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Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins

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We identify berberine (BBR), a compound isolated from a Chinese herb, as a new cholesterol-lowering drug. Oral administration of BBR in 32 hypercholesterolemic patients for 3 months reduced serum cholesterol by 29%, triglycerides by 35% and LDL-cholesterol by 25%. Treatment of hyperlipidemic hamsters with BBR reduced serum cholesterol by 40% and LDL-cholesterol by 42%, with a 3.5-fold increase in hepatic *LDLR* mRNA and a 2.6-fold increase in hepatic LDLR protein. Using human hepatoma cells, we show that BBR upregulates LDLR expression independent of sterol regulatory element binding proteins, but dependent on ERK activation. BBR elevates LDLR expression through a post-transcriptional mechanism that stabilizes the mRNA. Using a heterologous system with luciferase as a reporter, we further identify the 5′ proximal section of the *LDLR* mRNA 3′ untranslated region responsible for the regulatory effect of BBR. These findings show BBR as a new hypolipidemic drug with a mechanism of action different from that of statin drugs.

The expression of liver low-density lipoprotein receptor (LDLR) regulates human plasma LDL cholesterol (LDL-c) homeostasis[1,2]. Increased hepatic LDLR expression results in improved clearance of plasma LDL-c through receptor-mediated endocytosis, which has been strongly associated with a decreased risk of developing cardiovascular disease in humans[3,4]. LDLR expression is predominantly regulated at the transcriptional level through a negative feedback mechanism by the intracellular cholesterol pool. This regulation is controlled through specific interactions of sterol-regulatory element (SRE-1) of the LDLR promoter[5,6] and SRE binding proteins (SREBPs)[7–9]. In the inactive state, SREBP resides in the endoplasmic reticulum (ER) and associates with another transmembrane protein, SREBP-cleavage activating protein (SCAP) which provides conditional chaperone activity to the SREBP[10–12]. SCAP contains a cholesterol-sensing domain, which responds to the depletion of sterol with activation of the SCAP-SREBP transporting activity[13–15]. Under cholesterol-depleted conditions, SCAP transports SREBP to the Golgi apparatus, where the N-terminal transcription activation domain of the SREBP is released from the precursor protein through specific cleavages[11]. The active form of the SREBP translocates to the nucleus, binds to its cognate SRE-1 site and activates transcription of the LDLR gene. In contrast, under cholesterol-replete conditions, the SCAP-SREBP complex remains in an inactive form in the ER through active repression by sterols and *LDLR* gene transcription is maintained at a minimal constitutive level.

Clinically, statins have been the most widely prescribed drugs for hypercholesterolemia[3,4]. Statins inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Inhibition of cholesterol biosynthesis leads to a depletion of intracellular cholesterol and an activation of the SCAP-SREBP transporting activity, thereby resulting in upregulation of the LDLR and subsequent lowering of the LDL-c in blood. Statins effectively lower the plasma concentration of LDL-c and reduce mortality and morbidity from coronary artery disease[16,17]. Recent studies showed additional benefits of statin beyond its cholesterol-lowering effects[18]. But despite the success of treatment with statins, there is a need for new therapies to reduce LDL-c. Some patients do not tolerate statins well, and more importantly, many patients under statin treatment alone do not achieve the LDL-c goal suggested by the US National Institutes of Health guidelines[19]. Therefore, for the treatment of hypercholesterolemia, it is desirable to develop other therapeutic interventions that increase hepatic LDLR expression by mechanisms distinct from the current statin therapy.

BBR as a new upregulator of liver LDLR expression

We have conducted a rationalized screening to search for new LDLR upregulators from natural resources. Two criteria were applied to select the screening candidates. First, the herb samples have had major applications in Chinese medicine and have safety records in the clinic. Second, the chemical structures of active ingredients of the herbs are already defined. Cells from a human hepatoma–derived cell line, HepG2, were treated for 24 h with different compounds isolated

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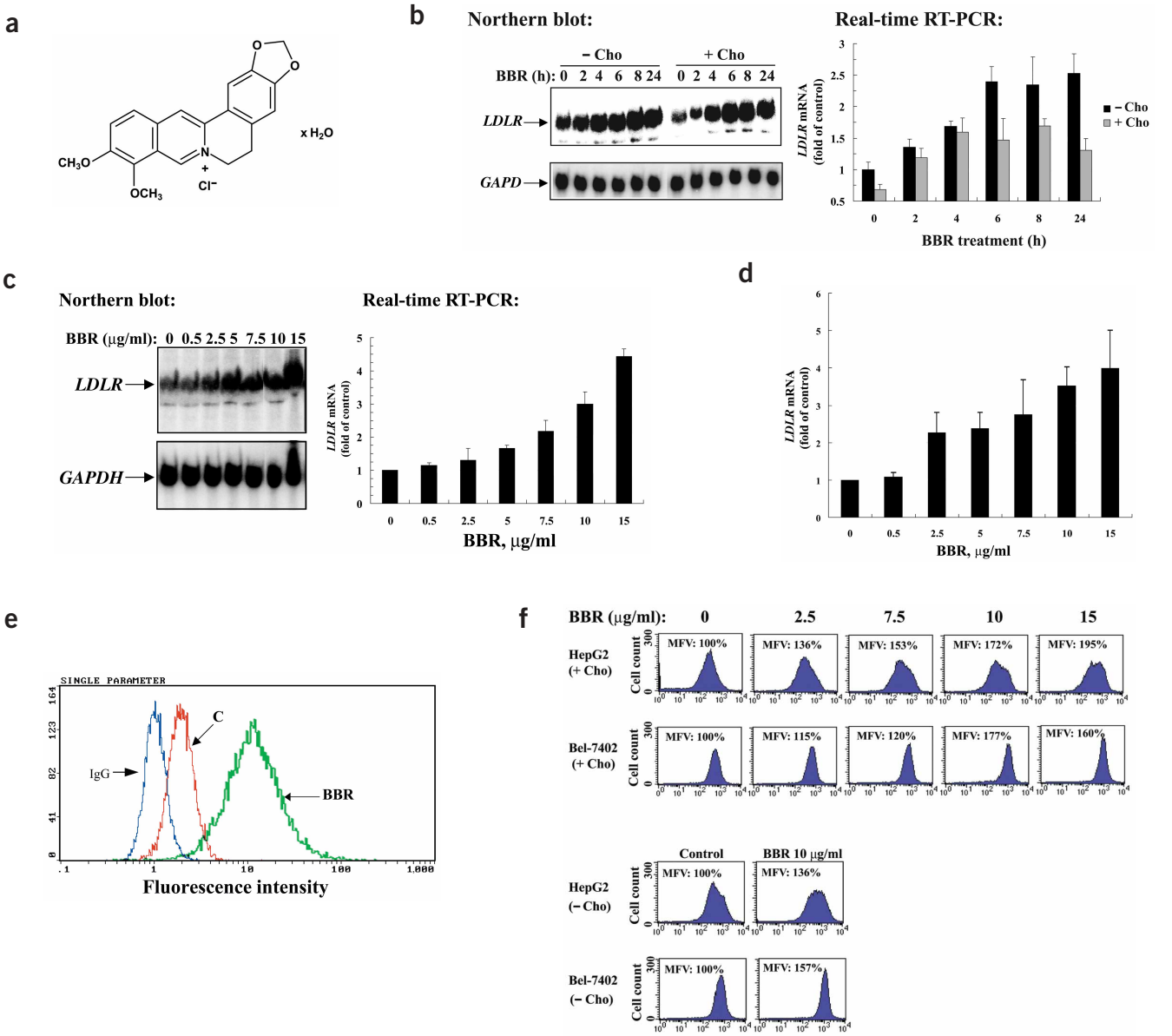

