# Gene-selective modulation by a synthetic oxysterol ligand of the liver X receptor

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**Abstract** **Liver X receptors (LXRs) play key roles in the regulation of cholesterol homeostasis by limiting cholesterol accumulation in macrophages within arterial wall lesion sites by a mechanism that includes the upregulation of ATP binding cassette transporters. These atheroprotective properties distinguish LXRs as potential targets for pharmaceutical intervention in cardiovascular disease. Their associated activity for promoting lipogenesis and triglyceride accretion through the activation of sterol-response element binding protein 1c (SREBP-1c) expression, however, represents a potential proatherogenic liability. A newly characterized synthetic oxysterol, *N,N*-dimethyl-3β-hydroxycholenamide (DMHCA), represents a gene-selective LXR modulator that mediates potent transcriptional activation of ABCA1 gene expression while exhibiting minimal effects on SREBP-1c both in vitro and in vivo in mice. DMHCA has the potential to stimulate cholesterol transport through the upregulation of LXR target genes, including ABCA1, in liver, small intestine, and peritoneal macrophages. Compared with known nonsteroidal LXR agonists, however, DMHCA exhibits only limited activity for increasing hepatic SREBP-1c mRNA and does not alter circulating plasma triglycerides. Cell-based studies also indicate that DMHCA enhances cholesterol efflux in macrophages and suggest a mechanism whereby this selective modulator can potentially inhibit cholesterol accumulation. DMHCA and related gene-selective ligands of LXR may have application to the study and treatment of atherosclerosis.**—Quinet, E. M., D. A. Savio, A. R. Halpern, L. Chen, C. P. Miller, and P. Nambi. **Gene-selective modulation by a synthetic oxysterol ligand of the liver X receptor. *J. Lipid Res.* 2004.** 45: **1929–1942.**

**Supplementary key words** nuclear receptors • ATP binding cassette transporter A1 • lipoproteins • macrophages • cholesterol efflux • sterol-response element binding protein 1c • atherosclerosis

Liver X receptors (LXRs) are members of the nuclear receptor superfamily defined as ligand-activated transcription factors. LXRα (NR1H3) and LXRβ (NR1H2) (1–3) belong to a subclass that form obligate heterodimers with retinoid X receptors (RXRs). They share significant amino acid identity in their DNA and ligand binding domains and are highly conserved between human and rodent species (3). The elucidation of their role as key regulators of cholesterol homeostasis followed from the recent identification of oxysterols as physiological ligands for LXR (4–7).

LXR nuclear receptors act as a transcriptional master switch for the coordinated regulation of genes involved in cellular cholesterol homeostasis, cholesterol transport, catabolism, and absorption (8, 9). Several LXR-responsive target genes, ATP binding cassette proteins, ABCA1 (8, 10–12) and ABCG1 (11, 13), cholesteryl ester transfer protein (CETP) (14), and apolipoprotein E (apoE) (15), with defined roles in the reverse cholesterol transport (RCT) process, govern the transport of excess cholesterol for eventual uptake and elimination by the liver. The ultimate disposal of this excess cholesterol returning to liver, its conversion to bile acids for secretion into bile, and its final elimination in feces are also under LXR control in some species. LXR-mediated upregulation of cholesterol 7α-hydroxylase (cyp7a), the rate-limiting enzyme for bile acid synthesis, correlates with resistance to dietary cholesterol and atherosclerosis (5, 16) in mice. The proximal promoter of the human gene does not contain an LXRE and consequently is not regulated by oxysterols or LXR. Thus, the regulation of murine and human CYP7A1 genes differs. A role for LXR target genes in the modulation of cholesterol absorption has also been implicated for ABCA1, ABCG5, and ABCG8 (8, 17, 18).

The identification of LXR nuclear receptors as direct regulators of ABC transporter gene expression focused attention on their potentially critical role in peripheral macrophages (8, 10–13). Here, the membrane-bound ATP binding cassette protein (ABCA1) initiates the process of cholesterol elimination from peripheral tissues. The ABCA1

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Abbreviations: ACC, acetyl-CoA carboxylase; acLDL, acetylated low density lipoprotein; apoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; cyp7a, cholesterol 7α-hydroxylase; DMHCA, *N,N*-dimethyl-3β-hydroxycholenamide; LXR, liver X receptor; RCT, reverse cholesterol transport; RXR, retinoid X receptor; SREBP-1c, sterol-response element binding protein 1c.

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cholesterol transporter promotes cellular cholesterol efflux, facilitates HDL formation, and allows for the removal of excess cholesteryl ester from cells, including macrophages of atherosclerotic lesion sites. The importance of its role in mediating the process of RCT is underscored in Tangier disease, a rare genetic disorder of HDL deficiency caused by mutations in the gene encoding ABCA1 (19–21). Tangier patients and, more commonly, heterozygous individuals for ABCA1 mutations, phenotypically have low HDL, accumulate lipids, and are at increased risk for atherosclerosis (22, 23). Furthermore, macrophage-selective ABCA1 knockouts created through bone marrow transplantation have defined an essential role for leukocyte ABCA1 expression in the control of atherosclerosis susceptibility in established models of the disease (24, 25). A similar analysis of LXR function using genetic knockouts and bone marrow transplantation approaches established macrophage-selective LXR expression as protective against lesion progression (26). In these studies, mice receiving LXR-negative macrophages developed a disorder similar to Tangier disease, emphasizing the importance of LXR-mediated regulation of macrophage ABCA1 in promoting cholesterol efflux from lesion sites within the arterial wall.

LXR regulation of ABCA1, CETP, apoE, and several other key genes in the biological process of RCT make it an attractive therapeutic target for the modulation of lipid metabolism. Potent synthetic LXR agonists increase HDL-cholesterol promisingly (8, 9, 27) and activate RCT, promoting the transfer of excess intracellular cholesterol to extracellular acceptors such as high density lipoprotein particles. Unfortunately, known ligands share the undesirable side effect of inducing lipogenesis and hypertriglyceridemia by transactivating genes involved in fatty acid biosynthesis, including that encoding sterol regulatory binding element protein 1c (SREBP-1c) (28, 29). The pharmacological challenge, therefore, is to identify LXR agonists with selective activity for specific LXR target genes. In this paper, we describe a compound that exhibits a highly desirable profile as a strong inducer of ABCA1 expression yet with diminished activity for increasing SREBP-1c or fatty acid synthase (FAS) (30). Cellular and in vivo studies provide critical proof of the concept of divergent gene regulation by LXR, in support of the search for more potent ligands with similar properties.

## EXPERIMENTAL PROCEDURES

### Materials and ligands

Oxysterols were purchased from Sigma (St. Louis, MO). 25-Hydroxycholesterol (H1015), 22(*R*)-hydroxycholesterol (H9384), 22(*S*)-hydroxycholesterol (H5884), *N,N*-dimethyl-3β-hydroxycholenamide (DMHCA) (6, 7), and the nonsteroidal LXR agonists Tularik TO901317 [*N*-(2,2,2,-trifluoro-ethyl)-*N*-[4-(2,2,2,-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]benzenesulfonamide] (8, 9) and Glaxo GW3965 [3-(3-(2-chloro-3-trifluoromethylbenzyl-2,2-diphenylethylamino)proproxy)phenylacetic acid] (27, 31) were prepared following standard chemical syntheses from the published literature. See **Fig. 1** for compound structures. Human apoA-I and BSA (essentially fatty acid free) were purchased from Sigma. Human LDL was obtained from Wake Forest University, School of Medicine (Wake Forest, NC). [1,2-$^3$H(N)]cholesterol was purchased from Perkin-Elmer (NET-139).

### Cell culture

Human HepG2, hepatoma (ATCC HB 8065), THP-1 macrophage (ATCC TIB-202), and J774.A1 macrophage-like (ATCC TIB-67) cell lines were obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, and the antibiotics 100 U/ ml penicillin and 100 μg/ml streptomycin (Gibco). THP-1 cells were maintained in RPMI 1640 medium (Gibco) containing 10% FBS, 2 mM L-glutamine, and 55 μM β-mercaptoethanol. Confluent THP-1 cells were differentiated with 50–100 ng/ml phorbal 12,13-dibutyrate (Sigma) suspended in ethanol for 3 days to induce differentiation into adherent macrophages. Murine J774.A1 macrophages were maintained in RPMI 1640 medium supplemented with 10% FBS, 4 mM L-glutamine, and antibiotics. HepG2 and J774 cells were plated on 96-well plates 18–24 h before ligand addition. LXR ligands were dissolved in ethanol and added to cells for 18 h. Control cells were treated with vehicle. Murine peritoneal macrophages were isolated using the procedure described below, although for culturing purposes they were obtained from thioglycolate-injected mice (25 ml/kg 4% Brewer's thioglycolate solution) from nonfasted animals (32). Cells were pelleted by centrifugation at 1,500 rpm for 15 min (4°C) and dispensed onto 6-well plates (2–3 million cells/well) or on a 96-well format in DMEM supplemented with 10% FBS. Nonadherent cells were removed after 5 h. The medium was either replaced or alternatively cells were treated with 50 μg/ml acetylated LDL (acLDL) (33) for 24 h before preloading the macrophages with lipid. Peritoneal macrophages were treated for 24 h with ligands in DMEM containing 5% lipoprotein-deficient serum (Intracel, Frederick, MD).

