Kakkalide and Irisolidone: HMG-CoA Reductase Inhibitors Isolated from the Flower of *Pueraria thunbergiana*

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As part of our search for anti-arteriosclerosis agents from traditional Chinese medicines, the 3-hydroxy-3-methylglutaryl (HMG-CoA) reductase (HCR)-inhibitory constituent, kakkalide, was isolated from the flower of *Pueraria thunbergiana* (PT, family Leguminosae). The anti-hyperlipidemic effects of kakkalide and its metabolite, irisolidone, which may be a bioactive form in *vivo* and potently inhibit the HCR activity, were investigated in *vivo*. Both the oral and interperitoneal administrations of kakkalide and irisolidone, with the exception of intraperitoneally treated kakkalide, potently lowered the serum levels of total cholesterol (TC) and triglyceride (TG) in Triniton WR1339-induced hyperlipidemic mice. The oral administrations of kakkalide and irisolidone in hyperlipidemic mice induced, by the long-term feeding of a high fat diet, also potently reduced the serum levels of TC and TG and epididymal fat pad weight. These findings suggest that PT can improve hyperlipidemia, and the hypolipidemic effect may be due to HMG-CoA reductase.

Key words  *Pueraria thunbergiana*; kakkalide; irisolidone; 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase; hypolipidemic activity

Lipid metabolism normally maintains an elegant balance between its synthesis and degradation. When the balance is disrupted, hyperlipidemia, such as hypertriglyceridemia and hypercholesterolemia, may develop. This can cause a variety of serious diseases, such as arteriosclerosis, hypertension, obesity and diabetes, etc. The rate-limiting enzyme for the biosynthesis of cholesterol from acetate is 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (HCR). HCR plays an important role in the biosynthesis of cholesterol; therefore, many researchers have been developing inhibitors of HCR.

The flower of *Pueraria thunbergiana* (PT, family Leguminosae), which contains kakkalide (>2%), is used to counteract problems associated with alcohol drinking, liver injury and weight loss. Niiho et al. reported that the isoflavonoid fraction of *P. lobata* suppressed the increases in the concentrations of blood ethanol, acetaldehyde and ketones induced by ethanol administration, and that its isoflavonoid and triterpenoid saponin fractions improved the abnormal metabolism induced by either carbon tetrachloride or high fat foods. Han et al. reported that, when kakkalide isolated from PT was metabolized to irisolidone by human intestinal microflora, the metabolite irisolidone reduced the mortality associated with the administration of ethanol in mice, and also showed hepatoprotective activity. However, the hypolipidemic effects of PT and its constituents have not been thoroughly studied.

As part of our continuing search for anti-arteriosclerosis agents from natural herbal resources, the HCR-inhibitory activity of PT was measured, kakkalide isolated as a HCR inhibitor, and its anti-hyperlipidemic effect investigated.

MATERIALS AND METHODS

**Materials** Cholestyramine, Triton WR-1339, RS-HMG-CoA, NADPH, dl-dithiothreitol (DTT), EDTA-K and lovastatin were purchased from Sigma Co. (St. Louis, MO, U.S.A.), triglyceride (TG), total cholesterol (TC) and high density lipoprotein (HDL) cholesterol assay kits were from Asan Pharmaceutical Co., Ltd. (Seoul, Korea). Orlistat (Xenical) was kindly donated by Dr. B. W. Song of Kyung Hee Medical Center, Kyung Hee University (Seoul, Korea). A high fat diet, containing 25% beef tallow [American Institute of Nutrition (AIN)-76 fat diet #180337], was purchased from Dyets, Inc. (Bethlehem, PA, U.S.A).

**Isolation of Kakkalide and Irisolidone from PT** PT was purchased from Kyung Dong Market (Seoul, Korea), and identified by Dr. Nam-Jae Kim, East-West Medical Center, Kyung Hee University. A voucher specimen (KHUP-01059) was deposited at the Herbarium of the College of Pharmacy, Kyung Hee University.

The flowers of PT (2 kg) were extracted twice with 101 of boiling water. After evaporation of the solvent, the extract (510 g) was reextracted in 500 ml of water. The suspended extract was further stepwise extracted with ethyl ether, ethyl acetate and butanol. Of these fractions, the most potent HCR-inhibitory ethyl acetate fraction (22 g) was subjected to silica gel column chromatography, and eluted with CHCl3:MeOH (20:1→10:1). Three compounds (PT-1, 2, 3) were isolated. Of them, PT-3 (3.5 g) exhibited the most potent HCR inhibition. The active constituent was subjected to further chromatography on a silica gel column, eluted with CHCl3:MeOH (20:1), and recrystallized from ethanol to yield kakkalide (1.9 g: purity, >95%).

