Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative[S]

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Abstract   The vitamin D receptor (VDR), a member of the nuclear receptor superfamily, mediates the biological actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D$_3$. It regulates calcium homeostasis, immunity, cellular differentiation, and other physiological processes. Recently, VDR was found to respond to bile acids as well as other nuclear receptors, farnesoid X receptor (FXR) and pregnane X receptor (PXR). The toxic bile acid lithocholic acid (LCA) induces its metabolism through VDR interaction. To elucidate the structure-function relationship between VDR and bile acids, we examined the effect of several LCA derivatives on VDR activation and identified compounds with more potent activity than LCA. LCA acetate is the most potent of these VDR agonists. It binds directly to VDR and activates the receptor with 30 times the potency of LCA and has no or minimal activity on FXR and PXR. LCA acetate effectively induced the expression of VDR target genes in intestinal cells. Unlike LCA, LCA acetate inhibited the proliferation of human monoblastic leukemia cells and induced their monocytic differentiation.[S] We propose a docking model for LCA acetate binding to VDR. The development of VDR agonists derived from bile acids should be useful to elucidate ligand-selective VDR functions.—Adachi, R., Y. Honma, H. Masuno, K. Kawana, I. Shimomura, S. Yamada, and M. Makishima. **Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative.** *J. Lipid Res.* **2005.** 46: 46–57.

Supplementary key words   nuclear receptor • structure-function relationship • colon cancer • intestine • leukemia

The active form of vitamin D, 1α,25-dihydroxyvitamin D$_3$ [1,25(OH)2D3], regulates calcium homeostasis, immunity, and cellular growth and differentiation (1). 1,25(OH)2D3 has been demonstrated to be able to inhibit the proliferation and/or to induce the differentiation of various types of malignant cells, including breast, prostate, colon, skin, and brain cancer cells, as well as myeloid leukemia cells in vitro (2). The administration of 1,25(OH)2D3 or its analogs has therapeutic effects in mouse models of malignancies such as leukemia and colon cancer (3, 4). The biological activities of 1,25(OH)2D3 are mediated through binding to its intracellular receptor, the vitamin D receptor (VDR; NR1I1), a member of the nuclear receptor superfamily. Additional nongenomic mechanisms of action may be mediated by a poorly characterized membrane receptor (5). Structure-function relationship studies of the interaction of vitamin D analogs with VDR reveal different binding modes in the ligand-binding pocket of VDR (6). These differences in ligand-receptor interaction may contribute to the differential recruitment of coactivators to VDR and selective biological actions (7).

Nuclear receptors are ligand-inducible transcription factors that are involved in many biological processes, including cell growth and differentiation, embryonic development, and metabolic homeostasis (8). Recently, nuclear receptors belonging to the NR1H and NR1I subfamilies, including VDR, have been shown to control cholesterol

Abbreviations: BSEP, bile salt export pump; CAR, constitutive androstane receptor; ER, estrogen receptor; FXR, farnesoid X receptor; IBABP, ileal bile acid binding protein; 3-keto-LCA, 3-keto-cholanic acid; LCA, lithocholic acid; LXR, liver X receptor; NBT, nitroblue tetrazolium; N-CoR, nuclear receptor corepressor; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D$_3$; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator-1; TR, thyroid hormone receptor; VDR, vitamin D receptor.
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[S] The online version of this article (available at http://www.jlr.org) contains an additional figure.

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and bile acid metabolism (9). Liver X receptor α (LXRα; NR1H3) and LXRβ (NR1H2) function as oxysterol receptors and regulate cholesterol metabolism in liver, intestine, adipose tissue, and macrophages. Bile acids, which are major metabolites of cholesterol in the body, bind to farnesoid X receptor (FXR; NR1H4) and induce negative feedback regulation of their synthesis from cholesterol. Primary bile acids, produced in the liver, are excreted in the bile after conjugation with taurine and glycine and are subsequently reabsorbed in the intestine. Bile acids that escape reabsorption are converted to secondary bile acids by the intestinal microflora. Pregnane X receptor (PXR; NR1I2), which acts as a receptor for various xenobiotics, senses the levels of secondary bile acids and induces their metabolism in the liver (10, 11). VDR was also found to function as a receptor for secondary bile acids such as lithocholic acid (LCA) and to be involved in bile acid metabolism by inducing a LCA detoxification mechanism in the liver and intestine (12).

Previously, we analyzed the structure-function relationships of the endocrine [1,25(OH)2D3] and xenobiotic (LCA) ligands with VDR and revealed that 1,25(OH)2D3 and LCA interact with a different set of amino acids in the ligand binding pocket of VDR (13, 14). These results suggest the possibility that VDR adopts distinct conformations in response to 1,25(OH)2D3 and LCA binding and provides a possible mechanism for the compounds' different biological actions. The docking models of LCA and 3-keto-cholanic acid (3-keto-LCA), which is a metabolite of LCA, reveal that these compounds are accommodated in the VDR ligand binding pocket more weakly than 1,25(OH)2D3 (13, 14), suggesting that modification of these bile acids can increase the VDR transactivation activity. In this study, we examined the ability of several LCA analogs to activate VDR and found that modification of the 3 position of LCA increased VDR transactivation by more than 30-fold. Furthermore, the LCA acetate analog can induce differentiation of myeloid leukemia cells.

MATERIALS AND METHODS

Chemical compounds

LCA formate and LCA isobutyrate were synthesized in our laboratory (H. Masuno and S. Yamada, unpublished results), and other bile acids and derivatives were purchased from Sigma-Aldrich (St. Louis, MO), Wako (Osaka, Japan), Nacalai (Kyoto, Japan), or Steraloids (Newport, RI). 1,25(OH)2D3 was obtained from Calbiochem (San Diego, CA).

Plasmids

Fragments of human VDR (GenBank accession number NM_000376), FXR (accession number NM_005123), and PXR (accession number NM_022002) were inserted into the pCMX vector to make pCMX-VDR, pCMX-FXR, and pCMX-PXR, respectively (12, 14, 15). The ligand binding domains of human VDR, FXR, thyroid hormone α1 (TRα1) (accession number NM_199334), retinoic acid receptor α (RARα) (accession number NM_000964), LXRα (accession number NM_005693), constitutive androstane receptor (CAR) (accession number NM_005122), estrogen receptor α (ERα) (accession number NM_000125), ret-

inoid X receptor (RXRα; accession number NM_002957), and mouse peroxisome proliferator-activated receptor α (PPARα; accession number NM_011144), PPARδ (accession number NM_011145), and PPARγ (accession number NM_011146) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-VDR, pCMX-GAL4-FXR, pCMX-GAL4-TRα, pCMX-GAL4-RARα, pCMX-GAL4-LXRα, pCMX-GAL4-CAR, pCMX-GAL4-ERα, pCMX-GAL4-RXRα, pCMX-GAL4-PPARα, pCMX-GAL4-PPARδ, and pCMX-GAL4-PPARγ, respectively. A full-length fragment of human VDR was inserted into the pCMX-VP16 vector to make pCMX-VP16-VDR. Nuclear hormone receptor-interacting domains of steroid receptor coactivator-1 (SRC-1) (amino acids 595–771; GenBank accession number U90661) and nuclear receptor corepressor (N-CoR) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-SRC-1 and pCMX-GAL4-N-CoR (14). Mutations were introduced into pCMX-GAL4-VDR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). hCYP3A4-ER-6x3-tk-LUC, IR-1x3-tk-LUC, and GAL4-responsive MH100 (UAS)x4-tk-LUC reporters were used to evaluate the activities of VDR and PXR, FXR, and GAL4-chimera receptors, respectively. All plasmids were sequenced before use to verify DNA sequence fidelity.

Cell lines and cell culture

HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and antibiotic-antimycotic (Nacalai) at 37°C in a humidified atmosphere of 5% CO_2 in air. Human hepatoblastoma HepG2 cells and colon cancer SW480 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotic-antimycotic (Nacalai) at 37°C in a humidified atmosphere of 5% CO_2 in air. Human myeloid leukemia THP-1 cells were cultured in suspension in RPMI 1640 medium containing 10% fetal bovine serum and 80 µg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air (16).

Cotransfection assay

Transfections were performed by the calcium phosphate coprecipitation assay as described previously (14). Eight hours after transfection, test compounds were added. Cells were harvested 16–24 h later, and luciferase and β-galactosidase activities were assayed using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). Cotransfection experiments used 50 ng of reporter plasmid, 20 ng of pCMX-β-galactosidase, 15 ng of each receptor and/or cofactor expression plasmid, and pGEM carrier DNA to give a total of 150 ng of DNA per well of a 96-well plate. Luciferase data were normalized to the internal β-galactosidase control and represent means ± SD of triplicate assays.

Competitive ligand binding assay

Human VDR protein was generated using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI). The protein was diluted 5-fold with ice-cold TEGWD buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20 mM sodium tungstate, and 10% glycerol). The diluted lysate was incubated with 1 nM of [26,27-methyl-^{3}H]1,25(OH)2D3 for 16 h at 4°C in the presence or absence of nonradioactive competing compounds (14). Bound and free compounds were separated by the dextran-charcoal method (17). Bound and labeled 1,25(OH)2D3 was quantitated using scintillation counting.