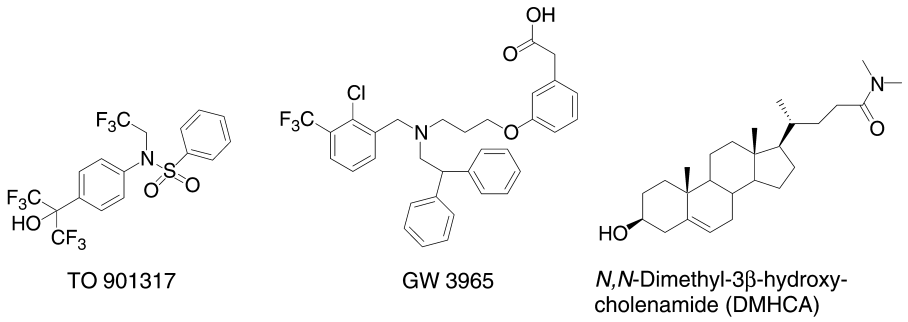



**Fig. 1.** Chemical structures of the synthetic ligands TO901317, GW3965, and *N,N*-dimethyl-3β-hydroxycholenamide (DMHCA).

### Measurement of cholesterol efflux

Differentiated THP-1 macrophage monolayers containing acLDL (25 μg/ml) and [1,2-$^3$H]cholesterol (2 μCi/ml) were preincubated for 48 h. Cells are washed with PBS and preincubated in RPMI 1640 containing 0.2% BSA in the presence or absence of compounds for 6 h. After an additional wash, human apoA-I (10 μg/ml) acceptor protein was added to the RPMI 1640 medium minus FBS and BSA, along with vehicle or ligands. Cellular cholesterol was quantified after 24 h. Replicate 80 μl aliquots of the incubation medium were removed and filtered through a 0.45 μm multiscreen plate (Millipore Corp., Bedford, MA). The radioactivity in the incubation medium was determined by liquid scintillation counting, and the percentage of radiolabeled cholesterol released (percent efflux) was calculated as follows: (treatment cpm in medium/control cpm) × 100.

### RNA isolation and quantitation

Total cellular RNA was isolated from treated cells cultured on 96-well plates using PrepStation 6100 (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Total RNA was isolated from tissues, and cells were plated on six-well format using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Glycogen (10 μg/ml; Ambion, Inc., Austin, TX) was added to facilitate the recovery of nucleic acid from murine peritoneal macrophages. RNA was resuspended in RNase-free water and stored at −70°C before analysis. RNA concentrations were quantitated with RiboGreen assay R-11490 (Molecular Probes, Eugene, OR).

### Gene expression analysis

Gene-specific mRNA quantitation was performed by real-time PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Samples (50–100 ng) of total RNA were assayed in duplicate or triplicate in 50 μl reactions using one-step RT-PCR and the standard curve method to estimate specific mRNA concentrations. Sequences of gene-specific primer and probe sets designed with Primer Express Software (Applied Biosystems) are shown in **Table 1**. RT and PCR procedures were performed according to PE Applied Biosystem's protocol for Taqman Gold RT-PCR. PCR results were normalized to GAPDH mRNA or 18S rRNA levels. Rodent GAPDH and human 18S probe and primers were purchased commercially (Applied Biosystems).

### In vivo studies: animals and diets

Male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and weighed 25–30 g at the initiation of treatment. Mice were maintained on a 12 h light/12 h dark cycle and fed a normal chow diet (Rodent Diet 5001; PMI Nutritionals, St. Louis, MO) ad libitum. Ligands were administered once per day in the morning either orally or by intraperitoneal injection in 1.3% Tween 80 and 0.25% sodium carboxymethylcellulose. Control animals were dosed with vehicle. At study termination, mice were fasted for 4 h, blood was recovered, and plasma was prepared using standard centrifugation techniques. Tissues were collected for RNA preparation and frozen in liquid $N_2$ before storage at −70°C. Murine peritoneal macrophages were isolated from animals euthanized with $CO_2$. For this purpose, the peritoneal cavity was flushed with 10 ml of ice-cold DMEM containing 10% FBS, and cells were pelleted from the medium by centrifugation at 1,500 rpm for 15 min (4°C). Cell pellets were resuspended in TRIzol for RNA isolation. Animal experiments were approved by the Institutional Animal Care and Use Committee of Wyeth Research.

### Plasma lipid and lipoprotein analysis

Plasma lipid, total cholesterol, and triglyceride concentrations were analyzed using a Hitachi 911® Clinical Autoanalyzer with Boehringer Mannheim cholesterol and triglyceride (glycerol-blanked) reagents (Roche Diagnostics, Indianapolis, IN). Plasma lipoprotein concentrations were determined for individual animals by fast-protein liquid chromatography (FPLC) analysis using Superose 6 columns (Pharmacia, Peapack, NJ). Cholesterol concentrations in column fractions were measured enzymatically with the Boehringer Mannheim reagent. Approximately 160 μl of plasma was required for both analyses. Hepatic lipid, cholesterol, and triglyceride levels in liver were determined by Anilytics (Gaithersburg, MD).

### Statistical analysis

Means, standard deviations, and statistical significance were determined by ANOVA or one-way ANOVA using Statistical Analysis Software (SAS , Cary, NC).

## RESULTS

### Differential induction of ABCA1 and SREBP-1c gene expression in hepatic and macrophage cell lines by endogenous oxysterols, DMHCA, and synthetic LXR agonists

To determine whether differences in LXR-mediated gene regulation exist for different ligands, agonists representative of both natural and synthetic oxysterols (25-hydroxycholesterol, 22*R*-hydroxycholesterol, 22*S*-hydroxycholesterol, and DMHCA) as well as the synthetic nonsteroidal LXR agonist TO901317 were profiled for comparative purposes. DMHCA, a cholenamide, was selected for its potency from among the published sterol-like agonists. Its reported

TABLE 1. Sequences of primers and probes for real-time quantitative PCR

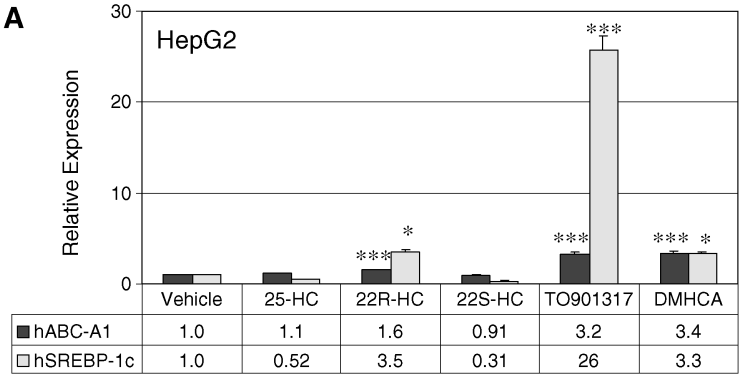
| Gene | Species | Forward Primer (5′ to 3′) | Reverse Primer (5′ to 3′) | Probe (5′ to 3′) |
|---|---|---|---|---|
| hABCA1 | Human | CAACATGAATGCCATTTTCCAA | ATAATCCCCTGAACCCAAGGA | TAAAGCCATGCCCTCTGCAGGAACA |
| hSREBP1c | Human | AGGGCGGGCGCAGAT | GGTTGTTGATAAGCTGAAGCATGT | TCGAAAGTGCAATCCATGGCTCCG |
| hFAS | Human | GCAGGAGTTCTGGGACAACCT | TCCAGCGACGGTCATCGT | TGACCATGTCCACACCGCCGA |
| hLXRα | Human | GCCCTGCATGCCTACGTCT | CATTAGCATCCGTGGGAACA | ATCCACCATCCCCATGACCGACTG |
| mABCA1 | Mouse | GAAGCCAGTTGTGCAAAACTAAATT | GCAACACTGTGGTGGCTTCA | CACATCTCATCTCCCGACCCAGCA |
| mABCG1 | Mouse | TTCATCGTCCTGGGCATCTT | CGGATTTTGTATCTGAGGACGAA | ATCTCCCTGCGGCTCATCGCCT |
| mCyp7A | Mouse | AGACCTCCGGGCCTTCCT | ATCACTCGGTAGCAGAAGGCAT | AATCAAAGAGCGCTGTCTGGGTCACG |
| mSREBP1c | Mouse | AGCCATGGATTGCACATTTGA | CAAACAGGCCAGGGAAGTCA | ACATGCTCCAGCTCATCAACAACCAAGA |
| mFAS | Mouse | AGAGATCCCGAGACGCTTCTG | AGCCGGTTGGCCATCATT | CTACAGCATGGTGGGCTGCCAGC |
| mACC | Mouse | GCCATTGGTATTGGGGCTTAC | CCCGACCAAGGACTTTGTTG | CTCAACCTGGATGGTTCTTTGTCCCAGC |

