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Combination of simvastatin with berberine improves the lipid-lowering efficacy

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Abstract

We have identified berberine (BBR) as a novel cholesterol-lowering drug acting through stabilization of the low-density lipoprotein receptor (LDLR) messenger RNA. Because the mechanism differs from that of statins, it is of great interest to examine the lipid-lowering activity of BBR in combination with statins. Our results showed that combination of BBR with simvastatin (SIMVA) increased the LDLR gene expression to a level significantly higher than that in monotherapies. In the treatment of food-induced hyperlipidemic rats, combination of BBR (90 mg/[kg d], oral) with SIMVA (6 mg/[kg d], oral) reduced serum LDL cholesterol by 46.2%, which was more effective than that of the SIMVA (28.3%) or BBR (26.8%) monotherapy ($P < .01$ for both) and similar to that of SIMVA at 12 mg/(kg d) (43.4%). More effective reduction of serum triglyceride was also achieved with the combination as compared with either monotherapy. Combination of BBR with SIMVA up-regulated the LDLR messenger RNA in rat livers to a level about 1.6-fold higher than the monotherapies did. Significant reduction of liver fat storage and improved liver histology were found after the combination therapy. The therapeutic efficacy of the combination was then evaluated in 63 hypercholesterolemic patients. As compared with monotherapies, the combination showed an improved lipid-lowering effect with 31.8% reduction of serum LDL cholesterol ($P < .05$ vs BBR alone, $P < .01$ vs SIMVA alone). Similar efficacies were observed in the reduction of total cholesterol as well as triglyceride in the patients. Our results display the rationale, effectiveness, and safety of the combination therapy for hyperlipidemia using BBR and SIMVA. It could be a new regimen for hypercholesterolemia.

1. Introduction

Atherosclerosis and coronary heart disease (CHD) have been leading causes of morbidity and mortality in the Western countries for decades [1]. It is widely acknowledged that lowering serum low-density lipoprotein cholesterol (LDL-c) can interfere with the progression of atherosclerosis and reduce cardiovascular events in CHD patients [2-4].

Among the currently available lipid-altering agents, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are most widely prescribed [5]. Statins inhibit the endogenous cholesterol biosynthesis in hepatocytes, thereby increasing the transcription of the low-density lipoprotein receptor (LDLR) gene [6]. In general, statins are safe, well tolerated, and highly efficient in reducing LDL-c. More importantly, statins reduce cardiovascular events of CHD patients, as supported by numerous well-designed studies [7,8].

In clinical practice, however, more than half of the CHD patients do not reach their LDL-c goal; and many of them undergo cardiovascular events, even with statins as their first-line therapy [9]. There are 2 reasons. Firstly, statins are frequently not titrated to their optimal dosages in practice, mainly because of concerns about their adverse effects, which include myopathy and/or liver function abnormalities and are dose dependent [10,11]. Secondly, a small portion of

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patients have refractory hypercholesterolemia and cannot reach their goal even when treated with very high doses of statins [12].

Under the circumstances mentioned above, combination therapy of statins with other kinds of lipid-altering agents is a choice in clinic. The advantages of combination therapy are the enhanced lipid-lowering effect, reduced dosage of statins, and decreased risk of adverse effects [13-15]. Currently available combination alternatives to treat hypercholesterolemia include statins combined with bile acid sequestrants [14,16], niacin [17,18], or ezetimibe [19,20]. Their effectiveness and safety have been validated in large-scale of clinical trials. Two of the combination regimens, the extended-release niacin plus lovastatin (known as *Advicor*; Kos Pharmaceuticals, Cranbury, NJ) and ezetimibe plus simvastatin (known as *Vytorin*; Merck/Schering Plough Pharmaceuticals, North Wales, PA), have been approved for clinical use by the United States Food and Drug Administration. More combination regimens are under development.

Berberine (BBR) is a natural compound isolated from the traditional Chinese herb *Coptidis Rhizoma*. We have identified BBR as a promising cholesterol-lowering agent with satisfying clinical effectiveness and safety. Berberine increases the expression of the LDLR gene in hepatocytes at the posttranscriptional level by stabilizing its messenger RNA (mRNA), a mechanism distinct from that of statins [21]. Because of its unique action and safety record, BBR is an ideal candidate for the combination therapy with statins to treat hypercholesterolemia. The present study was designed to examine the activity of BBR in combination with simvastatin (SIMVA).

2. Subjects, materials, and methods

2.1. Cell culture

HepG2 cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics at 37°C with 5% CO_2. Twenty-four hours before drug treatment, cells were trypsinized and grown in DMEM containing 10% lipoprotein-deficient serum (LPDS; Sigma Chemical, St Louis, MO). When cells reached about 80% confluence, media were switched to DMEM containing 0.5% LPDS and supplemented with BBR (Sigma), SIMVA (Merck, Rahway, NJ), or BBR plus SIMVA. Cells were treated for 18 hours before harvest.

2.2. RNA extraction and Northern blot analysis

Total RNAs were isolated from HepG2 cells or animal livers using the Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, TX) according to the supplier's protocol. Forty micrograms of total cellular RNA from each sample was run on a 1% agarose/formaldehyde gel before transferring to a nylon membrane and cross-linked by UV waves. The expression levels of LDLR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were detected by ^{32}P-labeled gene-specific probes as described previously [21].

2.3. Reverse transcription and quantitative real-time polymerase chain reaction

For reverse transcription, 0.5 μg of total cellular or liver RNA from each sample was used as template in a 20-μL reaction system that also contained Oligo(dT)$_{15}$ primer and the AMV reverse transcriptase (Promega, Madison, WI). The reverse transcription reactions were conducted at 42°C for 30 minutes and then inactivated at 95°C for 5 minutes. Quantitative real-time polymerase chain reaction (PCR) was performed with these complementary DNAs (cDNAs) using the Applied Biosystems 7500 Real-Time PCR System and the 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). All of the 20× TaqMan Gene Expression Assay Reagents containing specific primers and TaqMan probes for human or rat LDLR, as well as HMG-CoA reductase and GAPDH genes, were purchased from Applied Biosystems. In each of the 20-μL real-time PCR reaction, 2 μL of cDNA, 10 μL of the Universal PCR Master Mix, and 1 μL of the TaqMan Gene Expression Assay Reagent were used, with a reaction condition exactly the same as described previously [22]. Each experiment was repeated at least 3 times. The comparative threshold cycle method was used in relative gene quantification using the TaqMan SDS analysis software with GAPDH as the endogenous control.

