# Modified methylenedioxyphenol analogs lower LDL cholesterol through induction of LDL receptor expression<sup>®</sup>

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Abstract Although statin therapy is a cornerstone of current low density lipoprotein (LDL)-lowering strategies, there is a need for additional therapies to incrementally lower plasma LDL cholesterol. In this study, we investigated the effect of several methylenedioxyphenol derivatives in regulating LDL cholesterol through induction of LDL receptor (LDLR). INV-403, a modified methylenedioxyphenol derivative, increased LDLR mRNA and protein expression in HepG2 cells in a dose- and time-dependent fashion. These effects were apparent even under conditions of HMG-CoA reductase inhibition. Electrophoresis migration shift assays demonstrated that INV-403 activates SREBP2 but not SREBP1c, with immunoblot analysis showing an increased expression of the mature form of SREBP2. Knockdown of SREBP2 reduced the effect of INV-403 on LDLR expression. The activation of SREBP2 by INV-403 is partly mediated by Akt/GSK3ß pathways through inhibition of phosphorylation-dependent degradation by ubiquitin-proteosome pathway. Treatment of C57Bl/6j mice with INV-403 for two weeks increased hepatic SREBP2 levels (mature form) and upregulated LDLR with concomitant lowering of plasma LDL levels. Transient expression of a LDLR promoterreporter construct, a SRE-mutant LDLR promoter construct, and a SRE-only construct in HepG2 cells revealed an effect predominantly through a SRE-dependent mechanism. INV-403 lowered plasma LDL cholesterol levels through LDLR upregulation. These results indicate a role for small molecule approaches other than statins for lowering LDL cholesterol.—Ying, Z., R. Desikan, X. Xu, A. Maiseyeu, C. Liu, O. Sun, O. Ziouzenkova, S. Parthasarathy, and S. Rajagopalan. Modified methylenedioxyphenol analogs lower LDL cholesterol through induction of LDL receptor expression. J. Lipid Res. 2012. 53: 879-887.

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Elevated levels of plasma low density lipoprotein (LDL) cholesterol is a primary determinant of atherosclerosis (1, 2). In humans, the majority of plasma cholesterol is carried by the LDL fraction with cellular uptake of LDL being mediated through LDL receptor (LDLR). The circulating level of LDL is determined in large part by its rate of uptake through the hepatic LDLR pathway, as evidenced by the hypercholesterolemia in patients with defective LDLR or ApoB-100 or elevated LDL levels in mice with a homozygous deletion of the LDL receptor (3, 4).

Studies have shown that LDLR expression is finely attuned to changes in intracellular cholesterol (5). A transcription factor known as the sterol-responsive element binding protein 2 (SREBP2) plays a critical role in LDLR mRNA expression (6-8). After synthesis, SREBP2 forms a complex with the SREBP cleavage-activating protein (SCAP) and is localized in the endoplasmic reticulum (ER) as an inactive precursor (pro-SREBP2). Sterol deficiency results in the release of SREBP2/SCAP complex from ER and transport to the Golgi, where pro-SREBP2 is processed further, allowing the N-terminal fragment to enter the nucleus and upregulate transcription of LDLR (9). In addition to SREBP2, other transcription factors may be involved in a context-dependent fashion in regulating LDLR expression. For example, a mutation at the Sp1 binding site (-49C>T) in the promoter of LDLR gene is associated with familial hypercholesterolemia (10). Upregulation of LDLR may thus represent an attractive strategy to control plasma LDL cholesterol levels.

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Various studies have shown that sesame oil can lower LDL levels in animal models (11-13). The effects of sesame oil have been attributed to both the fatty acid and nonsaponifiable components (11-13). Recent structure-activity relationship analysis of berberine, a small molecule derived from a plant, has demonstrated that the methylenedioxyphenyl group of berberine is critical for the induction of

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Abbreviations: LDLR, LDL receptor; MDP, methylenedioxyphenol; PPAR, peroxisome proliferator-activated receptor; SRE, serum response element; SREBP2, sterol-responsive element binding protein 2. To whom correspondence should be addressed.

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LDLR (14). We previously synthesized an analog of methylenedioxyphenol (MDP), INV-403, and we demonstrated that, in a model of LDLR deficiency, this small molecule inhibited atherosclerosis, paralleled by a decrease in aortic inflammation oxidative stress and improvement in endothelial function without overt change in plasma lipid profile and LDL (15). In the present study, we assess the effects of INV-403 and related analogs on cholesterol metabolism and LDLR expression, and we show that treatment with INV-403 unexpectedly and dramatically reduced plasma LDL, paralleled by an increase in the hepatic LDLR.