Graphic manipulation and docking

Graphic manipulations were performed using SYBYL 6.8 (Tripos, St. Louis, MO) (13, 14). The atomic coordinates of the human VDR ligand binding domain (Δ165–215) crystal structure were retrieved from the Protein Data Bank (PDB #1DB1). LCA

acetate was docked into VDR using the docking software FlexX (version 1.10; Tripos) (18).

Animal studies

C57BL/6J mice were obtained from Japan SLC (Hamamatsu, Japan) and were maintained under controlled temperature (23 ± 1°C) and humidity (45–65%) with free access to water and chow (Oriental Yeast, Tokyo, Japan). Experiments were conducted with male mice between 8 and 9 weeks of age. Mice were treated orally with LCA or LCA acetate in a polyethylene glycol-Tween 80 (4:1) formulation or with vehicle alone (19). Mice were analyzed 12 h after treatment under fasting conditions. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Quantitative real-time RT-PCR analysis

Total RNAs from samples were prepared with an RNA STAT-60 kit (Tel-Test, Friendswood, TX). The cDNA was synthesized using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). Real-time PCR was performed on a LightCycler using the Fast-Start DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan) according to the instructions provided by the manufacturer (19). Primers were as follows: human VDR, 5′-GCTGACCTGGT-CAGTTACAGCA-3′ and 5′-CACGTCACTGACGCGGTACTT-3′; RXRα, 5′-AAGATGCGGGACATGCAGAT-3′ and 5′-CAGGCGGA-GCAAGAGCTTAG-3′; cyclophilin, 5′-CCCACCGTGTTCTTCGA-CAT-3′ and 5′-CCAGTGCTCAGAGCACGAAA-3′; CYP24A1, 5′-TGA-ACGTTGGCTTCAGGAGAA-3′ and 5′-AGGGTGCCTGAGTGT-AGCATCT-3′; CYP3A4, 5′-AGTGTGGGGCTTTTATGATG-3′ and 5′-ATACTGGGCAATGATAGGGA-3′; CaT1, 5′-AGCCTACATGA-CCCCTAAGGACG-3′ and 5′-GTAGAAGTGGCCTAGCTCCT-CGG-3′; E-cadherin, 5′-GAAGGTGACAGAGCCTCTGGATAG-3′ and 5′-CTGGAAGAGCACCTTCCATGA-3′; bile salt export pump (BSEP), 5′-TCTTTACTGGATTCGTGTGG-3′ and 5′-TGACACT-GAGGAAAATCTGG-3′; mouse cyclophilin, 5′-CAGACGCCACT-GTCGCTTT-3′ and 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; Cyp24a1, 5′-CCCATTACTCAGGGAAGCAC-3′ and 5′-CCACTCA-GACAATGAAGCCA-3′; Cyp3a11, 5′-CCAACAAGGCACCTCCC-ACG-3′ and 5′-TGGAATTCTTCAGGCTCTGA-3′; ileal bile acid binding protein (IBABP), 5′-GGTACCACCATGGCCTTCAGTG-GCAAATAT-3′ and 5′-GCTAGCTCAAGCCAGCCTCTTGCTTAC-3′. The RNA values were normalized to the amount of cyclophilin mRNA and are represented in arbitrary units.

Growth and differentiation of myeloid leukemia cells

Cell suspensions were cultured with or without test compound. The cells were counted in a model ZM Coulter Counter (Coulter Electronics, Luton, UK). Cell morphology was examined in cell smears stained with May-Gruenwald-Giemsa. α-Naphthyl acetate esterase was determined cytochemically, nitroblue tetrazolium (NBT) reduction was assayed colorimetrically, and expression of monocytic antigens CD11b and CD14 on the cell surface was determined using indirect immunofluorescent staining and a flow cytometer (Epics XL; Coulter Electronics) (16, 20).

RESULTS

Transactivation of VDR by LCA derivatives

To elucidate the structure-activity relationship of LCA derivatives (**Fig. 1A**) on VDR function, we transiently transfected HEK293 cells with a VDR expression vector and a luciferase reporter containing a VDR-responsive everted repeat-6 element from the CYP3A4 promoter (12). Cells were treated with test compounds and the induced luciferase

activities were compared (Fig. 1B). Transcriptional activation by 1,25(OH)2D3 and LCA was similar to previous reports (12). Esterification of the side chain carboxyl group of LCA with methyl, ethyl, and benzyl groups drastically decreased the activity on VDR (Fig. 1B). Next, we examined the effects of LCA derivatives modified at the 3α-hydroxyl group (Fig. 1A). LCA formate and LCA acetate were able to activate VDR as efficiently as LCA at the concentration of 10 μM. LCA isobutyrate activated VDR moderately, whereas LCA hemisuccinate was not an effective VDR agonist. The data indicate that addition of a large acyl group at the 3α-hydroxyl group of LCA abolishes VDR activation. The stereochemistry, as well as the substituent of the 3-hydroxyl group, is also important for LCA activity. Iso-LCA with a 3β-hydroxyl group and ursocholanic acid with no hydroxyl group at C-3 (Fig. 1A) have little activity on VDR (Fig. 1B). Interestingly, although the effect of LCA methyl ester on VDR activation was weak, LCA acetate methyl ester was able to induce VDR activation effectively. 3-Keto-LCA, a metabolite of LCA, is another potent bile acid for VDR (12). The esterification on the side chain of 3-keto-LCA modestly decreased its activity on VDR (Fig. 1B). 6-Keto-LCA is a very weak VDR agonist, and 7-keto-LCA and 12-keto-LCA were not able to activate VDR (12). Transactivation of VDR by 3,6-diketo-LCA, 3,7-diketo-LCA, and 3,12-keto-LCA was almost absent (Fig. 1B). These data indicate that addition of a ketone moiety at position 6, 7, or 12 to LCA or 3-keto-LCA disturbs the interaction with VDR.

LCA acetate is a potent agonist for VDR

We compared VDR dose-response curves for LCA, LCA formate, LCA acetate, LCA acetate methyl ester, and 3-keto-LCA. LCA acetate activated VDR with an EC_{50} of 0.40 μM, followed in rank order by LCA formate (EC_{50} = 4.0 μM), 3-keto-LCA (6.8 μM), and LCA (12.1 μM) (**Fig. 2A**). Notably, the potency of VDR activation by LCA acetate on VDR was 30-fold greater than that of LCA.

Upon ligand binding, nuclear receptors undergo a conformational change that induces recruitment of coactivators, such as SRC-1, and dissociation of corepressors, such as N-CoR (21). To assay ligand-dependent interactions of VDR with cofactors, the receptor-interacting domains of SRC-1 and N-CoR were fused to the GAL4 DNA binding domain (14). Cotransfection of GAL4-cofactors with VDR fused to the transactivation domain of herpesvirus VP16 protein allowed for the detection of ligand-dependent cofactor interaction (15). Although there was no association between control GAL4 protein and VP16-VDR, LCA acetate at 10 μM and 1,25(OH)2D3 at 100 nM strongly induced the association of VDR with SRC-1 (Fig. 2B). The effects of LCA formate and LCA acetate methyl ester on this interaction were modest, and activation by LCA and 3-keto-LCA were weak at 10 μM concentration. LCA acetate, LCA formate, LCA acetate methyl ester, and 3-keto-LCA dissociated N-CoR from VDR as effectively as 1,25 (OH)2D3 (Fig. 2B). The effects of these LCA derivatives on N-CoR dissociation were stronger than that of LCA. Thus, LCA acetate is a potent regulator of VDR-cofactor interaction.