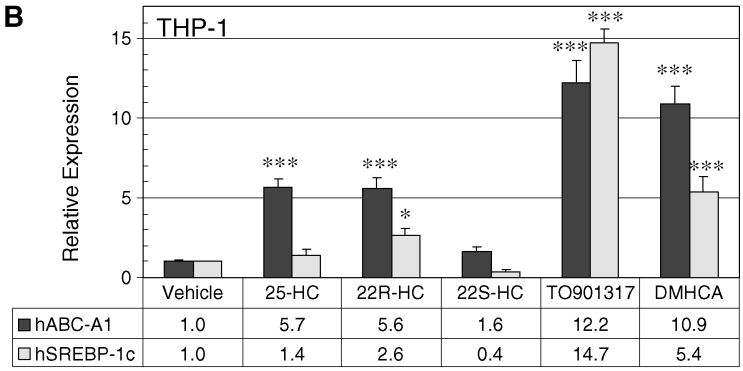
binding affinity (6), $K_i$ values of 100–200 nM measured by scintillation proximity assay for both LXR isoforms, is consistent with our unpublished binding data. TO901317, a more potent LXR binder, also shows no selectivity for either LXRα or LXRβ (9). Three cell lines, one of hepatic origin and two macrophage-like lines (**Fig. 2**), were dosed with 10 μM concentrations of LXR ligands to evaluate relative differences in LXR-regulated ABCA1 and SREBP-1c gene expression. All mRNA levels were measured by quantitative real-time PCR.

In the human hepatoma HepG2 cell line (Fig. 2A), 22*R*-hydroxycholesterol induced ABCA1 mRNA levels 1.6-fold. Moderate stimulation of ABCA1 mRNA was observed for both the synthetic Tularik agonist (TO901317; 3.2-fold) and the oxysterol analog (DMHCA; 3.4-fold). This blunted efficacy of LXR agonists for ABCA1 induction in hepatocytes is consistent with published observations in liver (8). By contrast, SREBP-1c mRNA was significantly induced by TO901317 (26-fold), whereas DMHCA increased SREBP-1c levels only moderately (3.3-fold).

In differentiated THP-1 human macrophage-like cells (Fig. 2B), ABCA1 expression was significantly induced by all compounds with the exception of the oxysterol 22*S*-hydroxycholesterol, which was shown to be an LXR antagonist (7). SREBP-1c gene activation was significantly lower than the ABCA1 increase for sterol agonists and was reduced compared with TO901317 for DMHCA. In THP-1 macrophages, like HepG2 cells, the synthetic agonist and DMHCA showed similar efficacy for increasing ABCA1 mRNA but differed in their ability to stimulate SREBP-1c.

This apparent differential transcriptional regulation becomes even more apparent in the murine J774 cells, a macrophage-like line exhibiting abundant levels of LXRβ relative to the LXRα isoform (data not shown). In J774 cells (Fig. 2C), ABCA1 mRNA increases for DMHCA exceeded that of TO901317. The induction of SREBP-1c by TO901317 was reduced relative to ABCA1 compared with a significant reduction in SREBP-1c mRNA observed for DMHCA and 25-hydroxycholesterol. The results from J774 cells showing diminished SREBP-1c induction by all



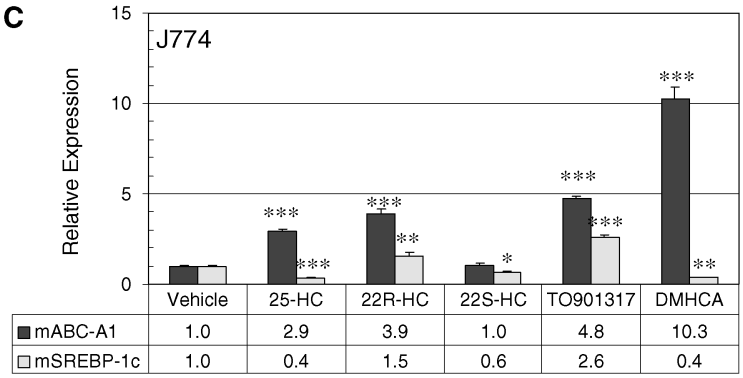




**Fig. 2.** Differential regulation of ABCA1 and sterol-response element binding protein 1c (SREBP-1c) gene expression in hepatic and macrophage cell lines by DMHCA, oxysterols, and synthetic agonists. Expression levels of ABCA1 and SREBP-1c are shown in HepG2 (A), THP-1 (B), and J774 (C) cells. Cells were treated with vehicle (ethanol) or 10 μM of the liver X receptor (LXR) agonists 25-hydroxycholesterol (25-HC), 22*R*-hydroxycholesterol (22*R*-HC), 22*S*-hydroxycholesterol (22*S*-HC), TO901317, and DMHCA for 18 h, and gene expression was analyzed by quantitative real-time PCR. RNA amounts were normalized with GAPDH, and the value was arbitrarily set to 1 in control cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (n = 3, mean ± SEM).

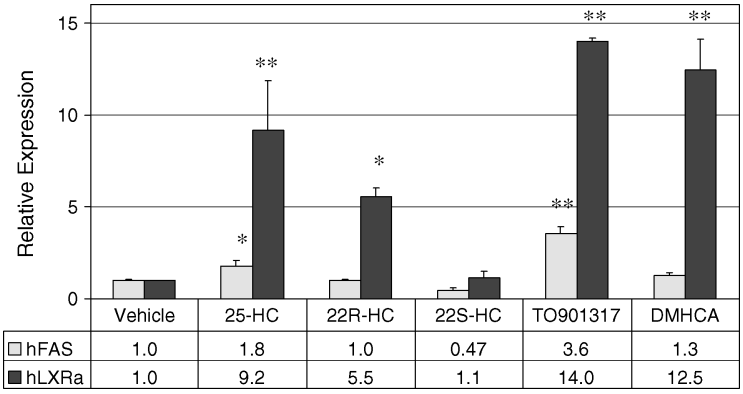



**Fig. 3.** DMHCA induces LXRα autoregulation in THP-1 macrophages but fails to upregulate fatty acid synthase. The relative amounts of FAS and LXRα mRNA were measured by real-time PCR in RNA isolated from differentiated THP-1 macrophages incubated with vehicle or 10 μM concentrations of LXR ligands, as described for Fig. 1. RNA amounts were normalized with GAPDH, and the value was arbitrarily set to 1 in control cells. * $P < 0.05$ and ** $P < 0.001$ (n = 3, mean ± SEM).

LXR agonists are consistent with literature showing that LXRα rather than LXRβ may preferentially mediate SREBP-1c gene activation (16, 34).

### Fatty acid synthase and LXRα autoregulation in differentiated THP-1 cells

Two additional LXR target genes, LXRα and fatty acid synthase (**Fig. 3**), were profiled for comparative purposes using RNA prepared from differentiated THP-1 cells. LXRα represents a direct target of LXR activation in human macrophages, but its autoregulation has been documented in several other cell types as well (31, 35, 36). FAS, an established target of the SREBP-1 pathway (37–39), is subject to both direct and indirect targeting by LXR (30). Their evaluation allowed further profiling of gene-specific effects associated with DMHCA.

In this analysis, autoregulation, restricted to the LXRα isoform was observed for all compounds (Fig. 3), with no change in LXRβ expression (data not shown) in these cells. In addition, alterations in LXRα expression were well correlated with changes in the LXR target gene, ABCA1 (Figs. 2, 3). In contrast, FAS gene expression was only modestly upregulated by 25-hydroxycholesterol and TO901317. The phenotype of divergent gene activation was again observed for DMHCA, as it induced LXRα mRNA to levels comparable with TO901317 but failed to upregulate FAS, either directly through LXR or indirectly through SREBP-1c activation.

