Biochimica et Biophysica Acta 877 (1986) 50–60
Elsevier

BBA 52250

CS-514, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue-selective inhibition of sterol synthesis and hypolipidemic effect on various animal species

Yoshio Tsujita [a,*], Masao Kuroda [a], Yoko Shimada [a], Kazuhiko Tanzawa [a], Mamoru Arai [a], Isao Kaneko [b], Minoru Tanaka [c], Hiroshi Masuda [d], Chitoshi Tarumi [d], Yoshio Watanabe [e] and Setsuro Fujii [f]

[a] *Fermentation Research Laboratories,* [b] *Bioscience Laboratories,* [c] *Analytical and Metabolic Research Laboratories,* [d] *Laboratory of Animal Science and Toxicology Laboratories, Sankyo Co. Ltd., Tokyo,* [e] *Institute for Experimental Animals, Kobe University School of Medicine, Kobe, and* [f] *Osaka University, Osaka (Japan)*

(Received November 25th, 1985)

Key words: Sterol synthesis; Hydroxymethylglutaryl-CoA reductase; Hypolipidemic drug; Lipoprotein; (Cell culture)

CS-514 is a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme in cholesterol synthesis. For the microsomal enzyme from rat liver, the mode of inhibition is competitive with respect to hydroxymethylglutaryl-CoA, and the K_i value is $2.3 \cdot 10^{-9}$ M. CS-514 also strongly inhibited the sterol synthesis from [^{14}C]acetate in cell-free enzyme systems from rat liver and in freshly isolated rat hepatocytes; the concentrations required for 50% inhibition were 0.8 ng/ml and 2.2 ng/ml, respectively. On the other hand, the inhibition by CS-514 was much less in the cells from nonhepatic tissues such as freshly isolated rat spleen cells, and cultured mouse L cells and human skin fibroblasts. In addition, the cellular uptake of ^{14}C-labeled CS-514 by isolated rat spleen cells or mouse L cells was less than one-tenth of that by isolated hepatocytes. These differences between hepatic and nonhepatic cells were further confirmed by the fact that CS-514 orally administered to rats inhibited sterol synthesis selectively in liver and intestine, the major sites of cholesterogenesis. CS-514 markedly reduced serum cholesterol levels in dogs, monkeys and rabbits, including Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model for familial hypercholesterolemia in man, but did not reduce those in rats and mice. In the former case, preferential lowering of atherogenic lipoproteins was observed in all of the animals tested. The biliary neutral sterols significantly decreased, whereas the amount of biliary bile acids was not affected by administration of the drug to dogs.

Introduction

In the development of atherosclerosis and coronary heart disease, which is the major cause of death in western and other developed countries, a high level of cholesterol in blood is considered to be an important risk factor [1]. Since more than 70% of the total input of body cholesterol is derived from de novo synthesis in humans [2], it is expected that serum cholesterol can be reduced as a result of inhibition of cholesterol biosynthesis. The most suitable target for this inhibitor is 3-hydroxy-3-methylglutaryl coenzyme A reductase (mevalonate : NADP + oxidoreductase (CoA-acylating), EC 1.1.1.34), the rate-limiting enzyme in the pathway of cholesterol biosynthesis [3].

On the basis of this concept, we carried out

* To whom correspondence should be addressed.

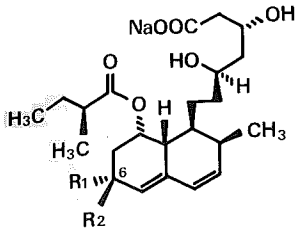

Fig. 1. Structure of CS-514 and related compounds. CS-514 (R_1 = H, R_2 = OH; molecular weight, 447), ML-236B sodium salt (R_1 = R_2 = H) and MB-530B (monacolin K or mevinolin) sodium salt (R_1 = CH_3, R_2 = H).

screening of the inhibitor of hydroxymethylglutaryl-CoA reductase and found several compounds in microbial products [4–12], including ML-236B and MB-530B [12]. The latter is an identical compound to monacolin K [13] or mevinolin [14]. Those compounds were potent competitive inhibitors of this enzyme.