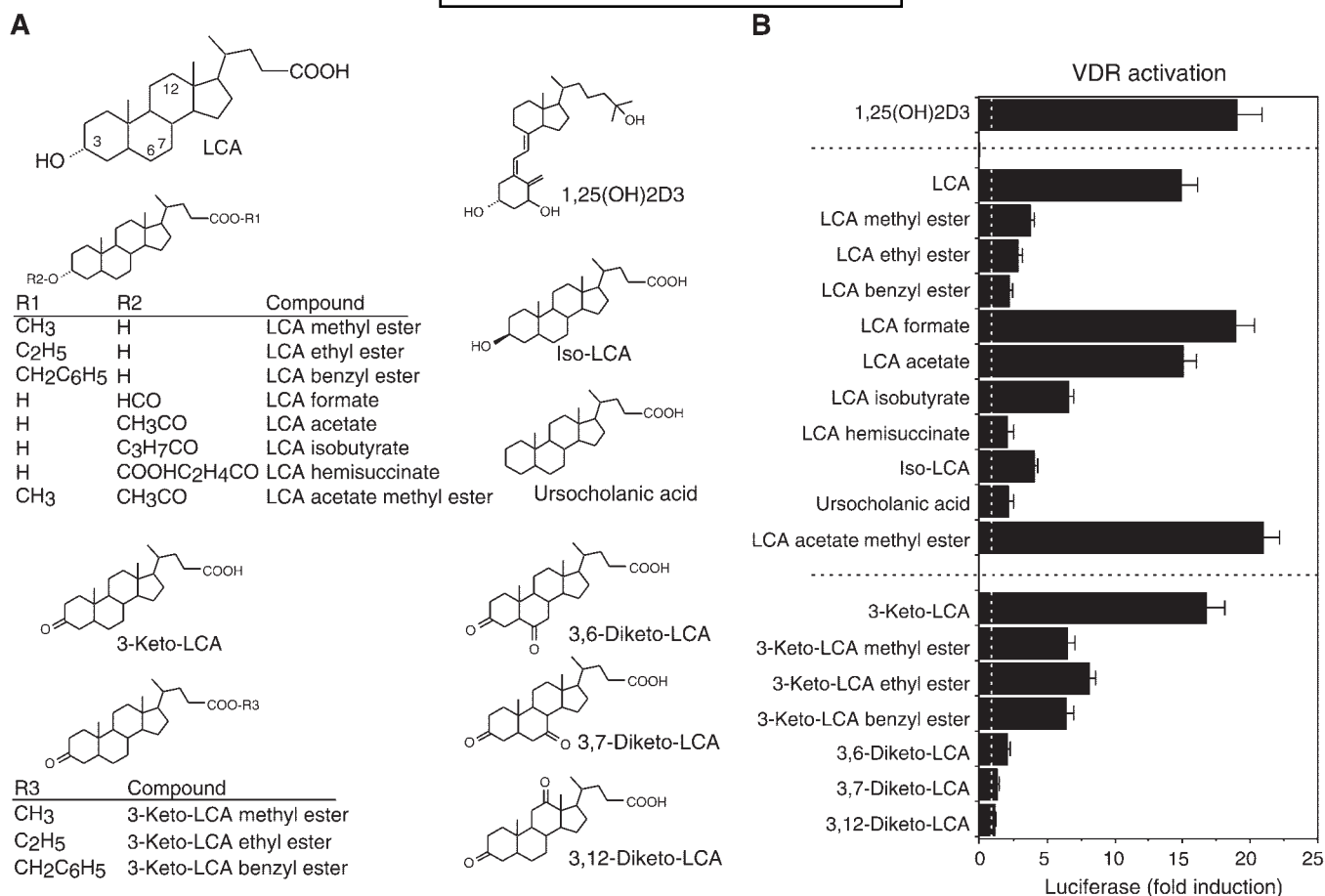Next, we assessed the ability of LCA derivatives to bind

Fig. 1. Lithocholic acid (LCA) derivatives activate vitamin D receptor (VDR). A: Structures of LCA, its derivatives, and $1\alpha,25$-dihydroxyvitamin D_3 [$1,25(OH)2D3$] are shown. B: Activation of VDR by LCA derivatives. HEK293 cells were cotransfected with CMX-VDR and CYP3A4-ER-6x3-tk-LUC and then treated with vehicle control (ethanol), $1,25(OH)2D3$ (100 nM), or bile acid derivatives (10 μM) for 24 h. Luciferase activity of the reporter is expressed as fold induction with compound treatment relative to vehicle control. The values represent means ± SD.

directly to VDR in vitro using the competitive binding assay. Isotopically labeled $1,25(OH)2D3$ was incubated with in vitro translated VDR protein in the absence or presence of test compounds. The binding of labeled $1,25(OH)2D3$ to VDR was competed by the addition of unlabeled $1,25(OH)2D3$ (Fig. 2C). LCA acetate and LCA formate also inhibited the binding of labeled $1,25(OH)2D3$ to VDR, indicating that these LCA derivatives directly bind to VDR. Competition with 3-keto-LCA and LCA was weaker than that of LCA acetate and LCA formate. Interestingly, although LCA acetate methyl ester showed enhanced activation of VDR compared with 3-keto-LCA in the luciferase reporter assay, as shown Fig. 2A, its direct interaction with VDR protein was weaker than those of LCA and 3-keto-LCA (Fig. 2C). LCA acetate did not inhibit the binding of labeled estradiol to ERα (data not shown). Taken together, these data indicate that LCA acetate activates VDR by direct binding.

LCA acetate is not a potent agonist for other bile acid receptors

The ligand binding domains of various nuclear receptors were fused to the DNA binding domain of the yeast transcription factor GAL4 to examine the effect of LCA acetate on these receptors. The GAL4-chimera receptors were cotransfected with a GAL4-responsive luciferase reporter into HEK293 cells (15). Because this reporter is activated only by GAL4-chimera receptors, the potentially confounding effects of endogenous receptors are eliminated. LCA acetate at 30 μM induced the activation of GAL4-VDR (**Fig. 3A**). It induced weak activation of FXR but was not effective on TRα, RARα, PPARα, PPARδ, PPARγ, LXRα, CAR, RXRα, or ERα. FXR has been previously shown to respond to various bile acids, such as chenodeoxycholic acid and deoxycholic acid (12, 15). Next, we determined FXR dose-response curves for LCA derivatives modified at position 3. As reported previously (15), chenodeoxycholic acid was a potent FXR agonist (Fig. 3B). Interestingly, ursocholanic acid and iso-LCA, which were not effective on VDR (Fig. 1B), strongly induced the activation of FXR (Fig. 3B). LCA formate and LCA acetate, as well as LCA, were weak FXR agonists. PXR was reported to respond to high concentrations of LCA (10, 11). To examine the effects of LCA derivatives on PXR, we transfected VDR or PXR expression vectors with a reporter containing a CYP3A4 element, which can be activated by both re-
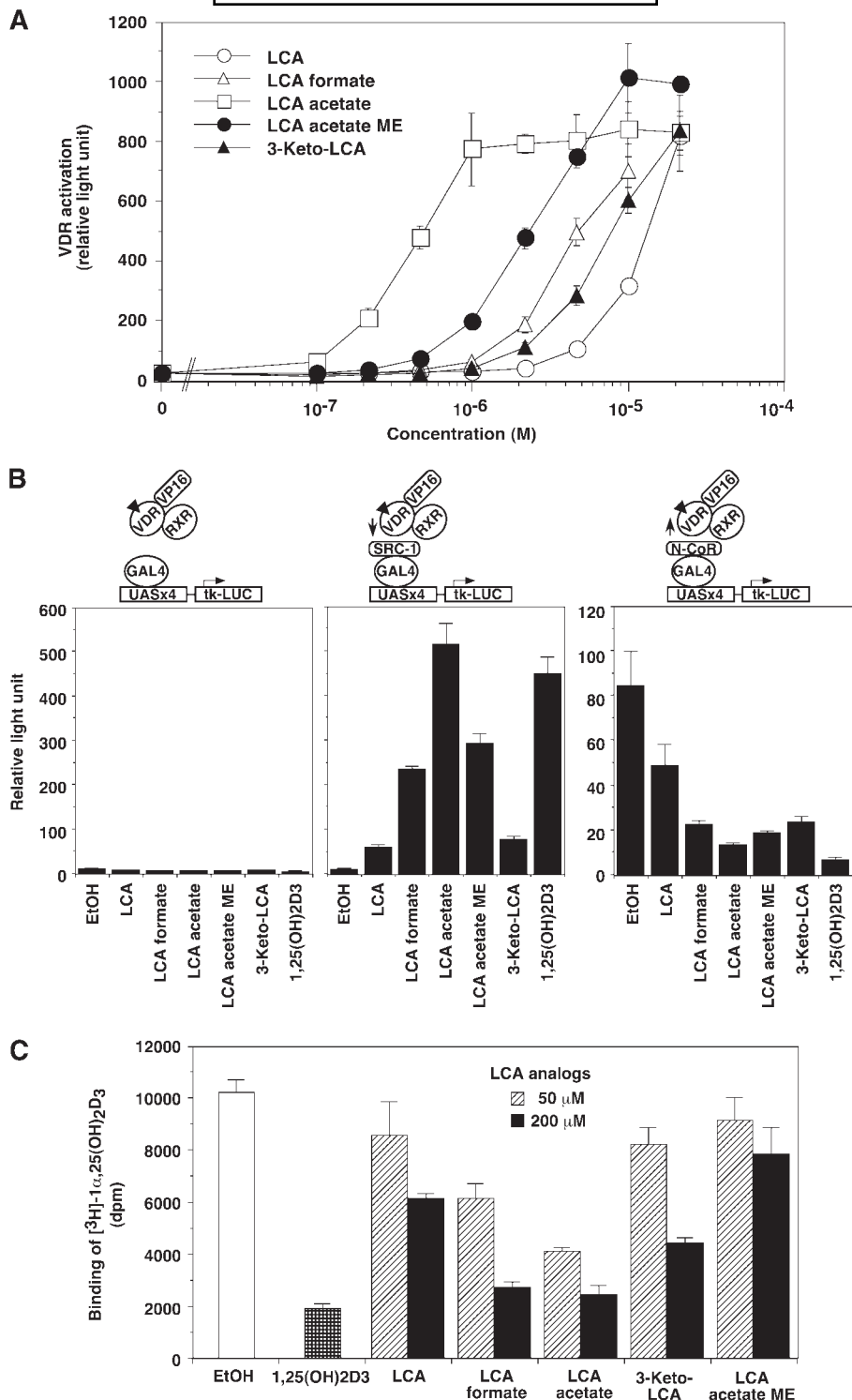
Fig. 2. LCA acetate is a potent VDR agonist. A: Concentration-dependent activation of VDR by LCA acetate and its related compounds. HEK293 cells were cotransfected with CMX-VDR and CYP3A4-ER-6x3-tk-LUC reporter and treated with several concentrations of LCA, LCA formate, LCA acetate, LCA acetate methyl ester (LCA acetate ME), and 3-keto-cholanic acid (3-keto-LCA) for 16 h. B: Interactions of VDR with steroid receptor coativator-1 (SRC-1) and nuclear receptor corepressor (N-CoR) induced by LCA acetate and its related compounds. HEK293 cells were cotransfected with GAL4 control vector or GAL4-chimera vectors for SRC-1 or N-CoR, in combination with VP16-VDR and MH100(UAS)x4-tk-LUC reporter, and were treated with ethanol (EtOH) control, 10 μM LCA acetate, or related bile acids. C: Direct binding of LCA acetate to VDR. In vitro translated VDR proteins were incubated with 1 nM [^{3}H]1,25(OH)2D3 in the presence or absence of nonradioactive 10 nM 1,25(OH)2D3 or 50 μM or 200 μM bile acid derivatives. The values represent means ± SD.
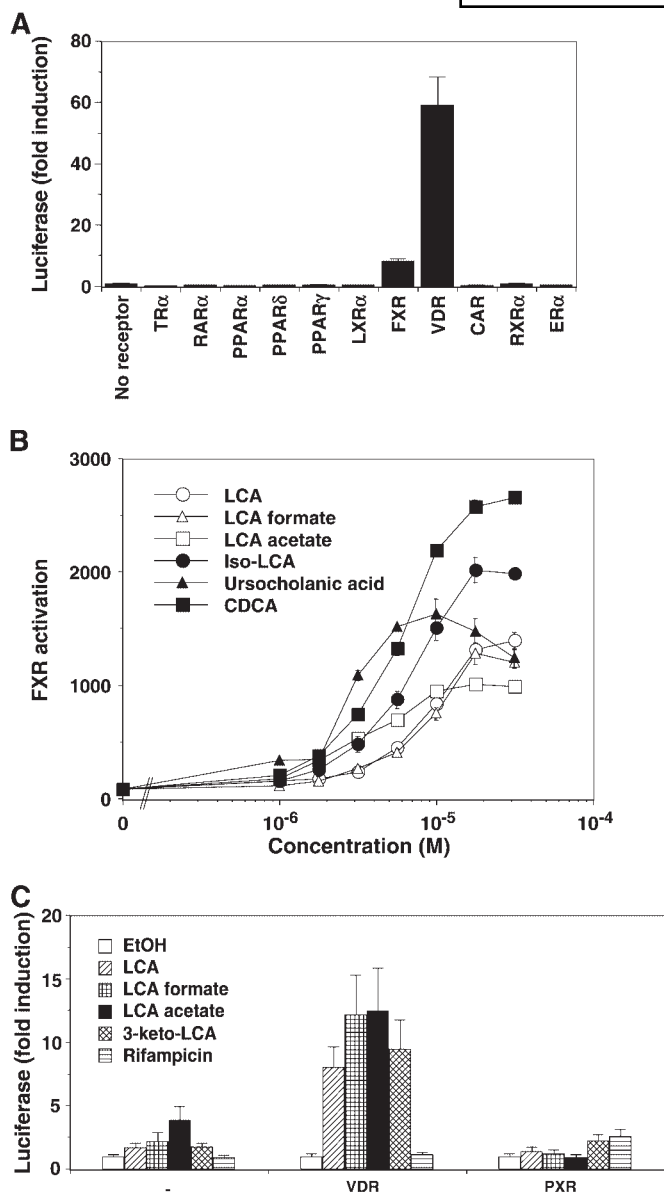
the LCA derivatives (Fig. 3C). This effect may be derived from endogenous receptors such as VDR. LCA acetate and LCA formate strongly induced the activity of transfected VDR, indicating that these LCA derivatives activate VDR in HepG2 cells. The PXR agonist rifampicin did not activate VDR. When HepG2 cells were cotransfected with PXR, rifampicin and 3-keto-LCA increased the reporter activity, but LCA acetate and LCA formate were not effective PXR ligands (Fig. 3C). These findings indicate that LCA acetate is selective for VDR activation.