Figure 1 Upregulation of LDLR expression by BBR in human hepatoma cell lines. (**a**) Chemical structure of BBR. (**b**) Time-dependent induction of *LDLR* mRNA expression. HepG2 cells cultured in EMEM containing 0.5% LPDS without cholesterol (–Cho) or LPDS + sterols including cholesterol (+Cho) were incubated with BBR (7.5 μg/ml) for the indicated times. The figure shown is representative of 3 separate kinetic studies. The abundance of *LDLR* mRNA in untreated cells in LPDS –Cho was defined as 1, and the amounts of *LDLR* mRNA from BBR-treated cells with or without sterols were plotted relative to that value. (**c**) Dose-dependent induction of *LDLR* mRNA expression in HepG2 cells. Cells were treated with BBR for 8 h at the indicated concentrations and total RNA was isolated for analysis of *LDLR* and *GAPD* mRNA expression by northern blot and real-time PCR assays. (**d**) Dose-dependent induction of *LDLR* mRNA expression in Bel-7402 cells examined by real-time RT-PCR. (**e**) BBR increased cell-surface LDLR expression. (**f**) The uptake of DiI-LDL was measured by FACScan with 2×10^4 cells per sample. The mean fluorescence value (MFV) of untreated cells is expressed as 100%. The data shown are representative of 3 separate assays.

from 700 Chinese herbs. The *LDLR* mRNA levels were subsequently determined by semi-quantitative RT-PCR assays. Among different compounds tested, BBR, an alkaloid originally isolated from Huanglian (*Coptis chinensis*), showed the highest activity in increasing *LDLR* expression. The chemical structure of BBR is benzyltetrahydroxyquinoline (molecular weight = 371.8; **Fig. 1a**). In China, BBR has been extensively used as a nonprescription drug to treat diarrhea caused by bacteria since the 1950s[20–23]. Treating HepG2 cells cultured in medium containing 0.5% lipoprotein-depleted fetal bovine serum (LPDS) or in LPDS supplemented with sterols (10 μg/ml cholesterol + 1 μg/ml 25-hydroxycholesterol) with BBR caused time-dependent increases in the expression of *LDLR* mRNA as determined by northern blot and real-time quantitative RT-PCR (**Fig. 1b**). Levels of *LDLR* mRNA, which reached a maximal level of 2.5-fold of control at 8 h, were increased as early as 2 h after the addition to the cells of BBR at a concentration of 7.5 μg/ml and expression of *LDLR* mRNA remained high throughout the 24-h treatment, regardless of the cholesterol concentration in the culture medium. The effect of BBR was also dose dependent. Northern blot showed a 50% increase in *LDLR* mRNA in cells treated with 2.5 μg/ml of BBR and a maximal increase of four-fold of control was seen with a concentration of 15 μg/ml. Similar magnitudes of increases in *LDLR* mRNA levels were confirmed by

quantitative real-time RT-PCR (**Fig. 1c**). The effect of BBR on LDLR was further confirmed in another human hepatoma cell line, Bel-7402. BBR at 2.5 μg/ml increased the *LDLR* mRNA in these cells by 2.3-fold (**Fig. 1d**). Accordingly, BBR increased LDLR protein on the cell surface, as determined by staining for antibodies to LDLR (**Fig. 1e**). The increase in *LDLR* mRNA expression directly translated into enhanced LDLR function. Uptake of 3,3′-dioctadecylindocarbocyanin-iodide (DiI)-LDL in hepatoma cells was increased in a dose-dependent manner in cells cultured with and without sterols (**Fig. 1f**).

BBR increases *LDLR* mRNA stability

The ability of BBR to increase *LDLR* mRNA expression independent of intracellular cholesterol levels suggests that SREBPs, the key transcription factors for the cholesterol-mediated feedback regulation, were not involved in the actions of BBR. To confirm this, we examined the maturation of endogenous SREBP-2 in HepG2 cells cultured with BBR, using the compound GW707 as a positive control. Previous studies have shown that GW707 can stimulate SREBP processing from the inactive precursor form (125 kDa) to the activated mature form (68 kDa), resulting in an increased LDLR transcription[24]. We harvested total cell lysates from untreated cells or cells treated with either BBR or GW707 for 8 h. Western blot showed that GW707 substantially increased the mature form of SREBP-2, whereas BBR had no effect (**Fig. 2a**).