Among these microbial products and their chemically synthesized and microbially transformed derivatives, CS-514 (Fig. 1) was selected because of its potency and tissue selectivity. This drug was found at first as a minor urinary metabolite of ML-236B in dogs and later was obtained by microbial transformation of ML-236B [8,9]. In this report, biochemical and pharmacological characteristics of CS-514 are described.

Materials and Methods

Materials. [1-^{14}C]Sodium acetate (58 Ci/mol), DL-[2-^{14}C]mevalonolactone (27.3 Ci/mol) and DL-[3-^{14}C]hydroxymethylglutaryl-CoA (55.1 Ci/mol) were obtained from New England Nuclear Corp. (Boston, MA, U.S.A.) and [*carboxyl*-^{14}C]sodium deoxycholate (52 Ci/mol) was purchased from Amersham International (Amersham, U.K.). CS-514 [8,9], ML-236B [5] and ^{14}C-labeled ML-236B [7] were prepared as described previously. ^{14}C-labeled CS-514 was prepared biosynthetically by adding ^{14}C-labeled ML-236B as a precursor to cultures of *Nocardia autotrophica* grown in Erlenmeyer flasks, and purified extensively as described [8]. MB-530B was prepared from the culture filtrate of *Monascus ruber* [12]. As internal standards for gas-liquid chromatography, neutral sterols, (5α-cholestane, cholesterol, desmosterol, sterol) and bile acids, (methyl esters of cholic, deoxycholic, lithocholic, chenodeoxycholic and 7-ketolithocholic acid) were obtained from Gasukuro Kogyo Co. (Tokyo, Japan) and 9-anthryl diazomethane was purchased from the Funakoshi Pharmaceutical Co. (Tokyo, Japan).

Animals. Wistar-Imamichi male rats weighing 120–140 g were used for usual experiments and rats weighing 250–330 g for the experiments of Triton-induced hyperlipidemia. The rats were maintained on a commercial rat chow (MM 1, Funabashi Farm Co, Chiba, Japan) for at least 1 week prior to use. Pure-bred beagle dogs weighing 8–10 kg were housed individually and fed a commercial dog food (Type DS, Oriental Yeast Co., Tokyo, Japan) at 200 g/day. Cynomolgus monkeys weighing 4.1–6.2 kg (male) and 3.2–4.1 kg (female) were housed individually and fed a commercial monkey food (Type AB, Oriental Yeast Co., Tokyo, Japan) at 80 g/day. Male Japanese white rabbits aged 11–13 months were housed individually and fed rabbit chow (RC-4, Oriental Yeast Co., Tokyo, Japan) at 150 g/day. Watanabe heritable hyperlipidemic (WHHL) rabbits weighing 3–3.5 kg were used for short-term experiments, and the rabbits at 3 months of age (weighing 1.8–2.2 kg) were used for a long-term experiment of 8 weeks. WHHL rabbits were housed individually and fed a commercial rabbit chow (Type GC, Oriental Yeast Co., Tokyo, Japan) at 120 g/day.

In animal experiments with dogs, monkeys and rabbits, blood samples were withdrawn between 9 and 9.30 a.m. on appropriate days before feeding.

Cells. All cultured cells used were maintained at 37°C in a 5% CO_2 incubator. Mouse L cells were seeded at about $2 \cdot 10^5$ cells per 60 mm dish and cultured for 3 days in Dulbecco's modified Eagle's minimum essential medium containing 5% fetal calf serum. Human skin fibroblasts from a normal subject (GM-442) and a homozygous familial hypercholesterolemic patient (GM-486) were obtained from the Institute of Medical Research (Camden, NJ, U.S.A.). These cells were grown in Dulbecco's modified Eagle's minimum essential medium containing 1% non-essential amino acids and 10% fetal calf serum. About $1 \cdot 10^5$ cells were seeded onto 60 mm dishes according to a standard protocol [15] and were

Rat hepatocytes were prepared by collagenase digestion according to the method of Moldéus et al. [16]. For preparation of rat spleen cell suspension, the spleen was cut into pieces with scissors and gently pressed on a stainless filter (100 mesh) using a silicon plug. The cells which filtered through were further treated twice on a 150 mesh filter and isolated by centrifugation at 1000 rpm for 5 min. The spleen cells thus obtained were completely disaggregated as single cells. Both hepatocytes and spleen cells were suspended at appropriate cell densities in serum-free Dulbecco's modified Eagle's minimum essential medium, and used for experiments within 3 h after preparation.