Effect of VDR mutation on LCA acetate response

To elucidate the structure-activity relationship of LCA acetate and VDR, we examined the effects of LCA acetate on the activation of several VDR mutants. Wild-type GAL4-VDR and several alanine mutants, Y143A, S237A, S275A, S278A, W286A, and H305A, were introduced into HEK293 cells and activation by LCA, LCA acetate, and $1,25(OH)_2D_3$ were compared (**Fig. 4A**). According to the crystal structure of the VDR-$1,25(OH)_2D_3$ complex (22), Y143 and S278 interact with the 3β-hydroxyl group of $1,25(OH)_2D_3$, S237 hydrogen bonds with the 1α-hydroxyl group, H305 coordinates the 25-hydroxyl group, and S275 and W286 mediate hydrophobic interaction with $1,25(OH)_2D_3$. The Y143A and W286A mutations inhibited activation by LCA acetate, LCA, and $1,25(OH)_2D_3$. The effect of S237A was modest on LCA, LCA acetate, and $1,25(OH)_2D_3$ activity. Whereas S275A and S278A almost abolished the activity of LCA, LCA acetate and $1,25(OH)_2D_3$ still activated these mutants. Interestingly, although H305A had significant effects on the activity of LCA and $1,25(OH)_2D_3$, this mutation had little effect on the activity of LCA acetate. Thus, LCA acetate is similar to 3-keto-LCA in its ability to activate VDR-H305A (13). In a previous study, we found that the VDR-S278V mutant is activated by $1,25(OH)_2D_3$ but not by LCA, whereas VDR-S237M can respond to LCA but not to $1,25(OH)_2D_3$ (14). We next examined the effects of LCA acetate on these mutants (Fig. 4B). The S237M mutation weakly affected the activity of LCA acetate as well as that of LCA. S278V drastically decreased LCA acetate activity. Based on these findings, we modeled LCA acetate in the VDR ligand binding domain (Δ165–215) (PDB #1DB1) using FlexX software (13). In contrast to the LCA docking model, the side chain of LCA acetate directs to the β-turn site (Fig. 4C, left panel) (13). The oxygen of the side chain carboxyl group and the carbonyl oxygen of the 3-O-acetyl acetate group nearly overlap with the 3β-hydroxyl oxygen and 25-hydroxyl oxygen, respectively, of $1,25(OH)_2D_3$ in the crystal structure of VDR-$1,25(OH)_2D_3$ (Fig. 4C, right panel). The proximity of these amino acid residues to hydrogen bond acceptors within $1,25(OH)_2D_3$ may be responsible for the strong activity of LCA acetate on VDR.

