Bile Acids and Their Derivatives as Vitamin D Receptor Agonists:

Molecular Mechanism and Biological Actions

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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 (1α, $25(OH)_2D_3$). It regulates calcium homeostasis, immunity, cellular differentiation, and other physiological processes. Secondary bile acids such as lithocholic acid (LCA) were identified as endogenous VDR agonists. The LCA binds to VDR and induces expression of an enzyme that metabolizes itself and reduces its toxicity.

I determined amino acid residues of VDR required for its activation by 1α, $25(OH)_2D_3$ and LCA by generating VDR mutants predicted to modulate ligand response based on sequence homology to pregnane X receptor, another bile acid-responsive nuclear receptor. In both vitamin D response element activation and mammalian two-hybrid assays, I clarified molecular mechanisms underlying VDR activation mediated by 1α, $25(OH)_2D_3$ and LCA.

To elucidate the relationship between chemical structures of bile acids and their agonistic activities for VDR, I examined the effect of several LCA derivatives on VDR activation. I determined structural elements required for potent activation of VDR and identified compounds with more potent activity than LCA. LCA acetate is the most potent of these VDR agonists with selectivity. LCA acetate induced VDR target genes in intestinal cells. Unlike LCA, LCA acetate inhibited the proliferation of human monoblastic leukemia cells and induced their

differentiation. These results clearly demonstrated LCA acetate exhibits more potent efficacies in cancer cells relative to LCA.

Molecular and biological analyses on bile acids and their derivatives as VDR agonists should be useful in the development of ligands that selectively target VDR function in cancer and immune disorders without inducing adverse effects.

Abbreviations

AF	activation function
CAR	constitutive androstane receptor
CDCA	chenodeoxycholic acid
CYP	cytochrome P450 enzyme
DR	direct repeat
ER	everted repeat
ERα	estrogen receptor alpha
GST	glutathione S-transferase
HEK293	human embryonic kidney 293
FXR	farnesoid X receptor
LBD	ligand-binding domain
LBP	ligand-binding pocket
LCA	lithocholic acid
LUC	luciferase
LXR	liver X receptor
N-CoR	nuclear receptor co-repressor
NBT	nitroblue tetrazolium
NR	nuclear receptor

PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
SERM	selective estrogen receptor modulator
SRC-1	steroid receptor coactivator-1
VDR	vitamin D receptor
VDRE	vitamin D response element

General Introduction

Structure and function of nuclear receptors

Nuclear receptors (NRs) function as ligand-inducible transcription factors. They are engaged in many biological processes such as cell growth and differentiation, embryonic development, and metabolism (Mangelsdorf *et al.*, 1995). Forty-eight nuclear receptors have been identified in human (Germain *et al.*, 2006), many of which are still classified as orphan receptors because their ligands are unknown (Gallastegui *et al.*, 2015). The primary structures of NRs are highly conserved among the family with six subregions from A to F based on degree of homology (Krust *et al.*, 1986) (**Fig. 1**). The divergent amino terminal A/B region possess ligand-independent transactivation function (activation function 1; AF-1). The most conserved C region is the deoxyribonucleic acid (DNA)-binding domain (DBD). The short D region is called hinge domain that connects the DBD and the following E region. The part of C and D regions encodes the nuclear localization signal. The E region acts as the ligand-binding domain (LBD) that serves ligand-dependent transactivation function (activation function 2; AF-2). A few receptors possess the F region. Intensive structural biology efforts have been made to reveal NR conformations in regulating their signal transductions (Rastinejad *et al.*, 2013).

The carboxyl-terminal LBD plays a pivotal role in ligand-dependent transcriptional control. The transcription activity of NRs is regulated by small molecule ligands such as steroids, retinoids, fatty acids, and other lipid-soluble compounds. On ligand binding, NRs

undergo a conformational change in the cofactor binding site and AF-2 domain, a structural rearrangement that results in the dynamic exchange of cofactor complexes (Glass and Rosenfeld, 2000). In the absence of ligand, corepressors bind to the AF-2 surface, that is composed of portions of helix 3, loop 3 – 4, helix 4, 5, and 11, and mediate silencing of gene transcription. Binding of a ligand mediates conformational changes; it alters the AF-2 surface by repositioning helix 12, reduces the binding affinity for corepressors and increases the affinity for coactivators, resulting in the stimulation of transcription (**Fig. 2**). These cofactors form complexes with transcription factors that induce chromatin remodeling or recruitment of the basal transcription machinery. These interactions allow NRs to modulate transcription of specific target genes and mediate dramatic changes of the transcription status within cells.

Though all NRs ultimately act to increase or decrease gene transcription, some (e.g., glucocorticoid receptor) reside primarily in the cytoplasm, whereas others (e.g., thyroid hormone receptor) are always located in the nucleus. After ligand binding, the cytoplasmically localized NRs translocate to the nucleus. The localization of NRs is controlled through the nuclear localization signal located between the C and D regions.

The DBD, consisting of two zinc fingers, contacts specific DNA recognition sequences in target genes. Most NRs bind to DNA as dimers. Consequently, each monomer recognizes an individual DNA motif, referred to as a "half-site". The steroid receptors, including

the glucocorticoid, estrogen, progesterone, and androgen receptors, bind to DNA as homodimers. Consistent with this twofold symmetry, their DNA recognition half-sites are palindromic. The thyroid, retinoid, peroxisome proliferator activated, and vitamin D receptors bind to DNA preferentially as heterodimers in combination with retinoid X receptors (RXRs). Their DNA half-sites are arranged as direct repeats (DRs).

NR family as drug target

Certain disease states are associated with defective regulation of gene transcription. A well-known instance is that, in promyelocytic leukemia, fusion of retinoic acid receptor (RAR) α to other nuclear proteins causes aberrant gene silencing and prevents normal cellular differentiation (de The *et al.*, 1990; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Pandolfi *et al.*, 1991; Chang *et al.*, 1992). Treatment with retinoic acid reverses this repression and allows cellular differentiation and apoptosis to occur (Fenaux and Degos, 1997). Not only the steroid hormone NRs such as estrogen receptor (Heldring *et al.*, 2007) and glucocorticoid receptor (Sundahl *et al.*, 2015), but also RXR-partnered NRs such as peroxisome proliferator-activated receptor (PPAR) γ (Spiegelman, 1998) have been intensively studied for their biological functions and their ligands have been under development or clinically used in diverse disease conditions (Evans and Mangelsdorf, 2014). Analyses of the gene-family distribution of targets

by drug substance for both small-molecule and biological drugs revealed that more than 50% of drugs target only four key gene families: class I G protein-coupled receptors (GPCRs), NRs, ligand-gated ion channels, and voltage-gated ion channels (Overington *et al.*, 2006). Collectively, NRs are considered to be a viable drug target class and is under active investigations.

NR modulators

Because many NRs play pivotal roles in diverse diseases, their synthetic ligands have been under active investigations to generate drugs either mimic the action of typical endogenous ligands (agonists) or block the action of endogenous ligands (antagonists). In order to design more suitable drugs with reduced side effects, selective modulators for NRs have been studied (Smith and O'Malley, 2004; Burris *et al.*, 2013). Selective NR modulators function as tissue and/or target gene selective ligands by exhibiting agonist, antagonist, or inverse agonist activity through interaction with coregulators.

The well-known examples of tissue-selective modulators were those for estrogen receptors, which are considered as a model case in studying NR modulators. Tamoxifen works as an antagonist in breast tissue but as an agonist in bone and uterus. Raloxifen was discovered as a selective estrogen receptor modulator (SERM) that is used in the prevention of osteoporosis

in postmenopausal women with reduced agonistic activity in endometrial growth in comparison to tamoxifen (Gottardis *et al.*, 1990). The structural analyses have revealed their distinctive linkage between structure and functions (**Fig. 2**). X-ray crystallography shows that various SERMs induce distinct estrogen receptor conformations (Heldring *et al.*, 2007). The tissue-specific responses caused by these agents in breast, bone, and uterus appear to reflect distinct interactions with coactivators. Moreover, there are examples of gene-selective modulators that exhibit target-gene selective actions within a single cell type (Bramlett and Burris, 2003; Quinet *et al.*, 2004), the mechanism of which cannot be ascribed to differential expression of cofactor proteins or receptor subtypes. Thus, diverse types of NR modulators have been studied for pursuing opportunities to develop novel pharmaceutical entities.

Vitamin D receptor

The vitamin D receptor (VDR [NR1I1]) is a member of the NR superfamily, which was originally identified as a receptor for the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α, $25(OH)_2D_3$] (**Fig. 3A**) (Baker *et al.*, 1988). 1α, $25(OH)_2D_3$ influences transcription of specific genes and this is mediated through the direct binding of the 1α, $25(OH)_2D_3$ to VDR/RXR heterodimeric complex (**Fig. 3B**). Activation of VDR results in the induction of the target genes, nearby which specific DNA sequences exist. This pathway is

called the genomic action of 1α, 25$(OH)_2D_3$. On the other hand, rapid signal transduction responses to 1α, 25$(OH)_2D_3$ have been observed within seconds to minutes, resulting in transcaltachia, insulin secretion, or ion channel regulation (Haussler *et al.*, 2011), which are called non-genomic actions. These are suggested to be mediated by plasma membrane-bound VDR.

VDR regulates many physiological processes including cell growth and differentiation, embryonic development, and metabolic homeostasis (Yamada *et al.*, 2003a). It has been reported that VDR binds more than 2700 sites in human genome (Ramagopalan *et al.*, 2010) and regulates over 900 genes (Wang *et al.*, 2005), reflecting its pleiotropic functions. Typically, target genes that have been studied include those for calbindin D_{9K} (Cao *et al.*, 2002), calbindin D_{28K} (Gill and Christakos, 1993), osteocalcin (Kerner *et al.*, 1989; Morrison *et al.*, 1989; Ozono *et al.*, 1990), *Cyp24A1* (Zierold *et al.*, 1995), transient receptor potential vanilloid type 6 (*TRPV6*) (Meyer *et al.*, 2006), *Rankl* (Kim *et al.*, 2006), and *CDKN1A* (*p21*) (Liu *et al.*, 1996). The genes for *Cyp27b1* (Murayama *et al.*, 1999) and *PTH* (Demay *et al.*, 1992) are reported to be regulated in negative ways. These target genes highlight the important roles of VDR in vitamin D metabolism, calcium homeostasis, and cell cycle regulation.

The active form of vitamin D, 1α, 25$(OH)_2D_3$, regulates calcium metabolism, cellular differentiation, and immunity through VDR activation (Bouillon *et al.*, 1995; Haussler *et al.*,

1998). Vitamin D is a secosteroid, in which the B ring of steroid structure is ruptured (Yamada *et al.*, 2003b). Ultraviolet irradiation induces a photochemical reaction of 7-dehydrocholesterol, which is synthesized from acetyl-coenzyme A and is a precursor of cholesterol, to produce the secosteroid vitamin D_3 in the skin. Vitamin D_3 is hydroxylated at the 25 position by vitamin D_3 25-hydroxylase (cytochrome P450 enzyme (CYP)27A1) in the liver to yield 25-hydroxyvitamin D_3, the major form of vitamin D in the circulation. The 25-hydroxyvitamin D_3 is further hydroxylated in the 1α position by CYP27B1. This reaction is tightly regulated and occurs exclusively in the kidney to yield the active metabolite, 1α, 25$(OH)_2D_3$.

1α, 25$(OH)_2D_3$ 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* (Brown *et al.*, 1999). The administration of 1α, 25$(OH)_2D_3$ or its analogs has therapeutic effects in mouse models of malignancies such as leukemia and colon cancer (Honma *et al.*, 1983; Kumagai *et al.*, 2003). Although vitamin D and its synthetic analogs have been used for the treatment of bone and skin disorders, clinical application to the management of cancer and leukemia has been limited by their adverse effects, especially hypercalcemia (Bouillon *et al.*, 1995). Therefore, development of synthetic vitamin D analogs that have efficient growth-inhibitory activity with low calcemic activity is underway in pharmaceutical point of view (Guyton *et al.*, 2003). Overall, modulating

the intracellular VDR activity would be beneficial for the treatment of cancer, infectious diseases, autoimmune disease, cardiovascular diseases, and androgenic alopecia. Three-dimensional modeling and the solution of a VDR crystal structure have yielded valuable insight into the mode of binding of 1α, 25$(OH)_2D_3$ in the VDR-ligand binding pocket (LBP) (Norman *et al.*, 1999; Rochel *et al.*, 2000; Yamamoto *et al.*, 2000).