2.4. Flow cytometry

After drug treatment, cells were detached and fixed in 4% paraformaldehyde at room temperature for 1 hour. After blocking, cells were incubated on ice for 1 hour with a rabbit-polyclonal antibody against LDLR (Santa Cruz Biotechnology, Santa Cruz, CA) with a dilution of 1:25. Normal rabbit immunoglobulin G (IgG) was used as a control for background staining. Afterward, cells were washed and stained on ice for 30 minutes with a fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Santa Cruz) with a dilution of 1:50. The fluorescent intensities on cell surface were analyzed in a FACSCalibur system (BD Biosciences, San Jose, CA).

2.5. Animals and diets

Male Wistar strain rats, weighing 180 ± 10 g, were purchased from the Institute of Laboratory Animal Science (Beijing, China). All of the animal experiment protocols followed the institutional guidelines of the Chinese Academy of Medical Sciences. Animals were housed in an air-conditioned room with 3 rats per cage and with a 12-hour–light and 12-hour–dark cycle. After 1 week of accommodation, all of the animals were then fed with a high-fat and high-cholesterol (HFHC) diet containing 2% cholesterol, 10% yolk powder, 15% lard, and 0.2% sodium cholate for 8 weeks. Five rats were fed with regular rodent chow, serving as normal-diet controls.

2.6. Animal experiments

Rats fed the HFHC diet were randomly divided into 5 groups. They were the HFHC diet control group treated with saline (n = 6), SIMVA at 6 mg/(kg d) (n = 9), BBR at 90 mg/(kg d) (n = 9), BBR (90 mg/[kg d]) plus SIMVA (6 mg/[kg d]) (n = 10), and SIMVA at 12 mg/(kg d) (n = 9), respectively. Berberine and SIMVA were both administrated orally for 30 days. The daily dose of BBR was divided between 2 servings each day at 8 AM and 5 PM, respectively, whereas SIMVA was given once a day at 5 PM. Blood samples were taken before, after, and at indicated days during the treatment by tail snipping after 6-hour fast. Serums were isolated by centrifugation. Serum LDL-c, total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-c) levels were assayed as described [22].

On the last day of treatment, the animals were killed and livers were dissected. A portion of every liver tissue sample was fixed in 10% formaldehyde at room temperature for hematoxylin and eosin (H&E) staining; the remaining tissues were quickly frozen at −80°C. Liver tissues were homogenized with the Ultraspec RNA lysis