Induction of VDR target genes by LCA acetate in intestinal cells

VDR is highly expressed in intestinal mucosa cells and regulates the expression of genes involved in calcium homeostasis and bile acid metabolism (1, 12, 23). We investi-
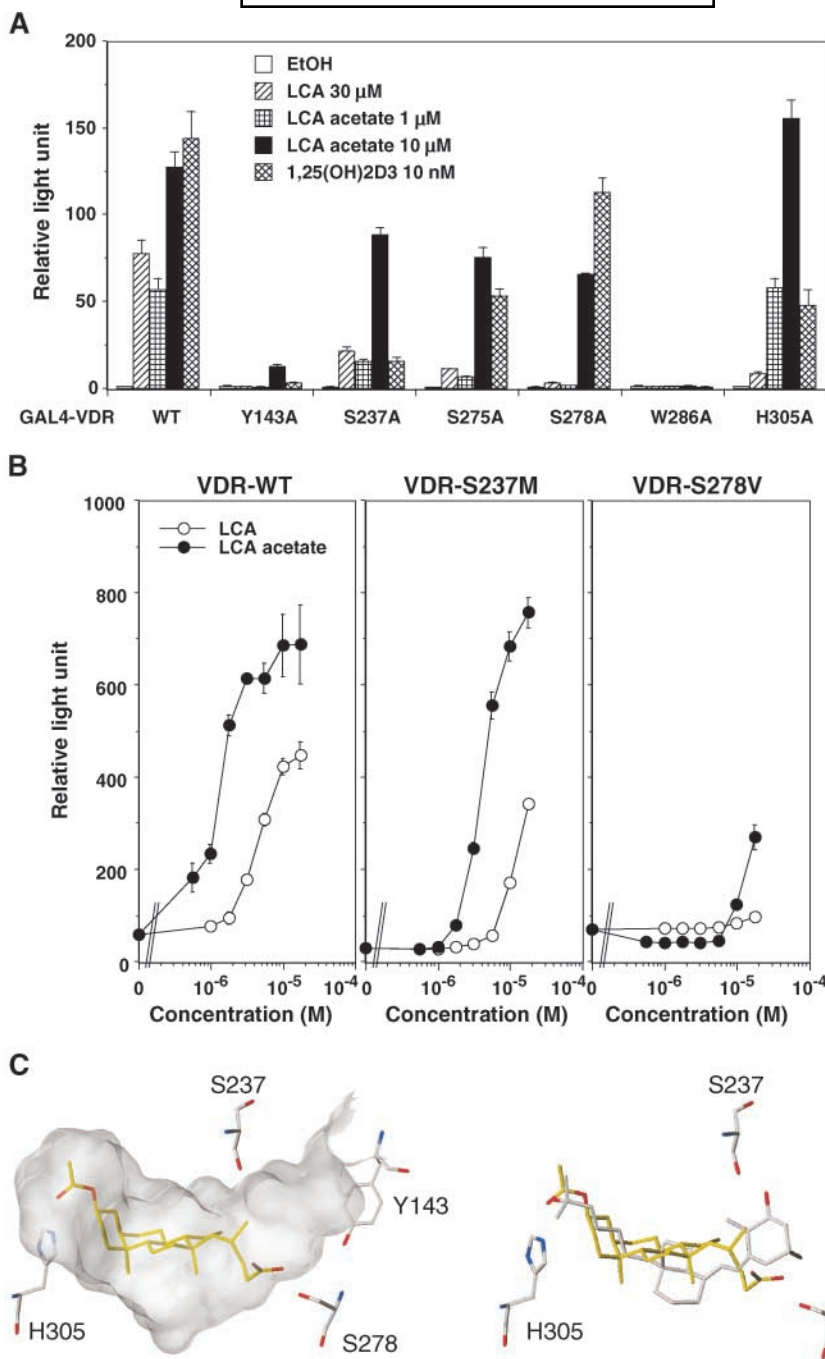
Fig. 3. LCA acetate is a selective agonist for VDR. A: Receptor-specific activation by LCA acetate. GAL4-chimera receptors for various nuclear receptors were expressed with MH100(UAS)x4-tk-LUC reporter in HEK293 cells and assayed for activation by 30 μM LCA acetate. Luciferase activity of the reporter is expressed as fold induction with compound treatment relative to vehicle control. PPAR, peroxisome proliferator-activated receptor. B: Concentration-dependent activation of farnesoid X receptor (FXR) by LCA acetate and its related compounds. HEK293 cells were cotransfected with CMX-FXR and IR-1x3-tk-LUC reporter and treated with several concentrations of LCA, LCA formate, LCA acetate, iso-LCA, ursocholanic acid, or chenodeoxycholic acid (CDCA). C: Comparative response of VDR and pregnane X receptor (PXR) to LCA acetate in liver HepG2 cells. HepG2 cells were transfected with CMX control vector (−), CMX-VDR, or CMX-PXR with CYP3A4-ER-6x3-tk-LUC and treated with vehicle control [ethanol (EtOH)], 30 μM LCA, LCA formate, LCA acetate, 3-keto-LCA, or rifampicin. The values represent means ± SD.

ceptors (12). Liver-derived HepG2 cells were used for this experiment, because PXR activation is cell type dependent (data not shown). In the absence of transfected receptors, the luciferase activity was increased by addition of

Fig. 4. Structure-function analysis of LCA acetate and VDR. A: Activation of VDR or its mutants by LCA acetate. GAL4-VDR and alanine mutants (Y143A, S237A, S275A, S278A, W286A, and H305A) were cotransfected with MH100(UAS)x4-tk-LUC reporter in HEK293 cells and treated with vehicle control [ethanol (EtOH)] or the indicated concentrations of test compounds. WT, wild type. B: Dose response of VDR S237M and S278V mutants for LCA acetate. HEK293 cells were cotransfected with GAL4-VDR, GAL4-VDR-S237M, or GAL4-VDR-S278V with MH100(UAS)x4-tk-LUC reporter in HEK293 cells. The values represent means ± SD. C: Docking model of VDR interaction with LCA acetate. Left panel: The side chain carboxyl group is directed to the β-turn site interacting with S278. The Connolly channel surface of the VDR ligand binding pocket is shown in translucent gray. Right panel: Overlay of LCA acetate (yellow) and 1,25(OH)2D3 (gray) accommodated in the VDR ligand binding pocket.

gated the ability of LCA acetate to activate endogenous VDR target genes in intestinal cells. Colon cancer-derived SW480 cells were incubated with LCA, LCA acetate, 1,25(OH)2D3, chenodeoxycholic acid, or rifampicin, and the expression of VDR target genes, including CYP24A1, CYP3A4, CaT1, and E-cadherin, was examined. CYP24A1 and CaT1 are involved in calcium homeostasis and CYP3A4 metabolizes LCA (1, 24). E-cadherin was reported to be
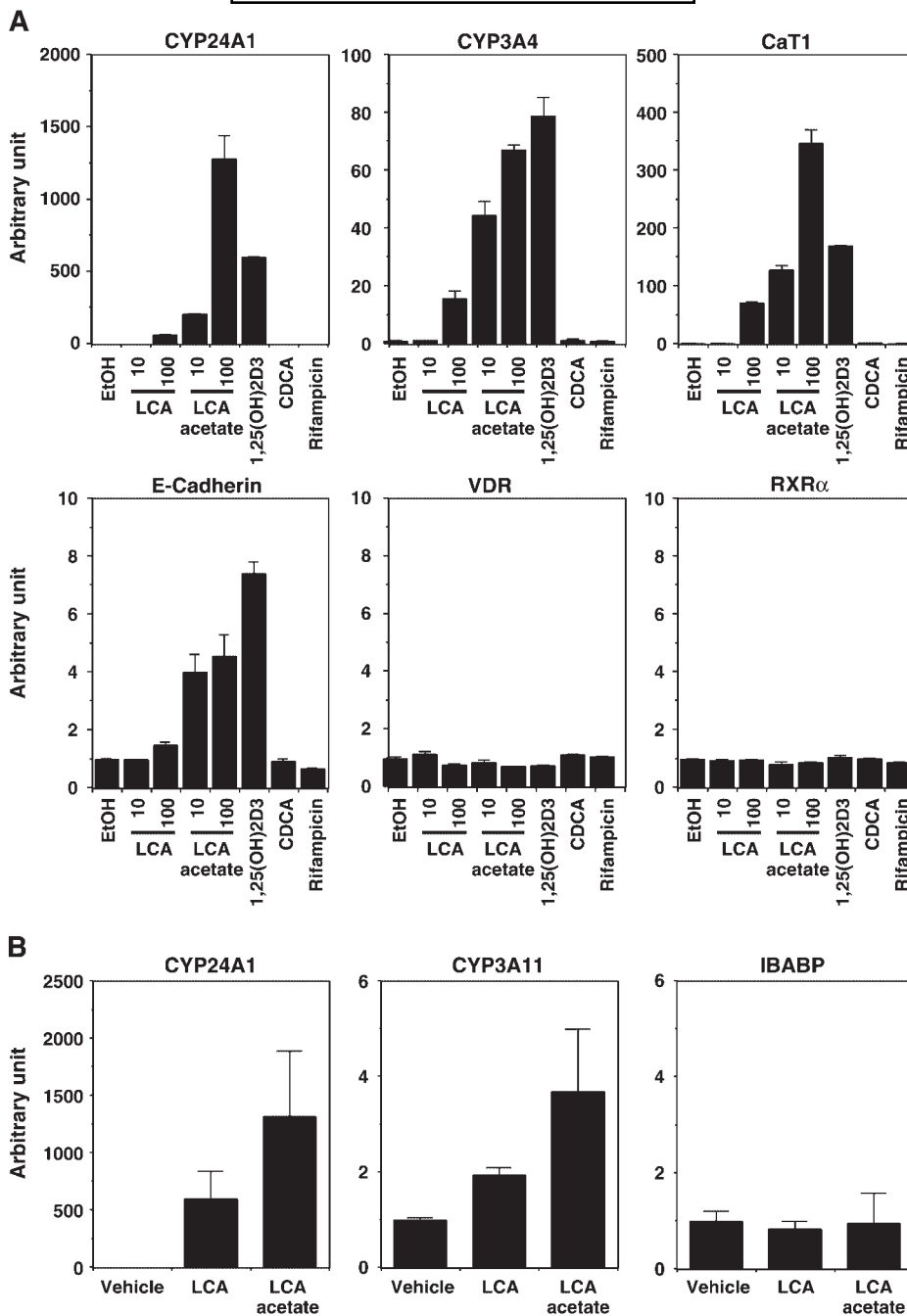
Fig. 5. Induction of VDR target genes by LCA acetate in intestinal cells. A: LCA acetate induced VDR target genes more effectively than LCA in colon cancer-derived SW480 cells. Cells were treated with vehicle control [ethanol (EtOH)], 10 or 100 μM LCA or LCA acetate, 100 nM 1,25(OH)2D3, 100 μM chenodeoxycholic acid (CDCA), or 30 μM rifampicin for 24 h. Quantitative real-time PCR from mRNA for CYP24A1, CYP3A4, CaT1, E-cadherin, VDR, and retinoid X receptor α (RXRα) was performed. B: LCA acetate increased VDR target genes in mouse intestine more effectively than LCA. Mice were orally administrated with 200 mg/kg LCA or LCA acetate. Twelve hours after administration, total RNA was extracted from intestinal mucosa and quantitative real-time PCR from mRNA for CYP24a1, Cyp3a11, and ileal bile acid binding protein (IBABP) was performed. The values represent means ± SD.

