J. Microbiol. Biotechnol. (2002), **12**(2), 222–227

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Hypocholestrolemic Effect of CJ90002 in Hamsters: A Potent Inhibitor for Squalene Synthase from *Paeonia moutan*

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Received: September 28, 2001
Accepted: February 8, 2002

Abstract Squalene synthase catalyzes the reductive dimerization of two molecules of farnesyl diphosphate to form squalene at the final branch point of the cholesterol biosynthetic pathway. Due to the unique position of this enzyme in the pathway, its inhibitors may have advantages as antihypercholesterolemic agents. Therefore, selective inhibitors of squalene synthase do not prevent the formation of the essential branch products of the isoprene pathway, such as dolichol, coenzyme-Q, and prenylated proteins, as might be expected for inhibitors of enzymes earlier in the pathway; for example, lovastatin and mevalotin. The current study reports that CJ90002, a pentagalloylglucose isolated from *Paeonia moutan* SIM (Paeoniaceae), which is an important Chinese crude drug used in many traditional prescriptions, was a potent inhibitor of rat microsomal squalene synthase, and also a potent inhibitor of cholesterol biosynthesis *in vitro*. In addition, the intraperitoneal and oral administration of CJ90002 had a significant lowering effect on plasma cholesterol levels in hamsters.

Key words: Cholesterol lowering agent, squalene synthase, *Paeonia moutan*, pentagalloylglucose

Elevated serum cholesterol is well established as a risk factor for coronary disease. Thus, reducing elevated levels of serum cholesterol in humans leads to a reduction in the incidence of coronary-related death. Studies in the last few years have shown that one of the more effective ways of reducing serum cholesterol is by inhibiting sterol biosynthesis [6, 13], and consequently, a number of therapeutic agents such as lovastatin and mevalotin are currently available that work by inhibiting HMG-CoA reductase. However, the product of this step, mevalonate, is also used for the synthesis of nonsterol products such as dolichol and ubiquinone, as well as for protein isoprenylation. Accordingly,

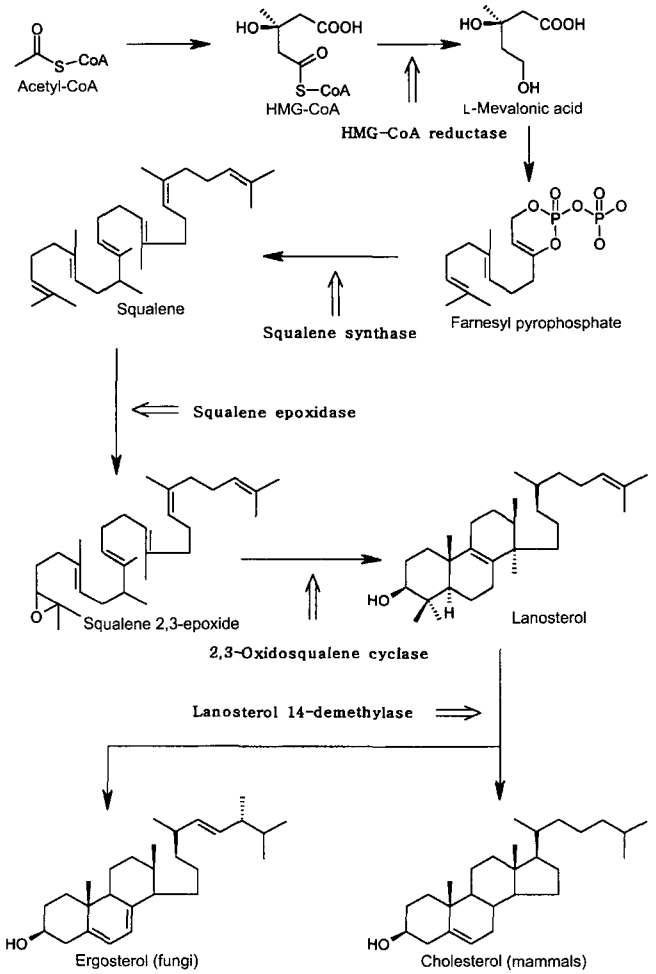

Fig. 1. Enzymatic biosynthesis of cholesterol.