Rabbit aortic fibroblasts were obtained from explants of aortic adventitia and cultured in Dulbecco's modified Eagle's minimum essential medium containing 5% fetal calf serum. About $1 \cdot 10^5$ cells were seeded onto 60 mm dishes and used for experiments after 3 days cultivation.

Cellular uptake of CS-514. Suspensions of freshly isolated rat hepatocytes (10^7 cells/ml) or spleen cells ($2 \cdot 10^8$ cells/ml) were incubated with various concentrations of ^{14}C-labeled CS-514 or ^{14}C-labeled ML-236B sodium salt at 37°C for 30 min in O_2/CO_2 (95 : 5) gas with shaking. 80 μl of cell suspension were layered on 250 μl of the solvent mixture (butyl phthalate/dinonyl phthalate, 7 : 3, v/v) in a polyethylene tube (1A tube, Sanko Plastic Co., Osaka, Japan) and centrifuged at 3000 rpm for 2 min. The bottom of tube containing the cells was cut and put into a vial for determination of cellular radioactivity.

Mouse L cells grown to form a monolayer in plastic dishes received 1 ml of serum-free Dulbecco's modified Eagle's minimum essential medium containing various concentrations of ^{14}C-CS-514 of ^{14}C-ML-236B sodium salt. After incubation for 30 min at 37°C, cells in the medium were scraped with a rubber policeman. After the cell suspension was centrifuged at 1000 rpm for 5 min, about 900 μl of the medium were removed and cells were resuspended with the remaining medium. The cell suspension thus obtained was assayed for cellular radioactivity by the same procedure as that employed for rat hepatocytes. After measurement of cellular radioactivity, the pellet of mouse L cells was taken out from the counting times, dissolved with 4 M NaOH at 80°C for 1 h, and assessed for protein content.

Sterol synthesis in the cell-free enzyme system from rat liver, cultured cells and tissue slices from various organs of rats. [1-^{14}C]Sodium acetate was used as a precursor for sterol synthesis in these experiments. For the measurement of sterol synthesis in the cell-free enzyme system from rat liver, the reaction mixture was incubated for 2 h at 37°C and incorporation of radioactivity into non-saponifiable lipids was assayed by the method described previously [5]. In the experiments with cultured cells, monolayers of mouse L cells, human skin fibroblasts and rabbit aortic fibroblasts were incubated with [^{14}C]sodium acetate at 37°C for 2 h. In the experiments with freshly isolated cells from rats, [^{14}C]sodium acetate was added to 1 ml of suspension culture containing $2 \cdot 10^6$ hepatocytes or $5 \cdot 10^7$ spleen cells, and the incubation was carried out at 37°C for 2 or 4 h, respectively. Incorporation of radioactivity into digitonin-precipitable sterols was measured by the method described previously [15]. In the experiment using tissue slices, rats were killed by carotid puncture and various organs were rapidly removed. The reaction mixture containing tissue slices and [^{14}C]sodium acetate was incubated for 2 h at 37°C in Krebs-Ringer bicarbonate buffer (pH 7.4) and incorporation of radioactivity into digitonin-precipitable sterols was measured as described previously [7]. All experiments were done by duplicate assays, unless stated otherwise.

Assay of hydroxymethylglutaryl-CoA reductase activity. Hydroxymethylglutaryl-CoA reductase activities in rat liver microsome [6] and in cultured cells [15] were measured as described previously.

Measurement of hypolipidemic activity of CS-514 in Triton WR-1339-induced hyperlipidemic rats. The experiment was carried out as described previously [17,18] except that the drug was administered orally at the time of Triton injection.

Measurement of lipids and fractionation of serum lipoproteins. Serum lipids were measured by enzymatic methods using the following assay kits unless otherwise stated: cholesterol, Determiner TC (Kyowa Hakko Co., Tokyo, Japan); triacylglycerol and phospholipid, Triglyceride G-Test Wako and Phospholipid B-Test Wako, respec-

Japan). Serum cholesterol levels in a Triton-induced hyperlipidemic rats were assayed by a slightly modified method of Zak [19] and Henly [20].