The lack of a sterol-regulatory effect through the SREBP pathway suggests that BBR increases LDLR expression by a mechanism distinct from statins. We were interested in determining the functionality of BBR in the presence of statins that inhibit HMG-CoA reductase. HepG2 cells cultured in LPDS medium were untreated, treated with lovastatin at 0.5 and 1 μM concentrations with or without BBR for 24 h, or treated with BBR alone. The results clearly showed that BBR and lovastatin had additive stimulating effects on *LDLR* mRNA expression; BBR activity was not diminished at all by lovastatin (**Fig. 2b**).

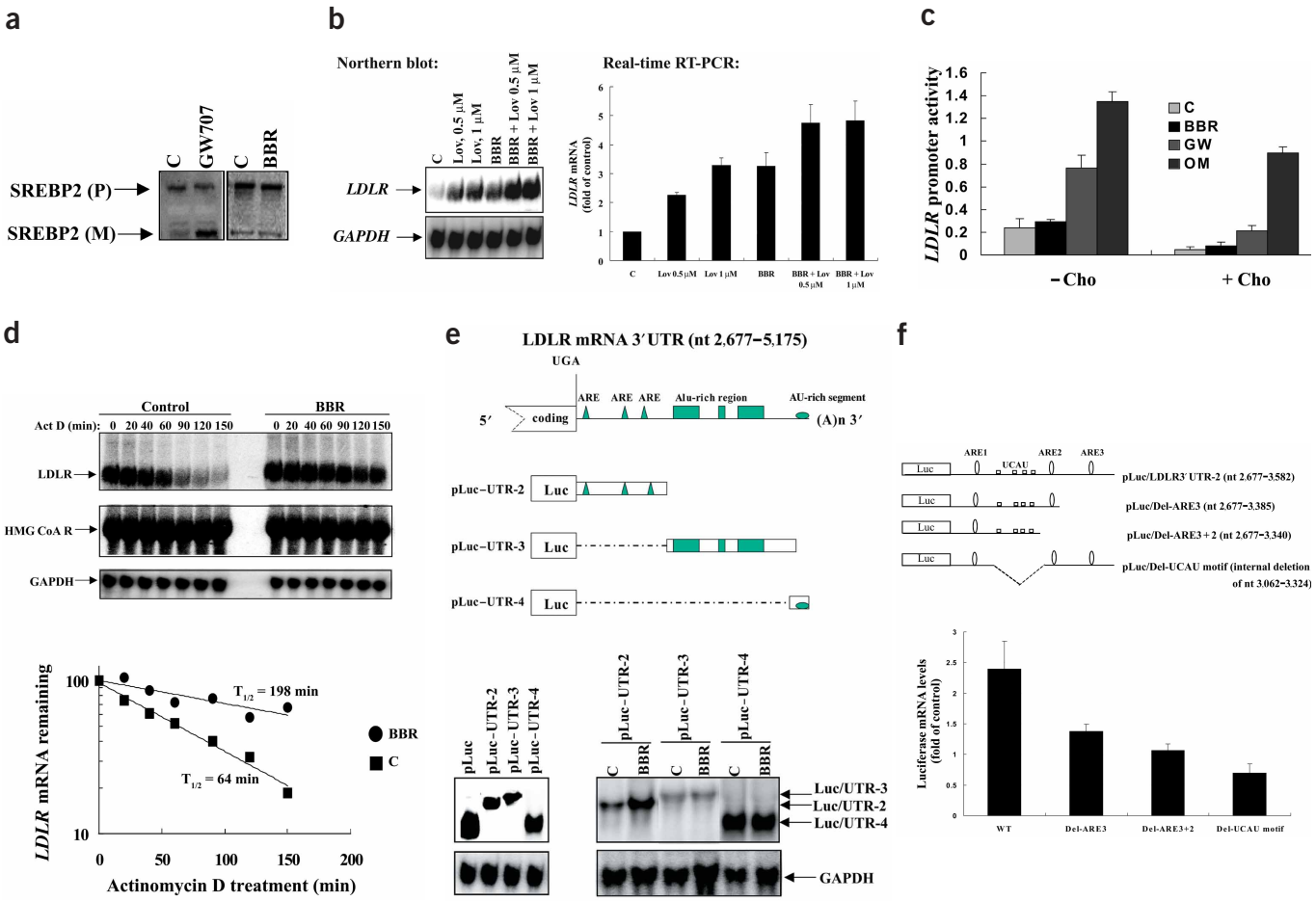

Figure 2 BBR increases LDLR expression by stabilizing *LDLR* mRNA through the 5′ proximal section of the *LDLR* mRNA 3′UTR. (**a**) Analysis of the precursor (P) and mature (M) forms of SREBP2 using a monoclonal antibody to SREBP2 in HepG2 cells. (**b**) HepG2 cells were treated either with lovastatin (Lov) alone at indicated concentrations or BBR alone, or with Lov plus 10 μg/ml of BBR, for 24 h and total RNAs were harvested and analyzed for *LDLR* and *GAPD* mRNA expression. (**c**) pLDLR234Luc and pRL-SV40 were cotransfected into HepG2 cells cultured in LPDS (–Cho) or LPDS (+Cho) medium for 24 h. BBR (10 μg/ml), GW707 (2 μm) and oncostatin M (50 ng/ml) were added to cells for 8 h prior to cell lysis. The firefly luciferase and renilla luciferase actvities were measured. (**d**) Cells were untreated or treated with BBR for 15 h. Actinomycin D (5 μg/ml) was added to cells for different intervals. Total RNA was isolated and analyzed for the amount of *LDLR* mRNA by northern blot. The normalized *LDLR* mRNA signals were plotted as the percentage of the *LDLR* mRNA remaining. Decay curves were plotted versus time. (**e**) Schematic presentation of the *LDLR* mRNA 3′ UTR and the chimeric Luc-LDLR 3′ UTR fusion constructs and the northern blot analysis of Luc-LDLR fusion mRNA. Plasmids pLuc and pLuc/UTRs were transfected (left panel) or were treated with BBR or BBR dilution buffer dimethylsulfoxide as control (right panel). The expression levels of *Luc* mRNA were determined by northern blot. (**f**) ARE and UCAU motifs are involved in BBR-induced *LDLR* mRNA stabilization. The responses of the wt pLuc/UTR-2 and deletion constructs to BBR treatment were examined by transient transfection followed by quantitative real-time RT-PCR analysis of *Luc* mRNA expression levels in transfected cells untreated or treated with BBR. The amount of *Luc* mRNA in untreated cells was defined as 1, and the amount of *Luc* mRNA in BBR-treated cells was plotted relative to that value.