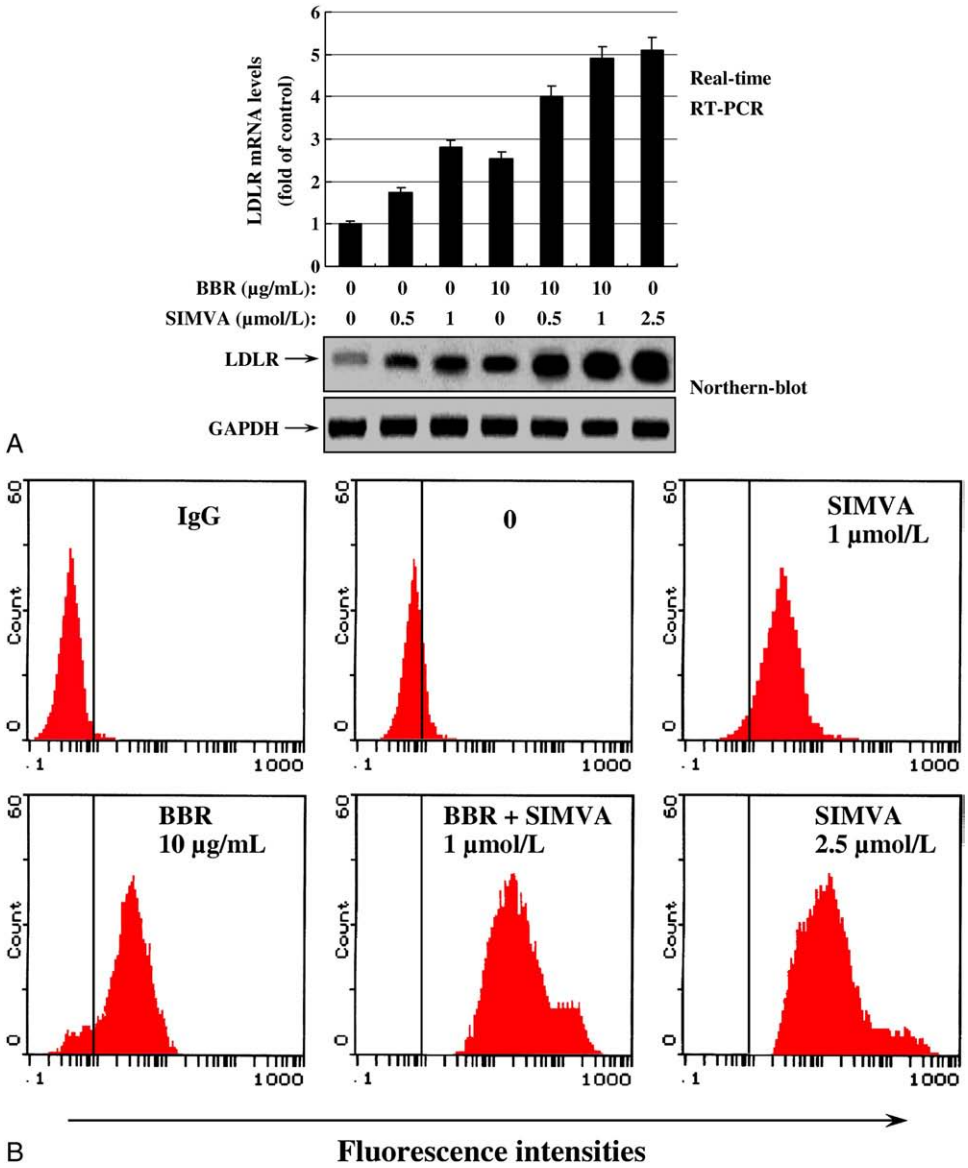

Fig. 1. Up-regulation of LDLR expression by coadministration of BBR and SIMVA in vitro. A, Low-density lipoprotein receptor mRNA expression levels. HepG2 cells cultured in LPDS containing media were left untreated or treated for 18 hours with SIMVA, BBR, or their combinations as indicated. The LDLR mRNA levels were analyzed by real-time RT-PCR (upper panel) and Northern blot (lower panel), respectively. For real-time RT-PCR, the LDLR mRNA levels were normalized to that of GAPDH and plotted as fold of untreated control, which was designated as 1. Values are mean ± SEM of at least 3 repeated experiments. For Northern blot, the presented is a representative of 3 separate experiments. Results of ANOVA: BBR alone, $P < .001$; SIMVA alone, $P < .001$; BBR × SIMVA, $P < .0001$. B, Low-density lipoprotein receptor protein expression on HepG2 cell surface. After treatment for 18 hours, cells were analyzed by flow cytometry for cell surface LDLR protein expression. Normal rabbit IgG was used as control for the background staining. Fluorescence intensities on cell surface were analyzed in a FACSCalibur system.

solution, and total RNAs were isolated. After reversely transcribing into cDNAs, the liver mRNA levels of LDLR and HMG-CoA reductase were analyzed by real-time PCR. Liver total lipids were extracted, and TC and TG contents in the liver were measured [22]. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were also determined.

2.7. Clinical trial

Sixty-three outpatients from the Nanjing First Hospital who were diagnosed with hypercholesterolemia (serum TC >5.2 mmol/L) and have had no previous treatments were enrolled in this study. The patients were randomly divided into 3 groups, receiving 1 g/d of BBR hydrochloride (Nanjing Second Pharmaceutics, Nanjing, China), 20 mg/d of SIMVA (Merck), or their combination regimen for 2 months. Berberine was given twice a day orally, whereas SIMVA was administered once a day. Before and after treatment, fasting blood samples were taken; and serum LDL-c, TC, TG, and HDL-c levels were assayed. Liver and kidney functions before and after treatment were also monitored. The clinical study was approved by the Ethics Committees of the Institute of Medicinal Biotechnology and Nanjing First Hospital. All patients have given informed consent.

2.8. Statistical analysis

After validation of the test for homogeneity of variance, the in vitro gene expression data were analyzed by 2-way analysis of variance (ANOVA); the animal experiment results were examined by 1-way ANOVA followed by the Newman-Keuls test for multiple comparisons. Differences between the baseline and end-point lipid values in patients were analyzed by the paired *t* test. For percentage changes of serum lipids from baselines to end points in the patients, 1-way ANOVA and the Newman-Keuls test were used. $P < .05$ was considered as statistically significant.

3. Results

3.1. Coadministration of BBR with SIMVA enhanced the up-regulatory effect on LDLR expression

To determine the in vitro LDLR up-regulatory activity of SIMVA in combination with BBR, HepG2 human hepatocytes were used. As shown in Fig. 1A, when treated alone, SIMVA up-regulated LDLR gene expression dose dependently, as determined by both real-time RT-PCR and Northern blot assays. At concentrations of 0.5, 1, and 2.5 μmol/L, SIMVA increased LDLR mRNA expression in HepG2 cells to 1.8-, 2.8-, and 5.1-fold of the untreated control, respectively. Berberine at 10 μg/mL increased LDLR mRNA to 2.5-fold of the control, similar to that of SIMVA at 1 μmol/L. Coadministration of BBR with SIMVA up-regulated LDLR mRNA to a level significantly higher than that of the monotherapies ($P < .0001$). When 10 μg/mL of BBR was combined with 1 μmol/L of SIMVA, a 4.9-fold