### Dose-response comparison of DMHCA in HepG2 cells

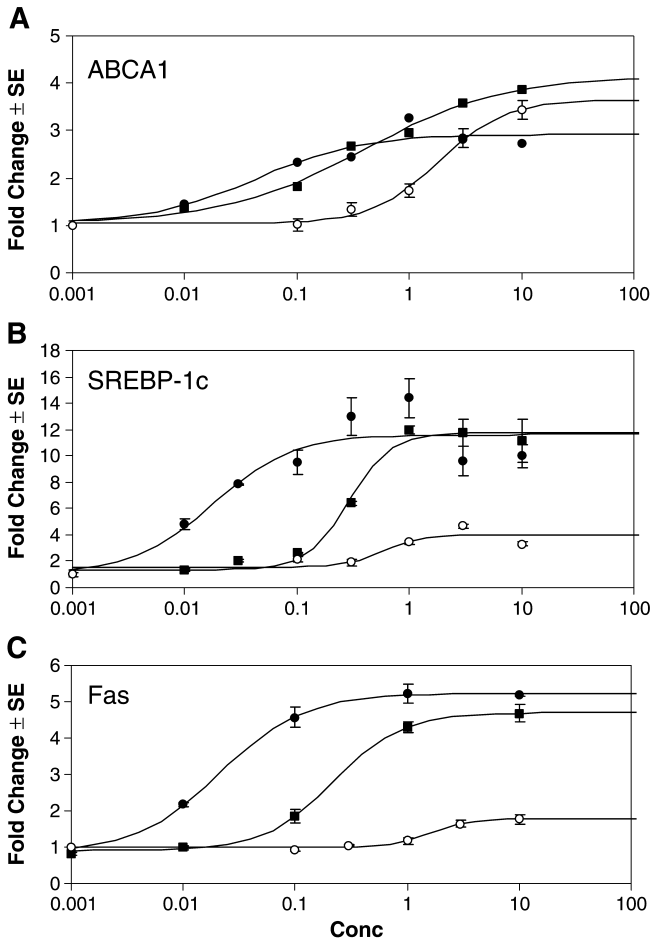
Additional experiments were designed to define the relative potency of various ligands for stimulating unfavorable lipogenesis in a liver cell line. For these studies, TO901317, GW3965, and DMHCA were analyzed in dose-response format and ABCA1, SREBP-1c, and FAS mRNA expression were quantitated by real-time PCR. The results (**Fig. 4**) indicate that significant potency differences exist for the three ligands in HepG2 cells. In these functional studies of endogenous gene regulation, DMHCA, with an $EC_{50}$ value of 1.6 μM for FAS and 0.6 μM for SREBP-1c induction (Fig. 4), was ~5- to 10-fold less potent than the GW3965 agonist. Both nonsteroidal reference compounds showed greater efficacy for stimulating SREBP-1c and FAS compared with DMHCA, which was 80% and 70% less efficacious for FAS and SREBP-1c stimulation while maintaining similar efficacy for ABCA1 expression (Fig. 4).

### Characterization of DMHCA partial agonist activity

Because DMHCA was less efficacious in stimulating FAS and SREBP-1c gene expression, it was of interest to test whether this compound can act as a selective antagonist in the presence of a full agonist. For the experimental data shown in **Fig. 5**, differentiated THP-1 macrophages were dosed with increasing concentrations of DMHCA in combination with GW3965 at its $EC_{50}$ of 150 nM. The results confirm gene-selective antagonist activity for SREBP-1c, with an $IC_{50}$ of 3.7 μM and 75% antagonism. Significantly, there was no antagonist activity observed for ABCA1 but rather a slight additive effect; no EC value could be generated. Similar results have been generated in J774 macrophages, with 100% antagonism for SREBP-1c gene transcription at the highest dose of 30 μM, and HepG2 cells (data not shown). The profile of DMHCA in the antagonist mode is therefore entirely consistent with its analysis in the agonist mode, demonstrating full-agonist properties for ABCA1 but partial agonism for the lipogenic LXR target genes SREBP-1c and FAS.

### Induction of ABCA1 mRNA in cultured peritoneal macrophages

The efficacy of DMHCA for mediating transcriptional activation of ABCA1 in lipid-loaded macrophages was determined in isolated murine peritoneal macrophages (**Fig. 6**). For these studies, mice were injected with thioglycolate 3 days before cell collection to elicit macrophages for primary culture in vitro. Adherent macrophages were either maintained in control medium (unloaded condition) or preloaded with acLDL for 24 h before ligand treatments. In this model, LXR agonists must exceed the activity of increased oxysterol activators present in lipid-loaded cells to impact cellular cholesterol efflux. Treatment of macrophages with acLDL alone increased ABCA1 mRNA by 3-fold (Fig. 6A). In unloaded cells, treatment with any of the nonsteroidal agonists, TO901317, GW3965, or DMHCA, stimulated ABCA1 mRNA expression by 43-, 32-, or 22-fold, respectively. All induced ABCA1 mRNA concentrations significantly more than acLDL. Pretreatment of thioglyco-




| Compound | hABCA1 | | hFAS | | hSREBP-1c | |
|---|---|---|---|---|---|---|
| | % ag | EC 50 μM | % ag | EC 50 μM | % ag | EC 50 μM |
| TO901317 | 100 | 0.043 | 116 | 0.023 | 100 | 0.013 |
| GW3965 | 166 | 0.397 | 101 | 0.224 | 100 | 0.150 |
| DMHCA | 139 | 1.825 | 21 | 1.617 | 33 | 0.579 |

**Fig. 4.** Dose-response comparison of DMHCA, GW3965, and TO901317. HepG2 cells were incubated with vehicle or increasing amounts of ligand: DMHCA (open circles), GW3965 (closed squares), and TO901317 (closed circles). Relative amounts of ABCA1 (A), SREBP-1c (B), and FAS (C) mRNA were measured by real-time PCR. RNA amounts were normalized with 18S rRNA, and the data are expressed relative to the vehicle control as fold change (n = 2, mean ± SEM). The activity of the agonists is profiled in the table at bottom. % ag, percentage agonism (maximal fold activation relative to TO901317); EC 50, effective concentration for 50% maximal activation.

late-elicited macrophages with acLDL (Fig. 6B) diminished their relative efficacy for ABCA1 activation as a result of the higher basal concentration of ABCA1 mRNA in the lipid-loaded cells; however, all four LXR ligands were still able to increase ABCA1 transcription.

The efficacy for SREBP-1c gene activation contrasts sharply with ABCA1 modulation in these primary cells. DMHCA failed to activate SREBP-1c gene expression and actually inhibited SREBP-1c in unloaded macrophages. By contrast, dramatic increases in SREBP-1c, some exceeding 100-fold, were observed for the other LXR agonists under either condition. The regulation of SREBP-1c in these primary cells further substantiates the differences observed in the established cell lines and the unique, selective properties of DMHCA.

### Dose-response effects of DMHCA and TO901317 in murine peritoneal macrophages

Dose-response studies were performed in cultured thioglycolate-elicited murine macrophages to evaluate potency in murine cells before in vivo characterization. DMHCA and TO901317 were dosed at concentrations from 0.003 to 10 μM and ABCA1 and SREBP-1c mRNA were measured by real-time PCR (Taqman) analysis (**Fig. 7**). Both ligands are similar in their ability to stimulate ABCA1 mRNA by ~30-fold, as observed in Fig. 6. An $EC_{50}$ value of 127 nM was determined for DMHCA from the dose-response curve of cell-based ABCA1 activation. Under these dosing conditions, the Tularik compound was ~10- to 20-fold more potent. Interestingly, DMHCA showed a biphasic response for SREBP-1c induction, with $EC_{50}$ and $IC_{50}$ values of 57 nM and 1 μM, respectively (Fig. 7B). Although the maximum induction of SREBP-1c obtained with DMHCA was 30-fold, it was as high as 100- to 200-fold in response to TO901317. Interestingly, the selectivity displayed by this compound increased dramatically at higher doses. For example, at 3 μM, the ABCA1 mRNA increase was equivalent to TO901317 but SREBP-1c induction was ~40-fold lower. This high-dose, gene-specific activation for DMHCA is graphically illustrated in Fig. 7B.

### In vivo effects of DMHCA on plasma lipids and lipoproteins

DMHCA was tested in male C57BL/6 mice maintained on chow diets to evaluate its in vivo efficacy. For these ex-

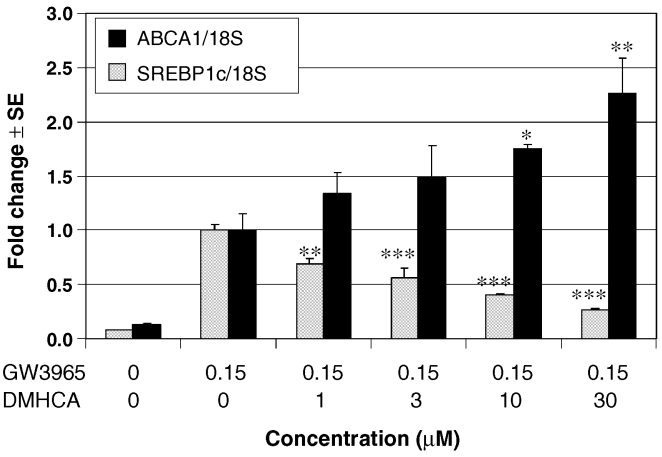



**Fig. 5.** DMHCA represses LXR-induced SREBP-1c expression but not ABCA1. Dose-response analysis for antagonism of LXR-mediated gene expression effects by DMHCA. Differentiated THP-1 cells were treated with 150 nM GW3965 and increasing doses of DMHCA. The relative amounts of ABCA1 and SREBP-1c mRNA were quantitated in cellular RNA isolated 18 h later by real-time PCR. RNA amounts were normalized with 18S rRNA, and the value was arbitrarily set to 1 in control cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (n = 2, mean ± SEM).