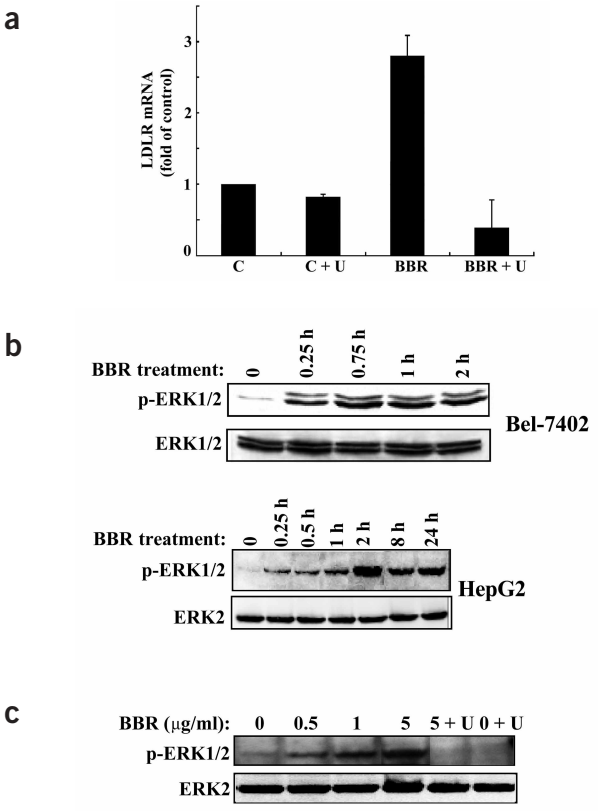

Figure 3 Blocking ERK activation abolished the regulatory effect of BBR on LDLR. (**a**) Relative amount of *LDLR* mRNA from total RNA from HepG2 cells treated with 5 μg/ml of BBR for 8 h with or without 0.5 μM U0126 was measured by a quantitative real-time RT-PCR. The amount of *LDLR* transcript in untreated cells was defined as 1, and amounts of *LDLR* mRNA from BBR- or U0126-treated cells were plotted relative to that value. (**b**) Total cell lysates were harvested from Bel-7402 cells or HepG2 cells that were untreated or treated with BBR at a dose of 5 μg/ml for different intervals as indicated. Total cellular proteins of 50 μg/lane were subjected to SDS-PAGE and western blotting using antibodies specific for either the activated and phosphorylated forms of ERK1/2, or total ERK 1/2. (**c**) HepG2 cells were treated with BBR for 1 h at the indicated concentrations and total cell lysates were used to detect phosphorylated and nonphosphorylated ERK by western blotting. Data represent mean ± s.d.

To elucidate the mechanism by which BBR increases LDLR expression, we analyzed LDLR promoter activity by transfection of HepG2 cells with the reporter construct pLDLR234Luc, which contains the SRE-1 motif and the sterol-independent regulatory element that mediates the cytokine oncostatin M–induced transcription of the LDLR gene[25,26]. After transfection, cells were cultured in LPDS or LPDS + cholesterol medium followed by an 8-h treatment with BBR, GW707 or oncostatin M. *LDLR* promoter activity was strongly elevated by GW707 and oncostatin M under both culturing conditions (**Fig. 2c**), consistent with previous studies[24,27]. Notably, BBR had no effect.

These results prompted us to examine the possibility that BBR treatment may stabilize *LDLR* mRNA, resulting in higher expression levels. Actinomycin D was added to control and BBR-treated HepG2 cells for the indicated period, and the half-lives of *LDLR* mRNA were determined by northern blot. Using the observed decay values, we showed that BBR prolonged the turnover rate of LDLR transcript by approximately threefold (198 versus 64 min; **Fig. 2d**). In contrast, the mRNA stability of HMG-CoA reductase was not altered by BBR.