The kakkalide (1 g) was incubated with intestinal microflora (1 g wet weight), as previously reported, with irisolidone (0.3 g) subsequently isolated using silica gel column chromatography.

Kakkalide: Pale yellowish needles, mp 251—253°C. IR (KBr) νmax cm⁻¹: 3500, 3350, 3200 (OH), 1635 (C=O), 1605, 1580, 1510 (phenyl). FAB-MS: 609 [M+H]+.

Irisolidone: Pale yellowish amorphous powder, mp 189—190°C. IR (KBr) νmax cm⁻¹: 3447 (OH), 1648 (C=O) and 1023 cm⁻¹. FAB-MS: 315 [M+H]+.

**Partial Purification of HCR and Its Activity Assay** HCR was partially purified from the liver of male

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Sprague–Dawley rats (250—300 g body weight) stimulated by 5% cholesterylamine, according to the previously reported method. 8) The inhibitory activity assay of HCR was performed according to the previous method. 8) Its activity was determined at 37 °C in a total volume of 0.5 ml using a Beckman spectrophotometer (Shimadzu Co.). The oxidation rate of NADPH was initially determined in the absence of HMG-CoA, and this blank value was subtracted from the rate obtained with both substrates. The activity assay reaction mixture contained 0.2 mM KCl, 0.16 M potassium phosphate, 4 mM EDTA and 1 mM DTT, at pH 6.8, and 0.2 mM NADPH and 0.1 mM RS-HMG-CoA.

Animals Male ICR mice (20—25 g) were supplied from the Orient Experimental Animal Breeding Center (Seoul, Korea). All the animals were housed in wire cages at 20—22 °C and 50±10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center, Seoul, Korea) and allowed water ad libitum. All the procedures relating to the animals and their care conformed to the international guidelines ‘Principles of Laboratory Animals Care’ (NIH publication no. 85-23, revised 1985). To evaluate the hypolipidemic effect, three hyperlipidemic animal models were established.

First, a hyperlipidemic mouse model, based on Triton WR-1339, was established according to the method of Lee et al. 9) Triton WR-1339 was injected at the end of a regular 16 h fasting period, as a solution in saline, at a dose of 200 mg/kg body weight into the tail veins of mice under light ether anesthesia. Eighteen hours after the Triton WR-1339 injection, 1—1.5 ml of blood was withdrawn by cardiac puncture. Sera were obtained by centrifugation (1500×g, 10 min). The test agents, lovastatin and orlistat, were administered orally or intraperitoneally once a day for 3 d. The final administration of the agents was performed 1 h before Triton WR-1339 injection.

Second, a hyperlipidemic mouse model, based on a high fat diet, was established. Six mice were used per group: the control group was fed an AIN-76 high fat diet for 5 weeks. The normal group received a solid normal diet only. Kakkalide and irisolidone, at a dose of 25 mg/kg/d, the water extract of PT, at a dose of 50 mg/kg/d, and orlistat and lovastatin, at doses of 10 mg/kg/d, were orally administered for 5 weeks. After a 16 h fasting period following the final administration of the agents, blood samples were withdrawn by cardiac puncture under ether anesthesia.

Determination of Serum TC, TG, and HDL Cholesterol TC was measured using the enzyme method of Allain et al. 10) The serum TG was measured using the method designed by Sardesai and Manning. 11) HDL cholesterol was measured using the method of Lee et al. 9)

Statistical Analysis All the data is expressed as a mean±standard deviation, and statistical significance was analyzed using one-way ANOVA followed by Student–Newman–Keuls test.

RESULTS

As part of our search for the anti-hyperlipidemic effects of traditional Chinese medicines, the HCR-inhibitory effect of PT was measured (Fig. 2), and was found to potently inhibit the HCR activity. Therefore, HCR-inhibitory activity-guided fractionation was performed against the PT extract. Kakkalide was isolated as a potent inhibitor, with an IC_{50} value of 48 μM. The metabolite of kakkalide, irisolidone, was also measured because kakkalide may be metabolized to irisolidone by intestinal microflora prior to its absorption into the blood. 7) The metabolite, irisolidone, was also found to be a potent inhibitor, with an IC_{50} value of 36 μM.