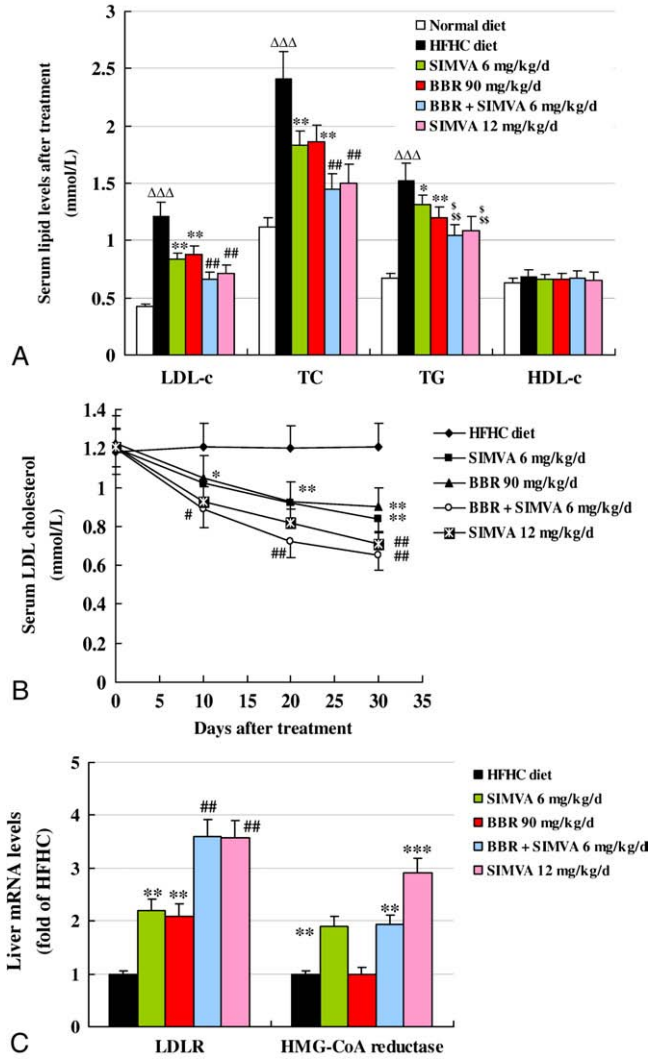

Fig. 2. Lipid-lowering and LDLR–up-regulating effects in hyperlipidemic rats. Male Wistar strain rats were fed with an HFHC diet for 8 weeks. Afterward, the rats were treated, respectively, with saline (n = 6, HFHC-diet control group), 6 mg/(kg d) of SIMVA (n = 9), 90 mg/(kg d) of BBR (n = 9), BBR (90 mg/[kg d]) plus SIMVA (6 mg/[kg d]) (n = 10), or 12 mg/(kg d) of SIMVA (n = 9) for 30 days. A, End point lipid levels. Serums were isolated from animals after 30 days of treatment. The LDL-c, TC, TG, and HDL-c levels were determined. Values are mean ± SEM of all of the animals in each group. For LDL-c, TC, and TG, $P < .001$ among groups by 1-way ANOVA. In the Newman-Keuls test, $^{\Delta\Delta\Delta}P < .001$ vs that of the normal diet group; $^{*}P < .05$, $^{**}P < .01$ vs that of the HFHC group; $^{\#\#}P < .01$ vs that of BBR or SIMVA (6 mg/[kg d]) monotherapy; $^{\$}P < .05$, $^{\$\$}P < .01$ vs TG of BBR or SIMVA (6 mg/[kg d]) monotherapy, respectively. B, Time-dependent LDL-c reduction after drug treatment. Values are mean ± SEM of all of the animals in each group. $^{*}P < .05$, $^{**}P < .01$ vs the HFHC group at the same time point; $^{\#}P < .05$, $^{\#\#}P < .01$ vs BBR or SIMVA (6 mg/[kg d]) monotherapy at the same time point (by 1-way ANOVA and the Newman-Keuls test). C, Low-density lipoprotein receptor–up-regulating activity in vivo. After 30 days of treatment, rats were killed and their livers were dissected. Liver total RNAs were isolated for real-time RT-PCR assay. The expression levels of LDLR and HMG-CoA reductase mRNAs were normalized to that of GAPDH and presented as fold of the HFHC group. Values are mean ± SEM of all of the animals in each group. For both of the LDLR and HMG-CoA reductase mRNAs, $P < .001$ among groups by 1-way ANOVA. In the Newman-Keuls test, $^{**}P < .01$, $^{***}P < .001$ vs that of the HFHC group; $^{\#\#}P < .01$ vs that of BBR or SIMVA (6 mg/[kg d]) monotherapy.

increase of LDLR mRNA was achieved, comparable with that of SIMVA at 2.5 μmol/L (Fig. 1A).

The LDLR protein expression on cell surface was examined next. As shown in Fig. 1B, SIMVA at 1 μmol/L or BBR at 10 μg/mL caused a similar degree of increase of LDLR protein expression. However, combination of BBR (10 μg/mL) with SIMVA (1 μmol/L) resulted in a significant rise of LDLR protein on the cell surface, close to that achieved when using 2.5 μmol/L of SIMVA. The flow cytometry results fully agreed with that of LDLR mRNA expression and showed that, when coadministered in vitro, BBR and SIMVA worked complimentarily to each other and up-regulated the LDLR gene expression to higher levels with a combined activity no less than that of a much higher concentration of SIMVA.

3.2. SIMVA in combination with BBR enhanced LDL-c–lowering effect in vivo

The increased effect of the coadministration on LDLR expression in vitro suggests an in vivo potential of enhanced LDL-c–lowering efficacy. To validate this hypothesis, we used a hyperlipidemic rat model [23] to test the therapeutic efficacy of SIMVA, BBR, and SIMVA in combination with BBR. The rats were first fed with an HFHC diet for 8 weeks. As shown in Fig. 2A, with respect to the normal-diet group, approximately 2.9-fold increase in LDL-c, 2.2-fold increase in TC, and 2.3-fold increase in TG levels were observed in the HFHC group of animals ($P < .001$). No alteration of the HDL-c level was found in this model. Serum lipid levels of the untreated HFHC group as well as the normal-diet group remained unchanged during the 30-day treatment.

Drug intervention reduced the LDL-c levels in HFHC diet–fed rats effectively and in a time-dependent manner (Fig. 2A, B), with significant reduction observed on day 10 of the monotherapy using 6 mg/(kg d) of SIMVA or 90 mg/(kg d) of BBR. At all of the time points, the LDL-c–lowering efficacy of the combination therapy was statistically superior to that of the monotherapies ($P < .05$ or $P < .01$). At the end of the treatment, SIMVA alone at 6 mg/(kg d) and BBR at 90 mg/(kg d) lowered serum LDL-c by 28.3% and 26.8%, respectively (Fig. 2A, B), as compared with the HFHC control group ($P < .01$ for both agents). Meanwhile, a 46.2% reduction of the LDL-c was observed when SIMVA (6 mg/[kg d]) was combined with BBR (90 mg/[kg d]); the reduction was significantly greater than that with either monotherapy ($P < .01$). Considering that SIMVA at 12 mg/(kg d) for 30 days reduced LDL-c in the hyperlipidemic rats by 43.4% (Fig. 2), adding BBR to SIMVA regimen could reduce the dose of SIMVA by at least 50%. These results could be well explained with the data presented in Fig. 1.