Development of VDR agonists

Synthetic vitamin D analogs have been used successfully in the treatment of bone and skin disorders. However, their adverse effects, including hypercalcemia, bone resorption, and soft tissue calcification, limit the clinical application of VDR agonists in the management of malignant tumors and immune disorders (Bouillon *et al.*, 1995). The need for VDR ligands with potent anticancer activity that lack adverse effects on calcium metabolism has led to a major synthetic chemistry effort (Nagpal *et al.*, 2005). Extensive researches have been made for development of potential VDR modulators. Several analogs exhibit efficient anti-proliferation and pro-differentiation activities with fewer calcemic side effects than 1α, 25$(OH)_2D_3$, but the underlying molecular mechanism of this functional specificity is still not understood (Bouillon *et al.*, 1995). Structure-function analyses of vitamin D analogs suggest that these secosteroids also act on a membrane receptor and that adverse effects are at least partly due to a poorly

characterized non-genomic mechanism of action (Norman *et al.*, 2001). There are the examples of VDR modulators that escaped their calcemic actions via modulation on the pharmacokinetic exposures (Burris *et al.*, 2013). Alfacalcidol (alpha D3) is a prodrug that is enzymatically converted to the active form 1α, 25$(OH)_2D_3$ in the liver through the metabolism by 25-hydroxylase. Thus, alfacalcidol exhibits its tissue-selectivity after the metabolic activation without affecting the enterocytes found in the small intestine. Alfacalcidol has been developed for the treatment of osteoporosis with less calcemic activity (Menczel *et al.*, 1994). Topical maxacalcitol has been clinically developed for the treatment of psoriasis vulgaris (Barker *et al.*, 1999). It is another example of VDR modulator that exhibited pharmacokinetic tissue selectivity with reduced binding to the serum vitamin D binding protein and its application has been approved for secondary hyperparathyroidism in patients (Brown *et al.*, 2002). In the medicinal chemistry efforts for pursuing the VDR modulators, Ro-26-9228 was identified that exhibits cell-type selective action and has a bone-protecting effect without inducing hypercalcemia with preferred gene regulation effects on osteoblasts over intestinal cells (Ismail *et al.*, 2004). Another example of tissue-selective VDR modulator is 2MD. It displays potent stimulating effects on bone formation with a selective activity on bone over intestine (Sicinski *et al.*, 1998). However, vitamin D analogues that possess cellular or tissue selectivity caused by distinctive transcriptional regulation have not been fully understood, while tissue selective estrogen

receptor modulators have been discovered and well characterized (Heldring *et al.*, 2007). Thus, multiple vitamin D derivatives have been in active research and development.

Researches seeking synthetic VDR agonists are also under active investigations by exploring a chemical space that is different from vitamin D_3. Non-secosteroidal VDR agonists have been subject to major medicinal chemistry efforts in pharmaceutical industries. Non-secosteroidal VDR ligands have been reported with less calcium mobilization side effects than $1\alpha,25(OH)_2D_3$ (Boehm *et al.*, 1999). As examples, LY2108491 and LY2109866 have been reported to function as potent and efficacious agonists to VDR in multiple cell types but exhibit attenuated transcriptional activity in intestinal cells (Ma *et al.*, 2006). VDRM2 has a nonsecosteroidal scaffold and acts as a tissue-selective and orally bioavailable VDR ligand that induces the expression of the bone genes. Animal experiments demonstrated its wider safety margin between bone efficacy and hypercalcemia (Sato *et al.*, 2010). Although these are the examples of VDR modulator with distinctive cell/tissue selective actions, they are still in preclinical investigations.

VDR as a bile acid sensor

It has been reported that VDR has dual functions as an endocrine receptor for 1α, $25(OH)_2D_3$ and as a metabolic sensor for bile acids (Makishima *et al.*, 2002). NRs belonging to

the NR1H and NR1I subfamilies, including VDR, have been shown to control cholesterol and bile acid metabolism (Lu *et al.*, 2001). 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 the feedback mechanism in the liver bile acid synthesis from cholesterol. Bile acids produced in the liver, called primary bile acids, are excreted in bile after conjugation with taurine and glycine, and most of them are reabsorbed in the intestine. Bile acids that escape reabsorption are converted to the secondary bile acids by the intestinal microflora. Pregnane X receptor (PXR, also called as steroid and xenobiotic sensing nuclear receptor (SXR); NR1I2), which acts as a receptor for various xenobiotics, responds to the secondary bile acids and induces their metabolism in the liver. Like other members from NR1H and NR1I subfamilies, VDR was also found to function as a receptor for the secondary bile acids such as lithocholic acid (LCA) (**Fig. 3A**) and be involved in bile acid metabolism by inducing detoxyfication mechanism for the toxic LCA in the liver and intestine (Makishima *et al.*, 2002).

These findings provided a rationale for the development of novel VDR ligands derived from bile acids and from other non-secosteroid compounds that regulate non-canonical VDR functions (Choi and Makishima, 2009). To develop new VDR ligands as therapeutic agents, it is

crucial to separate the desired biological functions of 1α, $25(OH)_2D_3$ that exhibits various functions including calcium regulation, cell differentiation, anti-proliferation, and immune modulation.

Diverse VDR analogs created an additional cavity in the VDR-LBP to accommodate their side chains and thus changed the structure of the LBP. Intriguingly, the VDR ligands acted as an agonist, a partial agonist, or an antagonist depending on the structure of the side chain (Yamamoto *et al.*, 2014). These results demonstrate that modifications of the VDR ligands change the pocket structure and provide a new perspective for the development of VDR modulators that exhibit a specific biological activity. In terms of development of pharmaceutical agents for modulating VDR activity, changing the chemical scaffold could provide novel chemical spaces and generally could have large impacts (Böhm *et al.*, 2004) on "chemotype"-related risks. Identifying the optimal starting point is very important to commence chemical optimization campaigns. Thus, bile acid-derived VDR agonists could be an alternative ligands to mitigate the limitations observed for 1α, $25(OH)_2D_3$ and its derivatives. In addition, it is of note that VDR acts as an anticancer transcription factor by upregulating expression of detoxifying enzymes for LCA that cause cancer whereas a physiological link between bile acids and calcium homeostasis has not been clarified. Thus, I hypothesize that bile acid-derived VDR

ligands may provide novel opportunities for development of VDR ligands and function as selective VDR modulators without causing hypercalcemia.

Objectives of this research

The aims of this research are to deeply analyze the interaction between the VDR-LBP and its ligands such as 1α, 25$(OH)_2D_3$ and bile acids, provide the fundamental information regarding the relationship between VDR structure and its function, and develop novel VDR ligands that could lead to efficient VDR-targeting therapy.

Part 1

Structural determinants for vitamin D receptor response to endocrine and xenobiotic signals

ABSTRACT

VDR responds to endocrine and xenobiotic signals that are mediated by $1\alpha,25(OH)_2D_3$ and secondary bile acids such as LCA, respectively, and regulates diverse physiological processes. To identify structural determinants required for VDR activation by $1\alpha,25(OH)_2D_3$ and LCA, I generated VDR mutants predicted to modulate ligand response based on sequence homology to PXR, another bile acid-responsive nuclear receptor. In both vitamin D response element activation and mammalian two-hybrid assays, I found that a VDR mutant with S278V is activated by $1\alpha,25(OH)_2D_3$ but not by LCA, whereas another VDR mutant with S237M can respond to LCA but not to $1\alpha,25(OH)_2D_3$. Based on mutagenesis data, I propose a docking model for LCA binding to VDR-LBP in which LCA interacts with amino acids of VDR in a different mode from $1\alpha,25(OH)_2D_3$. Comparative analysis of the VDR-LCA and VDR-$1\alpha,25(OH)_2D_3$ structure-activity relationships should be useful in the development of bile acid-derived synthetic VDR ligands that selectively target VDR function in cancer and immune disorders without inducing adverse hypercalcemic effects.

INTRODUCTION

VDR functions as a receptor for a number of secondary bile acids including LCA (Makishima *et al.*, 2002). LCA is also a weak agonist for the nuclear receptors FXR and PXR. The primary bile acids, cholic acid and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the liver and secreted in bile as glycine or taurine conjugates (Nagengast *et al.*, 1995). After assisting in the digestion and intestinal absorption of lipids and fat-soluble vitamins, including dietary vitamin D, the majority of bile acids are reabsorbed and returned to the liver through the enterohepatic circulation. Bile acids that escape reabsorption in the ileum are converted to the secondary bile acids deoxycholic acid and LCA by intestinal microflora. While FXR serves as a sensor for both primary and secondary bile acids (Makishima *et al.*, 1999a; Parks *et al.*, 1999; Wang *et al.*, 1999), PXR and VDR are selectively activated by secondary bile acids (Staudinger *et al.*, 2001; Xie *et al.*, 2001; Makishima *et al.*, 2002). PXR, which shows the highest sequence identity with VDR, responds to steroid hormone metabolites and xenobiotics, but not to $1\alpha,25(OH)_2D_3$ (Blumberg *et al.*, 1998; Lehmann *et al.*, 1998). The crystal structures of the VDR and PXR LBDs reveal strong structural conservation (Rochel *et al.*, 2000; Watkins *et al.*, 2001). In this study, I compared the LBD structures of VDR and PXR, generated multiple VDR point mutants, and analyzed their responses to $1\alpha,25(OH)_2D_3$ and LCA. I also examined

the LCA responses of VDR mutants that are observed in patients of vitamin D-resistant rickets (Haussler *et al.*, 1998) to gain insights into the potentially different functions of $1\alpha,25(OH)_2D_3$ and LCA. These experiments led to the identification of VDR mutants that selectively respond to $1\alpha,25(OH)_2D_3$ or LCA. Computational docking analysis was utilized to model the structural requirement for the mutated residues in ligand discrimination. Identification of critical residues for response to endocrine and bile acid ligands should aid the development of VDR agonists with improved pharmacological specificity.

MATERIALS AND METHODS

Chemical compounds

1α,25$(OH)_2D_3$ and LCA were obtained from Calbiochem (San Diego, CA) and Nacalai (Kyoto, Japan), respectively.

Graphical manipulation and docking

Graphical manipulations were performed using SYBYL 6.7 (Tripos, St. Louis, MO) (Yamamoto *et al.*, 2000; Choi *et al.*, 2001). The atomic coordinates of the crystal structures of hVDR-LBD (Δ165-215) (1DB1) and hPXR-LBD (1ILH) were retrieved from the Protein Data Bank. LCA was docked into VDR and PXR using the docking software FlexX (version 1.11.0) (Rarey *et al.*, 1996). The active site (in the case of NRs, the LBP) was defined to include all amino acids within 6.5 Å of the cocrystallized ligand.

Plasmids

A fragment of hVDR (GenBank Accession # J03258) was inserted into pCMX-flag vector to make pCMX-VDR (Willy and Mangelsdorf, 1997; Makishima *et al.*, 2002). The LBD

of hVDR was inserted into pCMX-GAL4 vector to make pCMX-GAL4-VDR, and full-length hVDR was inserted into pCMX-VP16 vector to make pCMX-VP16-VDR (Makishima *et al.*, 2002). In the mammalian two-hybrid assay, the NR-interacting fragment of SRC-1 exhibited more robust ligand-dependent interaction with VDR than full-length SRC-1. Therefore, nuclear hormone receptor-interacting domains of SRC-1 (amino acid 595-771; GenBank Accession # U90661) and N-CoR (amino acid 1990-2416; # U35312) were inserted into pCMX-GAL4 vector for pCMX-GAL4-SRC-1 and pCMX-GAL4-N-CoR, respectively. Mutations were introduced into pCMX-VDR, pCMX-GAL4-VDR and pCMX-VP16-VDR using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). VDR-responsive hCYP3A4-everted repeat (ER)-6x3-tk-luciferase (LUC) and GAL4-responsive MH100 (upstream activating sequence; UAS)x4-tk-LUC reporters were utilized (Willy and Mangelsdorf, 1997; Makishima *et al.*, 2002). All plasmids were sequenced prior to use to verify DNA sequence accuracy.

Cell culture and cotransfection assay

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and Antibiotic-Antimycotic (Nacalai) at 37ºC in a humidified atmosphere of 5% CO_2 in air. Transfections were performed by the calcium phosphate coprecipitation assay as previously described (Lu *et al.*, 2000). Eight

hours after transfection, cells were treated with ligands. Then, cells were harvested 16~20 hr after the treatment and luciferase and β-galactosidase activities were measured using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). DNA cotransfection experiments included 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 for a total of 150 ng of DNA per well in a 96-well plate. Luciferase data were normalized to an internal β-galactosidase control and represent the mean (± standard deviation) of triplicate assays.