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a more selective inhibition of cholesterol synthesis may be achieved by inhibiting any steps beyond the branch in the pathway (Fig. 1).

Squalene synthase (farnesyl-diphosphate : farnesyl-diphosphate farnesyl-transferase, EC 2.5.1.21) catalyzes the reductive dimerization of farnesyl diphosphate (FPP) to form squalene at the final branch point of the cholesterol biosynthetic pathway [1, 10]. Due to the unique position of squalene synthase in the pathway, inhibitors of this enzyme are expected to have advantages as antihypercholesterolemic agents, because selective inhibitors of squalene synthase do not interfere with the formation of the essential branch products of the isoprene pathway, such as dolichol, coenzyme-Q, and prenylated proteins, as might be expected for inhibitors of enzymes earlier in the pathway [3]. The substrate for squalene synthase, FPP, is the last water-soluble intermediate in the pathway and its metabolic routes are known in the event of intracellular accumulation [4].

Paeonia moutan SIM (Paeoniaceae) has long been used in the indigenous system of traditional medicines in Korea and China. This medicinal plant is known to exhibit various pharmacological actions [2, 5, 7-9, 11, 14], such as an antiproliferative effect, anti-inflammation, thrombocyte agglutination, antihemorrhage, and antioxidant agents, and gastric secretion; however, no study on its cholesterol-lowering action is available. In the course of screening for squalene synthase inhibitors from various traditional plants, a potent inhibitor, CJ90002 (1,2,3,4,6-*o*-pentagalloylglucose) (Fig. 2), was found in *Paeonia moutan* SIM extracts. In the current study, the *in vitro* inhibitory activity of CJ90002 against rat microsomal squalene synthase and cholesterol biosynthesis was studied along with its *in vivo* hypocholesterolemic activities in hamsters.

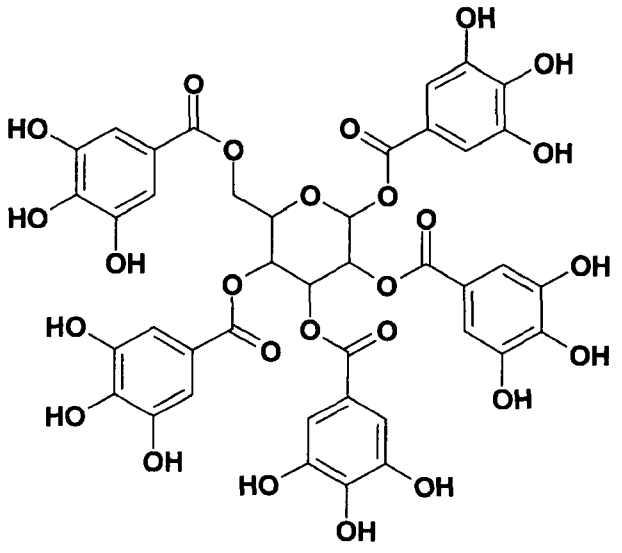

Fig. 2. Structure of CJ90002.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats and male Syrian golden hamsters (110–140 g) were purchased from Sam Yuk Laboratory Animal, Inc. (Osan, Korea) and Charles-River Laboratories, Inc. (Wilmington, MA, U.S.A.), respectively.

Isolation and Structure Elucidation of CJ90002

The dry powder of *Paeonia moutan* SIM was extracted in 80% methanol solution at 70°C for 3 h under reflux. The methanol was removed under reduced pressure and further extraction was carried out using *n*-buthanol. The resulting extracts were chromatographed on a silica gel, and eluted with *n*-hexane/ethanol (8:2) to yield the active fraction, which was then run on a preparative HPLC (LiChrosorb® RP-18 column, Merck KGaA, Darmstadt, Germany) to isolate the CJ90002 ($C_{41}H_{32}O_{26}$, MW 940.70). The structural elucidation of the purified CJ90002 was carried out on a Bruker ARX400 NMR spectroscopy (Karlsruhe, Germany) and Micromass Autospec Mass-spectroscopy (Manchester, U.K.).

