21 days. Twenty-four hr after the last dose of the drugs, the rats were anesthesized with ether, and the animals (whole) were digested in a mixture of 400 ml ethanol and 100 g KOH/200 g of tissue. Saponification under a reflux was continued for 4 hr. The digest was filtered on a coarse sintered filter, and bone fragments and insoluble residues were washed with water. The digest and washings were collected and the volume was brought to 1000 ml with water. An aliquot of the aqueous digest was extracted three times with petroleum ether and the unsaponifiable fraction was obtained. Isolation of cholesterol digitonide and count of radioactivity were performed as described above.

Assay of lipids. Tissue cholesterol was determined by the AutoAnalyzer method [10] after an aliquot of total lipid extract was dried and the dried lipids were re-extracted by isopropyl alcohol.

Chemicals. S-8527 and its Na salt were synthesized in this laboratory. Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) was obtained from Imperial Chemical Industries, Ltd., in England and the Na salt of *p*-chlorophenoxyisobutyric acid (CPIB) was prepared in this laboratory. All radiochemicals were purchased from Daiich Pure Chemicals, Ltd., Tokyo, Japan.

RESULTS

Effects on incorporation of [^{14}C]*acetate or* [^{14}C]*-mevalonate into cholesterol.* In experiments *in vivo*, the rats were given a daily oral dose of S-8527 or clofibrate for 8 days. Table 1 shows the effects of the drugs on the incorporation of [^{14}C]acetate or [^{14}C]mevalonate into liver cholesterol. S-8527 at 30 mg/kg did not cause a significant reduction in [^{14}C]-acetate incorporation into liver cholesterol. Higher dose of S-8527 (300 mg/kg) reduced the incorporation of [^{14}C]acetate into cholesterol by about 50 per cent/g of liver and 45 per cent/mg of cholesterol. Clofibrate (300 mg/kg) resulted in a decrease of labeled liver cholesterol of about 60 per cent/g of liver and 50 per cent/mg of cholesterol. Neither drug caused a statistically significant reduction in the incorporation of [^{14}C]mevalonate into cholesterol.

Table 2. Effects of S-8527 and CPIB on the incorporation of [^{14}C]acetate into cholesterol in normal rat liver slices*

	n	Radioactivity of cholesterol (dis./min/g × 10^{-2})	
		2.5×10^{-3} M	2.5×10^{-4} M
Control	5	280 ± 59	413 ± 82
S-8527	5	117† ± 25(−58)	396 ± 80(−4)
CPIB	5	101† ± 13(−64)	312 ± 54(−25)

* Each value represents the means ± S. E. Figures in parentheses represent per cent change from control. CPIB, Na chlorophenoxyisobutyrate.

† Significantly different from control ($P < 0.05$).

Table 1. Effects of S-8527 and clofibrate on the incorporation of [^{14}C]acetate or [^{14}C]mevalonate into liver cholesterol in rats*

	Radioactivity of cholesterol			
	[^{14}C]acetate		[^{14}C]mevalonate	
Treatment	Liver (dis./min/g × 10^{-2})	Cholesterol (dis./min/mg × 10^{-2})	Liver (dis./min/g × 10^{-3})	Cholesterol (dis./min/mg × 10^{-3})
Control	58·7(6) ± 9·7	26·0(6) ± 4·9	33·8(6) ± 1·9	14·6(6) ± 6·2
S-8527 (30 mg/kg)	53·2(6) ± 7·9	24·2(6) ± 4·7		
S-8527 (300 mg/kg)	27·6(6)† ± 7·6	14·0(6) ± 4·1	33·8(5) ± 2·9	14·6(5) ± 6·7
Clofibrate (300 mg/kg)	24·0(6)† ± 6·0	13·0(6)† ± 2·8	30·7(5) ± 1·6	15·2(5) ± 8·1

* Each value represents the means ± S. E. Figures in parentheses indicate number of animals.

† Significantly different from control ($P < 0.05$).