Total cholesterol levels were lowered in the treated groups in a manner similar to that of LDL-c. As compared with the untreated HFHC diet group, TC levels of the rats at the end point were reduced by 25.2%, 24.5%, 40.7%, and 38.9%, respectively, after 30 days of treatment with 6 mg/(kg d) of SIMVA, 90 mg/(kg d) of BBR, combination therapy (BBR 90 mg/[kg d] + SIMVA 6 mg/[kg d]), or 12 mg/(kg d) of SIMVA (Fig. 2A). Combination therapy also showed an improved TG-modifying activity as compared with the individual agents. As shown in Fig. 2A, 6 mg/(kg d) of SIMVA reduced serum TG by 15.3% ($P < .05$) as compared with the untreated HFHC group; and 90 mg/(kg d) of BBR reduced serum TG by 21.3% ($P < .01$). When coadministered, serum TG at the end point was reduced by 31.4%, an efficacy significantly higher than that of either monotherapy ($P < .05$ vs 90 mg/[kg d] of BBR, $P < .01$ vs 6 mg/[kg d] of SIMVA). Simvastatin at 12 mg/(kg d) for 30 days reduced serum TG in the hyperlipidemic rats by 28.8%.

To examine the in vivo LDLR–up-regulating efficacy of the coadministration of SIMVA and BBR, total RNAs were isolated from the rat livers after treatment and underwent real-time RT-PCR analysis. As shown in Fig. 2C, compared with the HFHC group ($P < .01$), approximately 2.1- and 2.2-fold increases of the liver LDLR mRNA were observed in the BBR (90 mg/[kg d]) and low-dose SIMVA (6 mg/[kg d]) groups, respectively. Combining BBR with SIMVA significantly increased the LDLR mRNA–up-regulating activity ($P < .01$ as compared with both agents) with a total 3.5-fold increase in the liver, which was very close to that of SIMVA at 12 mg/(kg d). Liver HMG-CoA reductase mRNA levels were also determined. Whereas SIMVA increased the mRNA level of HMG-CoA reductase dose dependently in the rat livers (Fig. 2C), BBR had no effect on it. Therefore, HMG-CoA reductase mRNA in the combination group (SIMVA 6 mg/[kg d] plus BBR 90 mg/[kg d]) was exactly the same as that of the therapy using SIMVA (6 mg/[kg d]) alone.

Combination regimen using BBR and SIMVA was well tolerated in the hyperlipidemic rats, with no observation of fur change, body weight loss, or food intake reduction.

3.3. Coadministration of BBR and SIMVA reduced liver steatosis effectively

To examine the effect of antilipid therapy on liver histology in the hyperlipidemic rats, liver tissues were examined under H&E staining. As shown in Fig. 3, after feeding with an HFHC diet, liver cells became extremely swelled and ballooned with severe steatosis (Fig. 3B), as compared with those from the normal diet–fed rats (Fig. 3A). After 30 days of treatment with SIMVA at 6 mg/(kg d) (Fig. 3C) or BBR at 90 mg/(kg d) (Fig. 3D), liver pathological changes were partially improved as demonstrated by the decreased size of liver cells and reduction of liver steatosis. Combination of BBR (90 mg/[kg d]) with SIMVA (6 mg/[kg d]) however reduced liver steatosis and restored liver tissue morphology to greater degrees than the monotherapies did (Fig. 3E). Simvastatin at 12 mg/(kg d) for 30 days improved liver histology (Fig. 3F) similar to that of the combination therapy.

In addition, liver fat storage in the rats was also examined. As shown in Table 1, in the HFHC diet–fed rats, liver TC and TG concentrations were increased to 3.8- and 5.6-fold of the normal-diet group ($P < .001$), respectively. Treatment of the

rats with SIMVA (6 mg/[kg d]) or BBR (90 mg/[kg d]) for 30 days reduced liver TC by 32.1% and 32.1% and reduced liver TG by 25% and 30.1%, respectively ($P < .01$ as compared with the HFHC group). However, 52.1% reduction of liver TC and 47% reduction of liver TG were achieved when the combination was used ($P < .01$ as compared with the monotherapies). Treatment with SIMVA at 12 mg/(kg d) for 30 days reduced liver TC and TG by 50% and 44.1%, respectively, close to that of combination therapy.

Because liver fat storage is usually related to the abnormality of liver function, we assayed the serum aminotransferases at the end of the experiment. As shown in Fig. 4, compared with the normal-diet group, HFHC-diet feeding caused an elevation of about 80% in serum ALT and AST in the Wistar rats ($P < .01$). Lipid-lowering therapies using BBR, SIMVA, or their combination reduced serum aminotransferases to the baselines ($P < .01$ vs the HFHC group).

3.4. Combination therapy using BBR and SIMVA in hypercholesterolemic patients

Sixty-three patients with diagnosed hypercholesterolemia and no previous treatment were enrolled to receive 1 g/d of BBR, 20 mg/d of SIMVA, or both. As shown in Table 2, after 2 months of oral treatment, 1 g/d of BBR or 20 mg/d of SIMVA monotherapy reduced serum LDL-c by 23.8% and 14.3% ($P < .001$ and $P < .01$ vs before treatment), respectively. As compared with the monotherapies, combination therapy showed an improved antilipid effect with 31.8% reduction of serum LDL-c at the end point ($P < .001$ vs before treatment, $P < .05$ vs BBR monotherapy, $P < .01$ vs SIMVA monotherapy). Total cholesterol declined after the treatment, with a pattern similar to that of LDL-c. Berberine alone reduced serum TG by 22.1% ($P < .01$ vs before treatment); SIMVA at 20 mg/d decreased TG by 11.4% ($P < .05$ vs before treatment) in the patients. When SIMVA was coadministered with BBR, the reduction was 38.9% ($P < .001$ vs before treatment), significantly improved as compared with the monotherapies ($P < .05$ vs BBR alone, $P < .01$ vs SIMVA alone). Serum HDL-c was without significant change in this study.

Combination therapy of BBR and SIMVA was safe and well tolerated in the hypercholesterolemic patients' cohort. Adverse effects as well as liver or kidney function abnormalities were observed neither in the combination therapy nor in the monotherapy groups.