Preparation of Rat Liver Microsome Fraction

The rat liver microsomes were prepared from male rats weighing 120–140 g, which were kept on a reverse diurnal light cycle for 7 days and then sacrificed. The livers were homogenized at 4°C in a homogenization buffer (50 mM phosphate buffer, pH 7.4, 4.0 mM $MgCl_2$, 1.0 mM EDTA, and 1.0 mM DTT) using a Potter homogenizer. The homogenates were centrifuged twice at 15,000 ×g for 15 min, at 4°C, pouring the supernatants through cotton gauze after each spin. The microsomes were then isolated from the supernatant by centrifugation at 100,000 ×g for 60 min at 4°C, resuspended in the homogenization buffer, and stored in aliquots at −70°C for up to 2 months.

Measurement of Squalene Synthase Activity

Enzyme reaction mixtures were composed of potassium phosphate buffer (100 mM, pH 7.4), 5 mM $MgCl_2$, 10 mM KF, 1 mM NADPH, 30 mM nicotinamide, 0.01 mM tolnaftate, the rat liver microsomal fraction containing 0.05 mg proteins, and a methanol solution of CJ90002. After 5 min preincubation at room temperature, the reaction was initiated by adding 0.1 mM [1-^{3}H] FPP (58.4 μCi/μmol). After incubation of the mixture at 30°C for 20 min, the reaction was terminated by the addition of 400 μl of 5 M NaOH in 95% ethanol solution. The reaction mixture was saponified at 65°C for 30 min, then 200 μl of distilled water and 500 μl of petroleum ether were added to extract the squalene. The extracted squalene was concentrated and the residue dissolved in a small amount of chloroform. The resulting solution was thin layer chromatographed over a silica gel, developed with chloroform to isolate the squalene,

then radio activity was measured using a liquid scintillation counter. A control without CJ90002 was also prepared and measured using the same manner as described above.

The squalene biosynthesis inhibiting effect of CJ90002 was determined as an inhibition percentage using the following equation:

$$\frac{\text{Radioactivity of control} - \text{Radioactivity with CJ90002}}{\text{Radioactivity of control}} \times 100$$

Measurement of Cholesterol Biosynthesis from [1-^{14}C] Acetate

Enzyme reaction mixtures were composed of potassium phosphate buffer (100 mM, pH 7.4), 1 mM ATP, 6 mM glutathione, 10 mM glucose-1-phosphate, 6 mM $MgCl_2$, 0.04 mM Coenzyme A, 0.25 mM NAD, 0.25 mM NADP, the rat liver microsomal fraction containing 0.1 mg proteins, and a methanol solution of CJ90002. After 5 min preincubation at room temperature, the reaction was initiated by adding 1 mM [1-^{14}C] sodium acetate (3 μCi/μmol). After the incubation of the reaction mixture at 30°C for 2 h, the reaction was terminated by the addition of 250 μl of 15% KOH in 95% ethanol solution. The reaction mixture was saponified at 75°C for 1 h, then 250 μl of distilled water and 500 μl of petroleum ether were added to extract cholesterol. The extracted cholesterol was concentrated and then measured using a liquid scintillation counter. A control without CJ90002 was also prepared and measured, as described above. The cholesterol biosynthesis inhibiting effect of CJ90002 was determined as an inhibition percentage using the same equation as mentioned above.

Measurement of Serum Cholesterol Levels After Oral Administration of CJ90002 for 10 Days in Hamsters

Seven groups (n=6) of male Syrian golden hamsters, weighing 120±5 g and 8 to 10 weeks old, were kept on a reverse diurnal light cycle at 22°C and humidity of 55±5% for 7 days. Water and feed were supplied *ad libitum* during this period.