associated with cell growth inhibition induced by 1,25 (OH)2D3 (25). As shown in **Fig. 5A**, 1,25(OH)2D3 induced the expression of CYP24A1, CYP3A4, CaT1, and E-cadherin. LCA acetate induced these VDR target genes more effectively than LCA, indicating that LCA acetate

acts as a potent VDR agonist in colon cancer cells. There were no changes in the expression of VDR and RXRα, which forms a heterodimer with VDR, after treatment with the VDR ligands. The FXR agonist chenodeoxycholic acid and the PXR agonist rifampicin were not able to in-

duce the expression of these genes, although PXR was reported to be involved in CYP3A4 gene regulation (11). The inability of PXR agonist to increase gene expression is likely attributable to the fact that PXR is not expressed in SW480 cells (data not shown). Next, we examined the expression of VDR target genes in vivo. Mice were orally administered LCA or LCA acetate, and the expression of intestinal Cyp24a1 and Cyp3a11 genes was evaluated. Both LCA and LCA acetate increased the mRNA expression of Cyp24a1 and Cyp3a11 significantly, LCA acetate being more effective than LCA (Fig. 5B). LCA and LCA acetate did not induce the FXR target IBABP gene expression (Fig. 5B). These data indicate that LCA acetate is a potent agonist for endogenous VDR in intestinal cells.

LCA acetate induces the differentiation of monoblastic leukemia cells

1,25(OH)2D3 is known as an inducer of myeloid leukemia differentiation (1). We examined the effects of LCA acetate on the growth and differentiation of human monoblastic leukemia THP-1 cells. 1,25(OH)2D3 inhibited the proliferation of THP-1 cells and enhanced NBT-reducing activity, a differentiation marker of myeloid leukemia cells, as reported previously (26). LCA acetate inhibited cell proliferation more effectively than LCA and 3-keto-LCA (**Fig. 6A**), and it induced NBT-reducing activity in the cells. In contrast, LCA and 3-keto-LCA were not able to induce this activity even at concentrations that completely inhibit cell proliferation (Fig. 6B). Untreated THP-1 cells have large nuclei with visible nucleoli and basophilic cytoplasmic staining. LCA acetate induced a concentration-dependent increase in the percentage of differentiated cells (Fig. 6C). In cells treated with LCA acetate, the nuclei were condensed, nucleoli were no longer apparent, and the cytoplasm appeared gray, indicating monocytic differentiation (Fig. 6C). Esterase activity, a functional marker of monocytic differentiation, was also induced by LCA acetate (Fig. 6D). LCA and 3-keto-LCA did not induce morphological and functional differentiation of THP-1 cells. LCA acetate increased the expression of surface makers, such as CD11b and CD14, as effectively as 1,25(OH)2D3 (Fig. 6E). Therefore, the VDR agonist LCA acetate is a potent inducer of monocytic differentiation in THP-1 leukemia cells.

DISCUSSION

In this study, we found that the modification of the 3α-hydroxyl group of LCA increases the transactivation activity and selectivity on VDR. Structure-function relationship analysis of the VDR-LCA interaction using several VDR mutants shows that the side chain of LCA faces H12 of the receptor and 3-keto-LCA is directed toward the β-turn site (13). As shown in Fig. 1, esterification of the side chain carboxyl group of LCA abolished VDR activation. However, in 3-keto-LCA, the corresponding esterifications had only moderate effects. This may be ascribed to the opposing docking modes of LCA and 3-keto-LCA. The LCA de-

rivatives modified at position 3, such as LCA formate and LCA acetate, have stronger activity than LCA (Figs. 1, 2). The docking model shown in Fig. 4 indicates that LCA acetate is accommodated in the VDR ligand binding pocket in the same manner as 3-keto-LCA and that LCA acetate can form hydrogen bonds with the same amino acid residues that coordinate 1,25(OH)2D3 binding. LCA acetate methyl ester has much stronger activity than LCA methyl ester. This may be attributable to different docking modes of these two LCA esters. LCA acetate and LCA can activate VDR-S237M (Fig. 4B), which does not respond to 1,25 (OH)2D3 (14). S237 is located in H3 and may mediate allosteric communication with the cofactor interaction surface. These findings suggest the possibility that LCA acetate induces an alternative conformation in VDR, which results in differential cofactor recruitment and selective physiological function. Further study is required to elucidate the structure-function relationship of VDR and LCA derivatives such as LCA acetate.

FXR is activated by both primary bile acids (chenodeoxycholic acid and cholic acid) and secondary bile acids (LCA and deoxycholic acid) (15, 27, 28). In contrast, VDR responds to only LCA and its derivatives (12). In the previous study, 6-keto-LCA was identified as a selective ligand for VDR, but its activity was very weak (12). The potent VDR agonist LCA acetate activated FXR to low levels, similar to the weak FXR agonist LCA, and much more weakly than chenodeoxycholic acid (Fig. 3B). In HepG2 cells, chenodeoxycholic acid induced the expression of the BSEP gene, which is an FXR target (9), but LCA and LCA acetate were not effective in its induction, although LCA acetate increased the VDR target CYP24A1 expression (see supplementary data online). Although LCA and 3-keto-LCA were agonists for PXR at high concentrations, LCA acetate did not activate PXR (Fig. 3C). These data indicate that LCA acetate is a selective agonist for VDR. Interestingly, although iso-LCA and ursocholanic acid were not able to activate VDR, they were more potent FXR agonists than LCA. Recently, crystal structures of FXR and PXR have been reported (29–31). Mutational analysis of FXR and PXR should be useful in elucidating the structure-function relationship of these LCA derivatives and in the development of selective ligands for the bile acid receptors VDR, FXR, and PXR.

Vitamin D has been identified as a protective agent against the development of colorectal cancer (32). Epidemiological analysis revealed that solar exposure, which results in vitamin D production in the skin, or vitamin D uptake reduces the incidence of colorectal cancer (32). Protective effects of vitamin D in colon carcinogenesis are mediated through its receptor VDR. VDR activation induces the expression of genes involved in growth inhibition, differentiation, and apoptosis (1, 33). In contrast to vitamin D, the secondary bile acid LCA is considered to be a promoter of colon carcinogenesis (34). LCA induces DNA strand breaks, forms DNA adducts, inhibits DNA repair enzymes, and can promote colon cancer in rodent models (35). CYP3A was reported to detoxify LCA to a nontoxic hyodeoxycholic acid and is a VDR target gene
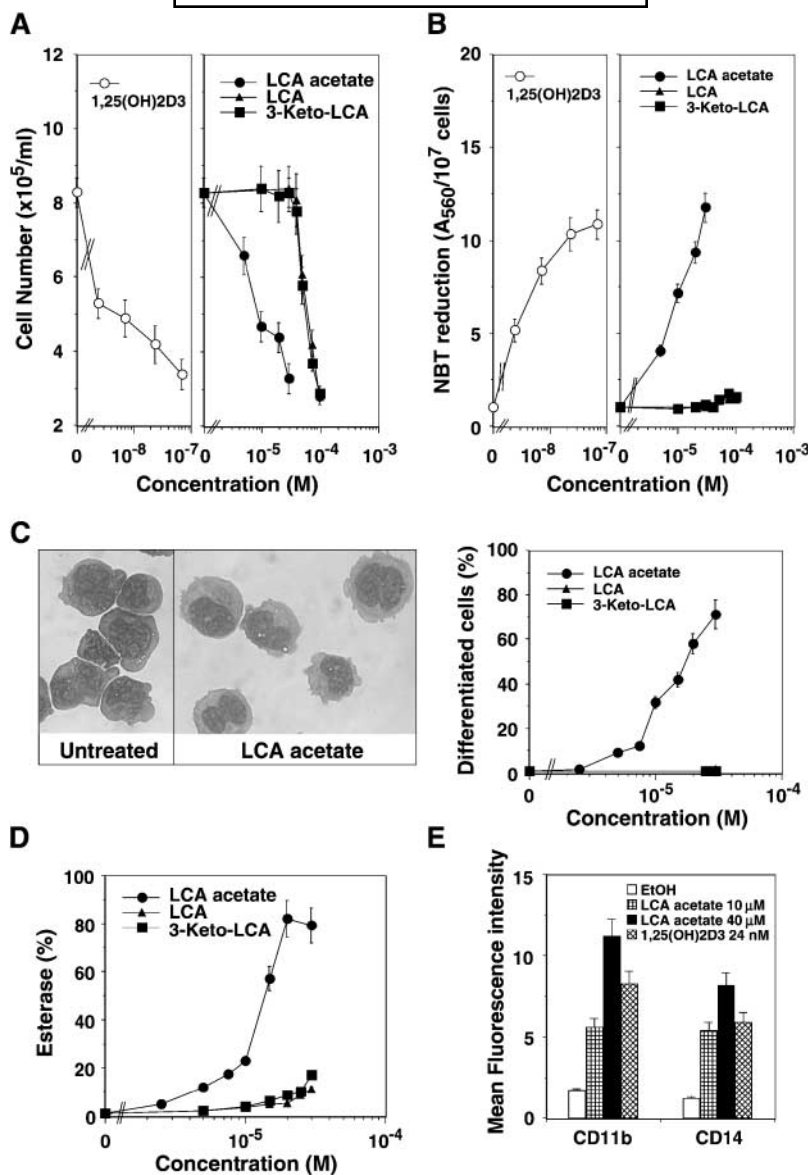
Fig. 6. Effects of LCA acetate on growth and differentiation of human myeloid leukemia THP-1 cells. A: Growth inhibition of THP-1 cells by 1,25(OH)2D3 and LCA acetate. B: Induction of nitroblue tetrazolium (NBT)-reducing activity in THP-1 cells by 1,25(OH)2D3 and LCA acetate. THP-1 cells were treated with 1,25(OH)2D3, LCA acetate, LCA, or 3-keto-LCA for 4 days. C: LCA acetate induces the morphological differentiation of THP-1 cells. Cells were treated with LCA acetate, LCA, or 3-keto-LCA for 6 days, and differentiated cells shown in the left panel were counted. D: LCA acetate induces monocyte-specific esterase activity. Cells were treated with LCA acetate, LCA, or 3-keto-LCA for 6 days. E: LCA acetate increases the expression of CD11b and CD14 surface antigens. Cells were treated with LCA or 1,25(OH)2D3 for 4 days and CD11b and CD14 expression was examined using monoclonal antibodies and flow cytometry. EtOH, ethanol. The values represent means ± SD.

