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Transport−Metabolism Interplay: LXRα-Mediated Induction of Human ABC Transporter ABCC2 (cMOAT/MRP2) in HepG2 Cells

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Abstract: Human ATP-binding cassette (ABC) transporter ABCC2 (cMOAT/MRP2) plays a crucial role in the hepatobiliary transport of sulfate-, glucuronide-, and glutathione-conjugated metabolites as well as a variety of amphiphilic organic anions derived from hepatic metabolism. Molecular mechanisms underlying the induction of this hepatic ABC transporter are of great interest to understand the transport−metabolism interplay *in vivo*. In the present study, to gain insight into the mechanism of ABCC2 induction, we tested a total of 46 structurally diverse compounds, including nuclear receptor ligands, antibiotics, bile salts, phytochemicals, and anticancer drugs. Among them, we found that LXRα ligands, i.e., T0901317, paxilline, and 22(*R*)-hydroxycholesterol, acted potently to induce the expression of ABCC2 at both mRNA and protein levels in human hepatocellular carcinoma HepG2 cells. The ABCC2 induction by T0901317 was dose- and time-dependent, where the induction pattern of ABCC2 was very similar to that of ABCG1, one of the target genes of LXRα. The ABCC2 induction by T0901317 was more strongly elicited when the LXRα gene was transiently transfected into HepG2 cells. In contrast, ABCC2 induction by T0901317 was attenuated by transient transfection of a dominant negative LXRα variant, suggesting that LXRα is involved in ABCC2 induction. Interestingly, RXR, a heterodimer partner of LXRα, affected the mRNA levels of ABCC2 and ABCG1 differently. ABCC2 induction by T0901317 was enhanced by RXR siRNA treatment, whereas ABCG1 induction was suppressed by the same treatment. This is the first report demonstrating that LXRα is potentially involved in ABCC2 induction.

Keywords: MRP2; cMOAT; ABC transporter; induction; nuclear receptor; liver X receptor

Introduction

The hepatic metabolism of xenobiotics including drugs is widely referred to as comprising phase I, phase II, and phase III systems.[1] Several transcription factors and nuclear receptors are involved in the induction or downregulation of various enzymes and transporters involved in those xenobiotic metabolizing systems. There is accumulating evidence to suggest that many inducers of drug metabolizing enzymes in phase I and phase II share common mechanisms of transcriptional activation.[2] Along with the induction of phase I and/or phase II enzymes, it has been shown that pretreatments with several types of inducers alter the expression of phase III transporter genes, suggesting that common regula-

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tory mechanisms may exist for those genes. Indeed, we first demonstrated that the phase III transporter ABCC1 (MRP1) and γ-glutamylcysteinyl synthetase (γ-GCS) were coordinately induced by cisplatin, heavy metals, nitrosoureas, or *tert*-butylhydroquinone (tBHQ) in cancer cells.[3−7]

Nrf2, an NF-E2-related transcription factor, plays a critical role in transcriptional upregulation of target genes, including those for metabolizing enzymes and transporters essential for cellular defense.[8,9] Oxidative stress and/or electrophilic attack can each lead to the dissociation of Nrf2 from Keap1 and thereby activate Nrf2 for transcriptional regulation of antioxidant response element (ARE)-dependent genes.[10] Hitherto many genes encoding detoxifying and antioxidant enzymes were found to be regulated by Nrf2.[11] Recently we have provided evidence that Nrf2 is involved in the induction of human ATP-binding cassette (ABC) transporter genes in HepG2 cells under oxidative stress. Among 48 human ABC transporters, ABCC2 (cMOAT/MRP2) and ABCG2 (BCRP) mRNA were found to be regulated by the Nrf2/Keap1 pathway.[12,13]

Nuclear receptors, on the other hand, form a family of ligand-activated transcription factors. These proteins modulate the regulation of target genes by contacting their promoter or enhancer sequences at specific recognition sites. These target genes include metabolizing enzymes and transporters. The liver X receptors LXRα (NR1H3) and LXRβ (NR1H2), which bind to the liver X receptor response element (LXRE) as heterodimers with RXR, are nuclear hormone receptors that play a key role in the regulation of lipoprotein metabolism. LXRα is expressed not only in the liver but also abundantly in other tissues associated with lipoprotein metabolism such as adipose tissue, macrophages, intestine, kidney, and spleen whereas LXRβ is ubiquitously expressed.[14−16] The LXRs act as cholesterol sensors to regulate the transcription of gene products that control intracellular cholesterol homeostasis though biosynthesis, catabolism, and transport. LXRs are bound and activated by naturally occurring oxysterols.[17] LXR activation can be antagonized by other small lipophilic agents, certain unsaturated fatty acid, and geranylgeranyl pyrophosphate.[18,19] LXRα regulates a number of genes involved in cholesterol and/or lipid homeostasis, including ABC transporters ABCA1, ABCG1, ABCG4, ABCG5, and ABCG8, as well as cholesterol ester transport protein (CETP), lipoprotein lipase (LPL),

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fatty acid synthase (FAS), and the sterol-regulating element binding protein-1 (SREBP-1).[20−25]

Hepatobiliary transport systems are essential for the excretion of a variety of organic anions including bile acids and bilirubin. Perturbation of this liver function can result in such pathological conditions as hyperbilirubinemia or cholestasis.[26] ABCC2 (cMOAT/MRP2) and ABCB11 (BSEP) are the major determinants of bile flow at the canalicular membrane of hepatocytes. Gene regulation of these hepatic ABC transporters is of great interest to understand the molecular mechanisms of toxic events in the liver. In the present study, to understand molecular mechanisms underlying the regulation of ABCC2 gene expression, we have selected structurally diverse compounds and examined their effect on the expression of this hepatic ABC transporter in human hepatocellular carcinoma HepG2 cells. We identified LXR ligands that up-regulate the expression of ABCC2 in HepG2 cells. Evidence is provided herein to show that induction of ABCC2 by the LXR ligand T0901317 is mediated by LXRα.