Table 3. Effects of S-8527 and clofibrate on the incorporation of [^{14}C]acetate into small intestine cholesterol in rats*

Treatment		Radioactivity of cholesterol	
		Intestine (dis./min/g × 10^{-2})	Cholesterol (dis./min/mg × 10^{-2})
Control	(5)	65·4 ± 5·7	26·1 ± 2·8
S-8527 (30 mg/kg)	(5)	61·0 ± 9·9	26·9 ± 3·7
Clofibrate (300 mg/kg)	(5)	55·3 ± 7·3	21·9 ± 2·4

* Each value represents the mean ± S. E. Figures in parentheses indicate number of animals

Table 2 shows the effects of adding the drugs to normal rat liver slices. S-8527 at concentrations of $2{\cdot}5 \times 10^{-3}$ M (1170 μg/ml) and $2{\cdot}5 \times 10^{-4}$ M reduced the incorporation of [^{14}C]acetate into cholesterol by about 58 and 4 per cent respectively. On the other hand, Na chlorophenoxyisobutyrate (CPIB) at concentrations of $2{\cdot}5 \times 10^{-3}$ M (478 μg/ml) and $2{\cdot}5 \times 10^{-4}$ M reduced the incorporation by about 64 and 25 per cent respectively. The inhibitory effect of clofibrate was more potent than that of S-8527.

Table 3 shows the effects on the incorporation of [^{14}C]acetate into cholesterol in the intestine. S-8527 at 30 mg/kg did not affect the incorporation of [^{14}C]acetate into cholesterol. Clofibrate at 300 mg/kg, which reduced the labeled cholesterol in the liver, did not cause a reduction of labeled cholesterol in the intestine.

Effects on incorporation of [^{14}C]leucine into lipoproteins. Table 4 shows the effects of the drugs on the incorporation of [^{14}C]leucine into serum lipoproteins. S-8527 at 30 mg/kg for 8 days lowered the incorporation of [^{14}C]leucine into the protein of VLDL, LDL and HDL fractions by about 40, 35 and 10 per cent respectively. Clofibrate at 300 mg/kg decreased the incorporation of [^{14}C]leucine into protein of the VLDL and HDL fractions by about 65 and 20 per cent respectively. The decrease of labeled protein in LDL was not statistically significant.

Effects on body retention of [^{14}C]cholesterol. The rats were injected with [^{14}C]cholesterol intravenously and the drugs were given once a day for 8 or 21 days. Table 5 shows the effects on the radioactivity (percentage of recovery of injected [^{14}C]cholesterol) existing in the body on day 9 or day 22 after injection of [^{14}C]cholesterol. There was no significant difference in the amount of radioactive cholesterol recovered from the body between control groups and S-8527 or clofibrate-treated groups. The data suggest that the excretion of sterol from the body may not be stimulated by both drugs.

DISCUSSION

Serum lipids can be lowered by an inhibition of cholesterol synthesis [11]. The results of our present studies showed that the incorporation of [^{14}C]acetate into liver cholesterol in rats was inhibited at the higher dose (300 mg/kg) of S-8527 but was not inhibited at the lower dose (30 mg/kg) of S-8527. Under these experimental conditions, clofibrate at 300 mg/kg inhibited the incorporation of [^{14}C]acetate into liver cholesterol as reported by others [4, 16]. It should be noted that S-8527 at 30 mg/kg did not affect the incorporation of [^{14}C]acetate into liver cholesterol. This dose was one-tenth that of clofibrate, but the hypolipidemic effect of 30 mg/kg of S-8527 was reported to be nearly equal to that of 300 mg/kg of clofibrate [1, 2]. These results show that the decrease of plasma cholesterol levels cannot be attributed simply to a decrease in the rate of cholesterol synthesis. In fact, the two drugs were reported to be different with regard to their effects on the concentration of liver cholesterol. Our investigation of the liver cho-

Table 4. Effects of S-8527 and clofibrate on the incorporation of [^{14}C]leucine into protein of serum lipoproteins in rats*

		Radioactivity of lipoproteins		
		VLDL(d < 1·019) (dis./min/ml)	LDL(1·019 < d < 1·063) (dis./min/ml)	HDL(d > 1·063) (dis./min/ml × 10^{-2})
Control	(6)	181 ± 22	740 ± 68	767 ± 20
S-8527 (30 mg/kg)	(6)	107† ± 12	482† ± 51	685† ± 21
Clofibrate (300 mg/kg)	(6)	63‡ ± 4	510 ± 65	606‡ ± 24

* Each value represents the mean ± S. E. of three pooled samples. Figures in parentheses indicate numbers of animals.