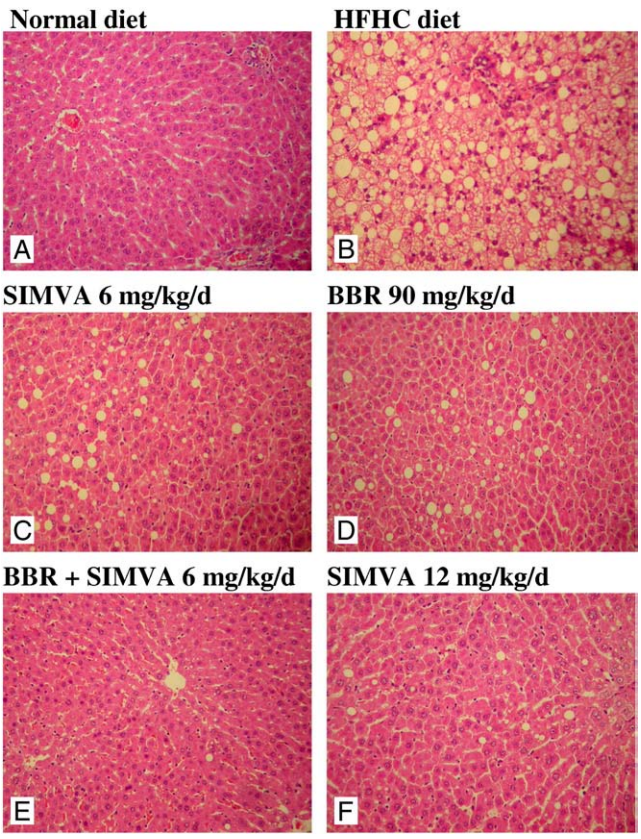

Fig. 3. Liver histology in the hyperlipidemic rats treated with study drugs. Liver tissue sections from the rats fed with normal diet (A), HFHC diet (B), or HFHC diet and treated with 6 mg/(kg d) of SIMVA (C), 90 mg/(kg d) of BBR (D), BBR (90 mg/[kg d]) + SIMVA (6 mg/[kg d]) (E), or 12 mg/(kg d) of SIMVA (F) were examined (H&E, × 200).

Table 1
Rat hepatic lipid contents after combination treatment

	TC		TG	
	μmol/g	% HFHC	μmol/g	% HFHC
Normal diet	3.72 ± 0.51	26 ± 3.5	2.16 ± 0.28	17.7 ± 2.3
HFHC diet	14.3 ± 1.62	100 *	12.2 ± 1.3	100 *
SIMVA 6 mg/(kg d)	9.72 ± 1.1	67.9 ± 7.8 †	9.15 ± 0.67	75 ± 5.7 †
BBR 90 mg/(kg d)	9.72 ± 0.87	67.9 ± 6.2 †	8.53 ± 0.58	69.9 ± 4.8 †
BBR + SIMVA 6 mg/(kg d)	6.86 ± 0.82	47.9 ± 5.1 ‡	6.47 ± 0.61	53 ± 5.6 ‡
SIMVA 12 mg/(kg d)	7.15 ± 0.68	50 ± 4.8 ‡	6.83 ± 0.46	55.9 ± 3.9 ‡

Total lipids were extracted from rat livers after treatment for determination of TC and TG contents. Values are mean ± SEM of all the animals in each group. For hepatic TC and TG, $P < .001$ among groups by 1-way ANOVA.

* $P < .001$ vs that of the normal-diet group.

† $P < .01$ vs that of the HFHC group.

‡ $P < .01$ vs that of BBR or SIMVA (6 mg/[kg d]) monotherapy by the Newman-Keuls test.

4. Discussion

In this study, we demonstrate that SIMVA in combination with BBR has significantly enhanced LDL-c–lowering efficacy both in preclinical and clinical studies as compared with therapies using either individual agent alone.

Combination therapy often takes advantage of complementary actions of different agents. For example, bile acid sequestrants block the enterohepatic circulation of bile acids; therefore, more cholesterol in the liver is converted into bile acids upon treatment [24]. Ezetimibe inhibits active cholesterol absorption in the intestine [25,26]. Berberine up-regulates the LDLR gene expression at the posttranscriptional level through activation of the extracellular signal–

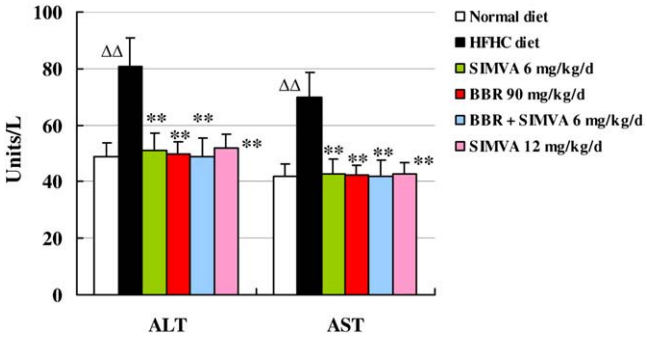

Fig. 4. Serum aminotransferase levels after treatment in rats. At the end of the animal experiment, blood samples were isolated and the ALT and AST levels were measured. Values are mean ± SEM of all the animals in each group. For both the ALT and AST, $P < .01$ among groups by 1-way ANOVA. $^{\Delta\Delta}P < .01$ vs that of the normal diet group; $^{**}P < .01$ vs that of the HFHC group by the Newman-Keuls test.

regulated kinase pathway. This mechanism is different from that of statins [27,28]. It suggests that enhanced LDLR–up-regulating activity might be achieved by combining BBR with statins. Indeed, our in vitro results showed that combining BBR with SIMVA increased the LDLR mRNA expression to a higher level as compared with the monotherapies. The concentration of SIMVA could be reduced by 50% to 60% to achieve a similar degree of LDLR up-regulation if combined with BBR.