# MATERIALS AND METHODS

### Synthesis of INV-403

The final compound was obtained in two-step process.

In step I, an intermediate compound [2] is made by the simple acetylation of methylenedioxyphenol [1]. Acetylation was achieved using acetic anhydride in 75–80% chemical yield. The starting material 3,4-methylenedioxyphenol (1.38 g, 0.01 mol) was dissolved in 10% sodium hydroxide solution (15 ml), and to the cooled solution, acetic anhydride (1.5 g, 0.015 mol) was added, with stirring over 10 min. The reaction mixture was extracted with carbon tetrachloride. The extract was neutralized with sodium carbonate solution and dried over magnesium sulfate. The solvent was removed under reduced pressure and directly purified over column chromatography, and purified product was used directly for next step. The acetylated product serves as the starting point of many other compounds, including nitro derivatives.



In step II, the compound [2] from step I (1.8 g, 0.01 mol) was dissolved in glacial acetic acid (15 ml) and was treated at room temperature gradually with a solution of concentrated nitric acid (1.1 g, 0.012 mol; specific gravity 1.42) in glacial acetic acid (5 ml). The solution was stirred for 3 h and then poured into cold water (50 ml). After 1 h, the pale-yellow crystalline precipitate was separated by filtration, washed with water, and dried. 3,4-methylenedioxy-6-nitrophenyl acetate (INV-403) was crystallized from ethanol, yield 81%, melting point 104–105°. The purity of obtained INV-403 was more than 99% as determined by nuclear magnetic resonance spectroscopy (supplementary Fig. III).



#### Animals

The Institutional Animal Care and Use Committee (IACUC) at Ohio State University approved the experimental animal protocols. Twelve male C57Bl/6j mice (8 weeks old) were obtained from the Jackson Laboratory and allowed to acclimate for two weeks. They were then intraperitoneally injected with vehicle (10% ethanol in phosphate buffered saline) or INV-403 (20 mg/kg/day for two weeks).

#### Plasma lipoprotein profile

Plasma lipoproteins were profiled by Cardiovascular Specialty Laboratories Inc. (Atlanta, GA). Total cholesterol and triglyceride concentrations were determined enzymatically with the CHOD-PAP (Roche Diagnostics) and lipase/GPO/PAP (Roche Diagnostics) methods, respectively. The LDL cholesterol was measured by Roche homogeneous LDL-C assay (Roche Diagnostics). The HDL cholesterol was subsequently measured by precipitation with phosphotungstic acid and MgCl2 (Roche Diagnostics). VLDL cholesterol was calculated by subtraction of HDL and LDL cholesterol from total cholesterol.

#### Western blot analysis

Tissue or cell lysates were prepared using radio-immunoprecipitation analysis (RIPA) buffer supplemented with protease and phosphatase inhibitors. Protein samples were then separated by 8% (LDLR) or 10% (proteins other than LDLR) sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electro-blotted onto polyvinylidene fluoride membranes. Target proteins were detected by primary antibodies as follows: mouse anti- $\beta$ -actin



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**Fig. 1.** INV-403 activates the LDLR promoter. (A) HepG2 cells were transfected with pLDLR-luc plasmid. These cells were then treated with the indicated compounds (10  $\mu$ M) for varying times, and then the luciferase activity in cell lysates was analyzed. n = 3. \**P* < 0.05 versus 0 h by one-way ANOVA. (B) HepG2 cells were transfected with pLDLR-luc plasmid. After 24 h, these cells were treated with the varying concentration of the indicated compounds for 4 h, and then the luciferase activity in cell lysates was analyzed. n = 6; \**P* < 0.05 versus vehicle by one-way ANOVA. (C) HepG2 cells were transfected with pLDLR-luc plasmid. After 24 h, these cells were transfected with pLDLR-luc plasmid. After 24 h, these cells were transfected with lovastatin (5  $\mu$ M) or vehicle for 20 h, and treated with INV-403 (10  $\mu$ M) for 4 h, and then the luciferase activity in cell lysates was analyzed. n = 4; \**P* < 0.05 versus vehicle; <sup>#</sup>*P* < 0.05 versus statin by one-way ANOVA.

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(Sigma), mouse anti-LDLR (R and D Systems), mouse anti-phosphoserine, rabbit anti-GSK3 $\beta$ (s9), rabbit anti-GSK3 $\beta$ (Y216), mouse anti-GSK3 $\beta$ , and anti-SREBP2 (Abcam). Secondary anti-bodies conjugated with horseradish peroxidase and chemiluminescence reagent (Amersham) were used to visualize the target proteins. Densities of target protein bands were determined with Quantity One<sup>®</sup> 4.4.1 (Bio-Rad). The internal control,  $\beta$ -actin, was used to normalize loading variations.