Experimental Section

Chemicals and Biochemicals. Antibiotic−antimycotic (100×) was purchased from Invitrogen Co. (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Protease inhibitor cocktail for general use was from Nacalai Tesque, Inc. (Kyoto, Japan). T0901317 and paxilline were purchased from ALEXIS Co. (Lausen, Switzerland). 22(*R*)-hydroxycholesterol was purchase from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals used were of analytical grade.

Cell Lines and Cell Culture Conditions. Human hepatocellular carcinoma HepG2 cells were obtained from the Riken Cell Bank (Tsukuba, Japan), and human epidermoid carcinoma KB3-1 cells were kindly provided by Prof. Kazumitsu Ueda (Kyoto University, Kyoto, Japan). Human breast adenocarcinoma MCF-7 cells were kindly provided by Prof. Masayuki Nakagawa. (Kagoshima University, Japan). HEK-293 cells carrying a hygromycin B-resistant gene (Flp-In-293 cells) were purchased from Invitrogen Co. (Carlsbad, CA). HepG2 and KB3-1 cells were maintained at 37 °C under 5% CO_2 gas in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ICN Biomedicals, Inc. Aurora, OH), glucose (1 g/L), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Throughout this study, the cells (1×10^6 cells) were placed in 6-well culture plates (Becton Dickinson and Company, Franklin Lakes, NJ).

Quantification of mRNA Levels by SYBR Green. Total RNA was extracted from HepG2, KB3-1, HEK-293, and MCF-7 cells with NucleoSpin RNA II (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions. cDNA was prepared from the extracted total RNA in a reverse transcriptase reaction with the High Capacity cDNA Archive Kit (Applied Biosystems, Lincoln Centre Drive, Foster City, CA) and oligo-dT primers according to the manufacturer's protocol. The RNA levels of ABC transporters, LXRs, and GAPDH were determined in a 7500 Fast Real Time-PCR System (Applied Biosystems) with SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio Inc., Otsu, Japan) and specific primer sets for real-time PCR (Table 1). The mRNA levels of genes were normalized against those of GAPDH.

Preparation of Cell Lysate Samples. Cell lysates were prepared as follows: after being cultured, cells were washed with ice-cold PBS(−), and then treated with the lysis buffer A [50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1% (v/v) Triton X-100, and protease inhibitor cocktail for general use (Nacalai Tesque, Inc., Kyoto, Japan)]. The cell lysate samples were homogenized by passage through a 27G needle 10 times. After centrifugation of the homogenate at 3000 rpm for 10 min at 4 °C, the resulting supernatant was collected as the cell lysate.

The protein concentration of the supernatant was determined by using the Protein Assay Bicinchoninate Kit with bovine serum albumin as a standard before mixing the supernatant with the SDS−PAGE sample buffer solution containing 10% (v/v) 2-mercaptoethanol.

Immunoblotting Analysis. For immunoblot analysis, samples were first treated with the SDS−PAGE sample buffer solution containing 10% (v/v) 2-mercaptoethanol. Thereafter, sample proteins were electrophoretically separated in 7.5% polyacrylamide gels and then electroblotted onto a Hybond-ECL (enhanced chemiluminescence) nitrocellulose membrane (GE Healthcare UK Ltd., Buckinghamshire,

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Table 1. PCR Primers To Quantitatively Measure mRNA Levels of ABCC2, ABCG1, LXRα, LXRβ, and GAPDH[a]

gene	F/R	primer sequence	position	T_m
ABCC2	forward	AGGTGGCTTGCAATTCGCCT	3650−3669	58.4
NM_000392	reverse	CCAATCTTCTCCATGCTACCGATGT	4005−4029	60.5
ABCG1	forward	CTTCATCTCCCTCCGCCTCATTGCC	2080−2104	65.4
NM_004915	reverse	GCTGAGCACGAGACACCCACAAACC	2273−2297	65.4
LXRα	forward	AGACTTTGCCAAAGCAGGG	1007−1025	56.1
NM_005693	reverse	ATGAGCAAGGCAAACTCGG	1096−1114	56.1
LXRβ	forward	TGATGTCCCAGGCACTGATG	408−427	58.4
NM_007121	reverse	CCTCTTCGGGATCTGGGAT	460−478	58.2
GAPDH	forward	ACTGCCAACGTGTCAGTGGTGGACCTGA	811−838	66.4
NM_002046	reverse	GGCTGGTGGTCCAGGGGTCTTACTCCTT	1102−1129	67.8

[a] F/R, forward or reverse primers; T_m, melting temperature.

England). The membrane was incubated in blocking solution containing 5% (w/v) skim milk in TTBS [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% (v/v) Tween 20] at 4 °C overnight.

We used the monoclonal antibody to human ABCC2 (MRP2) (ALEXIS Co., Lausen, Switzerland), or the monoclonal antibody against rabbit muscle GAPDH (American Research Products, Inc., Belmont, MA) (1:1000 dilution) as the first antibody, depending on the specific purpose of the immunoblot analyses. For the second antibody, we used antimouse IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology, Inc., Beverly, MA) at 1:3000 dilution.

HRP-dependent luminescence was developed by Western Lightning Chemiluminescent Reagent Plus (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) and detected with a Lumino Imaging Analyzer FAS-1000 (Toyobo, Osaka, Japan). Intensity of chemiluminescence was determined with a Gel-Pro Analyzer Version 3.1.00.00 (NIPPON ROPER Co., Ltd., Tokyo, Japan).

Generation of Dominant Negative Variant Form of Human LXRα. The dominant negative variant form of human LXRα (ΔAF2) was generated by cloning amino acids 1−435 of human LXRα according to the previous report.[27] Human LXRα expression vector (LXRα-pME18SFL3) (Toyobo, Osaka, Japan) was used as the template, and the ΔAF2 fragment was amplified by PCR with the forward primer 5′-TGACTGTTCTGTCCCCATAT-3′ and the reverse primer 5′-GAGCTTTTTGTCCTGCAGACGCAGTG-3′. The 5′-end of the amplicons was phosphorylated with T4 polynucleotide kinase (Toyobo, Osaka, Japan) and ligated by T4-DNA ligase (Roche Ltd., Mannheim, Germany). The DNA sequence of the resulting vector was examined to confirm the generation of the LXRα (ΔAF2)-pME18SFL3.