(11, 36). By binding to VDR, 1,25(OH)2D3 and LCA induce CYP3A expression in the intestine. VDR may serve as a sensor for LCA and function to protect intestinal mucosa from its harmful effects. Recently, a significant correlation between a VDR polymorphism and colorectal cancer risk was reported in a Singapore Chinese population (37). These findings suggest that VDR functions as an anticancer factor and indicate that it is a promising molecular target for chemoprevention against colorectal cancer.

Clinical trials of vitamin D and its analogs have been un-successful because of their hypercalcemic activities (38). Structure-function analysis of vitamin D analogs suggests that 1,25(OH)2D3 and its analogs also induce nonge-nomic VDR actions and that adverse effects are at least partly attributable to nongenomic mechanisms (5, 39). Ligand-dependent dissociation of nongenomic from ge-nomic activity was reported for the estrogen receptor (40). An estrogen receptor ligand, pyrazole, induced the transactivation of an estrogen receptor target gene but had weak nongenomic activity, whereas another ligand, es-

tren, induced strong nongenomic action of the estrogen receptor without altering gene expression. There has been no reported physiological correlation between bile acids and intestinal calcium absorption, suggesting that LCA or its derivatives may relatively induce genomic actions in the intestine, such as bile acid metabolism and cell growth control, without inducing hypercalcemia. LCA acetate induced VDR target genes via genomic action, including the LCA-detoxifying enzyme CYP3A, in colon cancer cells and mouse intestines more effectively than LCA (Fig. 4). Nongenomic action of bile acids and derivatives should be further investigated. The development of more potent LCA derivatives that are nontoxic and less hypercalcemic should be useful for chemoprevention against colon carcinogenesis.

1,25(OH)2D3 was found to induce the differentiation of mouse myeloid leukemia M1 cells more than 20 years ago (41). Treatment with 1,25(OH)2D3 or 1α-hydroxyvitamin D$_3$, which is rapidly metabolized to 1,25(OH)2D3, was reported to prolong survival in mice inoculated with M1 leukemia cells (3). The differentiation-inducing effects of 1,25(OH)2D3 were also demonstrated in human leukemia cells (42, 43). However, the molecular mechanisms of differentiation induced by 1,25(OH)2D3 have not been elucidated. We found that the potent VDR agonist LCA acetate was able to induce the differentiation of human monoblastic leukemia THP-1 cells at concentrations that induce VDR activation (Fig. 6). LCA and 3-keto-LCA inhibited proliferation but did not induce differentiation. The growth-inhibiting activity of these bile acids may be attributable to their cytotoxic effects. Zimber et al. (44) reported that bile acids, including deoxycholic acid, chenodeoxycholic acid, and LCA, induced the differentiation of human promyelocytic leukemia HL-60 cells. We did not observe differentiation-inducing activity of these bile acids in HL-60 cells (data not shown). This is probably because of differences between subclones of leukemia cell lines, which could affect sensitivity to the compounds. Regardless, LCA acetate did induce differentiation markers in HL-60 cells (data not shown). These findings indicate that LCA acetate is a more effective inducer of leukemia differentiation than bile acids such as LCA and chenodeoxycholic acid. Zimber et al. (45) reported that LCA alone did not induce the differentiation of THP-1 cells but that it enhanced the response to all-*trans*-retinoic acid, which is a potent differentiation inducer of myeloid leukemia cells. The combinational effects of LCA acetate and other differentiation inducers are now under investigation. The protein kinase C inhibitor sphingosine decreased the NBT-reducing activity induced by deoxycholic acid and chenodeoxycholic acid in HL-60 cells but did not alter the response to LCA (44), suggesting that the effect of LCA is mediated by mechanisms distinct from those used by deoxycholic acid and chenodeoxycholic acid. Expression of some VDR target genes was increased in THP-1 cells after treatment with LCA acetate (data not shown). The data indicate that LCA acetate functions as a VDR agonist in leukemia cells and induces cell differentiation. Further studies are required to elucidate the precise mech-

anisms of LCA acetate- and 1,25(OH)2D3-induced leukemia cell differentiation.▐▐

REFERENCES

1. Haussler, M. R., G. K. Whitfield, C. A. Haussler, J. C. Hsieh, P. D. Thompson, S. H. Selznick, C. E. Dominguez, and P. W. Jurutka. 1998. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J. Bone Miner. Res.* **13:** 325–349.
2. Brown, A. J., A. Dusso, and E. Slatopolsky. 1999. Vitamin D. *Am. J. Physiol. Renal Physiol.* **277:** F157–F175.
3. Honma, Y., M. Hozumi, E. Abe, K. Konno, M. Fukushima, S. Hata, Y. Nishii, H. F. DeLuca, and T. Suda. 1983. 1α,25-Dihydroxyvitamin D3 and 1α-hydroxyvitamin D3 prolong survival time of mice inoculated with myeloid leukemia cells. *Proc. Natl. Acad. Sci. USA.* **80:** 201–204.
4. Kumagai, T., J. O'Kelly, J. W. Said, and H. P. Koeffler. 2003. Vitamin D2 analog 19-nor-1,25-dihydroxyvitamin D2: antitumor activity against leukemia, myeloma, and colon cancer cells. *J. Natl. Cancer Inst.* **95:** 896–905.
5. Huhtakangas, J. A., C. J. Olivera, J. E. Bishop, L. P. Zanello, and A. W. Norman. 2004. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1α,25(OH)2-vitamin D3 in vivo and in vitro. *Mol. Endocrinol.* **18:** 2660–2671.
6. Yamada, S., M. Shimizu, and K. Yamamoto. 2003. Structure-function relationships of vitamin D including ligand recognition by the vitamin D receptor. *Med. Res. Rev.* **23:** 89–115.
7. Takeyama, K., Y. Masuhiro, H. Fuse, H. Endoh, A. Murayama, S. Kitanaka, M. Suzawa, J. Yanagisawa, and S. Kato. 1999. Selective interaction of vitamin D receptor with transcriptional coactivators by a vitamin D analog. *Mol. Cell. Biol.* **19:** 1049–1055.
8. Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell.* **83:** 835–839.
9. Lu, T. T., J. J. Repa, and D. J. Mangelsdorf. 2001. Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. *J. Biol. Chem.* **276:** 37735–37738.
10. Staudinger, J. L., B. Goodwin, S. A. Jones, D. Hawkins-Brown, K. I. MacKenzie, A. LaTour, Y. Liu, C. D. Klaassen, K. K. Brown, J. Reinhard, T. M. Willson, B. H. Koller, and S. A. Kliewer. 2001. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc. Natl. Acad. Sci. USA.* **98:** 3369–3374.
11. Xie, W., A. Radominska-Pandya, Y. Shi, C. M. Simon, M. C. Nelson, E. S. Ong, D. J. Waxman, and R. M. Evans. 2001. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc. Natl. Acad. Sci. USA.* **98:** 3375–3380.
12. Makishima, M., T. T. Lu, W. Xie, G. K. Whitfield, H. Domoto, R. M. Evans, M. R. Haussler, and D. J. Mangelsdorf. 2002. Vitamin D receptor as an intestinal bile acid sensor. *Science.* **296:** 1313–1316.
13. Choi, M., K. Yamamoto, T. Itoh, M. Makishima, D. J. Mangelsdorf, D. Moras, H. F. DeLuca, and S. Yamada. 2003. Interaction between vitamin D receptor and vitamin D ligands: two-dimensional alanine scanning mutational analysis. *Chem. Biol.* **10:** 261–270.