## Electrophoretic mobility shift assay

Nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) from mouse livers or HepG2 cells. The protein concentrations were determined by Bradford assay (Pierce). EMSAs were conducted with LightShift<sup>TM</sup> electrophoretic mobility shift assay (EMSA) kits (Pierce) per manufacturer's instructions. Briefly, 3 µg nuclear proteins/sample were incubated with 15 pmol of biotinylated oligonucleotide probe (5'-ATTTAGGTCCCTCCCCC CAACTTATGATTT-3') (16) in 25 mmol l-1 Hepes, pH 7.6, 100 mmol l-1 NaCl, 15% (v/v) glycerol, 0.1% (v/v) NP-40, and 0.5 mmol l-1 PMSF in a final volume of 20 µl. Incubations were conducted for 20 min at 18°C. After incubation, the samples were electrophoresed for approximately 2 h at 100 V with 5% acrylamide nondenaturing gels, and then transferred to nylon membranes via electroblotting at 380 milliamperes for 30 min. The DNA-protein complexes

were visualized by a chemiluminescent reaction with streptavidinhorseradish peroxidase and exposure of the blot to X-ray film (Kodak). Specificity of the SREBP2 probe was confirmed in assays in which unlabeled SREBP2 probe was added in excess as a competitor and by the supershift of SREBP2-DNA complexes in the presence of anti-SREBP2.

# **Real-time RT-PCR**

Total RNA was extracted with TRI-Reagent® (Sigma, St. Louis, MO) per the instruction. Real-time PCR was carried out using SYBR Green as described previously (17).  $2^{\Delta Ct}$  [target genes relative to the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control] were calculated as suggested by Schmittgen (18).

# LDL uptake assay

HepG2 cells were grown in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. When the cells were 70–80% confluent, fresh medium without FBS was added containing vehicle (1% DMSO) or different amounts of INV-403. After incubation for 12 h, medium was removed and cells were washed with PBS. Next, 25  $\mu$ g/ml of BODIPY-LDL (Molecular Probes) was added and incubated for 15 min. Cells were extensively washed three times with PBS, and LDL uptake was quantified on a fluorescence plate reader or visualized with confocal microscopy (following staining with DRAQ5 for nuclei). To quantify

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Fig. 2. INV-403 increases LDLR expression and function. (A) HepG2 cells were treated with INV-403  $(10 \,\mu M)$  for the indicated time, and the LDLR mRNA expression was then analyzed by real-time RT-PCR. n = 3; \*P < 0.05 versus 0 h by one-way ANOVA. (B) HepG2 cells were treated with the indicated concentration of INV-403 for 4 h, and the LDLR mRNA expression was then analyzed by real-time RT-PCR. n = 4; \*P < 0.05 versus vehicle by one-way ANOVA. (C, D). HepG2 cells were treated with INV-403 (10 µM) for the indicated time, and the LDLR protein expression was then analyzed by Western blot. n = 3; \*P < 0.05versus 0 h by one-way ANOVA. (E, F). HepG2 cells were treated with the indicated concentration of INV-403 for 8 h, and the LDLR protein expression was then analyzed by Western blot. n = 3; \*P < 0.05 versus 0 h; one-way ANOVA. (G, H) HepG2 cells were treated with vehicle or different concentrations of INV-403 for 16 h. After washing with PBS, cells were incubated with 25  $\mu$ g/ml of BODIPY-LDL for 15 min. Subsequent to extensively washing with PBS and staining with DRAQ5 for nuclei, LDL uptake was visualized with confocal microscopy (\*P < 0.05 versus vehicle by ANOVA; n = 3). The marked induction of LDLR by INV-403 indicated that HepG2 cells are an appropriate in vitro tool to define how INV-403 increases LDLR.

the confocal microscopy images, six images per slide were randomly chosen. The fluorescent density of each image was obtained and normalized by the density of DRAQ5 staining.