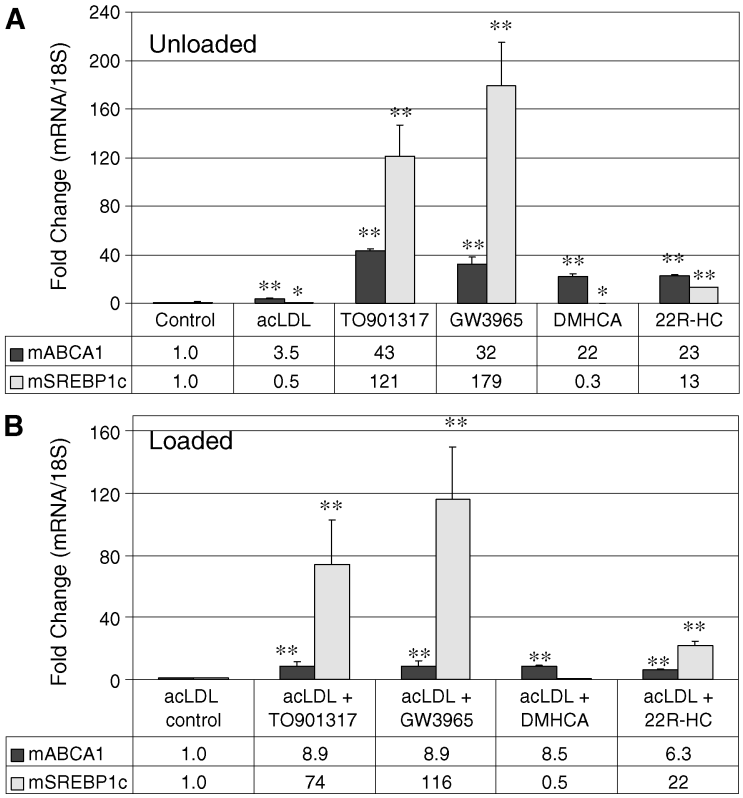



**Fig. 6.** DMHCA increases ABCA1 mRNA in both loaded and unloaded macrophages without stimulating SREBP-1c gene expression. Murine peritoneal macrophages were isolated from C57BL/6 mice injected with thioglycolate. A: Macrophages were treated with vehicle (ethanol), 50 μg/ml acetylated LDL (acLDL), 10 μM concentrations of the LXR agonists TO901317, GW3965, and DMHCA, or 22*R*-hydroxycholesterol (22*R*-HC). B: Cells were preloaded with 50 μg/ml acLDL for 24 h in culture and then treated for an additional 24 h with compound. Gene expression was quantitated by real-time PCR. RNA amounts were normalized with 18S rRNA, and the data are expressed relative to the vehicle control in A and to the acLDL control in B. * $P < 0.05$ and ** $P < 0.001$; data were log transformed (n = 2, mean ± SEM).

periments, mice were treated once daily for 6 days with ligand at a dose of 50 mg/kg. After the final dose, animals were fasted for 4 h before recovery of blood and tissues for analysis. Plasma lipids were measured and lipoproteins were analyzed by FPLC (**Table 2**). DMHCA was tested both by oral gavage and intraperitoneal injection initially, because of concerns about the potentially poor absorption properties of the chemical entity. No significant effects on either plasma lipids or lipoproteins were observed when mice were treated orally, with the exception of a small increase in triglycerides. Administration of DMHCA by intraperitoneal injection was associated with a small but significant decrease in both total cholesterol and HDL-cholesterol of 16–20%. Table 2 also compares lipid and lipoprotein changes characteristic of the orally dosed synthetic LXR agonists, TO901317 and GW3965. A lower dose of 10 mg/kg was included for GW3965. Both synthetic ligands increased HDL-cholesterol in the chow-fed mouse model but also increased plasma triglycerides rather significantly, by 3- and 2-fold, respectively. Total plasma cholesterol (mainly HDL-cholesterol) and VLDL-cholesterol were also increased. The data for the TO901317 reference agonist are consistent with the published literature (9, 30).

### Hepatic and duodenal gene expression profile of DMHCA and synthetic agonists

The gene expression profile of DMHCA was determined in two tissues of known physiological relevance for LXR-mediated gene regulation (**Fig. 8**). In liver, ABCA1, ABCG1, and SREBP-1c mRNA were significantly increased only in animals dosed by intraperitoneal injection, confirming suspicions that the compound may not be well absorbed by mice. The hepatic ABCA1 increase of 1.8-fold was small but comparable to that observed for either TO901317 or GW3965 in this tissue under oral dosing conditions (Fig. 8). All three compounds, DMHCA and the two reference ligands, stimulated the cholesterol efflux transporter ABCG1 to the same extent. Liver cyp7a, an LXR-responsive gene in the mouse, was also activated 4.5-fold by DMHCA treatment. By contrast, the 50% increase in SREBP-1c mRNA mediated by DMHCA was significantly smaller than that observed for either synthetic agonist, correlating with their respective capacity to increase circulating plasma triglycerides. In liver, the effect of DMHCA on SREBP-1c gene expression was approximately one-tenth that observed for either synthetic agonist at the same 50 mg/kg dose. GW3965 treatment at 10 mg/kg was used to establish an equipotent comparison, as it failed to upregulate ABCA1, ABCG1, cyp7a, FAS, or acetyl-CoA carboxylase (ACC) to the same extent as DMHCA at 50 mg/kg. Even at this lower dose, SREBP-1c regulation exceeded that observed for DMHCA by almost 5-fold, implying that gene selectivity is not just a potency issue. Additional hepatic gene expression profiling focused on genes involved in triglyceride synthesis, FAS and ACC. The influence of DMHCA on FAS and ACC mRNA regulation was found to be roughly equivalent to that of GW3965 at 50 mg/kg. Both of these ligands showed significantly lower activity than the more potent TO901317 agonists at the same concentration. Hepatic gene expression analysis implies that the upregulation of SREBP may be

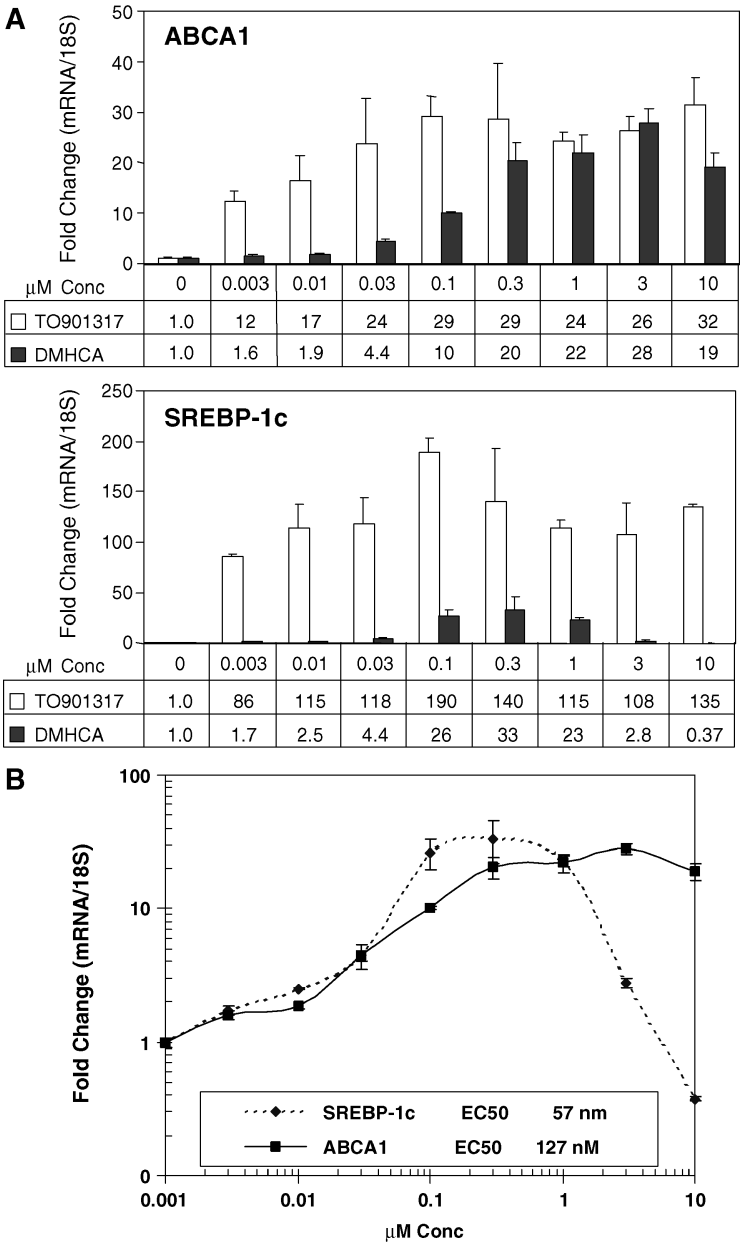



**Fig. 7.** DMHCA and TO901317 dose response in macrophages. Thioglycolate-elicited murine peritoneal macrophages were isolated from C57BL/6 mice. Macrophages were treated with vehicle (ethanol) or increasing concentrations of TO901317 and DMHCA in media containing 10% lipoprotein-deficient serum for 24 h. Gene expression was quantitated by real-time PCR, and RNA amount was normalized with 18S rRNA. Results are expressed relative to vehicle control (n = 2, mean ± SEM; data are representative of two experiments with similar results). A: Comparison of DMHCA- and TO901319-mediated regulation of ABCA1 and SREBP-1c mRNA levels. B: Double log plot of DMHCA data emphasizing selectivity at higher doses.

more tightly associated with plasma triglyceride increase in this model.