In accordance with the in vitro studies, we found that this combination significantly improved LDL-c–lowering efficacy both in rats and in the clinical study. Rodents with food-induced hyperlipidemia are widely used in animal experiments of lipid-altering drugs. Among the rodent models, hamsters are reported to develop marked and sometimes lethal adverse effects of severe liver damage and weight loss after statins treatment [29,30]. Rats fed with a diet rich in saturated fat and cholesterol are known to develop hyperlipidemia and are suitable for preclinical study of lipid-altering drugs [23,31]. Therefore, we used Wistar strain rats instead of hamsters in the present study. Simvastatin monotherapy at 6 mg/(kg d) or BBR at 90 mg/(kg d) for 30 days reduced serum LDL-c to similar levels in the animals. When combined, the cholesterol-lowering efficacy was significantly enhanced. Again, the dose of SIMVA could be reduced by 50% for an at least similar antilipid effect after its combination with BBR. Because BBR and SIMVA up-regulate LDLR mRNA through different operations, it partially explains the enhanced antilipid efficacy.

Coadministration of statins with other lipid-lowering drugs such as bile acid sequestrants or ezetimibe often lowers serum LDL-c additively [32]. To determine whether SIMVA and BBR reduce LDL-c in an additive manner when coadministered, the Schectman-Hiatt formula [32] was used. If SIMVA and BBR work additively, the end point LDL-c level in their combination therapy should equal to baseline × (1 − LDL-c reduction of SIMVA monotherapy percentage) × (1 − LDL-c reduction of BBR monotherapy percentage). In this study, SIMVA or BBR monotherapy reduced serum LDL-c by 28.3% and 26.8% in the animals, as well as 14.3% and 23.8% in the patients. According to the Schectman-Hiatt formula, serum LDL-c should then be reduced by 47.5% in animals and 34.7% in the clinical study after combination therapy if these 2 agents work additively. The theoretically predicted LDL-c–lowering efficacies of combination therapy are nearly the same as that actually observed in the study (46.2% for the animals and 31.8% for the patients), indicating that BBR and SIMVA appear to reduce serum LDL-c in an additive manner in this combination regimen.

Besides cholesterol, we also observed a beneficial effect of combination therapy on serum TG. Consistent with previous studies [13], SIMVA alone only had moderate TG-lowering effect. However, when coadministered with

Table 2
Lipid-lowering efficacies of the combination therapy using BBR and SIMVA in hypercholesterolemic patients

Measurement (reference range)	Treatment	BBR + SIMVA (n = 23)	SIMVA (n = 16)	BBR (n = 24)
LDL-c	Before	4.36 ± 0.97	4.28 ± 1	3.81 ± 0.56
(<3.1 mmol/L)	After	2.97 ± 0.93***	3.67 ± 0.8**	2.9 ± 0.7***
	% Change	31.8 ± 3.6†,‡	14.3 ± 4.6	23.8 ± 3.6
TC	Before	6.73 ± 0.98	6.56 ± 0.5	6.17 ± 0.56
(<5.2 mmol/L)	After	4.77 ± 0.82***	5.99 ± 0.6**	4.82 ± 0.65***
	% Change	29.1 ± 2.4†,‡	9.1 ± 1.5	21.8 ± 1.6
TG	Before	2.72 ± 0.61	2.28 ± 0.9	1.94 ± 1.05
(<1.7 mmol/L)	After	1.66 ± 0.35***	2.02 ± 0.7*	1.51 ± 0.77**
	% Change	38.9 ± 6.5†,‡	11.4 ± 3.5	22.1 ± 10
HDL-c	Before	1.46 ± 0.55	1.15 ± 0.5	1.21 ± 0.34
(>1.0 mmol/L)	After	1.34 ± 0.35	1.18 ± 0.4	1.14 ± 0.3
	% Change	8.2 ± 6.8	2.6 ± 0.9	5.8 ± 1.7

Hypercholesterolemic patients were treated with BBR 1 g/d, SIMVA 20 mg/d, or their combination orally for 2 months. Before and after treatment, fasting blood samples were taken for the measurement of serum LDL-c, TC, TG, and HDL-c levels. Percentage changes of serum lipids from baselines to end points were calculated. Values are mean ± SEM of all of the patients in each group. For percentage changes in LDL-c, TC, and TG, $P < .01$ among groups by 1-way ANOVA. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs that of before treatment by paired t test.
$^{\dagger}P < .05$ vs that of BBR alone, $^{\ddagger}P < .01$ vs that of SIMVA alone by the Newman-Keuls test.

BBR, serum TG significantly declined. This action is different from that of combination regimens using statins plus sequestrants or statins plus ezetimibe because adding sequestrants or ezetimibe to statins often compromises the TG-lowering activity of statins [13,14]. Berberine affects TG metabolism through more than one mechanism. Besides liver LDLR, a recent study demonstrates that BBR inhibits lipid synthesis in hepatocytes through activation of the adenosine monophosphate–activated protein kinase, which also partially explains the TG-lowering activity of BBR [33]. From this point of view, combination regimens containing BBR and statins could be particularly advantageous in treating hypercholesterolemic patients with elevated serum TG.

Combination treatment of BBR with SIMVA was safe and well tolerated. We did not find any adverse effects of this regimen in either animals or the patients. As mentioned above, this drug combination significantly improves the LDLR–up-regulating and LDL-c–lowering efficacies as compared with monotherapies, suggesting a good potential to reduce statins' dosage in clinic by adding BBR. This regimen could be particularly important when adverse effects of statins occur in patients. Considering the nonlinear LDL-c–lowering efficacy of statins [32], adding BBR to low dose of statins will be a good alternative to the monotherapy with high dose of statins.

In conclusion, our results demonstrate the rationale, effectiveness, and safety of a novel lipid-lowering combination regimen containing BBR and SIMVA. With the prevalence of CHD and in-depth understanding of the relationship between cholesterol and the development of atherosclerosis and CHD, more and more patients need aggressive cholesterol-lowering therapy. The present study provides a safe and effective option for the drug combination to treat patients with hypercholesterolemia.