Ligand binding assay

LBDs of hVDR and its mutants were cloned into the glutathione S-transferase (GST)-fusion vector pGEX-4T1 (Amersham, Piscataway, NJ). GST-VDR fusion proteins were expressed in BL21 DE3 cells (Promega, Madison, WI) and purified with glutathione sepharose beads (Amersham). Competitive ligand binding assay was performed by modification of previous reports (Nakajima *et al.*, 1994; Solomon *et al.*, 2001). Briefly, 500 ng of GST fusion proteins were bound to glutathione sepharose and incubated with [26,27-methyl-^{3}H] 1α,25$(OH)_2D_3$ (Amersham) in the presence or absence of nonradioactive ligand in a buffer (10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA), 300 mM KCl, 1 mM dithiothreitol, 10% glycerol) for 3 hr at 4°C. After washing

twice, the protein and bound 1α,25(OH)$_2$D$_3$ was resuspended in 200 µl of the binding buffer and 150 µl was assessed by liquid scintillation counting.

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.04 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

RESULTS

Comparison of LBDs of VDR and PXR

Since hVDR and hPXR share significant amino acid identity (44% in the LBDs) and PXR is responsive to LCA but not 1α,25(OH)$_2$D$_3$, I hypothesized that substitution of VDR residues with PXR amino acids at critical LBP positions which make hydrogen bonds with the hydroxyl groups of 1α,25(OH)$_2$D$_3$ might yield LCA-selective mutants. The VDR-1α,25(OH)$_2$D$_3$ co-crystal shows that the 1α-hydroxyl group of 1α,25(OH)$_2$D$_3$ contacts S237 and R274, the 3β-hydroxyl group is coordinated by S278 and Y143, and the 25-hydroxyl group makes hydrogen bonds to H397 and H305 (Rochel *et al.*, 2000). These amino acids, except for Y143, are conserved among human, mouse, rat, and chicken VDRs (**Fig. 4A**). Y143 is replaced with phenylalanine in cVDR, which is identical to the corresponding amino acid in

PXRs. R274 in hVDR is conserved in VDRs and PXRs, and H397 of hVDR is identical among other VDRs and hPXR. The alignment indicates that S237, S278, and H305 are unique to VDRs and that PXR has other amino acids at these positions. Although the Cα backbone of the VDR and PXR structures are very similar, PXR possess a flexible loop between the β-sheet and H7 instead of the H6 found in VDR, which is responsible for PXR's ability to respond to diverse ligands (Watkins *et al.*, 2001) unlike VDR (**Fig. 4B** and **C**). In addition, the crystal structures defined for the VDR-LBD (Δ165-215) and the PXR-LBD (Rochel *et al.*, 2000; Watkins *et al.*, 2001) indicate that small differences in the amino acids of LBD cause significant change in the shape of the LBP (**Fig. 4**). These findings suggest that mutational analysis of VDR based on an amino acid alignment and structural comparison with PXR should be very useful in elucidating the structure-function relationship of VDR and its ligands.

Functional analysis of VDR mutants

The structure-activity relationships for NRs and ligands have been elucidated by experiments based on site-directed mutagenesis. Replacement of a LBP residue with the corresponding residue of a closely related receptor was utilized to analyze PPARs and estrogen-related receptor-γ (Takada *et al.*, 2000; Greschik *et al.*, 2002). Alanine scanning mutagenesis was used to analyze the interaction of VDR with several vitamin D analogs and

bile acids (Choi *et al.*, 2003). To further analyze structure-activity relationships between VDR and two different natural ligands (1α,25(OH)$_2$D$_3$ and LCA), I replaced ligand-coordinating residues with the corresponding PXR amino acids. I generated hVDR mutants of Y143 and S278, which make hydrogen bonds with the 3-hydroxyl group of 1α,25(OH)$_2$D$_3$, and that of S237, which interacts with the 1α-hydroxyl group of 1α,25(OH)$_2$D$_3$. These amino acids were changed to the corresponding amino acids of hPXR (**Fig. 4A**) or alanine. Vitamin D-resistant rickets-associated mutants of VDR (R274L and H305Q) were also examined. Since F288 of hPXR contributes to structural differences between VDR and PXR (Watkins *et al.*, 2001), the corresponding amino acid in VDR (S275) was replaced with either phenylalanine or alanine (S275F and S275A). The crystal structure of VDR-LBD was determined in a deletion mutant (Δ165-215), because a long flexible loop between helices 1 and 3 prevents the preparation of stable crystals (Rochel *et al.*, 2000). This loop does not seem to contribute to ligand interaction, since VDR (Δ165-215) can be transactivated by 1α,25(OH)$_2$D$_3$, its synthetic analogs, and LCA as efficiently as wild-type VDR (Rochel *et al.*, 2001; Choi *et al.*, 2003).

I examined ligand-responsive transcriptional activation by the full-length VDR point mutants. HEK 293 cells were transfected with wild-type VDR or VDR mutants and a luciferase reporter containing a VDR-responsive element called everted repeat (ER)-6 from the CYP3A4 promoter (**Fig. 5A**). Because kidney-derived HEK293 cells express endogenous VDR, the

addition of 1α,25(OH)$_2$D$_3$ or LCA basally induced luciferase activity. Transfection of wild-type VDR effectively increased induction by both ligands. Y143A, Y143F, S278A, and S278V are the VDR mutants of Y143 or S278 that coordinate the 3-hydoxyl group of 1α,25(OH)$_2$D$_3$. They were activated by 1α,25(OH)$_2$D$_3$ but not by LCA. S237A and S237M are the VDR mutants of S237 that coordinate the 1α1-hydroxcyl group of 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ weakly activated S237A and had no effect on S237M activity, while LCA activated both mutants. These data suggested that the responses of VDR to 1α,25(OH)$_2$D$_3$ and LCA are changed by the mutations of the amino acid residues S237, Y143, and S278. The rickets-causing mutant R274L was unresponsive to both 1α,25(OH)$_2$D$_3$ and LCA, while another rickets-causing mutant H305Q exhibited the response to 1α,25(OH)$_2$D$_3$ but lost that to LCA, suggesting that R274L and H305Q mutants differently respond to LCA. S275A maintained responsiveness to 1α,25(OH)$_2$D$_3$ and LCA, but the S275F mutation abolished ligand response, suggesting that the substitution of S275 with a bulky phenylalanine hinders the ligand responses. The basal luciferase activity induced by 1α,25(OH)$_2$D$_3$ was repressed by transfection of S237M, R274L and S275F. This may be due to dominant negative effects of these mutants on endogenous VDR activity through sequestration of RXR or cofactors and competitive binding to the vitamin D response element (VDRE). The responses to LCA and 1α,25(OH)$_2$D$_3$ for S237A, S275A, and S278A mutants are consistent with a previous report utilizing a reporter with a direct repeat

(DR)-3 element from the osteopontin promoter (Choi *et al.*, 2003). In that study, Y143A did not respond to 10 nM 1α,25$(OH)_2D_3$, wheares I observed the response of Y143A to 1α,25$(OH)_2D_3$. This discrepancy may be due to the difference in ligand concentration or the response element construct tested. To evaluate the effects of mutations on the VDR responses without interference from the endogenous VDR, the following experiments were conducted using different assay systems.

Upon ligand binding, NRs undergo a conformational change that results in the dissociation of corepressors such as nuclear receptor corepressor (N-CoR) and recruitment of coactivators such as steroid receptor coactivator 1 (SRC-1) (Glass and Rosenfeld, 2000). Ligand-inducible cofactor recruitment was monitored to further examine ligand-response by VDR mutants in the mammalian two-hybrid assay using GAL4-SRC-1 receptor-interacting domain, containing the three LXXLL motifs, and VDR fused to the transactivation domain of herpesvirus VP16 protein. 1α,25$(OH)_2D_3$ induced association of SRC-1 strongly with wild-type VDR, Y143F, S275A and S278A, and moderately with Y143A, S237A, S278V, and H305Q. LCA was able to induce association of SRC-1 with wild-type VDR, Y143F, S237A, S237M, S275A (**Fig. 5B**). Compared to wild-type VDR, mutation of Y143 with phenylalanine (Y143F) retained both the responses to LCA and 1α,25$(OH)_2D_3$. Mutation with alanine (Y143A) eliminated the response to LCA, while it retained the 1α,25$(OH)_2D_3$ response, suggesting the

crucial role of Y143 for LCA response. Mutation of S237 with alanine (S237A) reduced the response to 1α,25$(OH)_2D_3$ but not to LCA. The bulky S237M mutation abolished the response to 1α,25$(OH)_2D_3$ but not to LCA, suggesting the crucial role of S237 for the 1α,25$(OH)_2D_3$ response. Mutation of S278 with alanine or valine abolished the response to LCA with retained response to 1α,25$(OH)_2D_3$, suggesting the crucial role of S278 for the LCA response. The S275A mutation did not affect the responses to LCA and 1α,25$(OH)_2D_3$, while more bulky S275F abolished both the responses, suggesting S275F hindered the conserved interactions for LCA and 1α,25$(OH)_2D_3$.

The mammalian two-hybrid assay using the GAL4-N-CoR chimeric corepressor showed 1α,25$(OH)_2D_3$-dependent dissociation of N-CoR from wild-type VDR, Y143A, Y143F, S237A, S275A, S278A, S278V, and S305Q, as well as LCA-dependent dissociation from wild-type VDR, Y143F, S237A, S237M, S275A, S278A, and H305Q (**Fig. 5C**). The dissociations of N-CoR from VDR and associations of SRC-1 with VDR were consistent except that LCA dissociated N-CoR from S278A and H305Q mutants. Difference in sensitivity of these assays may account for differential responsiveness of VDR mutants to ligands. The data indicate that S237 is more critical for induction by 1α,25$(OH)_2D_3$ and that Y143 and S278 are more important for LCA activation.

To exclude the possibility that R274L and S275F were not expressed or not able to associate with RXR, the functional expression of VDR mutants were examined in the cells using VP16-VDR chimeric mutants together with the VDRE-containing reporter. The luciferase activity gives an indirect measure of the protein expression levels of transfected receptors because VP16 chimeric receptors exhibit ligand-independent activity. All of the VDR mutants showed luciferase activities similar to wild-type VDR (**Fig. 5D**), indicating similar expression of functional VDR mutants in the cells.

The data shown in **Fig. 5** suggest that S278V and S237M respond selectively to $1\alpha,25(OH)_2D_3$ and LCA, respectively. To further investigate the preference of these VDR mutants for the ligands, dose-response curves were analyzed with the GAL4-VDR system, which eliminates the activity of endogenous VDR (**Fig. 6**). S278A was activated by $1\alpha,25(OH)_2D_3$ as effectively as wild-type VDR. The S278V mutation partially decreased $1\alpha,25(OH)_2D_3$ response. S237M completely abolished $1\alpha,25(OH)_2D_3$ response, while S237A had a more moderate effect (**Fig. 6A**). Importantly, LCA was able to activate S237M as effectively as S237A (**Fig. 6B**). LCA was unable to activate S278V and weakly activated S278A (**Fig. 6B**). The data indicate that the S278V mutant is unresponsive to LCA and the S237M mutant loses $1\alpha,25(OH)_2D_3$ response. Therefore, S278V selectively responds to $1\alpha,25(OH)_2D_3$ while S237M is specifically activated by LCA.

I examined direct binding of ligands to mutant VDRs *in vitro*. Isotopically-labeled $1\alpha,25(OH)_2D_3$ was incubated with GST-VDR proteins in the presence or absence of excess unlabeled $1\alpha,25(OH)_2D_3$ and specific binding of $1\alpha,25(OH)_2D_3$ was calculated. $1\alpha,25(OH)_2D_3$ effectively bound to wild-type VDR and S278V but only interacted weakly with S237M (**Fig. 7A**). $1\alpha,25(OH)_2D_3$ did not bind to GST control protein. The binding of labeled $1\alpha,25(OH)_2D_3$ to wild-type VDR and the S278V mutant was significantly reduced by addition of unlabeled $1\alpha,25(OH)_2D_3$ (**Fig. 7B**). Addition of unlabeled LCA inhibited the interaction of $1\alpha,25(OH)_2D_3$ with wild-type VDR at both 30 μM and 100 μM and with S278V at 100 μM, indicating that LCA binds more potently to wild-type VDR proteins than S278V.