The *LDLR* mRNA contains a 2.5-kb long segment of 3′UTR[28]. Three AU-rich elements (ARE) are located in the 5′ proximal region (**Fig. 2e**) and these AREs have been shown to be responsible for the rapid turnover of *LDLR* mRNA[29,30]. In the distal region of the UTR, three repeats of Alu-like sequences were reported to participate in the phorbol 12-myristate 13-acetate–induced mRNA stabilization[30]. Additionally, we have noticed that the nucleotide (nt) sequences (nt 5,085–5,175) at the extreme 3′ end of the UTR are highly AU-rich and we designated this region as the AU-rich segment. To determine whether regulatory sequences in the 3′UTR of *LDLR* mRNA are involved in the action of BBR on *LDLR* mRNA stability, we used luciferase (*Luc*) cDNA as a reporter gene. Three consecutive fragments of the *LDLR* 3′UTR were individually inserted into a cytomegalovirus promoter–driven *Luc* plasmid (pLuc) at the 3′ end of the *Luc* coding sequence before the SV40 polyadenylation signal. We transfected the wild-type (pLuc) and the chimeric plasmids (pLuc–UTR-2, UTR-3 and UTR-4) into HepG2 cells. After 48 h, we lysed the cells and isolated total RNAs. Expression of *Luc* mRNA and *Luc-LDLR* 3′UTR chimeric fusion RNAs were examined by northern blot analysis with a ^{32}P-labeld Luc cDNA as the probe. Inclusion of UTR-2 and UTR-3 sequences reduced expression levels of Luc mRNA by approximately 3–4-fold, indicating the presence of destabilization determinants within these regions, whereas the *Luc* mRNA level was only moderately reduced by fusing with UTR-4, containing the AU-rich segment sequence (**Fig. 2e**). BBR specifically increased the level of Luc–UTR-2 mRNA up to 2.5-fold without affecting expressions of Luc–UTR-3 and Luc–UTR-4 (**Fig. 2e**). BBR also did not affect the expression of the wild-type *Luc* mRNA (data not shown). Because all *Luc* constructs used the same cytomegalovirus promoter, these results clearly indicate that BBR affected the mRNA stability of the heterologous *Luc-LDLR* transcript and the stabilization is mediated through regulatory sequences present in the 5′ proximal region of the *LDLR* 3′UTR (nt 2,677–3,582). Without this region, stabilities of the chimeric transcripts were unchanged by BBR.

To narrow down the BBR-responsive region, we performed a detailed sequence analysis to search for potential regulatory motifs, which suggested that the BBR-responsive UTR-2 region (nt 2,677–3,582) contains three potential ARE sites and four repeats of the tetranucleotide UCAU (**Fig. 2f**). These UCAU repeats are clustered within sequences between ARE1 and ARE2. It was recently reported that the UCAU repeats in the 3′UTR of *Pmp1* mRNA (encoding MAPK phosphotase) are required for the regulation of *Pmp1* mRNA stability through the binding of a K-homology-type RNA binding protein in fission yeast[31]. To assess the potential roles of the ARE and UCAU motif in BBR-mediated *LDLR* mRNA stabilization, we made three more constructs that either delete the ARE3 (Del-ARE3), both ARE 3 and ARE2 (Del-ARE3 + 2), or eliminate the UCAU motif by an internal deletion. We transfected these constructs and the BBR-responsive wild-type construct into HepG2 cells, and then processed and treated the transfected cells (**Fig. 2e**). The effects of BBR on the chimeric *Luc* transcripts were determined by measuring *Luc* mRNA using a quantitative real-time RT-PCR assay. Deletion of ARE3 region resulted in a partial loss of BBR stimulation and deletion of both ARE3 and ARE2 rendered the construct unresponsive to BBR. Notably, the stabilizing effect of BBR on the *Luc* chimeric transcript was also abolished by deleting the UCAU motifs (**Fig. 2f**). Results of real-time RT-PCR were independently confirmed by northern blot analysis. These findings suggest that both ARE and UCAU motifs are involved in the stabilization of *LDLR* mRNA in BBR-treated HepG2 cells.

Activation of ERK is required for BBR to increase LDLR

Using different kinase inhibitors, including the inhibitor of MEK1 U0126, the p38 kinase inhibitor SB203580, the c-Jun N-terminal

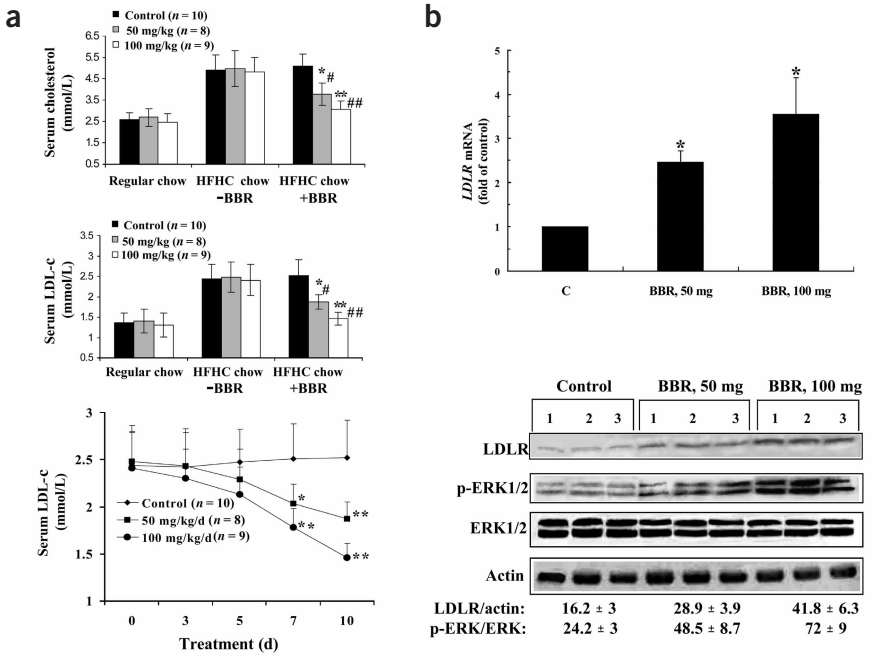

Figure 4 BBR reduces plasma LDL-c and increases liver LDLR expression in hamsters. (**a**) Serum was taken before and after a 2-week high-fat feeding, and was taken at the indicated times during and after BBR treatment at indicated daily doses. Results represent mean ± s.d. of 8–10 animals. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to untreated control; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ as compared to before treatment. (**b**) Four hours after the last drug treatment, three animals from each group were killed and liver total RNA and protein extracts were immediately prepared and analyzed for *LDLR* mRNA and protein by quantitative real-time RT-PCR (upper panel) and western blot (lower panel). The western blot membrane was stripped and reprobed sequentially with antibodies that recognize phosphorylated ERK, total ERK and β-actin.