After 7 days, all groups were supplied with conventional standard diet for 10 days. During this period, the first control group was orally administered once a day with 0.5% aqueous methylcellulose solution as a vehicle, while the other 3 groups were orally administered with CJ90002 in 0.5% methylcellulose, at a daily dosage of 30, 60, and 100 mg/kg body weight, respectively. The remaining 3 reference groups were orally administered with lovastatin in 0.5% methylcellulose as a reference compound, at a daily dosage of 30, 60, and 100 mg/kg body weight, respectively.

At the end of the period, the animals were fasted overnight and then anaesthetized with ether. Blood samples were taken by cardiac puncture, serum separated, and the levels of triglyceride (TG), total cholesterol (TC), and high-density lipoprotein-cholesterol (HDL-C) were measured. The low-density lipoprotein-cholesterol (LDL-C) level was calculated using the following equation:

$$\text{LDL-C} = \text{TC} - (\text{TG}/5 + \text{HDL-C})$$

Measurement of Serum Cholesterol Levels After Intraperitoneal Administration of CJ90002 for 3 Days in Hamsters

Three groups (n=5) of male Syrian golden hamsters, weighing 120±5 g and 8 to 10 weeks old, were kept on a reverse diurnal light cycle at 22°C and humidity of 55±5% for 7 days. Water and feed were supplied *ad libitum* during this period.

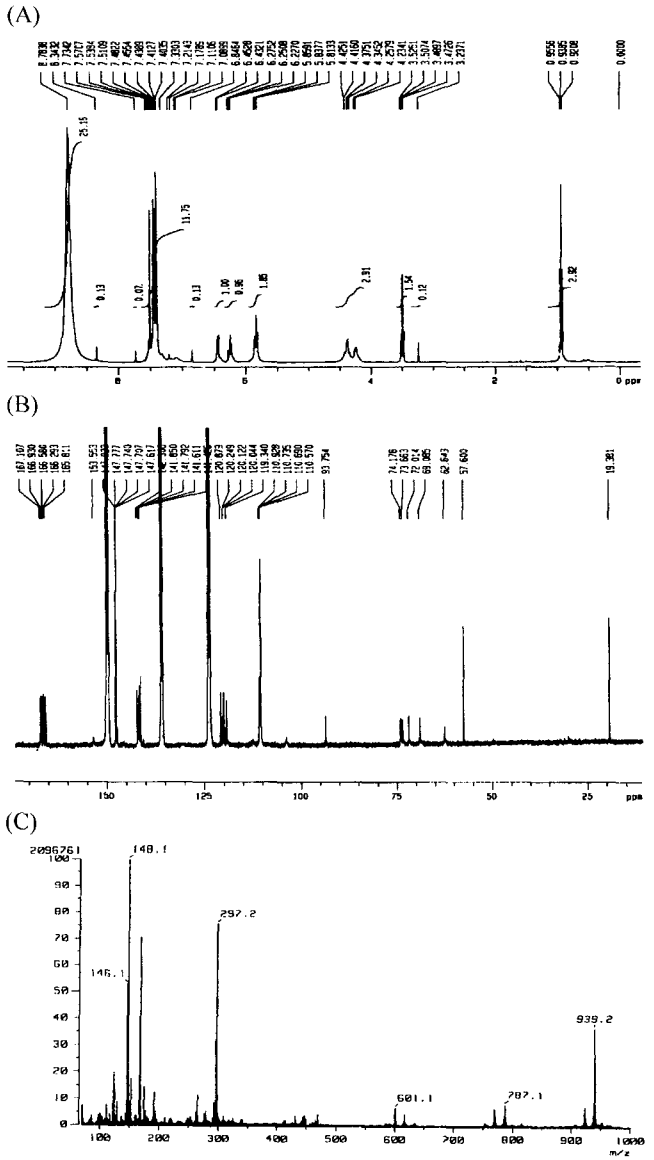

Fig. 3. Structural determination of CJ90002. (A) ^{1}H-NMR spectrum of CJ90002 in pyridine-d_5. (B) ^{13}C-NMR spectrum of CJ90002 in pyridine-d_5. (C) FAB-MS spectrum of CJ90002. The peak at 939.2 represents the molecular-ion peak [MH].