For the fractionation of serum lipoprotein, 0.5 ml (WHHL rabbit) or 1 ml (other animals) of serum was centrifuged according to the method of Hatch and Lees [21] using a Hitachi 65P ultracentrifuge equipped with an RPS-56T rotor (Hitachi Co., Tokyo, Japan). Each lipoprotein was fractionated as follows: very-low-density lipoprotein (VLDL, $d < 1.006$); intermediate-density lipoprotein (IDL, $1.006 < d < 1.019$); low-density lipoprotein (LDL, $1.019 < d < 1.063$); high-density lipoprotein (HDL, $1.063 < d < 1.21$) and infranatant ($d > 1.21$).

Analysis of biliary lipids. Bile samples for analysis were prepared as described previously [19]. For correcting recovery of neutral sterols and bile acids, 5α-cholestane and [^{14}C]deoxycholate were added to bile samples, respectively. Neutral sterols were determined by gas-liquid chromatography [22]. Bile acid was measured by high-performance liquid chromatography: the samples treated with 9-anthryl diazomethane were chromatographed on a 25 cm ODS C-18 column maintained at 35°C in a Hitachi Type 655 high-performance liquid chromatography (Hitachi Co., Tokyo, Japan) equipped with a fluorometric detector FP-110 (Japan Spectroscopic Co., Tokyo, Japan). Under these conditions, the overall recovery of bile acid varied between 65 and 95%.

The phospholipid in bile was assayed by phosphorus analysis using a kit, Phospholipid-Test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Other assay. Protein content was determined by the method of Lowry et al. [23] with bovine serum albumin as standard.

Results

Specific and tissue-selective inhibition of sterol synthesis

Fig. 2 shows the effect of CS-514 on the incorporation of various radiolabeled precursors into digitonin-precipitable sterols in the cell-free system from rat liver. The conversion of [^{14}C]acetate

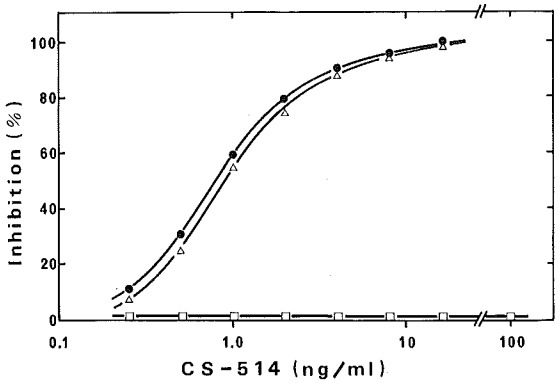

Fig. 2. Effect of CS-514 on cholesterol synthesis from acetate, hydroxymethylglutaryl-CoA or mevalonate in a cell-free enzyme system from rat liver. The assay method of sterol synthesis is described in Materials and Methods. The concentrations of the precursors were; 1 mM, 0.3 mM and 0.5 mM for [^{14}C]acetate (●), [^{14}C]hydroxymethylglutaryl-CoA (△) and [^{14}C]mevalonate (□), respectively. Each point is plotted as a percent inhibition to the respective control.

hibited to almost the same extent, i.e., the concentrations required for 50% inhibition (I_{50}) were 0.8 ng/ml and 0.9 ng/ml, respectively. In the case of ML-236B, the mother compound of CS-514, I_{50} for the conversion of [^{14}C]acetate was 10 ng/ml. On the other hand, the conversion of [^{14}C]-mevalonate was not affected by CS-514 at concentrations up to 100 ng/ml, indicating that this drug inhibited specifically the enzymatic step of the conversion of hydroxymethylglutaryl-CoA to mevalonate catalysed by hydroxymethylglutaryl-CoA reductase. The mode of inhibition of CS-514 for hydroxymethylglutaryl-CoA reductase was competitive with respect to hydroxymethylglutaryl-CoA CoA and non-competitive with respect to NADPH. The K_i values for the reductase from rat liver and mouse L cells were $2.3 \cdot 10^{-9}$ M and $2.2 \cdot 10^{-9}$ M, respectively.