# Transfection and luciferase assay

The SREBP trans-activation activity luciferase reporter plasmid (p6×SRE-Luc) was a gift from Dr. Andrew J. Brown (19). The luciferase reporter plasmids pLDLR-(wt)-Luc and pLDLR-(m)-Luc, respectively, contained a 335 bp fragment of the ldlr promoter with or without site-directed mutagenesis at SRE (ATCACCCCAC changed to ATAACCCCAC) (8). They were purchased from Addgene Inc. (Cambridge, MA). Transient transfection of semiconfluent HepG2 cells in 60 mm dishes was performed using the LipofectAMINE® reagent (Invitrogen, Carlsbad, CA) per manufacturer's instructions. After 24 h, these cells were digested and seeded into 96-well plates. After another 24 h, cells were treated and then collected for luciferase activity assay. Each condition was assayed in triplicate in every experiment, and each experiment was repeated at least three times. Luciferase assays were conducted using an assay kit from Sigma. Luciferase activities were expressed as relative units after normalization to cotransfected β-galactosidase (pcDNA3) activity using chlorophenol red-B-D-galactopyranoside substrate (Roche Diagnostics) as before (20). Results were combined from at least three independent experiments.

## Statistical analysis

Unless specifically mentioned, all results were expressed as mean  $\pm$  SEM. Probability values less than 0.05 were considered significant. Student *t*-test or ANOVA were used for statistical analysis with GraphPad InStat 5 software (GraphPad Software Inc., San Diego, CA).

## RESULTS

To investigate whether MDP or its analogs have the capacity to induce LDL receptor, we synthesized four molecules beginning with MDP by way of introducing acetyl group at phenolic OH and/or nitro group at the sixth position of MDP, and we compared their effects on LDLR promoter-controlled luciferase expression. Whereas INV-401 (I-acetoxymethylenedioxyphenol) did not activate the LDLR promoter, INV-402 (6-nitromethylenedixoyphenol) and INV-403 (O-acetoxynitromethylenedioxyphenol) timeand dose-dependently induced luciferase activity in HepG2 cells, indicating that the introduction of the nitro group at the sixth position may be essential for LDLR induction (Fig. 1A, B). There was no significant difference in activation of the LDLR promoter by INV-402 and INV-403. Given that we previously observed that the acetylation of phenolic OH increased the chemical stability (15), we used INV-403 in all subsequent studies to investigate the mechanisms of LDLR promoter activation. To determine the efficacy of INV-403 on LDLR expression, we compared the effects of INV-403 and a statin (lovastatin) on LDLR promoter activity. Fig. 1C shows that INV-403 and lovastatin had similar effects on LDLR promoter activity, with the combination increasing LDLR to levels higher than each compound individually. Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that play an important role in lipid metabolism (21). Supplementary Fig. I demonstrates that 10  $\mu$ M INV-403 did not activate PPAR $\alpha$ , PPAR $\Delta$ , or PPAR $\gamma$ 

in HepG2 cells using a yeast UAS-TK system, indicating that the effects of INV-403 on the LDLR promoter is specific and does not involve these pathways.

We next confirmed the effects of INV-403 on LDLR expression in HepG2 cells through assessment of LDLR mRNA expression in response to INV-403 treatment [Fig. 2A, B, half-maximal concentration ( $EC_{50}$ ): 20–800 nM]. Because posttranslational regulation plays a critical role in LDLR expression, we examined LDLR protein expression in response to INV-403 treatment. Fig. 2C–F revealed that consistent with the mRNA expression analysis, INV-403 time-and dose-dependently increased LDLR protein expression in HepG2 cells. The increased LDLR protein expression was confirmed by the increased uptake of fluorescently labeled cholesterol in HepG2 cells (Fig. 2G, H).

As LDLR expression is exquisitely sensitive to cholesterol levels and serum is the major source of exogenous cholesterol for cultured cells, we assessed the effect of serum on the induction of LDLR by INV-403. **Fig. 3A** reveals that INV-403 activated the LDLR promoter, even in the absence of serum, when it would be expected that there



**Fig. 3.** INV-403 increases LDLR expression whether or not there is exogenous cholesterol. (A) After 24 h of transfection with pLDLR-luc plasmid, HepG2 cells were cultured with the indicated concentration of serum overnight, and then treated with INV-403 (10  $\mu$ M) for 4 h. The luciferase activity in cell lysates was analyzed and presented. n = 3; \**P* < 0.05 versus vehicle by one-way ANOVA. (B, C) HepG2 cells were cultured in medium with the indicated concentration of serum overnight and then treated with the indicated concentration of INV-403 for 8 h, and the LDLR protein expression was analyzed by Western blot. N = 3; \**P* < 0.05 versus vehicle; one-way ANOVA.