Transient Transfection of LXRα and LXRα (ΔAF2). LXRα-pME18SFL3 (2 μg) (Toyobo, Osaka, Japan) or LXRα(ΔAF2)-pME18SFL3 (2 μg) vectors were incubated with LipofectAmine-2000 (Invitrogen, Carlsbad, CA) in Opti-MEM I Reduced-Serum Medium (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. HepG2 cells (1×10^6 cells) were placed in 6-well culture plates containing Opti-MEM I with LipofectAmine-2000−vector complex and incubated for 24 h. Then, the cell culture medium was replaced with fresh DMEM. The cells were further incubated with or without 10 μM T0901317 at 37 °C for 24 h.

Transfection of siRNA. RXRα siRNA (5′-GGUUCGUAAGCUCUUGCUTT-3′) (Ambion Inc., Austin, TX) (200 nM) was incubated with DharmaFECT4 (Dharmacon, Inc., Lafayette, CO) in Opti-MEM I Reduced-Serum Medium according to the manufacturer's instructions. HepG2 cells (1×10^6 cells) were placed in 6-well culture plates containing Opti-MEM I with DharmaFECT4-siRNA complex and incubated for 24 h. Then, the cell culture medium was replaced with fresh DMEM. After incubation for 24 h in fresh DMEM, the cells were further incubated with or without 10 μM T0901317 for 24 h

Statistical Analysis. Statistical analyses were performed by using Microsoft Excel 2003 software (Microsoft Co., Redmond, WA). The statistical significance of differences was determined according to the Student's *t*-test. *P* values <0.05 were considered statistically significant.

Results

Induction of ABCC2 Expression by LXR Ligands in HepG2 Cells. We have tested the effects of a total of 46 structurally diverse compounds on the mRNA levels of human ABC transporters in HepG2 cells. The test compounds included pharmaceutical drugs, natural compounds, and nuclear receptor ligands. Among the 48 different ABC transporters known, we focused in this study on the induction of ABCC2 (cMOAT/MRP2) and measured its mRNA levels by quantitative real-time PCR (Q-PCR) using specific primer sets (Table 1). HepG2 cells were incubated with each compound for 6 or 24 h. The concentrations of the compounds in the incubation medium were optimized to ensure that no cytotoxicity was exerted on the HepG2 cells over time. For example, a concentration of 1 μM was used for doxorubicin, cyclosporin A, rapamycin, vinblastine, pheophorbide a, novobiocin, nicardipine, FK506, prenylamine, fendiline, bepridil, quercetin, or rutin, whereas the

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Table 2. Function-Based Classification of the Test Compounds Used in the Present Study[a]

no.	compound	function	receptor	no.	compound	function	receptor
1	DMSO (control)	solvent		25	estrone	nuclear receptor ligand	ER
2	ATRA	nuclear receptor ligand	RAR	26	tBHQ	Nrf2 activator	
3	Wy-14643	nuclear receptor ligand	PPAR	27	doxorubicin	inhibitor or substrate	
4	clofibrate	nuclear receptor ligand	PPAR	28	cyclosporin A	inhibitor or substrate	
5	T0901317	nuclear receptor ligand	LXR	29	rapamycin	inhibitor or substrate	
6	paxillin	nuclear receptor ligand	LXR	30	verapamil	inhibitor or substrate	
7	22ROHCh	nuclear receptor ligand	LXR	31	vinblastine	inhibitor or substrate	
8	CDCA	nuclear receptor ligand	FXR	32	pheophorbide a	inhibitor or substrate	
9	CA	nuclear receptor ligand	FXR	33	novobiocin	inhibitor or substrate	
10	LCA	nuclear receptor ligand	PXR	34	nicardipine	inhibitor or substrate	
11	rifampin	nuclear receptor ligand	PXR	35	taurocholic acid	inhibitor or substrate	
12	paclitaxel	nuclear receptor ligand	PXR	36	SN-38	inhibitor or substrate	
13	sulfinpyrazone	nuclear receptor ligand	PXR	37	FK506	inhibitor or substrate	
14	clotrimazole	nuclear receptor ligand	CAR	38	prenylamine	inhibitor or substrate	
15	phenitoin	nuclear receptor ligand	CAR	39	fendiline	inhibitor or substrate	
16	9cisRA	nuclear receptor ligand	RXR	40	bepridil	inhibitor or substrate	
17	docosahexaenoic acid	nuclear receptor ligand	RXR	41	genisteine	phytochemical	
18	dexamethason	nuclear receptor ligand	GR	42	luteoline	phytochemical	
19	aldosterone	nuclear receptor ligand	MR	43	quercetin	phytochemical	
20	progesterone	nuclear receptor ligand	PR	44	rutin	phytochemical	
21	testosterone	nuclear receptor ligand	AR	45	protoporphyrin	phytochemical	
22	benzo(a)pyrene	nuclear receptor ligand	AhR	46	kaempferol	phytochemical	
23	β-naphthoflavone	nuclear receptor ligand	AhR	47	fisetin	phytochemical	
24	estradiol	nuclear receptor ligand	ER				

[a] ATRA, *all-trans* retinoic acid; 22ROHCh, 22(*R*)-hydroxycholesterol; CDCA, chenodeoxycholic acid; CA, cholic acid; LCA, lithocholic acid; 9cisRA, 9-*cis*-retinoic acid; tBHQ, *tert*-butylhydroquinone; AR, androgen receptor; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; ER, estrogen receptor; FXR, farnesoid X receptor; GR, glucocorticoid receptor; LXR, liver X receptor; MR, mineralcorticoid receptor; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor. Test compounds #27 to #40 are inhibitors or substrates for hepatobiliary ABC transporters, such as ABCB1 (P-gp/MDR1), ABCB11 (BSEP), ABCC2 (MRP2/cMOAT), or ABCG2 (BCRP).

SN-38 concentration was 10 nM. The other compounds were added to the incubation medium at 10 μM, since they were not toxic at this concentration over 24 h. Among those compounds thus tested (Table 2), clotrimazole, progesterone, and vinblastine were found to transiently enhance the mRNA of ABCC2 by more than 2-fold at the incubation time of 6 h. On the other hand T0901317 (T090), paxilline, 22(*R*)-hydroxycholesterol, rifampin, β-naphthoflavone, estradiol, and *tert*-butylhydroquinone (tBHQ) increased the mRNA level of ABCC2 at the incubation time of 24 h.