Table I summarizes the values of I_{50} for incorporation of [^{14}C]acetate into sterols either by CS-514 or by ML-236B in freshly isolated and cultured cells. Both CS-514 and ML-236B strongly inhibited sterol synthesis in freshly isolated rat hepatocytes. The values of I_{50} were 2.2 ng/ml and 7.0 ng/ml, respectively, both of which were close to the values obtained in the cell-free enzyme system from rat liver, suggesting that both drugs

in hepatocytes. In the cells from nonhepatic tissues, such as freshly isolated rat spleen cells and various cultured cells, ML-236B exerted the potent inhibitory activity as in rat hepatocytes. In contrast, the inhibitory activity of CS-514 was much less potent in these cells from nonhepatic tissues than in rat hepatocytes. In order to verify the assumption that the results mentioned above might be explained by poor permeability of CS-514 into the cells from nonhepatic tissues, cellular uptake of ^{14}C-CS-514 and ^{14}C-ML-236B sodium salt was compared among freshly isolated rat hepatocytes and spleen cells, and mouse L cells. As shown in Fig. 3, the cellular uptake of ^{14}C-CS-514 in hepatocytes occurred to almost the same extent as that of ^{14}C-ML-236B sodium salt. The uptake of ^{14}C-CS-514 in spleen cells and mouse L cells, however, was less than one-tenth of that of ^{14}C-ML-236B sodium salt. From these results, the less potent inhibitory activity of CS-514 in the cells from nonhepatic tissues can be ascribed to lower uptake of the drug by those cells.

After CS-514, ML-236B or MB-530B was administered orally to rats, the activity of sterol synthesis in slices of various organs was measured in vitro and inhibition in each organ was calculated (Table II). CS-514 inhibited the sterol synthesis selectively in liver and ileum (intestine), the major sites of cholesterogenesis, but only weakly inhibited that in other organs, including hormone-producing ones. In the cases of ML-236B and MB-530B, although the inhibition of sterol synthesis in liver and intestine was most potent, that in other organs was significant as well. These results indicate that the inhibitory activity of CS-514 is tissue selective, compared to those of ML-236B and MB-530B, and this observation is consistent with the results obtained with the isolated and cultured cells.

TABLE I

EFFECT OF CS-514 AND ML-236B ON STEROL SYNTHESIS IN VARIOUS ISOLATED AND CULTURED CELLS

The assay methods of sterol synthesis are described in Materials and Methods. The inhibitory activities of CS-514 and ML-236B on sterol synthesis are expressed by the concentrations required for 50% inhibition (I_{50}). n.t., not tested.

Cells	I_{50} (ng/ml)	
	CS-514	ML-236B
Freshly isolated rat hepatocytes	2.2	7.0 *
Freshly isolated rat spleen cells	70	1.3 *
Mouse L cells	600	1.4
Human skin fibroblasts		
(normal)	200	18.0
(homozygous familial hypercholesterolemia)	400	12.0
Rabbit aortic fibroblasts	750	n.t.

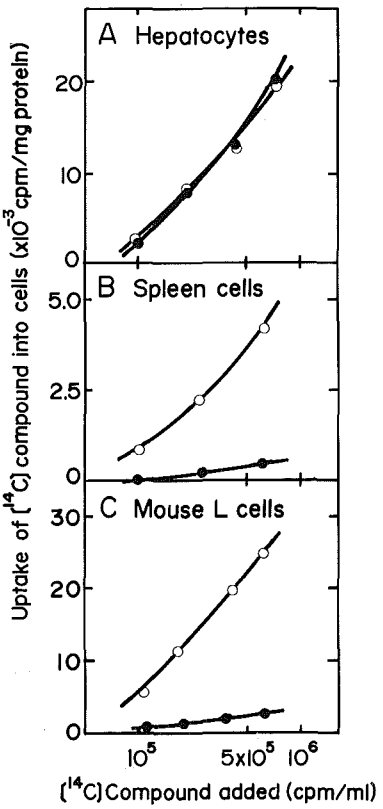

Fig. 3. Cellular uptake of ^{14}C-CS-514 and ^{14}C-ML-236B sodium salt into (A) freshly isolated rat hepatocytes, (B) freshly isolated spleen cells and (C) mouse L cells. The cells were incubated with the indicated concentrations of ^{14}C-CS-514 (●) or ^{14}C-ML-236B sodium salt (○) at 37°C for 30 min. Uptake of radioactivity into cells was determined as described in Materials and Methods. Each point expresses the average of duplicate (rat hepatocytes and spleen cells) or quadruplicate

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.