After 7 days, all groups were supplied with a conventional standard diet for 3 days. During this period, the first control group was intraperitoneally administered once a day with 4% DMSO solution as a vehicle, while the second group was given a daily dose of CJ90002 in 4% DMSO at 1 mg/kg body weight administered intraperitoneally. The last group was also given a daily dose of 10 mg/kg body weight CJ90002 in 4% DMSO administered intraperitoneally. At the end of the period, the animals were fasted overnight and then anaesthetized with ether. Blood samples were taken from the orbital plexus, serum separated, and the levels of triglyceride (TG), total cholesterol (TC), and high-density lipoprotein-cholesterol (HDL-C) measured. The low-density lipoprotein-cholesterol and very low-density lipoprotein-cholesterol levels were also calculated using the following equation:

$$(LDL+VLDL)\text{-}C=TC-HDL\text{-}C$$

Statistical Analysis

The data are expressed as mean±SEM. Where the analysis of variance showed a significant difference between the groups, the difference was assessed using the Student's *t*-test. Values of $p<0.05$ were considered to be statistically significant.

Results and Discussion

The Structure Elucidation of CJ90002

As shown in Fig. 3, the structural elucidation of the purified CJ90002 was carried out on a Bruker ARX400 NMR spectroscopy and Micromass Autospec Mass-spectroscopy.

CJ90002 is a well-known gallotannin (1,2,3,4,6-*o*-pentagalloylglucose), in which the glucose is saturated with five galloyl groups (Fig. 2). This compound is known to exhibit various pharmacological actions, such as an antiproliferative effect, anti-inflammation, thrombocyte agglutination, antihemorrhage, and antioxidant properties, and gastric secretion. However, there has been no report on its cholesterol-lowering action.

Table 1. Inhibitory effects of CJ90002 on rat liver microsomal squalene synthase and cholesterol biosynthesis.

Test compound	Concentration (μg/ml)	Inhibition (%)	
		Squalene synthase	Cholesterol biosynthesis
CJ90002	0.3	15	29
	1	57	63
	3	79	82
	10	92	93
	30	95	96

Inhibitory Effects of CJ90002 on Hepatic Microsomal Squalene Synthase and Cholesterol Biosynthesis *in vitro*

The *in vitro* inhibitory effects of CJ90002 on the hepatic microsomal squalene synthase and cholesterol biosynthesis at various concentrations are listed in Table 1. In both cases, CJ90002 exhibited strongly dose-dependent inhibition. As shown in Table 1, its IC_{50} values were less than 1 μg/ml, therefore, CJ90002 appears to be a potential candidate as a hypocholesterolemic agent. As such, further detailed *in vivo* experiments were carried out.

Effects of CJ90002 Administered Orally on Plasma Lipid Concentrations in Hamsters

The cholesterol-lowering activity of CJ90002 was studied in hamsters (Table 2), a species whose lipoprotein metabolism

Table 2. Effects of oral administrations of CJ90002 and lovastatin on hamster plasma lipoprotein cholesterol levels.

Test Compound	Dose (mg/kg/day)	TC	LDL-C	HDL-C	TG
		(Inhibition %)			
Control	0.5% Methylcellulose	149.9±28.7 (0)	44.4±31.9 (0)	49.8±16.2 (0)	268.5±129.7 (0)
CJ90002	30	136.0±56.0 (9.3)	41.9±25.4 (5.6)	54.8±27.5 (-10)	219.0±90.5 (18.4)
	60	139.3±56.6 (7.1)	47.8±27.5 (-7.7)	42.3±20.2 (15.1)	252.6±166.3 (5.9)
	100	115.5±47.8 (22.9)*	42.4±22.7 (4.5)	35.4±16.8 (28.9)*	248.1±173.3 (7.6)
Lovastatin	30	98.3±47.3 (34.4)**	48.8±28.8 (-9.91)	33.0±22.5 (33.3)	82.5±54.9 (69.3)**
	60	88.1±41.4 (41.2)***	28.4±19.4 (34.0)	28.6±15.3 (42.6)*	126.4±90.2 (52.1)*
	100	93.9±46.0 (37.4)**	38.2±22.0 (14.0)	33.3±18.6 (33.1)	93.1±61.2 (65.3)**

* : $p<0.05$, ** : $p<0.01$, *** : $p<0.001$.