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**Fig. 4.** INV-403 activates SREBP2. (A–D) HepG2 cells were treated with INV-403 (10  $\mu$ M) for the indicated time (A and B) or the indicated concentration of INV-403 for 1 h (C and D). Nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The DNA binding activity of SREBP2 was analyzed with a LightShift chemiluminescent EMSA kit (Pierce). n = 4; \**P* < 0.05 versus vehicle; one-way ANOVA. #, nonspecific band. (E–H) HepG2 cells were stimulated with INV-403 (10  $\mu$ M) for the indicated time (E and F) or the indicated concentration of INV-403 for 1 h (G and F). The SREBP2 proteins in cell lysates, including the precursor and mature forms, were analyzed by Western blot. n = 3; \**P* < 0.05 versus vehicle by one-way ANOVA.

would be maximal upregulation of LDLR expression. This was demonstrated on analysis of LDLR protein expression under the same conditions (Fig. 3B, C). Although the basal expression of LDLR was reduced, the relative increase in LDLR with INV-403 was preserved.

SREBP2 is essential for the activation of the LDLR promoter. INV-403 time- and dose-dependently increased SREBP2 DNA binding activity in HepG2 cells, indicating that SREBP2 plays a role in the induction of LDLR by INV-403 (**Fig. 4A–D**). In contrast, INV-403 did not affect the activation of SREBP1c, the homolog of SREBP2 (supplementary Fig. II). The activation of SREBP2 involves the cleavage of SREBP2 precursor, with the mature form of SREBP2 entering the nucleus to initiate transcription by binding to consensus elements on the LDLR promoter. To confirm the effect of INV-403 on SREBP2 activation, we analyzed the maturation of SREBP2 in HepG2 cells upon the treatment with INV-403. As shown in Fig. 4E–H, INV-403 time- and dose-dependently increased mature SREBP2 in HepG2 cells.

To verify the role of SREBP2 in the induction of LDLR by INV-403, we used the siRNA to knock down the expression of SREBP2 in HepG2 cells. SREBP2 siRNA markedly reduced the effect of INV-403 on LDLR expression, indicating that SREBP2 plays a role in the induction of LDLR by INV-403 (Fig. 5A, B). However, even when SREBP2 was knocked down with siRNA, a significant effect of INV-403 on LDLR expression was still observed. This was confirmed by a reporter assay (Fig. 5C), suggesting that INV-403 may also increase LDLR through SREBP2-independent mechanisms. To test whether INV-403 increases LDLR through SREBP2-dependent and independent mechanisms, we used various promoter-reporter constructs to assess the effects of INV-403. Fig. 5D reveals that INV-403 increased luciferase expression (albeit at much lower levels) under the control of the serum response element (SRE) alone. Consistent with the important role of SRE in the expression of LDLR, mutation of the SRE site of the LDLR promoter dramatically decreased the luciferase expression. However, a small but significant residual



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Fig. 5. INV-403 activates the LDLR promoter through SREBP2dependent and independent mechanisms. (A, B) After 24 h of transfection with control or SREBP2 siRNA. HepG2 cells were treated INV-403 (10 µM) for 1 h (SREBP2) or 8 h (LDLR). The expression of LDLR and SREBP2 was then analyzed and presented. n = 3; \*P < 0.05 versus vehicle by one-way ANOVA. (C) After 24 h of cotransfection with pLDLR-luc plasmid and control or SREBP2 siRNA, HepG2 cells were treated with INV-403 (10 µM) for 4 h. The luciferase activity in cell lysates was analyzed and presented. n = 3; \*P < 0.05 versus vehicle; one-way ANOVA. (D) HepG2 were transiently transfected with pLDLR-(wt)-Luc or p6×SRE-Luc, and then treated with INV-403 (10 µM) for 4 h. Cells were lysed and luciferase activity was analyzed. n = 3; \*P < 0.05 versus 0 h or vehicle, one-way ANOVA. (E) HepG2 were transiently transfected with pLDLR-(wt)-Luc or pLDLR-(m)-Luc, and then treated with INV-403 (10 µM) for 4 h. Cells were lysed and luciferase activity was analyzed. n = 3; \*P < 0.05 versus 0 h or vehicle, one-way ANOVA.

effect of INV-403 on luciferase expression remained (Fig. 5E).