T0901317 (T090), paxilline (Pax), and 22(R)-hydroxycholesterol (22(*R*)-OHCh) (Figure 2A) are ligands for liver X receptors (LXRs), and they were found to significantly increase the mRNA level of ABCC2 in HepG2 cells (P values <0.05 in Student's t-test) after a 24 h incubation at the concentration of 10 μM (Figure 2B). Induction of ABCC2 expression by T0901317 was the greatest among the LXR ligands tested (Figures 1 and 2B). As shown in Figure 3A, T0901317 enhanced the ABCC2 mRNA level in a dose-dependent manner in the concentration range of 0.5 to 50 μM. The ABCC2 mRNA level continuously increased over time during the incubation with 10 μM T0901317 (Figure 3B). On the other hand, the mRNA of ABCG1, which is one of the target genes of LXRs, was also found to increase during the incubation with 10 μM T0901317 (Figure 3C). Under the standard incubation condition without the LXR ligand, however, the mRNA level of ABCG1 was very low, whereas that of ABCC2 was moderately expressed. Therefore, the induction of ABCG1 by T0901317 appeared to be more prominent than that of ABCC2. Actually, the mRNA level of ABCG1 was increased by approximately 70-fold after the 24 h incubation with 10 μM T0901317 (Figure 3C).

To examine the effect of T0901317 on the induction of ABCC2 expression at the protein level, we detected the ABCC2 protein expressed in HepG2 cells by immunoblot analysis with the ABCC2-specific monoclonal antibody (ALEXIS Co., Lausen, Switzerland). As shown in Figure 4A, when the cell lysate sample was applied to SDS−PAGE without PNGaseF treatments, three bands were detected at molecular weights (MW) of 230,000, 200,000, and 190,000 in HepG2 cells under the standard incubation condition (left lane in Figure 4A). After N-linked glycan moieties of the ABCC2 protein were removed by PNGase F treatments, however, the two immunologically active bands detected at MW of 230,000 and 190,000 were diminished and one single band newly appeared at 150,000 as detected by immunoblotting with the monoclonal antibody (right lane in Figure 4A). These results strongly suggest that human ABCC2 (MW = 150,000) underwent N-linked glycosylation in HepG2 cells to make at least two glycosylated forms that were detected at MW of 230,000 and 190,000 by immunoblot analysis. To quantitatively analyze the ABCC2 protein level induced

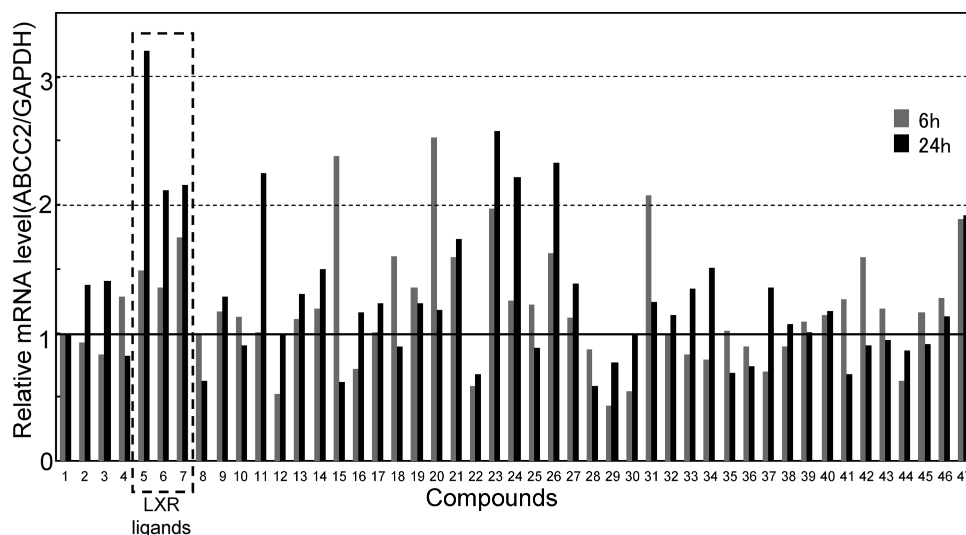

Figure 1. Screening of structurally diverse compounds to search for inducers of ABCC2 expression in HepG2 cells. Compounds tested are as follows: 1, DMSO as control; 2, ATRA (10 μM); 3, Wy-14643 (10 μM); 4, clofibrate (10 μM); 5, T0901317 (10 μM); 6, paxilline (10 μM); 7, 22ROHCH (10 μM); 8, CDCA (10 μM); 9, CA (10 μM); 10, LCA (10 μM); 11, rifampin (10 μM); 12, paclitaxel (10 μM); 13, sulfinpyrazone (1 μM); 14, clotrimazole (10 μM); 15, phenitoin (10 μM); 16, 9cisRA (10 μM); 17, docosahexaenoic acid (10 μM); 18, dexamethasone (10 μM); 19, aldosterone (10 μM); 20, progesterone (10 μM); 21, testosterone (10 μM); 22, benzo(a)pyrene (10 μM); 23, β-naphthoflavone (10 μM); 24, estradiol (10 μM); 25, estrone (10 μM); 26, tBHQ (10 μM); 27, doxorubicin (1 μM); 28, cyclosporin A (1 μM); 29, rapamycin (1 μM); 30, verapamil (10 μM); 31, vinblastine (1 μM); 32, pheophorbide a (1 μM); 33, novobiocin (1 μM); 34, nicardipine (1 μM); 35, taurocholic acid (10 μM); 36, SN-38 (10 nM); 37, FK506 (1 μM); 38, prenylamine (1 μM); 39, fendiline (1 μM); 40, bepridil (1 μM); 41, genisteine (10 μM); 42, luteoline (10 μM); 43, quercetin (1 μM); 44, rutin (1 μM); 45, protoporphyrin (10 μM); 46, kaempjerol (10 μM); 47, fisetin (10 μM). These compounds were dissolved in DMSO as stock solutions. HepG2 cells were incubated with a test compound in Dulbecco's modified Eagle's medium at 37 °C under 5% CO_2 gas for 6 or 24 h. The final concentration of each compound in the incubation medium is presented in parentheses. The concentrations were so optimized that those compounds did not exert any cytotoxicity to HepG2 cells over time. The mRNA levels of ABCC2 and GAPDH were determined as described in the Experimental Section. Data are expressed as relative values of ABCC2 mRNA/GAPDH mRNA and normalized to the level of the control (compound 1, DMSO).