Table 3. Effects of intraperitoneal administrations of CJ90002 on hamster plasma lipoprotein cholesterol levels.

Group	Dose	TC	TG	(LDL+VLDL)-C[a]	HDL-C
		(Inhibition %)			
Control	Vehicle (4% DMSO)	89.0±12.5 (0)	137.6±25.8 (0)	56.6±11.5 (0)	32.4±3.4 (0)
CJ90002	1 mg/kg/day	83.0±10.4 (6.8)	114.0±14.4 (17.2)	54.2±5.1 (4.2)	28.8±7.1 (11.1)
	10 mg/kg/day	68.8±13.3 (22.7)*	91.2±20.5 (33.3)*	42.4±7.8 (35.1)*	26.4±5.9 (18.5)

[a] : (LDL+VLDL)-C=TC−HDL-C ; * : $p<0.05$.

and circulating lipoprotein levels closely resembles that of humans [12]. The oral administration of CJ90002 with a dosage of 100 mg/kg/day for 10 days resulted in 22.9% lower plasma cholesterol (TC) level ($p< 0.05$). This result showed it to be slightly less efficacious than the cholesterol lowering caused by lovaststin, a potent inhibitor of HMG-CoA reductase. Its lowering cholesterol levels with dosages of 30, 60, and 100 mg/kg/day were 34.4% ($p<0.01$), 41.2% ($p<0.001$), and 37.4% ($p<0.01$), respectively. CJ90002 with dosages of 60 and 100 mg/kg/day lowered the cholesterol levels in the isolated HDL fraction (HDL-C) by 15.1 and 28.9%, respectively. However, this effect was not statistically significant ($p>0.05$). Unfortunately, while lovastatin significantly lowered the total triglyceride level (TG) at all doses tested, CJ90002 did not have any effect on lowering the TG. Consequently, the oral administration of CJ90002 exhibited a weaker activity than lovastatin. The reason for this might have been due to the low oral absorptivity of CJ90002. However, as mentioned above, since the results for LDL-C were not statistically significant, CJ90002 was intraperitoneally administered for 3 days to clarify whether or not the weaker activity of the agent was caused by its low oral absorptivity.

Effects of CJ90002 Administered Intraperitoneally on Plasma Lipid Concentrations in Hamsters

As shown in Table 3, the intraperitoneal injection of CJ90002 at 10 mg/kg/day for 3 days resulted in 22.7% lower plasma cholesterol level ($p>0.05$). Furthermore, CJ90002 at this same dose also lowered the TG level and cholesterol level in the isolated LDL plus VLDL fractions [(LDL+VLDL)-C] by 33.3 and 35.1%, respectively ($p>0.05$). In addition, lower cholesterol level in the isolated HDL fraction (HDL-C) was also detected, although the effect was not statistically significant (Table 3). Therefore, it would appear that low oral absortion of CJ90002 caused weak activity to lower hamster plasma lipoprotein cholesterol level.

In conclusion, the use of CJ90002 as a potent hypocholesterolemic agent may not be appropriate for hypolipidemic therapy; however, the current results provide a novel direction for further development of useful squalene synthase inhibitors with greater potential for clinical efficacy.

Acknowledgments

This work was supported by a research fund of Hanyang University (HY-2000-75). All NMR measurements using a Bruker Advance 400 spectrometer system were partially supported by the Shared Research Equipment Assistance Program (R23-2001-00002) of the Korea Basic Science Institute.

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