The levels of SREBP2 mature form are regulated through phosphorylation and subsequent ubiquitination. We investigated the role of GSK-3 $\beta$ , a constitutively active serine/ threonine kinase that posttranslationally modifies a number of cell signaling proteins. INV-403 time- and concentration-dependently increased GSK3 $\beta$  phosphorylation at Ser9 (the inhibitory phosphorylation site) and decreased GSK3 $\beta$ phosphorylation at Tyr216 (the activating autophosphorylaion site) (**Fig. 6A**, B). To verify the effect of INV-403 on GSK3 $\beta$ -mediated phosphorylation of SREBP2, we immunoprecipitated SREBP2 and analyzed the phosphorylation of SREBP2 in response to INV-403. Fig. 6C shows that INV-403

Fig. 6. Akt/GSK3β mediates LDLR induction by INV-403. (A) HepG2 cells were treated with INV-403 (10 µM) for the indicated time, and the phosphorylation of Akt and GSK3β was analyzed by Western blot. A representative blot from at least two independent experiments with similar results is presented. (B) HepG2 cells were treated with the indicated concentration of INV-403 for 4 h, and the phosphorylation of Akt and GSK3β was analyzed by Western blot. A representative blot from at least two independent experiments with similar results is presented. (C) HepG2 cells were treated with INV-403 (10 µM) or vehicle for 4 h, and anti-SREBP2 was used to precipitate SREBP2 from cell lysates. The serine phosphorylation level and total amount of SREBP2 were then analyzed by Western blot. (D) HepG2 cells were transiently transfected with reporter constructs, and following the indicated treatment, SREBP2 mature form and GSK3β were analyzed by Western blot. A representative blot from at least two independent experiments with similar results is presented. (E) HepG2 cells were transiently transfected with reporter constructs, and following the indicated treatment, cells were lysed and luciferase activity were analyzed. n = 3; \*P < 0.05 versus vehicle; \*P <0.05 versus INV-403, one-way ANOVA. iAkt, inhibitor of Akt.

reduced SREBP2 phosphorylation, suggesting that INV-403 may upregulate SREBP2 activity through inhibition of its phosphorylation by GSK3 $\beta$ . To further explore the signaling events upstream from GSK-3 $\beta$ , we investigated the effect of INV-403 on Akt, which is well known to phosphorylate GSK3 $\beta$  at Ser9, resulting in its inhibition. INV-403 time- and concentration-dependently activated Akt, suggesting that the effect of INV-403 on GSK3 $\beta$  activity may be mediated by Akt (Fig. 6A, B). To confirm the role of Akt in the inhibition of GSK3 $\beta$  by INV-403, we analyzed the effects of INV-403 on GSK3 $\beta$  activity in the presence of an Akt inhibitor. Fig. 6D shows that Akt inhibitor markedly increased INV-403–induced GSK3 $\beta$  phosphorylation at Ser9 and

reduced phosphorylation at Tyr216, paralleled by a decrease in mature SREBP2 level. Consistent with these observations, the inhibition of Akt significantly reduced INV-403–induced LDLR promoter activation, supporting that the Akt/GSK3 $\beta$ pathway plays a role in LDLR induction by INV-403.

Given the effects of INV-403 in promoting LDLR expression in HepG2 cells, we next investigated whether INV-403 can induce LDLR in vivo. Treatment with INV-403 significantly increased hepatic LDLR mRNA and protein levels in C57Bl/6j mice (Fig. 7A-C). EMSA revealed that INV-403 significantly increased the DNA binding activity and maturation of SREBP2 (Fig. 7D-G). Because hepatic LDLR levels play an important role in determining plasma LDL cholesterol, we next profiled plasma lipoproteins in INV-403-treated mice. Compared with vehicle, INV-403 decreased plasma LDL cholesterol level by about 60%, whereas it did not significantly affect other lipoproteins (Table 1). To confirm that INV-403 lowers LDL cholesterol level through LDLR-mediated pathways, we compared its effect on LDL lowering in wild-type and LDLR<sup>-/</sup> mice. Table 2 shows that, whereas INV-403 significantly lowered LDL cholesterol level in wild-type mice, it did not decrease LDL cholesterol level in LDLR<sup>-/-</sup> mice, strongly indicating that INV-403 lowers LDL cholesterol through induction of LDLR.

### DISCUSSION

The present study has several important findings regarding small molecule approaches to regulate LDLR, potential treatment strategies that may have important implications for cholesterol lowering. The main findings of our study are as follows: *i*) A novel small molecule analog of MDP may increase LDLR expression in cultured HepG2 cells, even under conditions of HMG-CoA reductase inhibition and sterol excess; *ii*) the increase in LDLR occurs predominately though SREBP2-dependent mechanisms; *iii*) Akt-mediated GSK3 $\beta$  inhibition is involved in the activation of SREBP2 by INV-403; *iv*) increase in LDLR in liver in C57BI/6 mice following a two-week treatment were paralleled by lower plasma LDL cholesterol levels and activation of SREBP2 in



**Fig. 7.** INV-403 activates SREBP2 and increases LDLR in C57B1/6j mice. Male C57B1/6J mice (12 weeks old; Jackson Laboratories) were intraperitoneally injected with INV-403 (20 mg/kg/day) or vehicle for 14 days. (A) Hepatic LDLR mRNA expression by real-time RT-PCR. (B, C) Hepatic LDLR mRNA expression by Western blot. (D, E) Hepatic SREBP2 DNA binding activity by EMSA. #, nonspecific band. (F, G) Hepatic SREBP2 expression. n = 6/group; \**P* < 0.05 versus control, Student *t*-test.