by T0901317, we treated the cell lysate samples with PNGase F prior to SDS−PAGE and then measured the immunologically active band at the MW of 150,000 upon immunoblotting. As demonstrated in Figure 4B lower panel, during the 48 h treatment with T0901317 (0 to 50 μM), the ABCC2 protein level increased in a dose-dependent manner in the concentration range of 0 to 50 μM, which is in accordance with the increase of ABCC2 mRNA levels (Figure 3A). In addition, it is important to note that the levels of glycosylated forms at the MW of 230,000 and 190,000 increased concomitantly (Figure 4B upper panel). Figure 4C lower panel depicts the ABCC2 protein levels in HepG2 cells incubated with 10 μM T0901317 for 0, 24, 48, and 72 h. The ABCC2 protein level increased during the initial incubation period of 0 to 48 h and then reached a steady-state level over 72 h. Throughout the experiment, there was no significant change in the level of GAPDH, an internal control protein (Figure 4B,C).

Involvement of LXRα in T0901317-Mediated Induction of ABCC2. To investigate the potential involvement of LXRα in the induction of *ABCC2* gene expression by T0901317, we constructed a human LXRα expression vector (LXRα-pME18SFL3) and used it to transiently transfect HepG2 cells. Q-PCR showed that the mRNA level of LXRα was increased about 20-fold at 48 h after the transient transfection (Figure 5A). The enhanced induction of ABCG1 and ABCC2 mRNA expressions were observed in the LXRα-transfected HepG2 cells when the cells were treated with T0901317 for 24 h (Figure 5B,C). On the other hand, the basal mRNA levels of both ABCC2 and ABCG1 did not change by the transfection of LXRα solely. These results suggest that the ABCC2 mRNA level enhanced by T0901317 is due to the interaction of T0901317 with LXRα from the human LXRα expression vector. To further examine the involvement of LXRα in the T0901317-mediated induction of ABCC2, we prepared LXRα dominant-negative expression vector to transiently transfect HepG2 cells. The dominant-negative LXRα used in this study is LXRα(ΔAF2) that lacks the AF2 transcriptional activation domain and has no transcriptional activity.[27] The T0901317-induced elevation in the mRNA levels of both ABCG1 and ABCC2 was significantly suppressed by transfection with the LXRα(ΔAF2) vector (Figure 6A,B). On the other hand, the basal expression levels of ABCG1 and ABCC2 were not affected by the transfection of the LXRα(ΔAF2) vector. These results

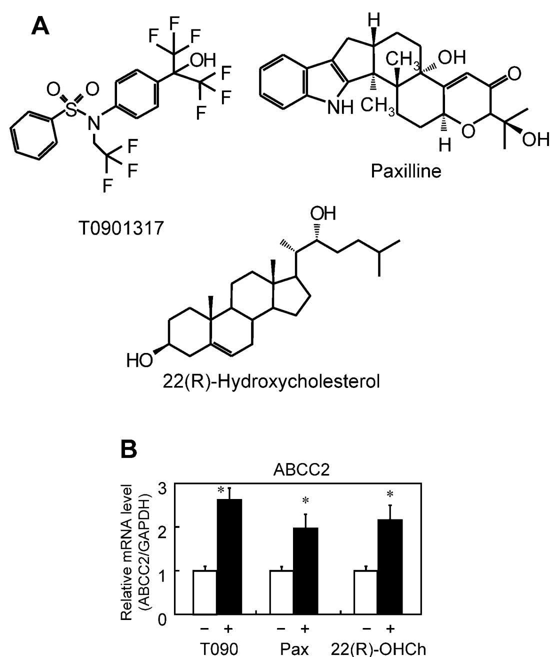

Figure 2. Effects of LXR ligands, T0901317, paxilline, and 22(R)-hydroxycholesterol, on the mRNA expression of human ABCC2. (A) Chemical structures of LXR ligands. (B) HepG2 cells were treated for 24 h with 10 μM T0901317 (T090), paxilline (Pax), or 22(R)-hydroxycholesterol (22(R)-OHCh). Data are expressed as a ratio of the expression levels in untreated cells and as the mean ± SE of quintuple determinations. *, $p < 0.05$ as compared with the untreated group.

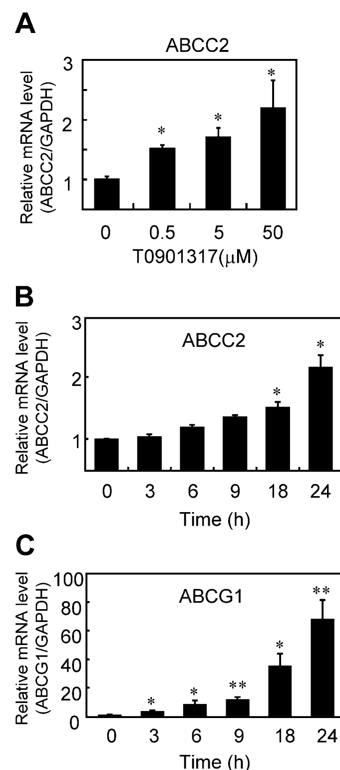

Figure 3. Effect of T0901317 on the mRNA expression levels of ABCC2 and ABCG1 in HepG2 cells. (A−C) HepG2 cells were cultured in the presence or absence of T0901317 (A, 0, 0.5, 5, or 50 μM; B and C, 10 μM) for the indicated period (A, 24 h; B and C, 0, 3, 6, 9, 18, 24 h). The mRNA levels of ABCC2, ABCG1, and GAPDH were determined by Q-PCR with specific primer sets. Data are expressed as a ratio of the expression levels in untreated cells and as the mean ± SE of quintuple determinations. *, $p < 0.05$; **, $p < 0.01$ as compared with the untreated group.

strongly suggest that LXRα is indeed involved in the induction of ABCC2 expression by T0901317.