Docking models of VDR interacting with LCA

To reveal the molecular basis for the mutated residues in mediating ligand specificity, I generated docking models. As shown by the 1α, $25(OH)_2D_3$ model and the VDR crystal structure, S237 makes a hydrogen bond with the 1α-hydroxyl group, Y143 and S278 interact with the 3-hydroxyl group, and H305 interacts with the 25-hydroxyl group (Rochel *et al.*, 2000) (**Fig. 8A**). The S237M mutation loses the ability to interact with $1\alpha,25(OH)_2D_3$ (**Fig. 5** and **6**). The model shows that a methionine residue at position 237 is too close to the 1α-hydroxyl group of $1\alpha,25(OH)_2D_3$ and would be expected to destabilize binding (**Fig. 8B**). Y143F and

S278V do not affect activation by 1α,25$(OH)_2D_3$. This may be due to the relatively conservative substitutions in these mutants and an apparent weak contribution of contacts between VDR and the 3-hydroxyl group of 1α,25$(OH)_2D_3$ to overall binding energy. S275F mutation causes a marked change in the VDR-LBP conformation such that it is unable to accommodate 1α,25$(OH)_2D_3$ (**Fig. 8B**). The mutational analysis in this study confirms the importance of contacts with the 1α-hydroxyl group of 1α,25$(OH)_2D_3$ in ligand binding and receptor activation.

Because no crystal structure data was available for VDR bound to secondary bile acid ligands when the study was conducted, a docking model was generated for LCA in the VDR-LBP (**Fig. 8C**). The carboxyl group of the LCA side chain is positioned within hydrogen bond distance of H305. Residues Y143 and S278 weakly interact with the 3-hydroxyl group of LCA. Mutation of H305 to glutamine decreased LCA response (**Fig. 5**), indicating that contacts of Y143 and S278 with LCA are insufficient for VDR activation. The S278V mutation abolished LCA response, although S278A maintained LCA response (**Fig. 6**). A valine residue at position 278 would be expected to sterically interfere with the 3-hydroxyl group of LCA, an unfavorable interaction that would be absent in S278A (**Fig. 8D**). Although the Y143F mutant was responsive, Y143A was not activated by LCA (**Fig. 5**). The conservative tyrosine to phenylalanine mutation maintains a large aromatic amino acid, while the alanine mutation is expected to more severely alter the VDR-LBP conformation. S237M mutation abolished

1α,25$(OH)_2D_3$ response, but did not affect activation by LCA (**Fig. 6**). As predicted by the docking model, S237 does not interact with LCA and the S237M mutation has little effect on LCA activity. S275F prevents both LCA and 1α,25$(OH)_2D_3$ from docking in the VDR-LBP (**Fig. 8D**). Taken together, these data indicate that LCA and 1α,25$(OH)_2D_3$ make non-overlapping critical interactions with residues of the VDR-LBP.

DISCUSSION

VDR (NR1I1) belongs to the NR1I subfamily along with PXR (NRI12) and constitutive androstane receptor (CAR; NR1I3) (Maglich *et al.*, 2001). PXR is activated by a broad variety of compounds such as xenobiotics, steroid derivatives and bile acids (Blumberg *et al.*, 1998; Lehmann *et al.*, 1998; Moore *et al.*, 2002). The PXR crystal structure reveals that polar residues spaced throughout the hydrophobic LBP modulate responsiveness of the receptor to various xenobiotics (Watkins *et al.*, 2001). CAR also functions as a xenobiotic receptor and shares some ligand selectivity with PXR (Moore *et al.*, 2002). Structural modeling shows that CAR and PXR have a relatively large internal LBP cavity (Dussault *et al.*, 2002). Despite these similarities, CAR has a more restrictive ligand selectivity profile than PXR. The ordered structure of H6 of CAR, which is similar to that of VDR, may impart more narrow ligand selectivity, because PXR has flexible loop 6 in that region (Moore *et al.*, 2002). Although the cavity of VDR-LBP is smaller than that of PXR or CAR, it is still larger than that of estrogen receptor (NR3A1), progesterone receptor (NR3C3), or RAR-γ (NR1B3) (Rochel *et al.*, 2000; Watkins *et al.*, 2001; Dussault *et al.*, 2002). CAR and PXR are functionally redundant xenobiotic sensors in that both can regulate common target genes encoding CYP3A and CYP2B (Xie *et al.*, 2000b). VDR is also able to regulate transcription of genes for CYP3A by binding to

the same response element as PXR and CAR (Xie *et al.*, 2000b; Makishima *et al.*, 2002), suggesting that NR1I receptors have evolved from a common ancestor and have a shared role in mediating the detoxification response to xenobiotics. These findings suggest a potential role of VDR as a xenobiotic sensor and the possibility that VDR responds to natural or synthetic compounds other than vitamin D and bile acid.

VDR is distinct from other NR1I receptors in that it interacts with bile acids with low affinity (at micromolar levels) like PXR and CAR but also responds to an endocrine ligand, $1\alpha,25(OH)_2D_3$, with high affinity, similar to steroid hormone receptors. In this study, I demonstrate that distinct amino acid residues in the VDR-LBP are important for interaction with $1\alpha,25(OH)_2D_3$ and LCA. This finding leads to the possibility that $1\alpha,25(OH)_2D_3$ and LCA induce different activated receptor conformations that might recruit distinct sets of cofactors (Takeyama *et al.*, 1999). Further analysis of ligand structure-function relationships should be helpful in elucidating the dual functions of VDR as an endocrine receptor for $1\alpha,25(OH)_2D_3$ and as a "xenobiotic sensor" for secondary bile acids produced by intestinal microflora.

I generated several VDR mutants at residues which make hydrogen bonds with hydroxyl groups of $1\alpha,25(OH)_2D_3$ and compared their responses to $1\alpha,25(OH)_2D_3$ and LCA in VDRE activation and mammalian two-hybrid assays (**Fig. 5**). There are some discrepancies in the behavior of VDR mutants in the three assays shown in **Fig. 5A-C**. $1\alpha,25(OH)_2D_3$ induced

the activation of Y143A as effectively as that of wild-type VDR, but the recruitment of SRC-1 by Y143A was weak. The effect of LCA on Y143F in VDRE activation was very weak, although LCA induced strong interaction with SRC-1 in Y143F as in wild-type VDR. Thus, the VDRE activation by full-length VDR was not completely correlated with recruitment of SRC-1. This may be because NR activation is mediated by sequential interaction with several sets of cofactor complexes (Glass and Rosenfeld, 2000). LCA induced N-CoR dissociation from Y143F, S278A, and H305Q. But these mutants showed low or undetectable transactivation by LCA in the VDRE-based assay. The data indicate the ligand-inducible dissociation of corepressor is not sufficient to induce VDR transactivation. Transfection experiments using the VDRE activation and mammalian two-hybrid assays in combination are useful in the detection of VDR-ligand interactions and ligand-induced receptor activation. The significance of interaction with particular cofactors on overall VDR transactivation potential requires further investigation.

I found that VDR mutants of S278V and S237M respond selectively to $1\alpha,25(OH)_2D_3$ and LCA, respectively. These mutants should be useful not only for analysis of structure-activity relationships but also for development of new synthetic VDR agonists. Selective screening using S278V and S237M may lead to discovery of a new synthetic ligand that lacks hypercalcemic activity. Mice with humanized PXR or CAR were generated because

human PXR and CAR have different ligand selectivity from their rodent homologues (Xie *et al.*, 2000a; Zhang *et al.*, 2002). Substitution of endogenous VDR with VDR possessing S278V may produce mice with defects in bile acid response with normal calcium metabolism while mice expressing VDR mutated with S237M may show selective dysfunction in vitamin D response. Development of ligand-specific VDR substituted mice should provide a valuable tool in clarifying VDR function *in vivo*.

Part 2

Selective activation of VDR by LCA acetate, a bile acid derivative

ABSTRACT

The VDR, a member of the nuclear receptor superfamily, mediates the biological actions of the active form of vitamin D, 1α, 25$(OH)_2D_3$. It regulates calcium homeostasis, immunity, cellular differentiation, and other physiological processes. VDR was found to respond to bile acids as well as other nuclear receptors, FXR and PXR. The toxic LCA induces its metabolism through VDR interaction. To elucidate the structure-function relationship between VDR and bile acids, I 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. I 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.

INTRODUCTION

In Part 1, I analyzed the structure-function relationships of the endocrine (1α, $25(OH)_2D_3$) and xenobiotic (LCA) ligands with VDR, and revealed that 1α, $25(OH)_2D_3$ and LCA interact with a different set of amino acids of the VDR-LBP. The results suggest the possibility that VDR adopts distinct conformations in response to 1α, $25(OH)_2D_3$ and LCA binding and provides a possible mechanism for the compounds' different biological actions. Encouraged by these considerations, I moved the study forward to elucidate the biological actions of bile acid-derived VDR agonists. However, application of LCA to further studies is not practical because it is cytotoxic and genotoxic and is a promoting factor for colon carcinogenesis (Nagengast *et al.*, 1995). 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 1α, $25(OH)_2D_3$ (Choi *et al.*, 2003), suggesting that modification of these bile acids can increase the agonistic activities for VDR.

To identify potent bile acid-derived VDR agonists, I 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 anti-proliferation and

pro-differentiation activities of the LCA analog, LCA acetate, were demonstrated in leukemia cells.

MATERIALS AND METHODS

Chemical compounds

LCA, 3-Keto-LCA, and their derivatives (LCA methyl ester, LCA ethyl ester, LCA benzyl ester, LCA acetate, LCA hemisuccinate, Iso-LCA, Ursocholanic acid, LCA acetate methyl ester, 3-Keto-LCA methyl ester, 3-Keto-LCA ethyl ester, 3-Keto-LCA benzyl ester, 3,6-Diketo-LCA, 3,7-Diketo-LCA, and 3,12-Diketo-LCA) were purchased from Sigma-Aldrich (St. Louis, MO), Wako (Osaka, Japan), Nacalai (Kyoto, Japan), or Steraloids (Newport, RI). 1α, 25$(OH)_2D_3$ was obtained from Calbiochem (San Diego, CA). LCA formate was synthesized and its purity was confirmed as described (Ishizawa *et al.*, 2008).

Plasmids

Fragments of human VDR (GenBank accession number NM_000376), FXR (accession number NM_005123), and PXR (accession number NM_022002) were inserted into

pCMX vector to make pCMX-VDR, pCMX-FXR, and pCMX-PXR, respectively (Makishima *et al.*, 1999a, 2002). The LBDs of human VDR, FXR, thyroid hormone receptor (TR) α1 (accession number NM_199334), RARα (accession number NM_000964), LXRα (accession number NM_005693), CAR (accession number NM_005122), estrogen receptor alpha (ERα) (accession number NM_000125), RXRα (accession number NM_002957), mouse PPARα (accession number NM_011144), PPARδ (accession number NM_011145), and PPARγ (accession number NM_011146) were inserted into 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. pCMX-GAL4, pCMX-GAL4-SRC-1, or pCMX-GAL4-N-CoR in combination with pCMX-VP16-VDR were used in the mammalian two-hybrid assays as described in Part 1. Mutations were introduced into pCMX-GAL4-VDR to make its point mutants as described in Part 1. hCYP3A4-ER-6x3-tk-LUC reporter was utilized to evaluate the activities of VDR or PXR. IR-1x3-tk-LUC reporter was utilized for FXR assay. GAL4-responsive MH100 (UAS)x4-tk-LUC reporter was utilized to evaluate the activities of GAL4-chimera receptors. All plasmids were sequenced prior to use to verify DNA sequence fidelity.

Cell lines and cell culture

HEK 293 cells were cultured in DMEM containing 5% fetal bovine serum and antibiotic-antimycotic (Nacalai). Human hepatoblastoma HepG2 cells and colon cancer SW480 cells were cultured in DMEM containing 10% fetal bovine serum and antibiotic-antimycotic (Nacalai). Human myeloid leukemia THP-1 cells were cultured in suspension in RPMI 1640 medium containing 10% fetal bovine serum and 80 μg/ml gentamicin (Makishima *et al.*, 1998). The cells were cultured at 37°C in humidified atmosphere of 5% CO_2 in air.

Cotransfection assay

Transfections, treatment with compounds, and the reporter gene assay were performed as described in Part 1.

Competitive ligand binding assay

Human VDR protein was generated using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) with a VDR expression vector. 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, 10% glycerol). The diluted protein was

incubated with 1 nM of [26,27-methyl-^{3}H]1α, 25$(OH)_2D_3$ for 16 h at 4^{o}C in the presence or absence of nonradioactive compounds for competition. Bound and unbound labeled 1α, 25$(OH)_2D_3$ were separated by the dextran-charcoal method (Yamamoto *et al.*, 2000). Bound 1α, 25$(OH)_2D_3$ was measured using scintillation counting.