Supplemental Material can be found at: http://www.jlr.org/content/suppl/2012/02/21/jlr.M022806.DC1

TABLE 1. The lipoprotein effects of INV-403 in C57/Bl6 mice

|                           | Vehicle         | INV-403           |
|---------------------------|-----------------|-------------------|
| Total cholesterol (mg/dl) | $70.7 \pm 21.5$ | $72.3 \pm 9.9$    |
| Total glyceride (mg/dl)   | $41.7 \pm 16.2$ | $41.4 \pm 11.7$   |
| HDL (mg/dl)               | $41.4 \pm 16$   | $52.4 \pm 5.2$    |
| LDL (mg/dl)               | $18.3 \pm 8.9$  | $7.3 \pm 0.9^{a}$ |
| VLDL (mg/dl)              | $9.7 \pm 7.3$   | $14.7\pm6.4$      |

Mice were intraperitoneally injected with INV-403 (20 mg/kg/day) for 14 days. Plasma were then collected and lipoproteins were profiled.  ${}^{a}P < 0.05$  versus vehicle; Student *t*-test. n = 6/group.

liver; and v) LDL-lowering effects were not demonstrable in the absence of LDLR.

The results in this study may provide additional targets for regulation of plasma LDL cholesterol. LDLR regulation by SREBP2 represents a potentially important therapeutic target in regulation of plasma LDL levels given its central role in regulating cellular and plasma cholesterol levels (6, 22, 23). In the present study, nitro derivatives of a small molecule derivative of sesame oil lignans are shown to have the potential to activate the LDLR promoter even under conditions where cholesterol levels within the cell are low (low sterol conditions) and during inhibition of HMG-CoA reductase by a statin. Indeed, the results indicate that the effects of INV-403 on LDLR promoter activity may be additive to that of statin therapy. Thus, there may be a potential to regulate LDLR expression overriding an endogenous feed-back loop that is remarkably efficient and sensitive to even small changes in intracellular cholesterol concentration.

Our data indicate that increased LDLR expression INV-403 is at least partly mediated by SREBP2 mediated activation of the LDLR promoter. This is based on the following observations: *i*) INV-403 increased LDLR promoter activity; *ii*) INV-403 Increased mature SREBP2 levels and upregulated its DNA binding activity; *iii*) LDLR promoter activity in response to INV-403 was substantially attenuated by a truncated SRE-only construct; and *iv*) loss of function of SREBP2 decreased the LDLR induction by INV-403. Notably, the increase in SREBP2 mature form was not proportional to the decrease in SREBP2 precursor (Figs. 4E, G and 5A), indicating that the stabilization of mature SREBP2 may be involved in the increase of SREBP2 activity in response to INV-403 treatment. The Proteasome-Glo<sup>TM</sup> cell-based assays

TABLE 2. The differential lipoprotein effects of INV-403 in C57/Bl6 and  $LDLR^{-/-}$  mice

|                             | Wt                             |                                      | LDLR <sup>-/-</sup>   |                                  |
|-----------------------------|--------------------------------|--------------------------------------|---|----------------------------------|
|                             | Vehicle                        | INV-403                              | Vehicle   | INV-403                          |
| Total cholesterol (mg/dl)   | 94.6 ± 11.8                    | $102.6 \pm 13.7$                     | 337.8 ± 44.8  | 389.4 ± 55.1                     |
| Total glyceride<br>(mg/dl)  | $67.4 \pm 22.8$                | $53.7 \pm 15.1$                      | $142.1 \pm 29.4$  | $125.5\pm20$                     |
| HDL (mg/dl)                 | $60.6 \pm 7.9$                 | $71.6 \pm 6.7$                       | $81.9 \pm 15$   | $92.6 \pm 8.7$                   |
| LDL (mg/dl)<br>VLDL (mg/dl) | $25.4 \pm 8.6$<br>$10.8 \pm 3$ | $14.6 \pm 1.1^{a}$<br>$13.4 \pm 5.5$ | $\begin{array}{c} 141.8 \pm 26.6 \\ 114.1 \pm 13.4 \end{array}$ | $167 \pm 24.6$<br>$121.8 \pm 29$ |

Mice were intraperitoneally injected with INV-403 (20 mg/kg/ day) for 14 days. Plasma samples were then collected and lipoproteins were profiled.