RXRα May Regulate ABCG1 and ABCC2 Genes Differently. To examine the expressions of ABCG1 and ABCC2 by T0901317 in more detail, we prepared siRNA against human RXRα to transiently transfect HepG2 cells. Q-PCR showed that the mRNA level of RXRα was significantly decreased at 48 h after the transient transfection (Figure 7A). The induction of ABCG1 mRNA expression by T0901317 was significantly suppressed when siRNA against RXRα was transfected into HepG2 cells before the T0901317 treatment (Figure 7B). In contrast, the mRNA level of ABCC2 was significantly increased by the transfection with siRNA against human RXRα rather than suppressed, where the basal mRNA level of ABCC2 was also significantly increased (Figure 7C). These results indicate that RXRα regulates the expression of *ABCG1* and *ABCC2* genes differently.

Induction of ABCC2 by T0901317 in HepG2, KB3-1, HEK-293, and MCF-7 Cells. We have further examined the effect of T0901317 on the mRNA levels of ABCC2 in human epidermoid carcinoma KB3-1 cells, human embryonic kidney HEK293 cells, and human breast adenocarcinoma MCF-7 cells. As shown in Figure 8A, LXRα mRNA was detected by RT-PCR and Q-PCR in all of the employed cells, whereas the mRNA level of LXRα in HepG2 cells was approximately 2-fold higher than that in the others. Accordingly, the induced level of ABCC2 mRNA by T0901317 was the highest in HepG2 cells as compared with levels in the other cell lines tested (Figure 8B).

Discussion

Induction of ACC2 Expression by LXRα Ligands. The multidrug resistance-associated protein (MRP) family plays a major role in the hepatic excretion of organic anions.[28,29] The expression, localization, and function of ABCC2 (cMOAT/MRP2) are critical for maintaining the bile flow and the hepatobiliary excretion of xenobiotics and drug

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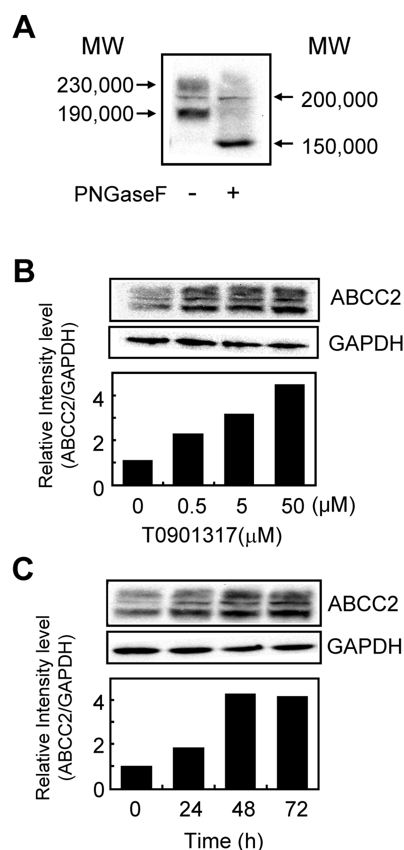

Figure 4. Effect of T0901317 on the protein expression levels of ABCC2 in HepG2 cells. (A−C) Cell lysate samples were prepared from HepG2 cells treated with T0901317 (B, 0, 0.5, 50 μM; C, 10 μM) for indicated period (B, 48 h; C, 0, 24, 48, 72 h) or not (A). Immunoblot analysis was performed with 30 μg of cell lysate samples and the monoclonal antibody against human ABCC2 or GAPDH as described in the Experimental Section. The intensity of the bands recognized by the monoclonal antibody against human ABCC2 was determined and expressed as a ratio of the bands in untreated cells (0 μM in B and 0 h in C). Similar results were obtained in more than two experiments carried out in the same way. The apparent molecular weights of glycosylated, intermediately glycosylated, and non-glycosylated ABCC2 were 230,000, 190,000, and 150,000, respectively.

metabolites.[30−32] Hitherto the PXR activator rifampicin was reported to induce ABCC2 in HepG2 cells and in primary human hepatocytes.[33,34]

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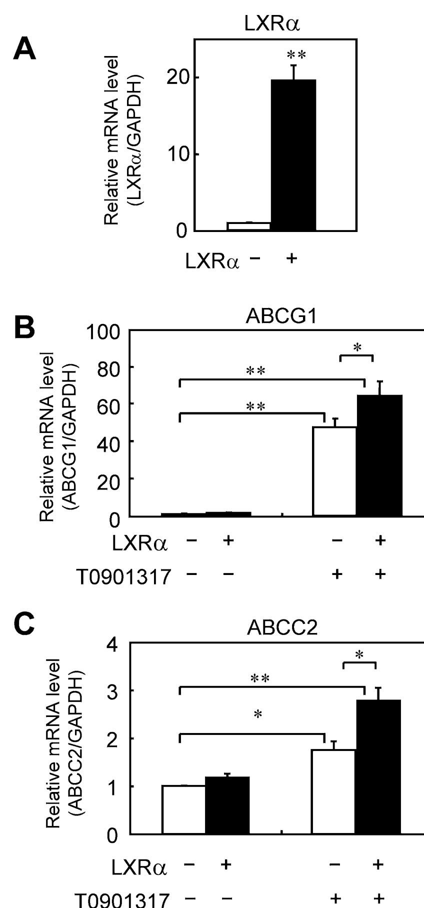

Figure 5. Effect of the LXRα expression vector on the T0901317-mediated induction of ABCC2 mRNA in HepG2 cells. HepG2 cells were cultured in the presence (B and C) or absence (A) of 10 μM T0901317 for 24 h after transient transfection of the LXRα-expression vector and then total RNA was extracted. The mRNA levels of LXRα (A), ABCG1 (B), ABCC2 (C), and GAPDH (A−C) were determined by Q-PCR using first strand cDNA from the total RNA and specific primer sets. Similar results were obtained in more than two other experiments. Data are expressed as the mean ± SE of quintuple determinations. *, $p < 0.05$; **, $p < 0.01$ as compared between the indicated groups.