One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.04 for Windows.

Graphical manipulation and docking

Graphical manipulations and docking of LCA acetate to hVDR-LBD (Δ165-215) (1DB1) were performed as described in Part 1.

Animal studies

C57BL/6J mice were obtained from Japan SLC (Hamamatsu, Japan) and housed in a room under controlled temperature (23 ± 1^{o}C) and humidity (45-65 %) and had free access to water and chow (Oriental Yeast, Tokyo). Experiments were conducted when the mice (males) were 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 vehicle alone. 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 reverse transcription (RT)-polymerase chain reaction (PCR) analysis

Total ribonucleic acids (RNAs) from samples were prepared with an RNA STAT-60 kit (Tel-Test, Friendswood, TX). The cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). Real time PCR was performed on a LightCycler using the FastStart DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan) according to the instructions provided by the manufacturer as described (Kaneko *et al.*, 2003). Primers for human cDNAs were as follows: *VDR*, 5'-GCTGACCTGGTCAGTTACAGCA-3' and 5'-CACGTCACTGACGCGGTACTT-3'; *RXRA* (RXRα), 5'-AAGATGCGGGACATGCAGAT-3' and 5'-CAGGCGGAGCAAGAGCTTAG-3'; *PPIA* (cyclophilin), 5'-CCCACCGTGTTCTTCGACAT-3' and 5'-CCAGTGCTCAGAGCACGAAA-3'; *CYP24A1*, 5'-TGAACGTTGGCTTCAGGAGAA-3' and 5'-AGGGTGCCTGAGTGTAGCATCT-3'; *CYP3A4*, 5'-AGTGTGGGGCTTTTATGATG-3' and 5'-ATACTGGGCAATGATAGGGA-3'; *TRPV6*, 5'-AGCCTACATGACCCCTAAGGACG-3' and 5'-GTAGAAGTGGCCTAGCTCCTCGG-3';

CDH1 (E-cadherin), 5'-GAAGGTGACAGAGCCTCTGGATAG-3' and 5'-CTGGAAGAGCACCTTCCATGA-3'. Primers for mouse cDNAs were as follows: *Ppia* (cyclophilin), 5'-CAGACGCCACTGTCGCTTT-3' and 5'-TGTCTTTGGAACTTTGTCTGCAA-3'; *Cyp24a1*, 5'-CCCATTACTCAGGGAAGCAC-3' and 5'-CCACTCAGACAATGAAGCCA-3'; *Cyp3a11*, 5'-CCAACAAGGCACCTCCCACG-3' and 5'-TGGAATTCTTCAGGCTCTGA-3'; *Fabp6* (ileal bile acid binding protein), 5'-GGTACCACCATGGCCTTCAGTGGCAAATAT-3' and 5'-GCTAGCTCAAGCCAGCCTCTTGCTTAC-3'. The RNA values were normalized to the amount of cylclophilin mRNA and are represented in arbitrary units.

One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.04 for Windows.

Growth and differentiation of myeloid leukemia cells

Suspensions of cells were cultured with or without the 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 as described (Makishima *et al.*, 1996). Briefly, cells were incubated with NBT

and added with HCl to stop the reaction. Formazan deposits were solubilized in DMSO, and the absorption of the formazan solution at 560 nm was measured in a spectrophotometer.

Expression of monocytic antigens CD11b and CD14 on the cell surface was determined using a flow cytometer (Epics XL; Coulter Electrinics) after indirect immunofluorescent staining. FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG and mouse monoclonal antibodies to CD11b (2LPM19c) and CD14 (TUK4) were used as described (Makishima *et al.*, 1998, 1999b).

RESULTS

Transactivation of VDR by LCA derivatives

To elucidate structure-activity relationship between VDR and LCA, I examined the effects of various LCA derivates on VDR activation (**Fig. 9A**). HEK293 cells were transfected with a VDR expression vector and a luciferase reporter containing a VDR-responsive ER-6 element from the CYP3A4 promoter. Cells were treated with test compounds and the induced luciferase activities were compared (**Fig. 9B**). 1α, $25(OH)_2D_3$ and LCA activated wild-type VDR as reported previously (Makishima *et al.*, 2002). The esterification of the side chain of LCA with methyl, ethyl, and benzyl groups drastically decreased the activity on wild-type VDR (**Fig. 9B**). Next, I examined the effects of LCA derivatives modified at position 3 (**Fig. 9A**). LCA formate and LCA acetate were able to activate wild-type VDR as efficiently as LCA at the concentration of 10 μM. LCA isobutyrate activated wild-type VDR moderately, whereas LCA hemisuccinate was not an effective agonist for wild-type VDR. The data indicate that addition of a large acyl group at the 3α-hydroxy group of LCA abolishes activation for wild-type VDR. 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. 9A**) have little activity on wild-type VDR (**Fig. 9B**). Interestingly, although the

effect of LCA methyl ester on wild-type VDR activation was weak, LCA acetate methyl ester was able to induce wild-type VDR activation effectively. 3-keto-LCA, a metabolite of LCA, is another potent bile acid for wild-type VDR (Makishima *et al.*, 2002). The esterification on the side chain of 3-keto-LCA modestly decreased its activity on wild-type VDR (**Fig. 9B**). 6-Keto-LCA is a very weak agonist for wild-type VDR, and 7-keto-LCA and 12-keto-LCA were not able to activate wild-type VDR (Makishima *et al.*, 2002). Transactivation of wild-type VDR by 3,6-diketo-LCA, 3,7-diketo-LCA, and 3,12-keto-LCA was almost absent (**Fig. 9B**). These data indicate that addition of a ketone group at position 6, 7, or 12 to LCA or 3-keto-LCA disturbs the interaction with wild-type VDR. Collectively, these data suggested that the position 3 of LCA could be modified to modulate VDR activation, while the esterifications of the carboxylic acid of LCA and modifications of 3-Keto-LCA could lose or diminish their agonistic activities.

LCA acetate is a potent agonist for VDR

To evaluate the potencies of LCA derivatives with the modifications at the position 3, I compared wild-type VDR dose-response curves for LCA, LCA formate, LCA acetate, LCA acetate methyl ester, and 3-keto-LCA. LCA acetate activated wild-type 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. 10A**). Notably, the potency of wild-type VDR activation by LCA acetate was 30-fold greater than that of LCA. These results demonstrated that the modifications of LCA at the position 3 lead to more potent VDR agonists in comparison with LCA.

To evaluate ligand-dependent interactions of VDR with cofactors, the mammalian two-hybrid assays were performed on LCA derivatives using receptor-interacting domains of SRC-1 and N-CoR that were fused to the GAL4 DNA-binding domain as described in Part 1. Cotransfection of GAL cofactors with VDR fused to VP16 allowed for detection of ligand-dependent cofactor interaction. Although there was no association between control GAL4 protein and VP16-VDR, LCA acetate at 10 μM and 1α, $25(OH)_2D_3$ at 100 nM strongly induced the association of wild-type VDR with SRC-1 (**Fig. 10B**). 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 wild-type VDR as effectively as 1α, $25(OH)_2D_3$ (**Fig. 10B**). The effects of these LCA derivatives on N-CoR dissociation were stronger than that of LCA. Thus, it was demonstrated that LCA formate and LCA acetate are potent regulators of VDR-cofactor interaction in comparison with LCA.

Next, to assess the ability of LCA derivatives to bind directly to wild-type VDR *in vitro*, I conducted the competitive binding assay. Isotopically labeled 1α, $25(OH)_2D_3$ was

incubated with *in vitro* translated wild-type VDR protein in the absence or presence of test compounds. The binding of labeled 1α, $25(OH)_2D_3$ to wild-type VDR was significantly reduced by the addition of unlabeled 10 nM 1α, $25(OH)_2D_3$ and 200 μM LCA (**Fig. 11**). LCA formate, LCA acetate, 3-keto-LCA, and LCA acetate methyl ester also inhibited the binding of labeled 1α, $25(OH)_2D_3$ to wild-type VDR, indicating that these LCA derivatives directly bind to wild-type VDR. Competition with 50 μM LCA was weaker than that of LCA formate and LCA acetate at 50 μM and 200 μM. Interestingly, although LCA acetate methyl ester showed enhanced activation of wild-type VDR compared with 3-keto-LCA in the luciferase reporter assay as shown **Fig. 10A**, its direct interaction with wild-type VDR protein was weaker than those of LCA and 3-keto-LCA (**Fig. 11**). LCA acetate did not inhibit the binding of labeled estradiol to ERα. Taken together, these data indicate that LCA formate and LCA acetate activates wild-type VDR by direct binding.

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

Bile acids are endogenous ligands for NRs including VDR, PXR, and FXR. Therefore, it is important to examine the selectivity of bile acid-derived VDR ligands over other NRs. The LBDs of various NRs were fused to the DBD 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. Because this reporter is activated only by the GAL4-chimera receptors, the potentially confounding effects of endogenous receptors are eliminated. LCA acetate at 30 μM induced the activation of wild-type GAL4-VDR (**Fig. 12A**). It induced weak activation of FXR but was not effective on TRα, RARα, PPARα, PPARδ, PPARγ, LXRα, CAR, RXRα, and ERα. FXR has been previously shown to respond to various bile acids, such as CDCA and deoxycholic acid (Makishima *et al.*, 1999a, 2002). Next, I determined FXR dose-response curves for LCA derivatives modified at position 3. As reported previously (Makishima *et al.*, 1999a), CDCA was a potent FXR agonist (**Fig. 12B**). Urosocholanic acid and iso-LCA, which were not effective on wild-type VDR (**Fig. 9B**), strongly induced the activation of FXR (**Fig. 12B**). LCA formate and LCA acetate, as well as LCA, were weak FXR agonists. These data indicate that structure-activity relationships of LCA derivatives are different between VDR and FXR. PXR was reported to respond to high concentrations of LCA (Staudinger *et al.*, 2001; Xie *et al.*, 2001). To examine the effects of LCA derivatives on PXR, I transfected wild-type VDR or PXR expression vectors with a reporter containing a CYP3A4 element, which can be activated by both receptors. Liver-derived HepG2 cells were used for this experiment, because PXR activation is cell type dependent. In the absence of transfected receptors, the luciferase activity was increased by addition of the LCA derivatives (**Fig. 12C**). Because a PXR agonist, rifampicin, did not activate the reporter,

this effect may be derived from endogenous receptors such as VDR but not PXR. LCA acetate and LCA formate strongly induced the activity of transfected wild-type VDR, indicating that these LCA derivatives activate VDR in HepG2 cells. Rifampicin did not activate wild-type 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. 12C**). Collectively, these findings indicate that LCA formate and LCA acetate are selective for VDR activation among the examined NRs. In the following experiments, the most potent LCA derivative, LCA acetate, was investigated in detail.