 ${}^{a}P < 0.05$  versus vehicle; Student *t*-test. n = 6/group.

with HepG2 cells did not reveal an inhibition of proteasome activity by INV-403 in vitro (data not shown), indicating that INV-403 may target upstream events.

GSK3β is an ubiquitously expressed Ser/Thr kinase involved in a variety of cellular processes, ranging from glycogen metabolism and insulin signaling to cellular processes, such as proliferation. It has been shown to phosphorylate SREBP2 and target it to proteasome through ubiquitination-dependent mechanism (24). Our data demonstrate that INV-403 activates GSK3B with a time course and a dose-dependent response similar to that of SREBP2 activation, indicating that it may play a role in the SREBP2-mediated LDLR induction by INV-403. Interestingly, GSK3β appears to phosphorylate SREBP2 mature form only (Fig. 6C). This is consistent with a previous study showing that the phosphorylation of SREBP2 by GSK3ß is DNA-binding-dependent (24). The inhibition of Akt decreased LDLR induction by INV-403 as shown by reporter assays, offering evidence that Akt/GSK3B pathway may mediate the LDL-lowering effect of INV-403. However, the extent to which Akt/GSK3<sup>β</sup> pathway contributes to the LDL-lowering effect of INV-403 remains to be determined.

Although SREBP-2 through binding of the SRE undoubtedly plays the predominant role in INV-403 effects, our data also suggest a role for SRE/SREBP-2-independent effects, albeit to a much lower level compared with that through the SREBP2-dependent pathway (Fig. 5D). This is consistent with a growing number of studies showing that transcription factors other than SREBPs and cis-elements other than SRE are involved in the LDLR transcription regulation. For example, both Sp1 and estrogen receptor a  $(ER\alpha)$  are required for the LDLR induction by estrogen (25). Inhibition of PPAR $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) can also contribute to LDLR induction by estrogen (26). The induction of LDLR by berberine appears to require c-jun (14). CCAAT-enhancer-binding protein (C/EBP) is necessary for the induction of LDLR by oncostatin M (27–29). Notably, INV-403 activated the LDLR promoter far more efficiently than a truncated SRE-only promoter, indicating that there may be an important contribution of other cisacting elements. However, because INV-403 activated the SRE-mutated LDLR promoter to a much lower level, it remains to be determined whether the SREBP2-independent mechanism is pharmacologically meaningful.

The present study also provides evidence that the effects of INV-403 are selective for LDL with no discernible effects on other lipoprotein fractions. We previously showed that INV-403 had no LDL-lowering effects in LDLR-deficient WHHL rabbits (15). Consistent with this data, our current study, while demonstrating an effect of LDL lowering in wild-type mice, was unable to discern an effect on LDL lowering in LDLR<sup>-/-</sup> mice, indicating that LDLR may be essential for the LDL-lowering action of INV-403. There were no effects on plasma triglycerides, consistent with an exclusive effect on LDL pathways as noted with the phenotype of PCSK9<sup>-/-</sup> mice (30). The effects of this molecule appear to be specific for SREBP2 with no evidence of activation of SREBP1c (supplementary Fig. II). The absence of effects on triglyceride levels is again reassuring



and supports the lack of effect on SREBP-1c. Further, there is no evidence that this activates other pathways, such as PPAR $\alpha$ , PPAR $\gamma$ , or PPAR $\Delta$  (supplementary Fig. I). These results are different from those noted with berberine, which has also been reported to lower LDL through upregulation of LDLR but which was accompanied by significant decrease in plasma TG level in humans (31). This may reflect LDLR-independent effects of berberine.

Previous studies have shown that the methylenedioxyphenyl group is essential for the induction of LDLR by berberine (14). However, our data demonstrate that the MDP in and of itself cannot induce LDLR, indicating that it may be essential but not sufficient for LDLR induction.

Our study has multiple important limitations that must be acknowledged. First, we have not provided LDL-lowering effects in a model of dyslipidemia with an intact LDLR (such as a hamster model of dyslipidemia) and this may require additional experimentation. We have not provided evidence that the LDL lowering results in reduction in atherosclerosis. Finally, we have not provided definitive evidence of the precise mechanism by which this drug posttranslationally regulates SREBP2 that is distinguishable from other classic triggers of SREBP2 activation, such as intracellular sterol deficiency, which is well known to activate not only LDLR but also HMG-COA reductase.

Our findings suggest that small molecule strategies that regulate LDLR and SREBP2 represent a promising area of investigation.

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