To evaluate the hypolipidemic effects of PT and its HCR inhibitor, kakkalide, the inhibitory effects of kakkalide and irisolidone in Triton WR-1339-induced hyperlipidemic mice were also measured. Orally administered PT, kakkalide and irisolidone in the Triton WR-1339-treated control group significantly decreased the serum TG and TC levels. However, PT and kakkalide did not show any significant increase of HDL-cholesterol level, although irisolidone increased its level (Table 1). Of the agents, irisolidone exhibited the most potent inhibition. However, when kakkalide and irisolidone were intraperitoneally administered, irisolidone potently lowered the levels of serum TG and TC induced by Triton WR-1339, but kakkalide showed only weak decreases.

The inhibitory effects of PT, kakkalide and irisolidone were measured in hyperlipidemic mice induced by long-term feeding of a high fat diet (Table 2). The TG, TC and LDL cholesterol levels in serum were increased by the 5 week treatment with a high fat diet. The administration of PT, kakkalide and irisolidone inhibited these levels, as well as the increase in body weight, compared with those of the control group (data not shown). The increased epididymal fat pad masses on administration of a high fat diet were also significantly reduced. However, the HDL-cholesterol levels in the PT, kakkalide and irisolidone-treated groups increased.
PT, which potently inhibited the HCR activity of rat livers, was selected for a preliminary study as an anti-arteriosclerosis agent from natural herbal resources. Kakkalide was isolated from PT as a HCR inhibitor using HCR-inhibitory activity-guided fractionation. If natural glycose constituents, such as kakkalide, are orally administered, they may not be easily absorbed due to their hydrophilicity. Therefore, these constituents will inevitably come into contact with intestinal microflora in the alimentary tract, where they may subsequently be transformed.\textsuperscript{12,13} Orally administered kakkalide may be transformed to irisolidone before its absorption from the gastrointestinal tract into the blood. Han et al.\textsuperscript{13} reported that irisolidone, and not kakkalide, was detected in the urine after the administration of kakkalide to rats. Therefore, the hypolipidemic effects of PT, kakkalide and irisolidone were investigated \textit{in vivo}. PT exhibited a hypolipidemic effect in hyperlipidemic mice induced by Triton WR-1339. When kakkalide and irisolidone were orally administered, these agents showed potent hypolipidemic effects. However, when kakkalide and irisolidone were intraperitoneally administered, kakkalide showed only a weak hypolipidemic effect, but irisolidone showed a potent hypolipidemic effect. These results suggest that the metabolite, irisolidone, may be the active form for the biological effects of kakkalide, as previously reported, where intraperitoneally and orally administered irisolidone exhibited potent hepatoprotective effects in t-butyl hydroperoxide (BHP)-induced experimental liver injury mice.\textsuperscript{14} Even if kakkalide exhibits an antihyperlipidemic effect by the inhibition of HCR, intraperitoneally administered kakkalide, which is a glycoside, may be easily excreted in the urine or bile due to its hydrophilicity compared to that of irisolidone. Kakkalide and irisolidone also significantly lowered the serum TC and TG levels in hyperlipidemic mice induced by long-term feeding of a high fat diet. These agents also significantly reduced the epididymal fat pad masses, with an increase in the cell size due to the high fat diet. Their potencies at doses of 25 mg/kg were comparable with that of Orlistat at a dose 10 mg/kg. These results suggest that kakkalide and irisolidone not only inhibit the HCR activity, but also reduce the increases in the levels of blood TG and TC.

If PT is orally administered to humans, its main constituent, kakkalide, may act in the intestine, where it can be metabolized to irisolidone by intestinal microflora. The metabolite, irisolidone, may be more easily absorbed into the blood compared to kakkalide, as it is more hydrophobic. The absorbed irisolidone may inhibit HCR in certain tissues, such as liver; thus, irisolidone may down-regulate the biosynthesis of triglycerides as well as that of cholesterol.

Based on these findings, the antihyperlipidemic effect of PT may be dependent on the metabolism of the main constituents, such as kakkalide, by intestinal microflora. Also, PT seems to be a potent hypolipidemic agent amongst traditional Chinese medicines.
REFERENCES