Effect of VDR mutation on LCA acetate response

To elucidate the structure-activity relationship of LCA acetate and VDR, I 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 1α, 25$(OH)_2D_3$ were compared (**Fig. 13A**). According to the crystal structure of the VDR-1α, 25$(OH)_2D_3$ complex (Rochel *et al.*, 2000), Y143 and S278 interact with the 3β-hydroxyl group of 1α, 25$(OH)_2D_3$, S237 makes a hydrogen bond with the 1α-hydroxyl group, H305 coordinates the 25-hydroxyl group, and S275 and W286 mediate hydrophobic interaction with 1α, 25$(OH)_2D_3$. The Y143A

and W286A mutations inhibited the responses to LCA acetate, LCA, and 1α, 25(OH)$_2$D$_3$, suggesting that Y143 and W286 interact with LCA acetate, LCA, and 1α, 25(OH)$_2$D$_3$ in similar ways. The effects of S237A was modest on LCA, LCA acetate, and 1α, 25(OH)$_2$D$_3$ activity. Whereas S275A and S278A almost abolished the activity of LCA, LCA acetate and 1α, 25(OH)$_2$D$_3$ still activated these mutants, suggesting that S275 and S278 are important for the activity of LCA but not for those of LCA acetate and 1α, 25(OH)$_2$D$_3$. Interestingly, although H305A had significant effects on the activity of LCA and 1α, 25(OH)$_2$D$_3$, 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 H305A (Choi *et al.*, 2003). In Part 1, I found that S278V mutant is activated by 1α, 25(OH)$_2$D$_3$ but not by LCA, whereas S237M can respond to LCA but not to 1α, 25(OH)$_2$D$_3$. I next examined the effects of LCA acetate on these mutants (**Fig. 13B**). 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, I modeled LCA acetate in the VDR LBD (Δ165-215) (PDB #1DB1) using FlexX software. In contrast to the LCA docking model, the side chain of LCA acetate directs to the β-turn site (**Fig. 13C**, left panel). 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 1α, 25(OH)$_2$D$_3$ in the crystal structure of VDR-1α, 25(OH)$_2$D$_3$ (**Fig. 13C**, right panel). The

proximity of these amino acid residues to hydrogen bond acceptors within 1α, 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 known to regulate the expression of genes involved in calcium homeostasis and bile acid metabolism (Berger *et al.*, 1988; Haussler *et al.*, 1998; Makishima *et al.*, 2002). To investigate the ability of LCA acetate to activate endogenous VDR target genes, I utilized colon cancer-derived SW480 cells that highly express *VDR* gene. SW480 cells were incubated with LCA, LCA acetate, 1α,25$(OH)_2D_3$, CDCA, or rifampicin, and the expression of the VDR target genes for *CYP24A1*, *CYP3A4*, *TRPV6*, and *CDH1* (E-cadherin) were examined. CYP24A1 and TRPV6 are involved in calcium homeostasis and CYP3A4 metabolizes LCA (Haussler *et al.*, 1998; Van Cromphaut *et al.*, 2001). *CDH1* is reported to be regulated by 1α, 25$(OH)_2D_3$ and its induction leads to cell growth inhibition (Pálmer *et al.*, 2001). As shown in **Fig. 14**, 1α, 25$(OH)_2D_3$ significantly increased the expression of *CYP24A1*, *CYP3A4*, *TRPV6*, and *CDH1*. LCA acetate significantly increased expression of *CYP24A1* at 100 μM and *CYP3A4*, *TRPV6*, and *CDH1* at both 10 μM and 100 μM, while LCA significantly increased expression of *CYP3A4* and *TRPV6* only at 100 μM. These results indicate that that LCA acetate acts as a more potent agonist in colon cancer

cells in comparison with LCA. To assess potential secondary effects, mRNAs for VDR and RXRα were monitored. The expression of *VDR* was reduced upon the treatments with 100 μM LCA (0.78-fold), 100 μM LCA acetate (0.71-fold), and 1α, 25$(OH)_2D_3$ (0.74-fold) in comparison with vehicle control, while the expression of *RXRA* was not significantly changed. These data indicate that the drastic increases observed for VDR target genes were not caused by the increase of *VDR* and *RXRA*. The FXR agonist CDCA and the PXR agonist rifampicin were not able to induce the expression of these genes, although PXR was reported to be involved in CYP3A4 gene regulation (Luo *et al.*, 2002). This inability of PXR agonist to increase gene expression is likely attributable to the fact that PXR is not expressed in SW480 cells. Collectively, these data indicate that LCA acetate is a potent agonist for endogenous VDR in intestinal cells.

Next, to assess the ability of LCA acetate to induce VDR target genes *in vivo*, I examined the expression of VDR target genes in the intestine where tested compounds can be delivered via oral administrations. Mice were orally administrated LCA or LCA acetate, and the expressions of intestinal *Cyp24a1* and *Cyp3a11* were evaluated. Both LCA and LCA acetate tended to increase *Cyp24a1* and *Cyp3a11* although the differences compared to the vehicle controls were not statistically significant (**Fig. 15**). LCA and LCA acetate did not increase the expression of an FXR target gene, *Fabp6* (**Fig. 15**). Therefore, it was not clear from this

experiment whether LCA acetate acts as VDR agonist in the mouse intestine. Further investigations are necessary to demonstrate the biological actions of LCA acetate *in vivo* in different experimental conditions.

LCA acetate induces the differentiation of monoblastic leukemia cells

1α, 25$(OH)_2D_3$ is known as an inducer of myeloid leukemia differentiation (Haussler *et al.*, 1998). To assess the ability of LCA acetate to induce cellular differentiation, I examined the effects of LCA acetate on the growth and differentiation of human monoblastic leukemia THP-1 cells. 1α, 25$(OH)_2D_3$ inhibited the proliferation of THP-1 cells and induced the NBT reduction, a differentiation marker of myeloid leukemia cells, as reported previously (Makishima *et al.*, 1996). LCA acetate inhibited cell proliferation more effectively than LCA and 3-keto-LCA (**Fig. 16A**), and it induced the NBT-reducing activity in the cells. In contrast, LCA and 3-keto-LCA were not able to induce the NBT-reducing activity even at concentrations that completely inhibit cell proliferation (**Fig. 16B**). 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. 16C**). In the cells treated with LCA acetate, the nuclei were condensed, nucleoli were no longer apparent, and the cytoplasm appeared gray, indicating monocytic differentiation (**Fig. 16C**). Esterase

activity, a functional marker of monocytic differentiation, was also induced by LCA acetate (**Fig. 16D**). 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 1α, 25$(OH)_2D_3$ (**Fig. 16E**). Therefore, the VDR agonist LCA acetate is a potent inducer of monocytic differentiation in THP-1 leukemia cells.

DISCUSSION

In this study I 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 (Choi *et al.*, 2003). As shown in **Fig. 9**, 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 derivatives modified at position 3, such as LCA formate and LCA acetate, have stronger activity than LCA (**Fig. 9** and **10**). The docking model shown in **Fig. 13** indicates that LCA acetate is accommodated in the VDR-LBP in the same manner as 3-keto-LCA. 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 S237M mutant (**Fig. 13B**), which does not respond to 1α, 25$(OH)_2D_3$. 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 (CDCA and cholic acid) and secondary bile acids (LCA and deoxycholic acid) (Makishima *et al.*, 1999a; Parks *et al.*, 1999; Wang *et al.*, 1999). In contrast, VDR responds to only LCA and its derivatives (Makishima *et al.*, 2002). In the previous study, 6-keto-LCA was identified as a selective ligand for VDR, but its activity was very weak (Makishima *et al.*, 2002). The potent VDR agonist LCA acetate activated FXR to low levels, similar to the weak FXR agonist LCA (**Fig. 12B**). The activity of LCA acetate for FXR was much weaker than CDCA (**Fig. 12B**). In HepG2 cells, CDCA induced the expression of the *BSEP* gene, which is an FXR target (Lu *et al.*, 2001), but LCA and LCA acetate were not effective in its induction, although LCA acetate increased the VDR target *CYP24A1* expression. Although LCA and 3-keto-LCA were agonists for PXR at higher concentrations, LCA acetate

did not activate PXR (**Fig. 12C**). 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. Crystal structures of FXR and PXR have been reported (Watkins *et al.*, 2001; Downes *et al.*, 2003; Mi *et al.*, 2003). 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 (Garland *et al.*, 1999). 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 (Garland *et al.*, 1999). 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 (Haussler *et al.*, 1998; Pálmer *et al.*, 2003). In contrast to vitamin D, the secondary bile acid LCA is considered to be a promoter of colon carcinogenesis (Nagengast *et al.*, 1995). LCA induces DNA strand breaks, forms DNA adducts, inhibits DNA repair enzymes, and can promote colon cancer in rodent models (Narisawa *et al.*, 1974). CYP3A was reported to detoxify LCA to a nontoxic hydodeoxycholic acid and is a VDR target gene (Thummel *et al.*, 2001; Xie *et al.*, 2001). By binding to VDR, 1α,

$25(OH)_2D_3$ and LCA induce the genes for CYP3A in the intestine. VDR may serve as a sensor for LCA and function to protect intestinal mucosa from its harmful effects. A significant correlation between a VDR polymorphism and colorectal cancer risk was reported in Singapore Chinese population (Wong *et al.*, 2003). 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 unsuccessful because of their hypercalcemic activities (Guyton *et al.*, 2003). Structure-function analysis of vitamin D analogs suggests that 1α, $25(OH)_2D_3$ and its analogs also induce non-genomic VDR actions and that adverse effects are at least partly attributable to non-genomic mechanisms (Huhtakangas *et al.*, 2004; Zanello and Norman, 2004). Ligand-dependent dissociation of non-genomic from genomic activity was reported for the estrogen receptor (Kousteni *et al.*, 2001). An estrogen receptor ligand, pyrazole, induced the transactivation of an estrogen receptor target gene but had weak non-genomic activity, whereas another ligand, estren, induced strong non-genomic 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-detoxyfying enzyme CYP3A, in colon cancer cells more effectively than LCA (**Fig. 14**). Non-genomic 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.

1α, 25$(OH)_2D_3$ was found to induce the differentiation of mouse myeloid leukemia M1 cells more than 30 years ago (Abe *et al.*, 1981). Treatment with 1α, 25$(OH)_2D_3$ or 1α-hydroxyvitamin D_3, which is rapidly metabolized to 1α, 25$(OH)_2D_3$, was reported to prolong survival in mice inoculated with M1 leukemia cells (Honma *et al.*, 1983). The differentiation-inducing effects of 1α, 25$(OH)_2D_3$ were also demonstrated in human leukemia cells (Miyaura *et al.*, 1981; Mangelsdorf *et al.*, 1984). However, the molecular mechanisms of differentiation induced by 1α, 25$(OH)_2D_3$ have not been elucidated. I 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. 16**). LCA and 3-keto-LCA inhibited the proliferation but did not induce differentiation. The growth-inhibiting activity of these bile acids may be attributable to their cytotoxic effects. Zimber *et al.* reported that bile acids, including deoxycholic acid, CDCA, and LCA, induced the differentiation of human promyelocytic leukemia HL-60 cells (Zimber *et al.*, 1994). I did not observe

differentiation-inducing activity of these bile acids in HL-60 cells. 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. These findings indicate that LCA acetate is a more effective inducer of leukemia differentiation than bile acids such as LCA and CDCA. Zimber et al. reported that LCA alone did not induce the differentiation of THP-1cells but that it enhanced the response to all-*trans*-retinoic acid, which is a potent differentiation inducer of myeloid leukemia cells (Zimber *et al.*, 2000). The combinational effects of LCA acetate and other differentiation inducers are to be investigated. The protein kinase C inhibitor sphingosine decreased the NBT-reducing activity induced by deoxycholic acid and CDCA in HL-60 cells but did not alter the response to LCA (Zimber *et al.*, 1994), suggesting that the effect of LCA is mediated by mechanisms distinct from those used by deoxycholic acid and CDCA. Expression of some VDR target genes was increased in THP-1cells after treatment with LCA acetate. The observation indicate that LCA acetate functions as a VDR agonist in leukemia cells and induces cell differentiation. Further studies are required to elucidate the precise mechanisms of LCA acetate- and 1α, $25(OH)_2D_3$-induced leukemia cell differentiation.

General Discussion

Structural determinants for VDR responses to its ligands

Structure-function relationship studies on the interaction of vitamin D analogs with VDR have revealed different patterns of docking of ligands into the VDR-LBP (Yamada *et al.*, 2003b). The difference in ligand-receptor interaction may contribute selective recruitment of coactivators to VDR, leading to selective biological actions (Takeyama *et al.*, 1999). Based on these considerations, I hypothesized that bile acid analogues function as novel VDR modulators. In Part 1, I determined important amino acids for VDR responses to LCA based on functional comparison with PXR amino acids. A docking model suggested LCA and 1α, 25$(OH)_2D_3$ differently interacted with the amino acid in helix 3 of VDR-LBP that form coactivator binding surface. In Part 2, I clarified structure-activity relationship for LCA derivatives and VDR response. Based on biological analyses, LCA acetate exhibited anti-proliferation and pro-differentiation activities for THP-1 cells. I believe that these fundamental results obtained in this study could facilitate the development of selective VDR modulators and further understanding of biological functions of VDR.