In the same study, quantitative increases in ABCA1 and SREBP-1c mRNA were observed for both dosing regimens in the small intestine with DMHCA (**Fig. 9A**). The level of duodenal gene induction with oral treatment was roughly equivalent to that exhibited by intraperitoneal injection. These results suggest a local effect within the gut, despite a lack of oral hepatic activity, and imply poor bioavailability or rapid metabolism of the compound within the enterocyte or shortly thereafter. The relative efficacy of DMHCA for LXR-mediated gene activation is comparable to that of GW3965 in this tissue and no more selective (Fig. 9A). The absolute expression level of SREBP-1c is extremely low [Ct (threshold cycle) value ⩾ 30] in these cells.

### DMHCA induces ABCA1 expression in murine peritoneal macrophages in vivo

The in vivo efficacy of DMHCA was also evaluated in peritoneal macrophages isolated from mice treated with either vehicle or 50 mg/kg/day DMHCA for 6 days as described in Experimental Procedures (Fig. 9). In macrophages, ABCA1 gene expression was induced 3.6-fold in the intraperitoneal treatment group. RNA isolated from duodenum served as a positive control in these experiments. RNA quantitation in the duodenal samples confirmed that both oral and intraperitoneal treatments induce ABCA1 and SREBP-1c gene expression locally in the intestine (data not shown). Oral dosing was not associated with alterations in macrophage gene expression (Fig. 9B), despite the local induction of both ABCA1 and SREBP-1c in the corresponding intestinal RNA samples. Oral admin-

TABLE 2. Plasma lipid and lipoprotein cholesterol concentrations in mice treated with the liver X receptor ligands DMHCA, TO901317, and GW3965

| Route | Treatment | Dose | *n* | TC | TG | VLDL-C | LDL-C | HDL-C |
|---|---|---|---|---|---|---|---|---|
| | | | | | | *mg/dl* | | |
| Oral | Vehicle | — | 6 | 79 ± 1.6 | 72 ± 3.9 | 3.9 ± 0.54 | 8.6 ± 0.96 | 66 ± 1.9 |
| | DMHCA | 50 mg/kg | 6 | 79 ± 1.2 | 90 ± 4.3[a] | 4.7 ± 0.46 | 8.7 ± 0.85 | 66 ± 2.0 |
| Intraperitoneal | Vehicle | — | 6 | 61 ± 1.2 | 56 ± 5.1 | 3.6 ± 0.58 | 4.4 ± 0.34 | 54 ± 1.7 |
| | DMHCA | 50 mg/kg | 6 | 51 ± 3.5[b] | 60 ± 7.1 | 3.0 ± 0.21 | 5.0 ± 0.52 | 43 ± 3.7[c] |
| Oral | Vehicle | — | 6 | 66 ± 4.2 | 67 ± 7.0 | 1.6 ± 0.4 | 9.1 ± 1.4 | 55 ± 3.6 |
| | TO901317 | 50 mg/kg | 6 | 149 ± 7.8[b] | 193 ± 34[b] | 10 ± 1.2[b] | 21 ± 2.3[b] | 118 ± 7.3[b] |
| Oral | Vehicle | — | 6 | 74 ± 4.9 | 59 ± 7.5 | 6.1 ± 1.1 | 10 ± 0.6 | 57 ± 4.9 |
| | GW3965 | 50 mg/kg | 6 | 115 ± 2.2[b] | 126 ± 7.3[b] | 11 ± 0.6[b] | 12 ± 0.7 | 92 ± 2.0[b] |
| | GW3965 | 10 mg/kg | 6 | 93 ± 3.9[c] | 105 ± 20[b] | 12 ± 1.7[b] | 11 ± 0.9 | 70 ± 5.8 |

Values represent means ± SEM. DMHCA, *N,N*-dimethyl-3β-hydroxycholenamide; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TC, total cholesterol, TG, triglycerides, VLDL-C, VLDL-cholesterol.

[a] $P < 0.05$ relative to vehicle control.

[b] $P < 0.005$ relative to vehicle control.

[c] $P < 0.01$ relative to vehicle control.

istration of the Tularik compound at the same dose for only 3 days increased ABCA1 expression by almost 7-fold ($P < 0.01$).

### DMHCA fails to stimulate lipid accumulation in liver

A dose-response study was performed in chow-fed mice to evaluate DMHCA effects in liver lipid accumulation. Plasma and liver lipids as well as gene expression were analyzed in mice dosed with 50, 25, and 10 mg/kg DMHCA (**Table 3**). In this study, liver weights were found to be unchanged, and no significant liver lipid accumulation was observed for either cholesterol or triglyceride at any dose. These data extend earlier findings and demonstrate no effect with lower doses of DMHCA.

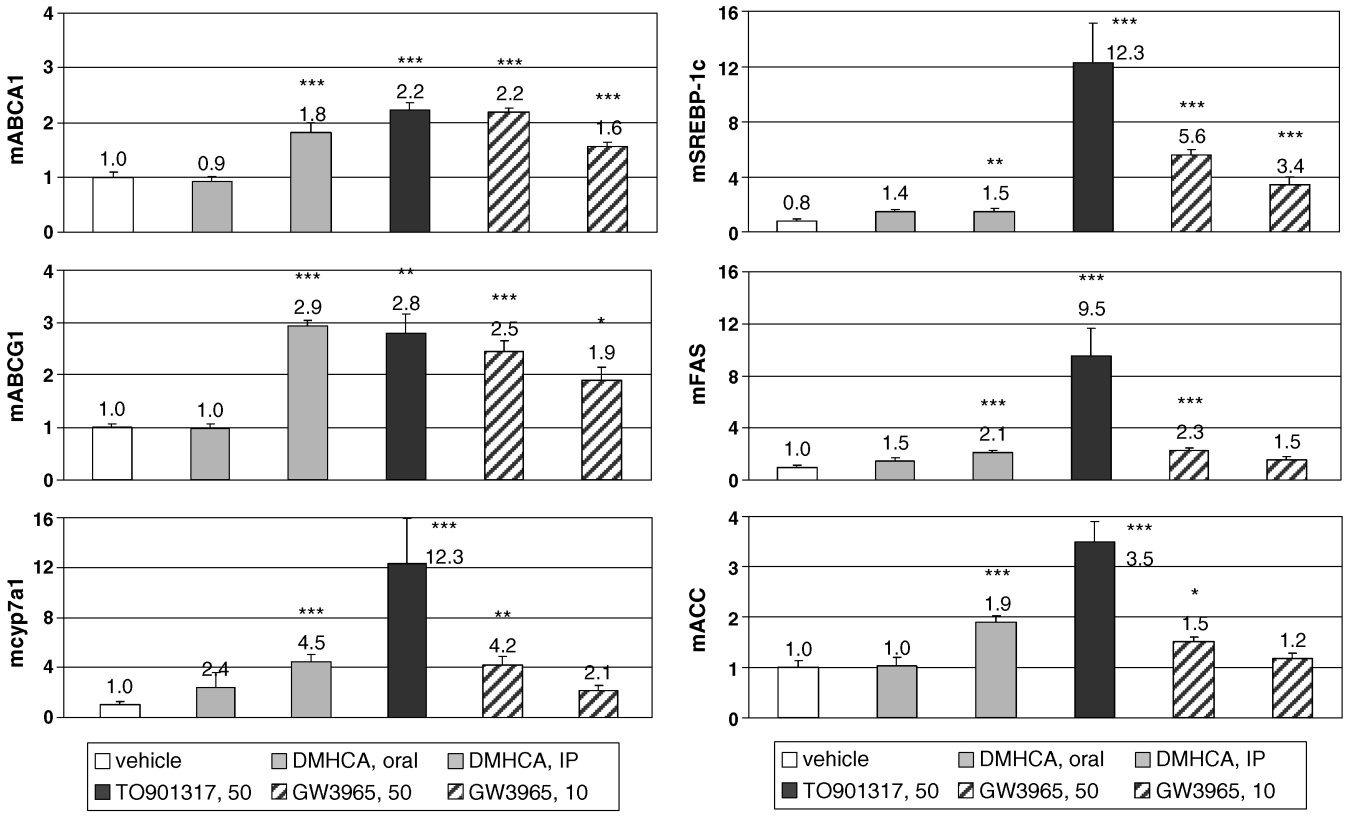



**Fig. 8.** DMHCA effects on ABCA1, ABCG1, cholesterol 7α-hydroxylase (cyp7a), SREBP-1c, FAS, and acetyl-CoA carboxylase (ACC) mRNA expression in mouse liver. Male C57BL/6 mice were treated with DMHCA (50 mg/kg/day) or vehicle for 6 days either orally or by intraperitoneal injection. Nonsterol synthetic agonists were dosed orally as indicated, TO901317 at 50 mg/kg/day and GW3965 at 50 and 10 mg/kg/day. Hepatic gene expression was analyzed by real-time PCR. RNA amounts were normalized with 18S rRNA, and the data are expressed relative to the vehicle control. Values represent means ± SEM, n = 6 mice per group except for TO901317, n = 4 mice. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.001$.