14. Adachi, R., A. I. Shulman, K. Yamamoto, I. Shimomura, S. Yamada, D. J. Mangelsdorf, and M. Makishima. 2004. Structural determinants for vitamin D receptor response to endocrine and xenobiotic signals. *Mol. Endocrinol.* **18:** 43–52.

15. Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science.* **284:** 1362–1365.

16. Makishima, M., J. Okabe-Kado, and Y. Honma. 1998. Growth inhibition and differentiation induction in human monoblastic leukaemia cells by 1α-hydroxyvitamin D derivatives and their enhancement by combination with hydroxyurea. *Br. J. Cancer.* **77:** 33–39.

17. Yamamoto, K., H. Masuno, M. Choi, K. Nakashima, T. Taga, H. Ooizumi, K. Umesono, W. Sicinska, J. VanHooke, H. F. DeLuca, and S. Yamada. 2000. Three-dimensional modeling of and ligand docking to vitamin D receptor ligand binding domain. *Proc. Natl. Acad. Sci. USA.* **97:** 1467–1472.

18. Rarey, M., B. Kramer, T. Lengauer, and G. Klebe. 1996. A fast flexible docking method using an incremental construction algorithm. *J. Mol. Biol.* **261:** 470–489.

19. Kaneko, E., M. Matsuda, Y. Yamada, Y. Tachibana, I. Shimomura, and M. Makishima. 2003. Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. *J. Biol. Chem.* **278:** 36091–36098.

20. Makishima, M., K. Shudo, and Y. Honma. 1999. Greater synergism of retinoic acid receptor (RAR) agonists with vitamin D3 than that of retinoid X receptor (RXR) agonists with regard to growth inhibition and differentiation induction in monoblastic leukemia cells. *Biochem. Pharmacol.* **57:** 521–529.

21. Glass, C. K., and M. G. Rosenfeld. 2000. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* **14:** 121–141.

22. Rochel, N., J. M. Wurtz, A. Mitschler, B. Klaholz, and D. Moras. 2000. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol. Cell.* **5:** 173–179.

23. Berger, U., P. Wilson, R. A. McClelland, K. Colston, M. R. Haussler, J. W. Pike, and R. C. Coombes. 1988. Immunocytochemical detection of 1,25-dihydroxyvitamin D receptors in normal human tissues. *J. Clin. Endocrinol. Metab.* **67:** 607–613.

24. Van Cromphaut, S. J., M. Dewerchin, J. G. J. Hoenderop, I. Stockmans, E. Van Herck, S. Kato, R. J. M. Bindels, D. Collen, P. Carmeliet, R. Bouillon, and G. Carmeliet. 2001. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc. Natl. Acad. Sci. USA.* **98:** 13324–13329.

25. Palmer, H. G., J. M. Gonzalez-Sancho, J. Espada, M. T. Berciano, I. Puig, J. Baulida, M. Quintanilla, A. Cano, A. G. de Herreros, M. Lafarga, and A. Munoz. 2001. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. *J. Cell Biol.* **154:** 369–388.

26. Makishima, M., Y. Kanatani, Y. Yamamoto-Yamaguchi, and Y. Honma. 1996. Enhancement of activity of 1α,25-dihydroxyvitamin D3 for growth inhibition and differentiation induction of human myelomonocytic leukemia cells by tretinoin tocoferil, an alpha-tocopherol ester of all-trans retinoic acid. *Blood.* **87:** 3384–3394.

27. Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, and J. M. Lehmann. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* **284:** 1365–1368.

28. Wang, H., J. Chen, K. Hollister, L. C. Sowers, and B. M. Forman. 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell.* **3:** 543–553.

29. Downes, M., M. A. Verdecia, A. J. Roecker, R. Hughes, J. B. Hogenesch, H. R. Kast-Woelbern, M. E. Bowman, J. L. Ferrer, A. M. Anisfeld, P. A. Edwards, J. M. Rosenfeld, J. G. Alvarez, J. P. Noel, K. C. Nicolaou, and R. M. Evans. 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. *Mol. Cell.* **11:** 1079–1092.

30. Mi, L. Z., S. Devarakonda, J. M. Harp, Q. Han, R. Pellicciari, T. M. Willson, S. Khorasanizadeh, and F. Rastinejad. 2003. Structural basis for bile acid binding and activation of the nuclear receptor FXR. *Mol. Cell.* **11:** 1093–1100.

31. Watkins, R. E., G. B. Wisely, L. B. Moore, J. L. Collins, M. H. Lambert, S. P. Williams, T. M. Willson, S. A. Kliewer, and M. R. Redinbo. 2001. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science.* **292:** 2329–2333.

32. Garland, C. F., F. C. Garland, and E. D. Gorham. 1999. Calcium and vitamin D. Their potential roles in colon and breast cancer prevention. *Ann. N.Y. Acad. Sci.* **889:** 107–119.

33. Palmer, H. G., M. Sanchez-Carbayo, P. Ordonez-Moran, M. J. Larriba, C. Cordon-Cardo, and A. Munoz. 2003. Genetic signatures of differentiation induced by 1α,25-dihydroxyvitamin D3 in human colon cancer cells. *Cancer Res.* **63:** 7799–7806.

34. Nagengast, F. M., M. J. Grubben, and I. P. van Munster. 1995. Role of bile acids in colorectal carcinogenesis. *Eur. J. Cancer.* **31A:** 1067–1070.

35. Narisawa, T., N. E. Magadia, J. H. Weisburger, and E. L. Wynder. 1974. Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N′-nitrosoguanidine in rats. *J. Natl. Cancer Inst.* **53:** 1093–1097.

36. Thummel, K. E., C. Brimer, K. Yasuda, J. Thottassery, T. Senn, Y. Lin, H. Ishizuka, E. Kharasch, J. Schuetz, and E. Schuetz. 2001. Transcriptional control of intestinal cytochrome P-4503A by 1α,25-dihydroxy vitamin D3. *Mol. Pharmacol.* **60:** 1399–1406.

37. Wong, H-L., A. Seow, K. Arakawa, H-P. Lee, M. C. Yu, and S. A. Ingles. 2003. Vitamin D receptor start codon polymorphism and colorectal cancer risk: effect modification by dietary calcium and fat in Singapore Chinese. *Carcinogenesis.* **24:** 1091–1095.

38. Guyton, K. Z., T. W. Kensler, and G. H. Posner. 2003. Vitamin D and vitamin D analogs as cancer chemopreventive agents. *Nutr. Rev.* **61:** 227–238.

39. Zanello, L. P., and A. W. Norman. 2004. Rapid modulation of osteoblast ion channel responses by 1α,25(OH)2-vitamin D3 requires the presence of a functional vitamin D nuclear receptor. *Proc. Natl. Acad. Sci. USA.* **101:** 1589–1594.

40. Kousteni, S., T. Bellido, L. I. Plotkin, C. A. O'Brien, D. L. Bodenner, L. Han, K. Han, G. B. DiGregorio, J. A. Katzenellenbogen, B. S. Katzenellenbogen, P. K. Roberson, R. S. Weinstein, R. L. Jilka, and S. C. Manolagas. 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell.* **104:** 719–730.

41. Abe, E., C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, and T. Suda. 1981. Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. USA.* **78:** 4990–4994.

42. Miyaura, C., E. Abe, T. Kuribayashi, H. Tanaka, K. Konno, Y. Nishii, and T. Suda. 1981. 1α,25-Dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells. *Biochem. Biophys. Res. Commun.* **102:** 937–943.

43. Mangelsdorf, D. J., H. P. Koeffler, C. A. Donaldson, J. W. Pike, and M. R. Haussler. 1984. 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *J. Cell Biol.* **98:** 391–398.

44. Zimber, A., A. Chedeville, C. Gespach, and J-P. Abita. 1994. Inhibition of proliferation and induction of monocytic differentiation in HL60 human promyelocytic leukemia cells treated with bile acids in vitro. *Int. J. Cancer.* **59:** 71–77.

45. Zimber, A., A. Chedeville, J-P. Abita, V. Barbu, and C. Gespach. 2000. Functional interactions between bile acids, all-trans retinoic acid, and 1,25-dihydroxy-vitamin D3 on monocytic differentiation and myeloblastin gene down-regulation in HL-60 and THP-1 human leukemia cells. *Cancer Res.* **60:** 672–678.