kinase inhibitor curcumin, the PI-3 kinase inhibitor wortmannin and the PKC inhibitor calphostin C, we found that the activity of BBR on *LDLR* expression was most sensitive to U0126. At 0.5 μM, U0126 abolished the activity of BBR on *LDLR* mRNA expression (**Fig. 3a**). To determine whether BBR directly activates the MEK1-ERK pathway, we treated cells with BBR for different intervals and assessed levels of activated ERK in control and BBR-treated cells by western blotting using antibodies that only recognize the activated (phosphorylated) ERK. In both hepatoma cell lines, BBR rapidly activated ERK and the kinetics of ERK activation preceded the upregulation of LDLR expression by BBR (**Fig. 3b**). The activation of ERK by BBR is also dose dependent (**Fig. 3c**). These data indicate that activation of ERK pathway is a prerequisite event in BBR-mediated stabilization of the LDLR transcript.

BBR lowered serum LDL-c in hypercholesterolemic people

We enrolled 91 hypercholesterolemic people (52 males and 39 females) and determined whether they could be considered hyperlipidemic (IIa and IIb) according to the 'Hyperlipidemia Diagnostic Criteria' recommended for the Chinese population[32]. We randomly divided the study participants into BBR ($n = 63$) and placebo treatment groups ($n = 28$).

Subjects in the BBR treatment group were given 0.5 g of BBR orally twice per day for 3 months. The same procedure was applied to the placebo group. Blood samples determined fasting serum concentrations of cholesterol, triglycerides, HDL-cholesterol (HDL-c) and LDL-c, as well as liver and kidney functions at baseline and at the end of treatment. BBR significantly lowered serum levels of cholesterol by 18% ($P < 0.001$), triglycerides by 28% ($P < 0.001$) and LDL-c by 20% ($P < 0.001$) in the 63 hypercholesterolemic subjects, although serum HDL-c values remained unchanged (**Supplementary Table 1** online). All subjects tolerated BBR well and we observed no side effects, with the exception of one subject having mild constipation during treatment, which was relieved after reducing the dose to 0.25 g twice per day. BBR did not change kidney functions, but improved liver function by reducing levels of alanine aminotransferase, aspartate aminotransaminase and gamma glutamyl transpeptidase. The placebo group showed no changes in these parameters.

Because some participants were taking other medications that could have influenced the results, we re-evaluated the results by analyzing the data only from those participants (32 of the BBR group and 11 of the placebo group) who were neither on other drugs or herbs nor on special diets before or during BBR therapy (**Supplementary Table 2** online). BBR significantly lowered the serum levels of cholesterol by 29% ($P < 0.0001$), triglycerides by 35% ($P < 0.0001$) and LDL-c by 25% ($P < 0.0001$), although again serum HDL-c level remained unchanged (**Table 1**). In this study cohort, BBR showed an equal cholesterol-lowering effect in Type IIa and IIb patients; however, we observed the triglycerides-lowering effect of BBR in Type IIb patients only, and not in IIa patients whose baseline values of triglycerides were not elevated (**Supplementary Table 3** online). BBR treatment also significantly improved liver function in this subgroup (**Table 2**).

Table 1 Effects of BBR on serum lipids in the subgroup of hypercholesterolemic patients who were not taking other medication before or during BBR treatment.

Treatment (3 months)		BBR[a] Hypercholesterolemia (>5.2 mmol/L, $n = 32$)	Placebo Hypercholesterolemia (>5.2 mmol/L, $n = 11$)
Serum level of cholesterol			
Cholesterol	Before	5.9 ± 0.7	6.1 ± 0.6
	After	4.2 ± 0.9*	6.0 ± 0.8
Triglyceride	Before	2.3 ± 1.8	2.2 ± 0.8
	After	1.5 ± 0.9*	2.1 ± 0.9
HDL-c	Before	1.1 ± 0.3	1.2 ± 0.5
	After	1.1 ± 0.3	1.2 ± 0.4
LDL-c	Before	3.2 ± 0.7	3.7 ± 0.7
	After	2.4 ± 0.6***	3.7 ± 0.8

[a]Statistical analysis of the baselines of cholesterol, triglyceride, HDL-c and LDL-c showed that there were no significant differences between the BBR and placebo groups before therapy ($P > 0.05$). $^{***}P < 0.0001$, as compared to the baselines of 'before treatment' group.

Lipid-lowering effects of BBR in hamsters

To verify that the increased hepatic LDLR expression by BBR was the primary reason for the reduced LDL-c in patients, an animal study was conducted in hamsters, in which the effects of added cholesterol and fat on the kinetics of hepatic LDLR-mediated LDL clearance have been well characterized[33–35]. Before BBR treatment, animals were fed

Table 2 Effect of BBR on liver and kidney functions of the subgroup of hypercholesterolemic patients who were not taking other medication before or during BBR treatment.

	n	ALT(U/L)	AST(U/L)	GGT (U/L)	Bil-T(μM/L)	Cr (μM/L)	BUN (mM/L)
BBR group:							
Before treatment	32	44.9 ± 21.8	39.3 ± 22.2	53.7 ± 24.4	17.4 ± 8.8	75.5 ± 14.6	5.76 ± 1.2
After treatment	32	23.6 ± 11.1**	26.6 ± 8.2*	31.7 ± 15.2**	13.8 ± 6.3**	72.6 ± 18.7	5.79 ± 1.2
Placebo group:							
Before treatment	11	45.7 ± 17	39.6 ± 19.2	52.2 ± 21.4	17.0 ± 6.0	72.6 ± 17.1	5.60 ± 1.4
After treatment	11	44.8 ± 10.2	38.8 ± 8.3	52.0 ± 14.8	17.3 ± 5.3	73.1 ± 19	5.66 ± 1.3
Normal range[a]		0–40.0	0–40.0	10.0–50.0	3.4–26.6	39.8–134.4	2.1–7.9