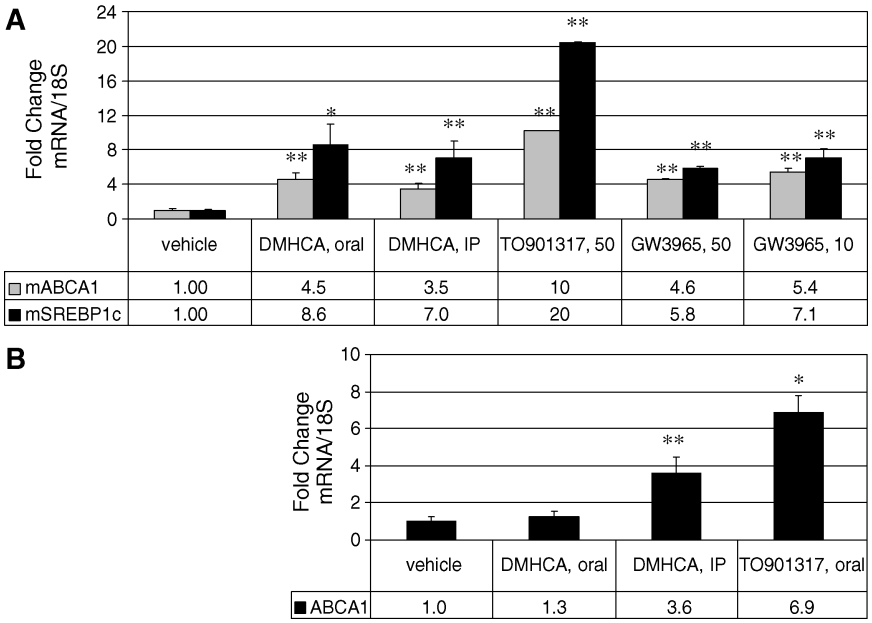



**Fig. 9.** DMHCA effects on ABCA1 and SREBP-1c mRNA expression in duodenum and murine peritoneal macrophages. A*:* Mice were dosed as described for Fig. 8. B*:* Mice were dosed for 6 days with DMHCA (50 mg/kg/day) orally or by intraperitoneal injection (IP) or with TO901317 (50 mg/kg/day) for 3 days. RNA was isolated from the duodenum and peritoneal macrophages for gene expression measurements by real-time PCR. RNA amounts were normalized with 18S rRNA, and expression in control mice was set to 1.0. Values represent means ± SEM, n = 6 mice per group. * $P < 0.01$ and ** $P < 0.001$.

Plasma HDL-cholesterol decreased by 21% in the 50 mg/kg group, consistent with the previous study, and smaller (11%) decreases were observed at two lower doses. VLDL- and LDL-cholesterol were unchanged, as were plasma triglycerides at the higher dose. Small, inconsistent effects on plasma triglycerides documented at lower doses are potentially the result of the short duration (4 h) fast and animal variation. The 1.5-fold increase in hepatic SREBP-1c mRNA failed to achieve significance in this study ($P = 0.056$). Importantly, no increase in SREBP-1c mRNA was observed at lower doses in vivo, as might be anticipated from the biphasic response observed in vitro. Changes in FAS or ACC mRNA were minimal and reduced slightly in this experiment relative to their previous evaluation (Fig. 8). No changes were observed for hepatic apoE mRNA.

TABLE 3. DMHCA dose-response study in C57BL/6 mice treated for 7 days

| Variable | Vehicle | DMHCA 10 mg/kg | DMHCA 25 mg/kg | DMHCA 50 mg/kg |
|---|---|---|---|---|
| Plasma parameters | | | | |
| Total cholesterol (mg/dl) | 68 ± 1.5 | 62 ± 2.7 | 63 ± 3.2 | 59 ± 1.8[a] |
| Triglycerides (mg/dl) | 56 ± 3.7 | 46 ± 4.5[a] | 74 ± 5.4[b] | 61 ± 2.5 |
| VLDL-C (mg/dl) | 3.2 ± 0.29 | 2.5 ± 0.48 | 4.5 ± 0.45[a] | 3.8 ± 0.42 |
| LDL-C (mg/dl) | 10 ± 0.53 | 10 ± 0.87 | 9.4 ± 0.94 | 12 ± 1.1 |
| HDL-C (mg/dl) | 55 ± 1.3 | 49 ± 1.9[a] | 49 ± 2.7[a] | 44 ± 1.4[c] |
| Liver lipids and clinical parameters | | | | |
| Liver weight (g) | 1.3 ± .023 | 1.3 ± 0.044 | 1.4 ± 0.046 | 1.4 ± 0.042 |
| Cholesterol (mg/g) | 2.7 ± .21 | 3.2 ± 0.17 | 2.8 ± 0.17 | 2.7 ± 0.33 |
| Triglycerides (mg/g) | 8.0 ± .45 | 7.8 ± 0.95 | 8.7 ± 0.56 | 6.5 ± 0.56 |
| Gene expression | | | | |
| SREBP-1c (fold change) | 1.0 ± 0.14 | 1.1 ± 0.18 | 1.3 ± 0.15 | 1.5 ± 0.24 |
| FAS (fold change) | 1.0 ± 0.08 | 1.3 ± 0.14 | 1.8 ± 0.24[a] | 1.6 ± 0.31[a] |
| Acetyl-CoA carboxylase (fold change) | 1.0 ± 0.05 | 1.4 ± 0.13 | 1.5 ± 0.14[a] | 1.4 ± 0.18[a] |
| Apolipoprotein E (fold change) | 1.0 ± 0.08 | 0.83 ± 0.02 | 0.89 ± 0.06 | 0.88 ± 0.06 |

Values represent means ± SEM (n = 6 per group). SREBP-1c, sterol-response element binding protein 1c.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.005$.

### DMHCA induces cholesterol efflux in differentiated THP-1 cells

As a result of DMHCA's interesting in vivo profile, we sought to verify that the transcriptional regulation of ABCA1 does indeed correlate to a diminished potential for cellular cholesterol accumulation. For these experiments, the efficacy of DMHCA for mediating cholesterol efflux was examined in human THP-1 cells preincubated with acLDL (25 μg/ml) and [1,2-$^3$H]cholesterol (2 μCi/ml). As shown in **Fig. 10**, the addition of human apoA-I protein to the cell media stimulates cholesterol efflux by ~2.0-fold compared with controls lacking apoA-I. In cells preincubated with DMHCA, cholesterol efflux was enhanced further, with a maximal increase of 65% at the 5 μM concentration ($P < 0.01$). TO901317 and the synthetic LXR agonist GW3965 also enhanced cholesterol efflux in this model. However, both nonsteroidal ligands showed reduced efficacy at concentrations of 3 μM or greater (data not shown), perhaps because of cellular toxicity. Collectively, these results establish that DMHCA enhances ABCA1 gene expression and macrophage cholesterol efflux via LXR activation.

## DISCUSSION

DMHCA has previously been extensively characterized for its efficacy and potency in vitro using competition binding assays (6), cell-based reporter assays (6, 7, 40), and, more recently, cell-free assays developed to more accurately measure these parameters by ligand-dependent recruitment of steroid receptor coactivator 1 (7). In these reports, it was identified as a potent LXR activator compared with 24,25(*S*)-epoxycholesterol and several cholenic acid analogs. For example, DMHCA binds LXRα with a $K_i$ value of 100 nM, half the concentration of the most potent endogenous oxysterol, 24,25(*S*)-epoxycholesterol (6), and its $EC_{50}$ for reporter gene transactivation (100 nM) was similar (41).

Despite these in vitro studies, little is known about the physiological effects of oxysterol or cholenamide LXR agonists on gene modulation. Notably, a selective LXRα receptor agonist, cholestenoic acid, which shares structural similarity with DMHCA, was profiled in vivo in hypercholesterolemic rats, mice, and hamsters without any supporting gene expression data (42). None of the literature, therefore, has specifically or rigorously addressed the potential differences in LXR-mediated endogenous gene regulation, the subject of these studies.

Gene expression profiling of the LXR agonist DMHCA in several cell lines as well as in murine primary macrophages demonstrated a unique differential gene regulation of ABCA1 and SREBP-1c. The most convincing evidence was observed in murine J774 cells and primary murine macrophages (Fig. 6), in which SREBP-1c expression was inhibited by treatment with DMHCA despite significant induction of ABCA1 mRNA levels. These in vitro cell-based studies suggest that DMHCA is a potentially novel LXR agonist with differential and selective activity for ABCA1 and SREBP-1c gene regulation.