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References

[1] Balbisi EA. Management of hyperlipidemia: new LDL-C targets for persons at high-risk for cardiovascular events. Med Sci Monit 2006;12: RA34-9.
[2] Stamler J, Daviglus ML, Garside DB, et al. Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. JAMA 2000;284:311-8.
[3] Anderson KM, Castelli WP, Levy D. Cholesterol and mortality: 30 years of follow-up from the Framingham study. JAMA 1987;257: 2176-80.
[4] Grundy SM, Cleeman JI, Merz CN, et al. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. Circulation 2004;110:227-39.
[5] Klotz U. Pharmacological comparison of the statins. Arzneimittelforschung 2003;53:605-11.
[6] Corsini A, Bellosta S, Baetta R, et al. New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharmacol Ther 1999;84:413-28.
[7] Moghadasian MH. A safety look at currently available statins. Expert Opin Drug Saf 2002;1:269-74.
[8] Stein EA. New statins and new doses of older statins. Curr Atheroscler Rep 2001;3:14-8.
[9] Mckenney J. Combination therapy for elevated low-density lipoprotein cholesterol: the key to coronary artery disease risk reduction. Am J Cardiol 2002;90:8K-20K.
[10] Farmer JA, Gotto Jr AM. Antihyperlipidaemic agents. Drug interactions of clinical significance. Drug Saf 1994;11:301-9.
[11] Jones P, Kafonek S, Laurora I, et al. Comparative dose efficacy of atorvastatin versus simvastatin, pravastatin, lovastatin, and fluvastatin in patients with hypercholesterolemia (the CURVES study). Am J Cardiol 1998;81:582-7.
[12] Brown AS, Bakker-Arkema RG, Yellen L, et al. Treating patients with documented atherosclerosis to National Cholesterol Education Program–recommended low-density–lipoprotein cholesterol goals with atorvastatin, fluvastatin, lovastatin and simvastatin. J Am Coll Cardiol 1998;32:665-72.
[13] Brown AS. Use of combination therapy for dyslipidemia: a lipid clinic approach. Am J Cardiol 2002;90:44K-9K.
[14] Hunninghake D, Insull Jr W, Toth P, et al. Coadministration of colesevelam hydrochloride with atorvastatin lowers LDL cholesterol additively. Atherosclerosis 2001;158:407-16.
[15] Davidson MH. Combination therapy for dyslipidemia: safety and regulatory considerations. Am J Cardiol 2002;90:50K-60K.
[16] Knapp HH, Schrott H, Ma P, et al. Efficacy and safety of combination simvastatin and colesevelam in patients with primary hypercholesterolemia. Am J Med 2001;110:352-60.
[17] Gupta EK, Ito MK. Lovastatin and extended-release niacin combination product: the first drug combination for the management of hyperlipidemia. Heart Dis 2002;4:124-37.
[18] Wolfe ML, Vartanian SF, Ross JL, et al. Safety and effectiveness of extended-release niacin when added sequentially to a statin for treatment of dyslipidemia. Am J Cardiol 2001;87:476-9.
[19] Pearson T, Denke M, McBride P, et al. Effectiveness of the addition of ezetimibe to ongoing statin therapy in modifying lipid profiles and attaining low-density lipoprotein cholesterol goals in older and elderly patients, subanalysis of data from a randomized, double-blind, placebo-controlled trial. Am J Geriatr Pharmacother 2005;3:218-28.
[20] Robinson JG, Davidson MH. Combination therapy with ezetimibe and simvastatin to achieve aggressive LDL reduction. Expert Rev Cardiovasc Ther 2006;4:461-76.
[21] Kong W, Wei J, Abidi P, et al. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat Med 2004;10:1344-51.
[22] Kong W, Abidi P, Kraemer FB, et al. In vivo activities of cytokine oncostatin M in the regulation of plasma lipid levels. J Lipid Res 2005; 46:1163-71.
[23] Mani UV, Sharma M, Waghray K, et al. Effect of colocasia leaves (*Colocasia antiquorum*) on serum and tissue lipids in cholesterol-fed rats. Plant Foods Hum Nutr 1989;39:245-55.
[24] Van Itallie TB, Hashim SA, Crampton RS, et al. The treatment of pruritus and hypercholesterolemia of primary biliary cirrhosis with cholestyramine. N Engl J Med 1961;265:469-74.
[25] Bruckert E, Giral P, Tellier P. Perspectives in cholesterol-lowering therapy: the role of ezetimibe, a new selective inhibitor of intestinal cholesterol absorption. Circulation 2003;107:3124-8.
[26] Sprecher DL, Abrams J, Allen JW, et al. Low-dose combined therapy with fluvastatin and cholestyramine in hyperlipidemic patients. Ann Intern Med 1994;120:537-43.
[27] Kong W, Liu J, Jiang JD. Human low-density lipoprotein receptor gene and its regulation. J Mol Med 2006;84:29-36.

[28] Abidi P, Zhou Y, Jiang JD, et al. Extracellular signal-regulated kinase–dependent stabilization of hepatic low-density lipoprotein receptor mRNA by herbal medicine berberine. Arterioscler Thromb Vasc Biol 2005;25:2170-6.

[29] Oms P, Assie N, Bruniquel F, et al. Biochemical changes and morphological alterations of liver and kidney in hamsters after administration of the HMG-coenzyme A reductase inhibitor, simvastatin: prevention and reversibility by mevalonate. Pharmacol Toxicol 1995;77:391-6.

[30] Morand OH, Aebi JD, Dehmlow H, et al. Ro 48-8071, a new 2,3-oxidosqualene:lanosterol cyclase inhibitor lowering plasma cholesterol in hamsters, squirrel monkeys and minipigs: comparison to simvastatin. J Lipid Research 1997;38:373-90.

[31] Yokozawa T, Ishida A, Cho EJ, et al. The effects of *Coptidis Rhizoma* extract on a hypercholesterolemic animal model. Phytomedicine 2003; 10:17-22.

[32] Schectman G, Hiatt J. Dose-response characteristics of cholesterol-lowering drug therapies: implications for treatment. Ann Intern Med 1996;125:990-1000.

[33] Brusq JM, Ancellin N, Grondin P, et al. Inhibition of lipid synthesis through activation of AMP kinase: an additional mechanism for the hypolipidemic effects of berberine. J Lipid Res 2006;47:1281-8.