The identification of potent VDR agonist provided a good research tool for the analyses of VDR structure and functions (Ikura and Ito, 2016). The crystal structures of the rat VDR-LBD in ternary complexes with a synthetic partial peptide of the coactivator MED1 (mediator of RNA polymerase II transcription subunit 1) and four ligands, LCA, 3-keto LCA,

LCA acetate, and LCA propionate were determined, elucidating a part of their agonistic mechanism (Masuno *et al.*, 2013). The structures reveal that LCA and its derivatives bind to the same VDR-LBP that $1\alpha,25(OH)_2D_3$ binds to. Interestingly, the crystal structures revealed that LCA bound to VDR in a reverse direction to that obtained from the docking model generated in this research (**Fig. 8**). The discrepancy might be explained by limitations of the docking method that was employed in this research. However, it is of note that the importance of the hydrogen-bonding network that I have demonstrated for agonistic activity of LCA was recaptured by the crystallographic analyses. Namely, the important interactions between LCA and Y143, R274, S278, and H305 of human VDR were suggested by the determined interactions between LCA and Y143, R270, S274, and H301 of rat VDR. Interestingly, interactions with S233, R270, and H301 of rat VDR were suggested to be water-mediated bondings, which highlights an unique binding mode of LCA different from that of $1\alpha,25(OH)_2D_3$. Collectively, the structure-function relationship analysis conducted in this research and the resolved VDR structures consistently demonstrated that LCA and its derivatives interact with the VDR-LBP in a mode different from $1\alpha,25(OH)_2D_3$, especially in interactions regarding helix 3 and 4/5 residues. Although the crystallographic analysis provides a concrete snapshot of ligand-receptor interactions, the drawback is that it is not able to visualize dynamic motions of interactions. For example, different VDR ligands exhibit agonistic

or antagonistic activities although the main chains including helix 12 of VDR-LBD are almost identical to those two crystal structures (Nakabayashi *et al.*, 2008; Inaba *et al.*, 2009; Kakuda *et al.*, 2010). Combinations of the other methodologies could support us to fully understand the dynamic actions of VDR modulators. The research strategy employed in my study that mutated unconserved amino acids of the most-related PXR would be one of the viable approaches because such VDR mutants could keep receptor functionality in comparison to random mutations. The functional analyses are complementary to the structural analyses for better understanding the molecular mechanism underlying the interaction between VDR and its ligands.

LCA acetate acted as a VDR modulator

Mutational analyses suggested that LCA and vitamin D interact with the different amino acids. Especially, S237 resides in a crucial helix that composes a molecular surface for the dynamic recruitment of cofactors (Yamada and Yamamoto, 2006; Yamamoto *et al.*, 2006). This strongly drove me to further investigate the biological actions of the bile acid-derived VDR ligands. There are multiple reports that describe the functional difference of LCA and vitamin D_3 in activating VDR functions. Thus, potent LCA derivatives are under active development to

identify novel VDR modulators. Of particular interest, it was reported that LCA acetate and its analogue effectively induced VDR target genes in the kidney without causing hypercalcemia (Ishizawa *et al.*, 2008). The effect of $1\alpha,25(OH)_2D_3$ on the induction of *TRPV6* was 4- to 7-fold greater than that on *CYP24A1* induction, while the potency of LCA derivatives on *TRPV6* induction was 2- to 3-fold greater than that on *CYP24A1* induction in intestinal SW480 cells (Ishizawa *et al.*, 2008). The result suggested that the vitamin D signal is amplified for the induction of *TRPV6* in intestinal cells, highlighting the gene-selective effects of VDR ligands. Their molecular mechanism of action is to be further investigated.

Possible molecular mechanism of VDR modulators

There could be several explanations for gene-selective action of the VDR modulators. The combination of cellular fluorescence resonance energy transfer (FRET) and chromatin immunoprecipitation (ChIP) assays have revealed that LCA induce unique cofactor complex formation differently from $1\alpha,25(OH)_2D_3$ and its analogue in a cell context-dependent manner, suggesting ligand-selective dynamic VDR conformations (Choi *et al.*, 2011). In addition, it was demonstrated that structurally diverse ligands could affect not only VDR-LBP but also DNA binding activity of VDR. Binding of an agonist to the VDR/RXR heterodimer alters the stability of the VDR-DBD, demonstrating that the ligand influences the DNA recognition (Zhang *et al.*,

2011). These data suggested a mechanism by which NRs show promoter specificity and have differential effects on various target genes. On the other hand, it has been proposed that the DNA response element functions as a sequence-specific allosteric ligand that modulates the activity of the NRs (Meijsing *et al.*, 2009). Based on these researches, it could be hypothesized that bile acid-derived ligands could regulate a distinctive target gene set different from 1α, $25(OH)_2D_3$ by binding to unique response elements in coordinated interactions with cofactors on the promoter regions. These potential mechanisms are to be investigated in the future studies for bile acid-derived ligands. Further investigations would provide insight into the function of gene-selective NR modulators and the functional difference between LCA derivatives and vitamin D_3.

Development of better bile acid-derived VDR modulators

To develop better bile acid-derived VDR modulators, further investigations are necessary to address the following concerns. First, several off-target effects other than NRs are to be clarified. Vitamin D binding protein (Verboven *et al.*, 2002) and bile acid binding proteins (Nakahara *et al.*, 2005; Monaco, 2009) are lipid binding proteins that exhibit binding activities to endogenous VDR ligands and versatile functions. LCA derivatives including LCA acetate were reported not to have significant activity for G protein-coupled bile acid receptor (GPBAR)

1 (Ishizawa *et al.*, 2008), a G-protein coupled plasma membrane receptor for bile acids that mediates many of the rapid, non-genomic actions of bile acids (Lieu *et al.*, 2014). Second, the pharmacokinetic profile needs to be determined and optimized so as to exhibit desirable efficacies in animal models. Together with the functional and structural analyses, rational designs could be employed to develop more potent and selective VDR modulators.

Application to studies on the biological function of VDR

Identification of potent and novel VDR ligands provided useful chemical tools to elucidate the function of VDR modulation *in vitro* and *in vivo*. As an example of noncanonical function of VDR, it is present in caveolae-enriched plasma membranes and binds 1α, 25$(OH)_2D_3$ (Huhtakangas *et al.*, 2004). VDR signaling was studied using VDR ligands including 1α, 25$(OH)_2D_3$, LCA, and its derivative in primary human hepatocytes (Han *et al.*, 2010), demonstrating that VDR ligands activate membrane VDR and activate the intracellular kinase pathway, which results in recruitment of corepressors to suppress *CYP7A1* gene transcription. This membrane VDR-signaling pathway may suppress bile acid synthesis as a rapid response to bile acids and protect hepatocytes from cholestatic liver injury. Thus, it is intriguing to examine whether the potent VDR agonists that were derived from bile acids could affect the non-canonical VDR signaling pathways.

The induction of genes encoding CYP3As has been studied *in vitro* and *in vivo* using LCA as a bile acid ligand (Matsubara *et al.*, 2008). The results indicated the selective engagement of VDR, but not PXR, in the LCA-mediated induction of CYP3As *in vivo*. In addition, LCA can substitute for a diverse 1α, $25(OH)_2D_3$ dependent actions in calcium and bone homeostasis, as shown by the increase in serum calcium levels in vitamin-D-deficient rats treated with LCA (Nehring *et al.*, 2007). However, utilization of LCA as a native VDR ligand still has limitations for its solubility or difficulty in application due to its low potency. Therefore, the potent VDR ligands could provide further insights into the bile-acid mediated and other physiological gene regulation in comparison to vitamin D.

Future directions for the study of NR modulators

Recent advancement in the technology has provided high-throughput sequencing capabilities in basic research areas. For example, ChIP-sequence analysis would facilitate further understanding of the functional characteristics mediated by VDR modulators.

As discussed above, although crystallography is a powerful technology to uncover the molecular basis for ligand-receptor interactions, the approach can capture only the snapshots. Thus, dynamic behavior of protein conformations is to be investigated by multiple approaches. Hydrogen/deuterium exchange (HDX) has been applied to determine molecular mechanism of

VDR modulators that could not be inferred from static crystal structures (Zhang *et al.*, 2010). NMR chemical shifts of rat VDR-LBD when bound to three distinctive ligands demonstrated the differential influence of these ligands on the solution conformation of the protein (Singarapu *et al.*, 2011). A combination of small-angle X-ray scattering and molecular dynamics simulations proposed a model for mechanism of agonism/antagonism of VDR-LBD (Anami *et al.*, 2016). HDX analyses has demonstrated the intra- and interdomain structural communications for VDR-RXR complex in the presence of target DNA sequences (Zhang *et al.*, 2011). These emerging technologies can provide fundamental information regarding dynamic interaction between ligand and receptors. Moreover, recent advancement in cryo-electron microscopy (Cryo-EM) prompted efforts towards understanding the full-length NR and provided transcriptional platforms. Notably, the structure of the full-length human RXR/VDR nuclear receptor heterodimer complex was determined in complex with its DR-3 response element (Orlov *et al.*, 2012). Application of these technologies to the analyses on the interaction between bile acid-derived ligands and full-length VDR as well as VDR-LBP would address the dynamic behavior of VDR modulators. On top of that, elucidation of the dynamic behaviors of full-length NRs along with coregulators and DNA are crucial for structural and functional understanding of the biology regarding NRs.

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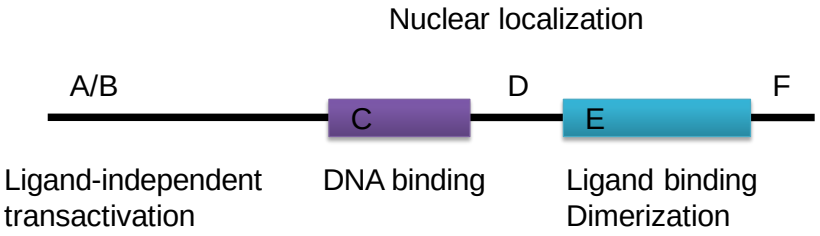

Fig. 1. Schematic representation of primary structure of the NR

Typical NR is comprised of A/B, C, D, E, and F regions. The amino terminal A/B region encode ligand-independent transactivation function. The C region encodes the DNA-binding domain (DBD). The D region connects the DBD and the following E region. The C and D regions encode nuclear localization signals. The E region encodes the ligand-binding domain (LBD) that serves ligand-dependent transactivation function and dimerization. A few receptors encode the F region in the C-terminal.

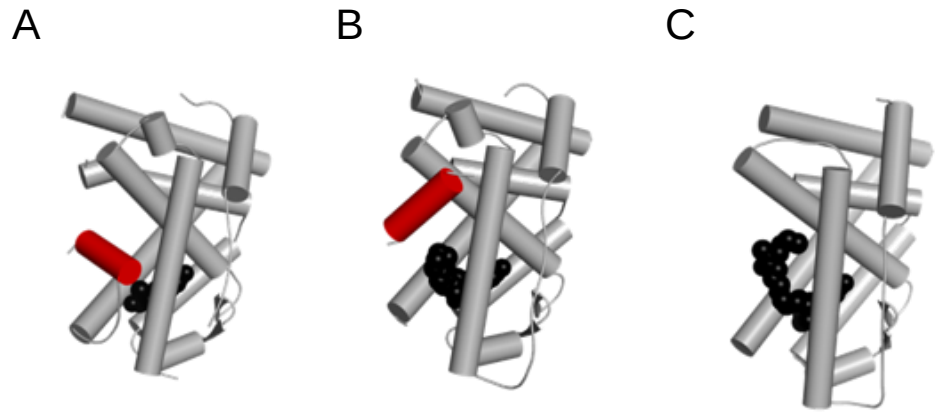

Fig. 2. Conformational change mediated by NR ligands observed in estrogen receptor crystal structures

A, B, and C: Agonist conformation in complex with estradiol (PDB 1ERE) (A), antagonist conformation in complex with raloxifen (PDB 1ERR) (B), and antagonist conformation in complex with ICI182780 (PDB 1HJ1) (C) are drawn using PyMol. Helix 12 is depicted as red syringes. In the presence of pure antagonist, helix 12 is disordered and does not appear in the structure.