† Significantly different from control ($P < 0{\cdot}05$).

‡ Significantly different from control ($P < 0{\cdot}01$).

Table 5. Effects of S-8527 and clofibrate on the body retention of [^{14}C]cholesterol in rats

	Recovery of [^{14}C]cholesterol(%) Days of drug treatment	
	8	21
Control	41·7(6) ±1·0	12·2(5) ±1·5
S-8527 (30 mg/kg)	43·0(5) ±3·8	15·9(5) ±1·4
S-8527 (300 mg/kg)	43·5(6) ±3·9	14·5(3) ±1·4
Clofibrate (300 mg/kg)	38·6(5) ±3·0	15·0(5) ±2·6

* Each value represents the mean ± S. E. Figures in parentheses indicate numbers of animals.

lesterol levels indicated that the lower dose (1–30 mg/kg) of S-8527 increased liver cholesterol concentration slightly, but the higher dose (100–300 mg/kg) of S-8527 tended to decrease the liver cholesterol concentration, while in clofibrate-treated animals, the decrease of liver cholesterol concentration was dose-dependent [2]. In addition, when the drugs were added to normal rat liver slices to determine whether a drug was a direct inhibitor of cholesterol synthesis, the effect of S-8527 was not so marked as that found with clofibrate. On a weight-for-weight basis, the inhibitory effect of S-8527 was about one-tenth that of clofibrate (CPIB). These results support the suggestion that S-8527 may lower plasma cholesterol by means other than the inhibition of cholesterol synthesis. S-8527 at 30 mg/kg, which lowered serum cholesterol without the inhibition of cholesterol synthesis, decreased the incorporation of [^{14}C]leucine into the protein of the VLDL, LDL and HDL fractions. It is conceivable, therefore, that S-8527 primarily inhibits either the secretion of lipoproteins containing cholesterol into plasma or inhibits the formation of lipoproteins containing cholesterol in the liver, or both, and secondarily interferes with cholesterol synthesis.

Clofibrate has been reported to have a number of effects on lipid metabolism of rats. Its mechanism of action is not completely clear [12], but studies have suggested that it acts at least in part by inhibiting cholesterol synthesis [4, 13–16]. The results of our present studies show that clofibrate interferes with the conversion of acetate to mevalonate, confirming the results of others [4, 16]. In S-8527-treated rats, serum cholesterol levels decreased without inhibition of cholesterol synthesis. In these respects, S-8527 and clofibrate have different modes of action. Kritchevsky and Tepper [17] reported that S-8527 lowered serum lipids by a mechanism similar to that of clofibrate. At the higher dose S-8527 seems to possess effects similar to those of clofibrate.

Of the nonhepatic tissues that can synthesize cholesterol, the intestine is thought to be a likely source of serum cholesterol [18]. Clofibrate did not affect the incorporation of [^{14}C]acetate into intestinal cholesterol, confirming results by Gould [4] but not Cheng [15]. S-8527 did not affect cholesterol synthesis in the intestine as found in clofibrate-treated animals.

Plasma cholesterol also can be lowered by other mechanisms, including increased cholesterol catabolism, inhibition of absorption of cholesterol, and a shift of cholesterol from plasma to the liver.

Grundy *et al.* [19] reported that clofibrate could cause marked reduction in body pools of cholesterol in patients with hyperlipidemia. The rats were injected with [^{14}C]cholesterol, and the body retention of labeled cholesterol was measured on days 9 and 22 after injection of cholesterol. Under these experimental conditions, clofibrate did not affect the body retention of cholesterol. S-8527 showed results similar to those with clofibrate. Therefore, it seems probable that S-8527 and clofibrate did not affect cholesterol catabolism in rats.

The other possible mechanism that has been considered is an inhibition of absorption of cholesterol. In cholesterol-fed rats, preliminary study showed that S-8527 lowered serum lipids but the effects were not so marked as those in animals fed normal chow. These results suggest the cholesterol absorption is not significantly influenced by S-8527.

In both S-8527- and clofibrate-treated animals, the total content of cholesterol in the whole liver was reported to be greater than that in the control [2, 20]. It is possible, therefore, that the lipids lost from the plasma pool may be shifted to the liver. The mechanism of action of clofibrate and S-8527 has not been determined.

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