In the present study, we report the potential involvement of the nuclear receptor LXR in the induction of the *ABCC2* gene in HepG2 cells. Under standard cell-culture conditions, the LXRα mRNA level in HepG2 cells was higher than those in KB3-1, HEK-293, and MCF-7 cells (Figure 8). Accordingly, ABCC2 mRNA levels were enhanced by the LXR ligand T0901317 in HepG2 cells, whereas ABCC2 induction by the same treatments was moderate or marginal in the other cells tested (Figure 8). Throughout this study, therefore, we investigated the LXR-mediated induction of ABCC2 in HepG2 cells as an *in vitro* model system.

The effects of LXRα-overexpression (Figure 5) and dominant negative LXRα expression (Figure 6) provide evidence that induction of ABCC2 and ABCG1 mRNA is regulated by the LXR pathway (Figures 5 and 6). LXRα and LXRβ, which are bound and activated by the cholesterol

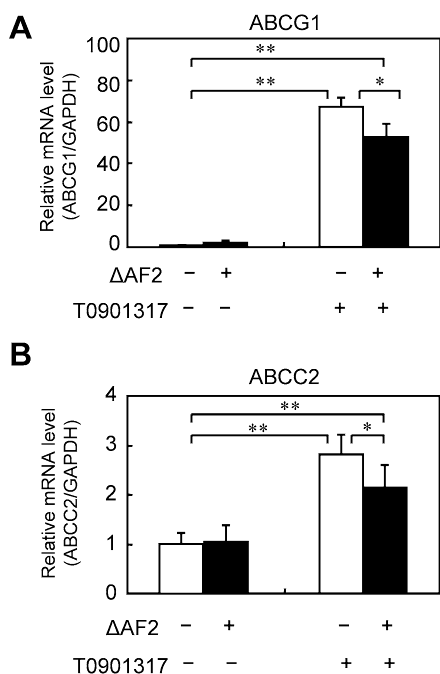

Figure 6. Effect of the dominant negative LXRα expression vector on T0901317-mediated induction of ABCC2 mRNA in HepG2 cells. (A and B) HepG2 cells were cultured in the presence or absence of 10 μM T0901317 for 24 h after transient transfection of the dominant negative LXRα-expression vector; and then total RNA was extracted. The mRNA levels of ABCG1 (A), ABCC2 (B), and GAPDH (A and B) were determined by Q-PCR using first strand cDNA from the total RNA and specific primer sets. Similar results were obtained in more than two other experiments. Data are expressed as the mean ± SE of quintuple determinations. *, $p < 0.05$; **, $p < 0.01$ as compared between the indicated groups.

metabolite oxysterol, regulate the transcription of the following gene products that control intracellular cholesterol homeostasis through catabolism and transport: SREBP, SHP, ABCA1, ABCG1, ABCG4, ABCG5, ABCG8, CETP, and LPL.[35] In the present study, ABCG1, one of the target genes of LXRα, was used as the positive control for the assessment of LXRα activation by T0901317. It appeared that T0901317 induced ABCG1 (Figure 6A) to a greater extent than it did ABCC2 (Figure 6B). This may be due to the presence of high background mRNA levels of ABCC2 in HepG2 as

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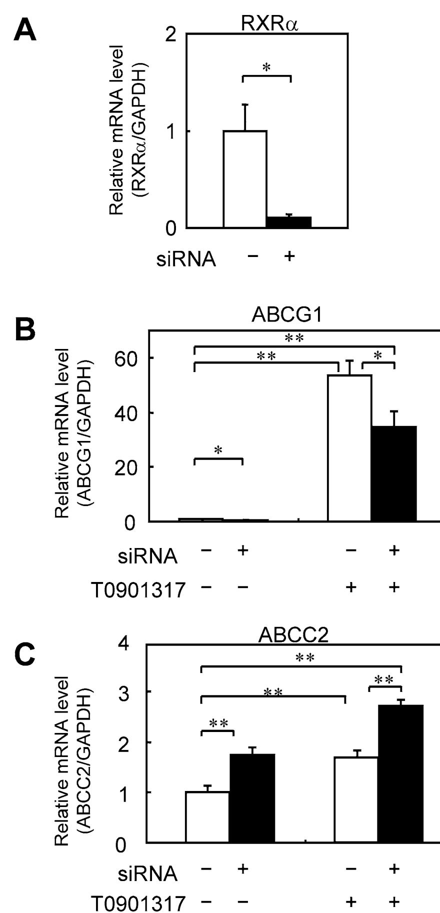

Figure 7. Effect of the RXRα expression vector on the T0901317-mediated induction of ABCC2 mRNA in HepG2 cells. HepG2 cells were cultured in the presence (B and C) or absence (A) of 10 μM T0901317 for 24 h after transient transfection of the RXRα-expression vector; and then total RNA was extracted. The mRNA levels of RXRα (A), ABCG1 (B), ABCC2 (C), and GAPDH (A−C) were determined by Q-PCR using first strand cDNA from the total RNA and specific primer sets. Similar results were obtained in more than two other experiments. Data are expressed as the mean ± SE of quintuple determinations. *, $p < 0.05$; **, $p < 0.01$ as compared between the indicated groups.

compared with ABCG1 mRNA levels before the T0901317 treatments. Interestingly, RXR, the heterodimer partner of LXRα, differently affected the mRNA levels of ABCC2 and ABCG1. ABCC2 induction by T0901317 was enhanced by RXR siRNA treatment, whereas ABCG1 induction was suppressed by the same treatment (Figure 7). At present, the reason for those different responses remains to be elucidated.