DMHCA dose response studies in a hepatocyte cell line demonstrate full efficacy for ABCA1 activation but only partial activity for the lipogenic gene targets SREBP-1c and FAS (Fig. 4) relative to the more potent TO901317 agonist. In addition, when profiled in the presence of a potent agonist, DMHCA acts as a gene-selective functional antagonist and partially blocks SREBP-1c mRNA increases induced by GW3965, whereas ABCA1 regulation is unaffected. This diminished efficacy for SREBP-1c would be expected to limit its lipogenic potential in hepatocytes. Equally important, however, the ligand retains agonist properties for ABCA1 necessary for mediating cholesterol efflux in macrophage-like cells.

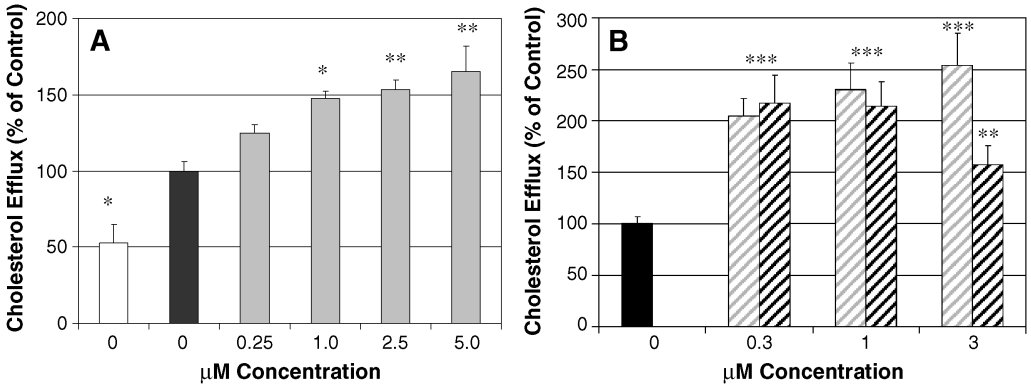



**Fig. 10.** Cholesterol efflux measured in THP-1 cells. Cells were incubated with acLDL (25 μg/ml) and [$^3$H]cholesterol (2 μCi/ml) for 48 h followed by 6 h of incubation in 0.2% BSA-containing medium with vehicle or drugs. Apolipoprotein A-I (apoA-I) protein (10 μg/ml) was added to the final 24 h incubation performed in the presence of vehicle or drug. Cholesterol efflux is presented as the percentage of [$^3$H]cholesterol in the media versus the apoA-I-containing control (black bars). The white bar represents the apoA-I-minus control. A: Dose-response treatment with DMHCA. B: Treatment with TO901317 (gray-striped bars) and GW3965 (black-striped bars). Values represent means ± SEM, n = 4 replicates; data are representative of duplicate experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

DMHCA was profiled in mice for comparison with two published synthetic LXR agonists, TO901317 and GW3965. In this model, gene expression changes in tissues, particularly liver, showed a good correlation with in vitro cellular profiles. An important separation of hepatic ABCA1 gene modulation relative to SREBP-1c was also achieved in vivo for DMHCA (Fig. 8). Unlike ligands possessing full-agonist properties, however, this selective LXR modulator demonstrates a distinct lipid and lipoprotein phenotype in the mouse model. In particular, DMHCA exhibits mild hypolipidemic properties and decreases both total plasma cholesterol and HDL-cholesterol, a profile contrasting sharply with that of either synthetic ligand, TO901371 or GW3965, which are distinguished for their ability to increase HDL-cholesterol (9, 27) (Table 2).

The present observations suggest that the hypocholesterolemic effect (and reduction in HDL-cholesterol) may result from coordinated regulation of LXR target genes such as those involved in *1*) metabolism and elimination of hepatic cholesterol, and/or *2*) decreases in cholesterol absorption by the duodenum, particularly in that several of these gene markers were upregulated by DMHCA in our studies in appropriate tissues. In the present study, DMHCA treatment was not associated with significant alterations in plasma VLDL- or LDL-cholesterol accumulation, so LXR-mediated changes seem to occur in the absence of significant increases in liver lipogenesis and export. The net result observed in the case of DMHCA is a decline in total plasma cholesterol and HDL.

Most significantly, circulating plasma triglycerides were unaffected by treatment with DMHCA, contrary to known effects of the synthetic LXR agonists but consistent with its reduced activity for hepatic SREBP-1c activation. In a recently published report (42), cholestenoic acid was shown to exhibit overall hypolipidemic effects in several animal models of hypercholesterolemia. Interestingly, the authors state that cholestenoic acid fails to induce liver SREBP-1c expression in hamsters, although the data are not shown. These authors suggest that hypocholamide is easily inactivated by glucuronidation and thereby loses its ability to stimulate LXRα transactivation.

Despite minimal lipid or lipoprotein effects, alterations in LXR-mediated gene expression profiled in liver, small intestine, and peritoneal macrophages confirm the efficacy of DMHCA for activating LXRs. In fact, DMHCA-induced hepatic ABCA1 and ABCG1 mRNA increases compare favorably with those observed for synthetic agonists (Fig. 8), and for several other LXR targets, cyp7a and fatty acid synthase were comparable to GW3965. In contrast, the increase in hepatic SREBP-1c mRNA abundance was only one-tenth of that observed for either TO901317 or GW3965, giving this compound a novel and selective in vivo profile. Importantly, neither the small 50% increase in hepatic SREBP-1c mRNA nor SREBP-1c upregulation in peripheral tissues was associated with changes in circulating plasma triglycerides. A dose-response comparison in mice confirms a lack of effect on triglycerides even at doses lower than 50 mg/kg and extends results to emphasize that liver lipid accumulation is not adversely affected. Comparative data with known reference agonists demonstrate a major qualitative difference in plasma triglycerides and hepatic SREBP gene expression. The data imply that the separation of ABCA1 and SREBP-1c regulation observed initially in vitro in cell lines has also been achieved in vivo with DMHCA.

In nonhepatic tissues such as the small intestine, DMHCA-induced ABCA1 gene expression increases were equivalent to those observed for the synthetic agonists. In enterocytes, several studies demonstrate that strong upregulation of ABCA1 gene expression in response to either cholesterol feeding or RXR/LXR agonist treatment accompanies a decrease in cholesterol absorption efficiency (8, 17). A role for the concurrent upregulation of ABCG5 and ABCG8 along with ABCA1 has also been proposed to inhibit lumenal cholesterol absorption by the small intestine (18). In our studies, the increase in intestinal ABCA1 mRNA abundance was exhibited by either oral or intraperitoneal dosing regimens of DMHCA.

In this tissue, significant SREBP-1c upregulation was also observed. DMHCA activity therefore appears to demonstrate tissue selectivity, potentially not unlike some of its selective estrogen receptor modulator counterparts (43). Nuclear receptors, for which estrogen receptor is prototypical, regulate transcription by binding specific response elements and recruiting transcriptional machinery in the form of coactivators to targeted promoters. Estrogen receptor ligands have also been shown to act as antagonists in tissues in which corepressor proteins predominate (i.e., tamoxifen in breast) but as agonists, by recruiting more highly expressed coactivators, in other cell types such as endometrial cells. The elucidation of the molecular mechanisms controlling DMHCA's selective effects warrants further study, as analogs of this class may hold promise for enhancing cholesterol elimination without promoting potentially harmful lipogenesis. In addition, one cannot preclude the possibility that some effects of DMHCA are attributable to physiochemical properties or even interaction with molecules other than LXR. For example, the TO901317 ligand has been shown to exhibit pregnane X receptor activity at micromolar concentrations (9).

The experimental evidence also confirms that DMHCA has the potential to induce cholesterol efflux through the induction of ABCA1 expression in peritoneal macrophages in vivo (Fig. 7B). Several recent studies suggest that ABCA1 expression is critical for the mobilization of cholesterol from lesion sites and that this process may occur with favorable results even in the absence of defined plasma HDL-cholesterol changes (24, 25). Specifically, the LXR ligand GW3965 has been shown to reduce atherosclerosis in two distinct models of atherogenic lesion development, LDL receptor$^{-/-}$ and apoE$^{-/-}$ mice (32). Interestingly, the reduction in atherosclerosis correlates with the induction of genes involved in RCT in the intestine and vessel wall but occurs in the absence of significant changes in HDL levels. Clearly, an analog with more appropriate pharmacodynamic parameters will be required for long-term in vivo testing.

This work provides an initial characterization of gene-

selective modulation previously not documented for LXR agonists and provides strong supporting data that in vitro selectivity translates into in vivo activity. Although DMHCA is a less potent activator compared with existing compounds, it may exhibit gene-regulatory properties desirable for an LXR modulator and may provide proof of the concept of selective agonist-mediated LXR gene modulation. Ongoing work will focus on improving the potency, selectivity, and mechanism of action of LXR modulators with profiles resembling this compound. Toward this end, we seek an orally active molecule that will segregate the beneficial antiatherogenic properties of LXR ligands from the potentially proatherogenic lipogenic effects common to this class of pharmacophores.

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