[a]"National Clinical Laboratory Manual" issued by The Ministry of Health of the People's Republic of China, with minor modifications. *$P < 0.01$; **$P < 0.001$, as compared to those 'before treatment.' ALT, alanine aminotransaminase; AST, aspartate aminotransaminase; GGT, gamma glutamyl transpeptidase; Bil-T, total bilirubin; Cr, creatinine; BUN, blood urea nitrogen.

a high-fat and high-cholesterol (HFHC) diet for 2 weeks, which significantly increased total serum cholesterol ($P < 0.001$) and LDL-c ($P < 0.001$). Treatment of these hyperlipidemic animals with BBR by oral administration for 10 d resulted in dose-dependent decreases in both serum total cholesterol and LDL-c (**Fig. 4a**). After the 10-d treatment, BBR at a dose of 50 mg/kg/d reduced LDL-c by 26%, and at a dose of 100 mg/kg/d, reduced LDL-c by 42% as compared to the control animals on the same HFHC diet. The BBR effect was also time dependent (**Fig. 4a**). Reductions in serum LDL-c were observed by day 5 and became significant by day 7 at both doses ($P < 0.01$). At the end of treatment, three animals from each group were killed and liver *LDLR* mRNA and protein expressions were examined by quantitative real-time RT-PCR and western blot. *LDLR* mRNA and protein levels were elevated in all BBR-treated hamsters in a dose-dependent manner. We detected a 3.5-fold increase in mRNA and a 2.6-fold increase in protein in hamster livers treated with 100 mg/kg/d of BBR (**Fig. 4b**). Notably, increased expressions of LDLR were concurrent with the activations of ERK from BBR-treated hamsters. These results provide a direct link of the cholesterol-lowering effect of BBR with its activity on upregulation of hepatic LDLR and confirm the participation of the ERK pathway in these processes *in vivo*.

DISCUSSION

Increasing hepatic LDLR expression by inhibition of cellular cholesterol biosynthesis through the SRE-1–SREBP pathway is the primary mechanism of statin therapy for hypercholesterolemia. Here, we identify a new cholesterol-lowering drug, BBR, that effectively lowers serum cholesterol, triglycerides and LDL-c to levels comparable to those of statins, but works through a different mechanism.

Using human hepatoma–derived cell lines, we show that BBR increases mRNA and protein as well as the function of hepatic LDLR. This activity is independent of intracellular cholesterol levels and has no effects on the activation process of SREBP or the activity of HMG-CoA reductase (data not shown). BBR does not stimulate the transcription of *LDLR*, as the *LDLR* promoter activity is not increased by this compound. The post-transcriptional regulation appears to be the main working mechanism underlying the effect of this drug on liver *LDLR* expression. In BBR-treated cells, the mRNA half-life of LDLR was considerably extended. We sought to understand how BBR affects the stability of the LDLR transcript by examining the LDLR mRNA 3′UTR. Using *Luc* mRNA as the reporter, we show that the 5′ proximal ARE-containing region, covering nt 2,677–3,582, responds to BBR with an increased stability of the fusion transcript. Within this region, deletion of ARE3 resulted in a partial loss of BBR-mediated stabilization and deletion of both ARE2 and ARE3 rendered the construct unresponsive to BBR stimulation. These data support the functional role of AREs in BBR-regulated *LDLR* mRNA stabilization. It is possible that an mRNA-binding protein may interact with AREs after BBR stimulation to protect these labile sequences from endonuclease-induced degradation, resulting in mRNA stabilization. In addition to AREs, we found that deletion of UCAU repeats located within the sequences between ARE1 and ARE2 also abolished BBR stimulation of the reporter construct. Notably, the UCAU motif has been recently linked to the MAP kinase pathway[31]. Because ERK activation is required for BBR to stabilize the *LDLR* mRNA, interactions of mRNA binding proteins with these motifs may be a direct downstream event of the ERK signaling cascade. Interestingly, a recent study on bile acid–mediated *LDLR* mRNA expression also reported that ERK activation was required for the stabilizing effect of bile acid on *LDLR* mRNA[36]. These results warrant further investigations to firmly define the precise roles of ARE and UCAU motifs in BBR-elicited *LDLR* mRNA stabilization.

The upregulatory effects of BBR on LDLR expression seen in human hepatoma cells and in hamsters are likely to be accountable for its LDL-c–lowering effects in people with hypercholesterolemia. A 29% reduction of serum cholesterol, 35% reduction of triglyceride and 25% reduction of LDL-c were achieved in hypercholesterolemic participants after a 3-month treatment. Compared to statin therapies that have shown to maximally lower LDL-c to 60%[16–18], the effects of BBR seem moderate. The regimen used in this study is a pioneer trial for hypercholesterolemia with a modest dose; larger cholesterol-lowering effects of BBR may be achieved by improvement of the treatment protocol. This notion is supported by our studies in hamsters. The dose-dependent effects of BBR on liver LDLR expression and plasma LDL-c reduction were observed in hyperlipidemic hamsters. BBR was well tolerated by all participants, consistent with other reports[20,21].

Based on the clinical outcomes, we assumed that reduced fat storage in liver might be responsible for the improved liver function observed in BBR-treated patients. This speculation is supported by our results showing that heavy hepatic fat staining in control animals fed a HFHC diet were greatly reduced by BBR treatment (data not shown). In addition, we believe that the systemic effect of BBR, but not an inhibition in the adsorption of lipid in gut, plays a major role in reducing serum lipids, because in the hamster experiments we found that intraperitoneal administration of BBR at 20 mg/kg had better lipid-lowering effect than oral administration at 100 mg/kg, and oral administration of BBR did not increase fecal lipids of the hamsters (data not shown).