To examine the direct or indirect involvement of an LXRα-dependent mechanism in the induction of ABCC2 expression in the human liver, further studies are needed. The sandwich-cultured human hepatocyte system, for example, is a good model that maintains a physiologically normal morphology of hepatocytes with higher expression and function levels of transporters, as compared with hepatocytes cultured in a conventional configuration.[36,37] The sandwich configuration facilitates repolarization of the cells,

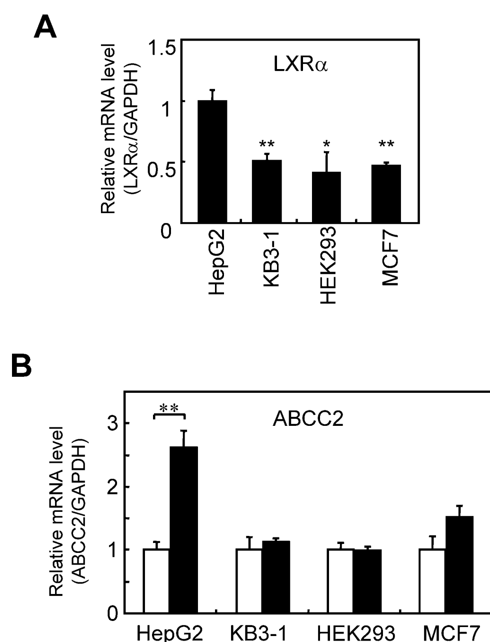

Figure 8. Comparison of the effects of T0901317 on the induction of ABCC2 mRNA among 4 cell lines. HepG2 and KB3-1 cells were cultured as described in the Experimental Section. HEK-293 cells were maintained at 37 °C under 5% CO_2 gas in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH), 4.5 g/L glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL amphotericin B, and 100 μg/mL hygromycin B. MCF-7 cells were maintained at 37 °C under 5% CO_2 gas in Dulbecco's modified Eagle's medium (4.5 g/L glucose) (DMEM) (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. In these experiments, HepG2, KB3-1, HEK-293, and MCF-7 cells were cultured in the presence (B and C) or absence (A) of 10 μM T0901317 for 24 h; and then total RNA was extracted. The mRNA levels of LXRα (A), ABCG1 (B), ABCC2 (C), and GAPDH (A–C) were determined by Q-PCR using first strand cDNA from the total RNA and specific primer sets. Similar results were obtained in more than two other experiments. Data are expressed as the mean ± SE of quintuple determinations. *, $p < 0.05$ **, $p < 0.01$ as compared between the indicated groups.

which is necessary for the formation of canalicular networks. In this context, it would be of interest to further evaluate the role of LXRα in the induction of ABCC2 by using the sandwich-cultured human hepatocytes.

Transporter–Metabolism Interplay. The metabolism of xenobiotics, including drugs, is widely referred to as comprising phase I and phase II systems, where the phase I system includes the oxidation of xenobiotics and the phase II system deals with the conjugation of phase I products. The oxidative metabolism process in the phase I system is mediated by cytochrome P-450 (CYP) or flavin mixed-function oxidase. Some activated xenobiotics can interact with DNA and/or proteins in cells to cause toxic effects. In the phase II system, on the other hand, activated hydrophobic xenobiotics are converted into hydrophilic forms via conjugation reactions with glutathione, sulfate, or glucuronide. This phase II metabolism is regarded as the detoxification process of xenobiotics. In some cases, however, the phase II system becomes a critical step in the formation of genotoxic electrophiles. Furthermore, the intracellular accumulation of the resulting metabolites can lead to a decrease in the detoxification activity of the phase II system. Therefore, a membrane transport system must perform the task of eliminating phase II metabolites from cells. In 1992, Ishikawa proposed a new concept for a "phase III" detoxification system by emphasizing the biological importance of ATP-dependent export pumps.[1] Since that time, a total of 48 different human ATP-binding cassette (ABC) transporter genes have been discovered, and some of them, e.g., ABCB1 (P-glycoprotein/MDR1), ABCB4 (MDR3), ABCB11 (BSEP), ABCC2 (MRP2/cMOAT), and ABCG2 (BCRP/MXR1/ABCP), are playing important roles in hepatobiliary transport of xenobiotic metabolites as well as bile salts and phospholipids in the liver. ABCC2 is localized in the apical surface, predominantly in the canalicular membrane of hepatocytes. The major physiological function of this protein is to transport conjugated metabolites into the bile canaliculus in the liver. Patients with impaired ABCC2 expression show defects in hepatobiliary extrusion of bilirubin glucuronide and develop Dubin-Johnson syndrome.[38] Besides those ABC transporters, currently accumulating evidence strongly suggests that other ABC transporters, such as ABCA1, ABCG1, ABCG5, and ABCG8, are also playing significant roles in the efflux of steroids and oxidized cholesterol derivatives (oxysterols) under regulation by nuclear receptors and their ligands. The cross-talk of nuclear receptors may mediate important metabolic deregulation. Correct understanding in detail of transcriptional regulation is the basic foundation for proper drug design and therapy planning.

Multiple Regulatory Mechanisms for ABCC2 Expression. Recent studies have demonstrated that ABCC2 was regulated by pregnane X receptor (PXR),[39,40] farnesoid X receptor (FXR),[40] constitutive androstane receptor (CAR),[40] Nrf2 activator,[12] and interferon regulatory factor 3.[41] There has been much progress made in our understanding of the

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transcriptional mechanisms that regulate the expression of drug metabolizing enzymes and transporter genes. It is now well established that a number of ligand-activated nuclear receptors and transcription factors may be important determinants of the interindividual variability in drug response and toxicity. These advances shall directly or indirectly impact the drug discovery and development process. Much remains to be clarified, however, regarding the spectrum of regulated nuclear receptor target genes, the precise molecular mechanisms governing the inductive response, the influence of coordinate nuclear receptor control, cross-talk and signaling pathways, and the impact of genetic and splice variants of nuclear receptors and their gene expression.

Abbreviations Used

ABC, ATP-binding cassette; ARE, antioxidant responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OHCh, hydroxycholesterol; LXR, liver X receptor; PBS(−), phosphate-buffered saline without both Ca^{2+} and Mg^{2+}; RT-PCR, reverse transcriptase-polymerase chain reaction; RXR, retinoid X receptor; Q-PCR, quantitative real-time PCR; TBS, Tris-buffered saline; T_m, melting temperature; TTBS, TBS with 0.05% (v/v) Tween 20.

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