These findings strongly suggest that BBR is a promising new hypolipidemic drug that acts through pathways distinct from those of

statins. We postulate that BBR can be used as a monotherapy to treat hypercholesterolemic patients or it may be explored in combination therapy with statins.

METHODS

Quantification of *LDLR* mRNA expression by northern blot analysis and real-time PCR. We performed isolation of total RNA and analysis of *LDLR* and *GAPD* mRNA by northern blot as previously described[27,37]. For quantitative real-time PCR assays, we conducted reverse transcription with random primers using Superscript II at 42 °C for 30 min with 1 μg of total RNA. We performed real-time PCR on the cDNA using the ABI Prism 7900-HT Sequence Detection System and Universal MasterMix (Applied Biosystems). We assessed *LDLR* and *GAPD* mRNA expression levels using the human *LDLR* and *GAPD* Pre-Developed TaqMan Assay Reagents (Applied Biosystems). Other predeveloped real-time PCR probes, including hamster LDLR, hamster GAPDH and firefly luciferase, were also purchased from Applied Biosystems.

Flow cytometry analysis (FACS). We treated Bel-7402 cells with BBR (10 μg/ml, 24 h). We detached cells with cell removal buffer containing EDTA[38], washed and resuspended the cells in FACS solution (PBS with 0.5% BSA and 0.02% sodium azide) at a density of 1×10^6 cells/ml. Cells were incubated with monoclonal antibody to LDLR (Santa Cruz) at a final dilution of 1:50 (room temperature, 1 h). We used an isotype-matched, nonspecific mouse IgG as a control for nonspecific staining. We washed and stained cells with FITC-conjugated goat antibody to mouse IgG (Santa Cruz, 1:100 dilution). The fluorescence intensity was analyzed by FACS (FACSort, Becton Dickinson).

Plasmid construction and northern blot analysis of Luc fusion transcripts. The wild-type *Luc* reporter plasmid pLuc was constructed by insertion of the *Luc* cDNA into the *Hind*III and Xba1 sites of pcDNA3.1/Zeo(+)[39]. Addition of the *LDLR* 3′UTR was accomplished by PCR amplifying different regions of the 2.5-kb 3′UTR of *LDLR* mRNA using XbaI-tailed primers and pLDLR3 as the template. We cut individual PCR fragments with XbaI and inserted them adjacent to the 3′ *Luc* coding region to yield pLuc/UTR vectors. To create ARE deletion constructs, we generated an Apa1 site at nt 3,384 for deleting ARE3, and we generated an Apa1 site at nt 3,334 for deleting ARE2 by site-directed mutagenesis using pLuc/UTR-2 as the template. We cut mutated plasmids with Apa1 to remove the ARE-containing region and religated the remaining vector with the 5′ proximal region of UTR-2. To create the UCAU motif deletion, we generated two SacII sites for internal deletion of nt 3,062–3,324 by using UTR-2 as the template. All constructs were sequenced and the correct clones were further propagated to isolate plasmid DNA. Efficient expressions of the wild-type and chimeric *Luc* transcripts were examined by northern blot after transfection of the plasmid DNA into HepG2 cells.

To examine the stabilizing activity of BBR on the fusion transcripts, cells seeded in culture dishes were transiently transfected with the chimeric plasmids. Twenty-four hours after transfection, cells were trypsinized and reseeded equally into two dishes for each plasmid transfection. After an overnight incubation, one dish was treated with dimethylsulfoxide as the solvent control and another was treated with BBR for 8 h. Total RNA was isolated from transfected cells that were untreated or treated with BBR.

To detect the presence of *Luc-LDLR* fusion transcripts, we performed a PCR reaction to amplify a 550-base pair fragment of *Luc* coding region with 5′ primer *Luc*-2up (5′-GCTGGAGAGCAACTGCATAAGGC-3′) and the 3′ primer *Luc*-2lo (5′-GCAGACCAGTAGATCCAGAGG-3′) using pGL3-basic as the template. The PCR fragment was labeled with ^{32}P and used as the probe in northern blot analysis.

Subjects and design. We enrolled 91 hypercholesterolemic participants (serum cholesterol >5.2 mmol/L) in this study at the First Hospital of Nanjing City, Nanjing, China. We randomly assigned 63 participants to the BBR treatment group and the other 28 participants to the placebo treatment group as study controls (**Supplementary Tables 1** and **2** online). Subjects in the BBR treatment group were assigned to take BBR hydrochloride (Nanjing Second Pharmaceutics, Inc.) orally at a dose of 0.5 g twice a day for 3 months. A similar procedure was applied to the placebo group. We took blood samples before and three months after therapy. We measured fasting serum concentrations of cholesterol, triglycerides, HDL-c and LDL-c before and after the 3-month treatment using standard methods routinely applied in hospitals. Liver and kidney functions were examined in subjects treated with BBR and placebo. All participants gave informed consent.

BBR *in vivo* activity in hamsters. Two weeks before treatment, the food of female golden hamsters (National Vaccine & Serum Institute) was switched to a HFHC diet (10% lard, 10% egg yolk powder and 1% cholesterol). Animals on the HFHC diet were given BBR orally twice a day at 50 mg/kg/d or 100 mg/kg/d for 10 d. Control animals received an equal volume of vehicle (0.9% saline). Serum cholesterol, triglyceride and LDL-c levels were measured after 4 h fasting before (day 0), during and after the course of treatment. The diet control group was comprised of five hamsters fed a normal diet without drug treatment. Four hours after the last treatment, all animals were killed and their livers removed and stored in liquid nitrogen for RT-PCR, northern and western blot analysis, and histological examinations.

The animal and human studies were approved by the Research Committees of the Institute of Medicinal Biotechnology and Nanjing First Hospital.

Statistics. To compare the values before and after BBR treatment, a paired *t*-test was used. For the animal experiment, differences of mean results among study groups were examined by student's *t*-test for equal or unequal variances depending on a preliminary F test for homogeneity of variance.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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