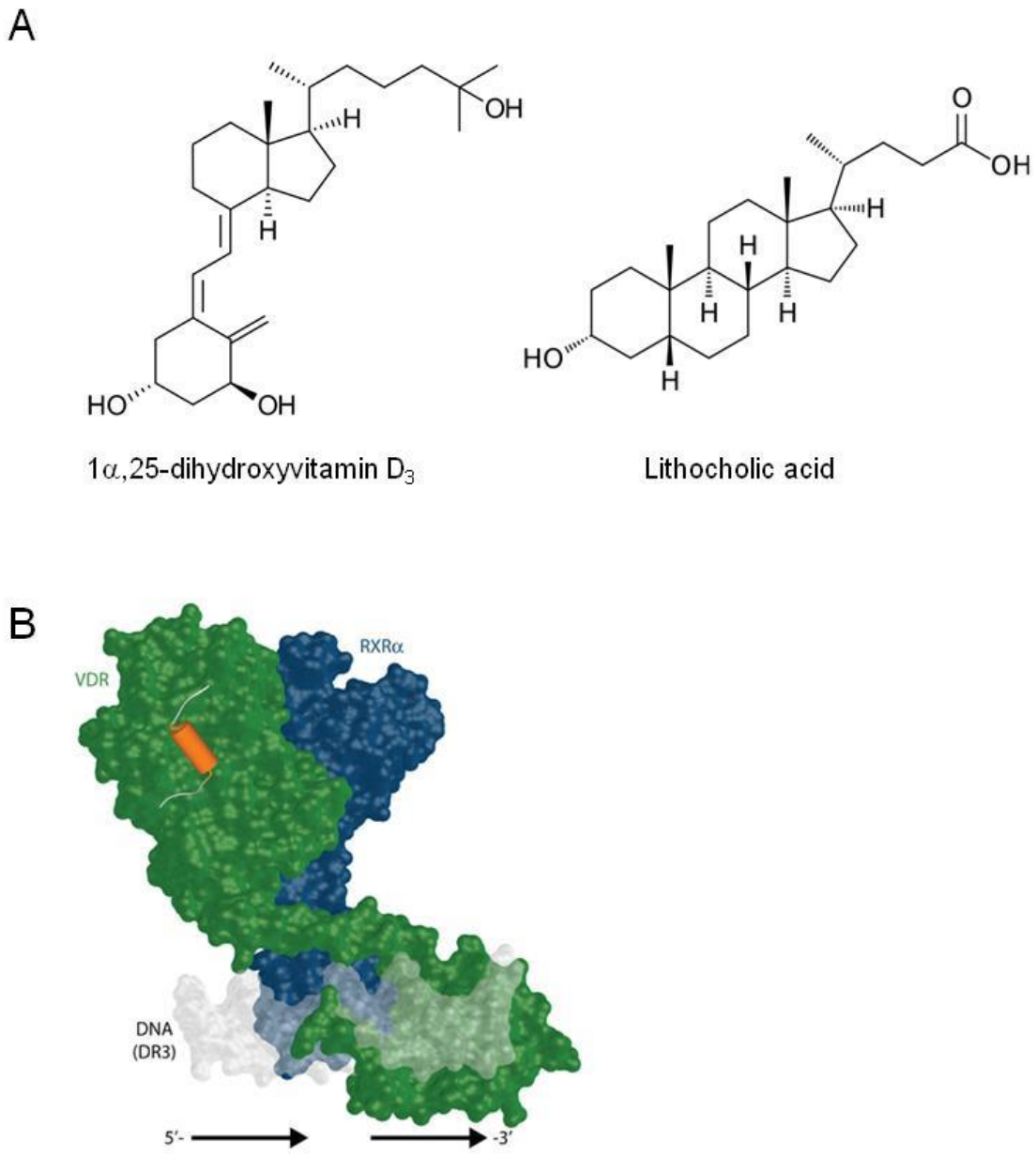

Fig. 3. The vitamin D receptor and its ligands

A: Chemical structures of 1α,25-dihydroxyvitamin D_3 and lithocholic acid (LCA). B: The full length RXR-VDR structural model derived from SAXS and cryo-EM experiments proposed by Molnar (Molnar, 2014). A surface representation of the RXR (blue)-VDR (green) heterodimer is

shown on direct repeat (DR)-3 VDRE. The possible location of the coactivator peptide (orange) is highlighted as well. The 5'- and 3'-prime orientation of the DR-3 is annotated.

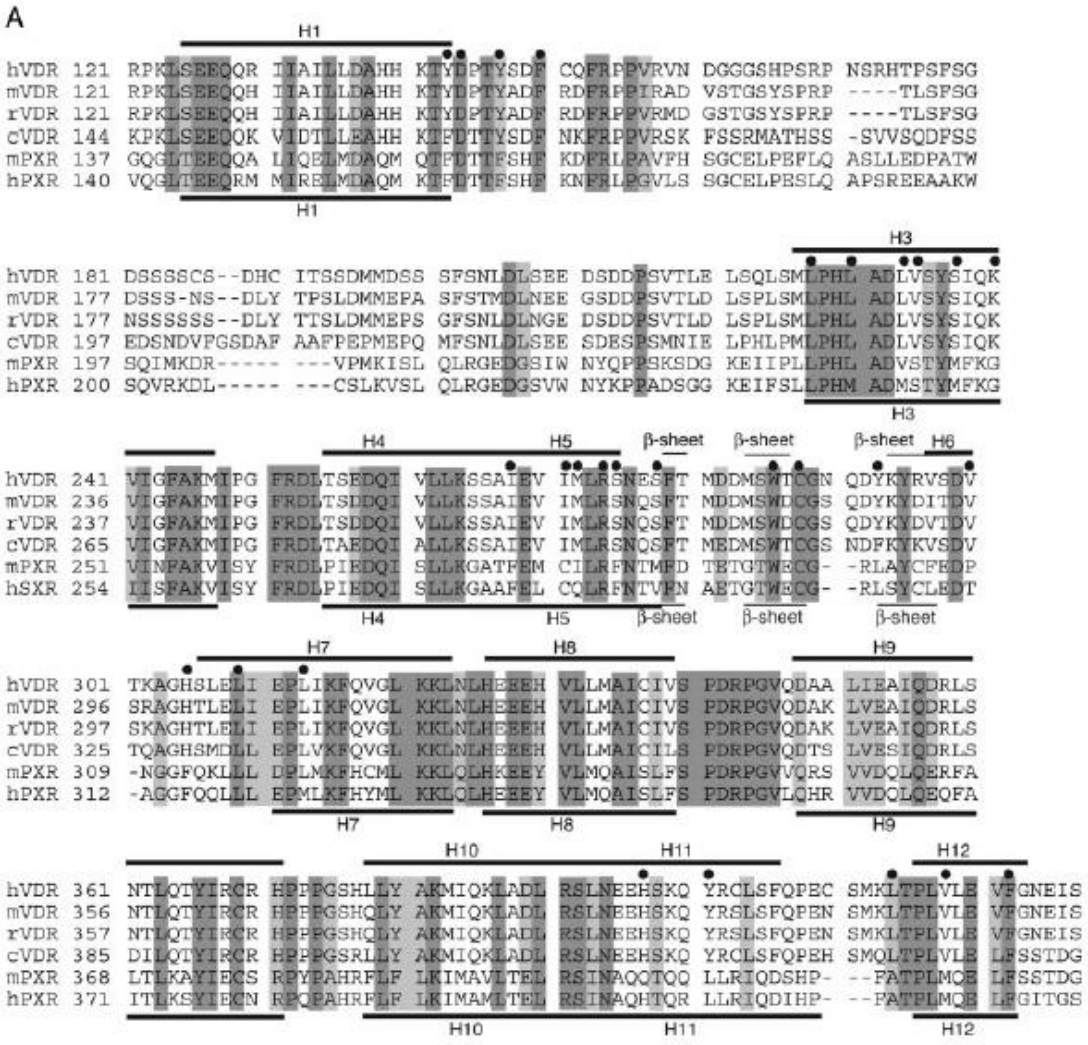
A
H1
hVDR 121 RPKLSEEQQR IIAILLDAHH KTYDPTYSDF CQFRPPVRVN DGGGSHPSRP NSRHTPSFSG
mVDR 121 RPKLSEEQQH IIAILLDAHH KTYDPTYADF RDFRPPIRAD VSTGSYSPRP ----TLSFSG
rVDR 121 RPKLSEEQQH IIAILLDAHH KTYDPTYADF RDFRPPVRMD GSTGSYSPRP ----TLSFSG
cVDR 144 KPKLSEEQQK VIDTLLEAHH KTFDTTYSDF NKFRPPVRSK FSSRMATHSS -SVVSQDFSS
mPXR 137 GQGLTEEQQA LIQELMDAQM QTFDTTFSHF KDFRLPAVFH SGCELPEFLQ ASLLEDPATW
hPXR 140 VQGLTEEQRM MIRELMDAQM KTFDTTFSHF KNFRLPGVLS SGCELPESLQ APSREEAAKW
H1
H3
hVDR 181 DSSSSCS--DHC ITSSDMMDSS SFSNLDLSEE DSDDPSVTLE LSQLSMLPHL ADLVSYSIQK
mVDR 177 DSSS-NS--DLY TPSLDMMEPA SFSTMDLNEE GSDDPSVTLD LSPLSMLPHL ADLVSYSIQK
rVDR 177 NSSSSSS--DLY TTSLDMMEPS GFSNLDLNGE DSDDPSVTLD LSPLSMLPHL ADLVSYSIQK
cVDR 197 EDSNDVFGSDAF AAFPEPMEPQ MFSNLDLSEE SDESPSMNIE LPHLPMLPHL ADLVSYSIQK
mPXR 197 SQIMKDR----- ---VPMKISL QLRGEDGSIW NYQPPSKSDG KEIIPLLPHL ADVSTYMFKG
hPXR 200 SQVRKDL----- ---CSLKVSL QLRGEDGSVW NYKPPADSGG KEIFSLLPHM ADMSTYMFKG
H3
H4
H5
β-sheet
β-sheet
β-sheet
H6
hVDR 241 VIGFAKMIPG FRDLTSEDQI VLLKSSAIEV IMLRSNESFT MDDMSWTCGN QDYKYRVSDV
mVDR 236 VIGFAKMIPG FRDLTSDDQI VLLKSSAIEV IMLRSNQSFT MDDMSWDCGS QDYKYDITDV
rVDR 237 VIGFAKMIPG FRDLTSDDQI VLLKSSAIEV IMLRSNQSFT MDDMSWDCGS QDYKYDVTDV
cVDR 265 VIGFAKMIPG FRDLTAEDQI ALLKSSAIEV IMLRSNQSFT MEDMSWTCGS NDFKYKVSDV
mPXR 251 VINFAKVISY FRDLPIEDQI SLLKGATFEM CILRFNTMFD TETGTWECG- -RLAYCFEDP
hSXR 254 IISFAKVISY FRDLPIEDQI SLLKGAAFEL CQLRFNTVFN AETGTWECG- -RLSYCLEDT
H4
H5
β-sheet
β-sheet
β-sheet
H7
H8
H9
hVDR 301 TKAGHSLELI EPLIKFQVGL KKLNLHEEEH VLLMAICIVS PDRPGVQDAA LIEAIQDRLS
mVDR 296 SRAGHTLELI EPLIKFQVGL KKLNLHEEEH VLLMAICIVS PDRPGVQDAK LVEAIQDRLS
rVDR 297 SKAGHTLELI EPLIKFQVGL KKLNLHEEEH VLLMAICIVS PDRPGVQDAK LVEAIQDRLS
cVDR 325 TQAGHSMDLL EPLVKFQVGL KKLNLHEEEH VLLMAICILS PDRPGVQDTS LVESIQDRLS
mPXR 309 -NGGFQKLLL DPLMKFHCML KKLQLHKEEY VLMQAISLFS PDRPGVVQRS VVDQLQERFA
hPXR 312 -AGGFQQLLL EPMLKFHYML KKLQLHEEEY VLMQAISLFS PDRPGVLQHR VVDQLQEQFA
H7
H8
H9
H10
H11
H12
hVDR 361 NTLQTYIRCR HPPPGSHLLY AKMIQKLADL RSLNEEHSKQ YRCLSFQPEC SMKLTPLVLE VFGNEIS
mVDR 356 NTLQTYIRCR HPPPGSHQLY AKMIQKLADL RSLNEEHSKQ YRSLSFQPEN SMKLTPLVLE VFGNEIS
rVDR 357 NTLQTYIRCR HPPPGSHQLY AKMIQKLADL RSLNEEHSKQ YRSLSFQPEN SMKLTPLVLE VFGNEIS
cVDR 385 DILQTYIRCR HPPPGSRLLY AKMIQKLADL RSLNEEHSKQ YRCLSFQPEH SMQLTPLVLE LFSSTDG
mPXR 368 LTLKAYIECS RPYPAHRFLF LKIMAVLTEL RSINAQQTQQ LLRIQDSHP- --FATPLMQE LFSSTDG
hPXR 371 ITLKSYIECN RPQPAHRFLF LKIMAMLTEL RSINAQHTQR LLRIQDIHP- --FATPLMQE LFGITGS
H10
H11
H12
B
H6
VDR
(Δ165-215)
C
loop
PXR

Fig. 4. Comparison of the LBD sequence of VDR and PXR

A: Sequence alignment of VDR-LBDs [h, human (NCB accession no. AAA61273); m, mouse (NP_033530); r, rat (NP_058754); c,chicken (O42392)] and PXR-LBDs [m, mouse (AAC39964); h, human (AAD05436)]. Bars show helices (H) and β-strands in hVDR and hPXR. Dark shadows show completely conserved residues in the alignment, and light shadows indicate partially conserved residues. Black circles show amino acid residues lining the LBP of hVDR. B and C: Ribbon loop presentations of hVDR-LBD (Δ165–215) (B) and hPXR-LBD (C). α-Helix, β-sheet, and loop are shown as ribbons, arrows, and tubes, respectively. The LBPs of VDR and PXR are shown as Connolly channel surfaces.

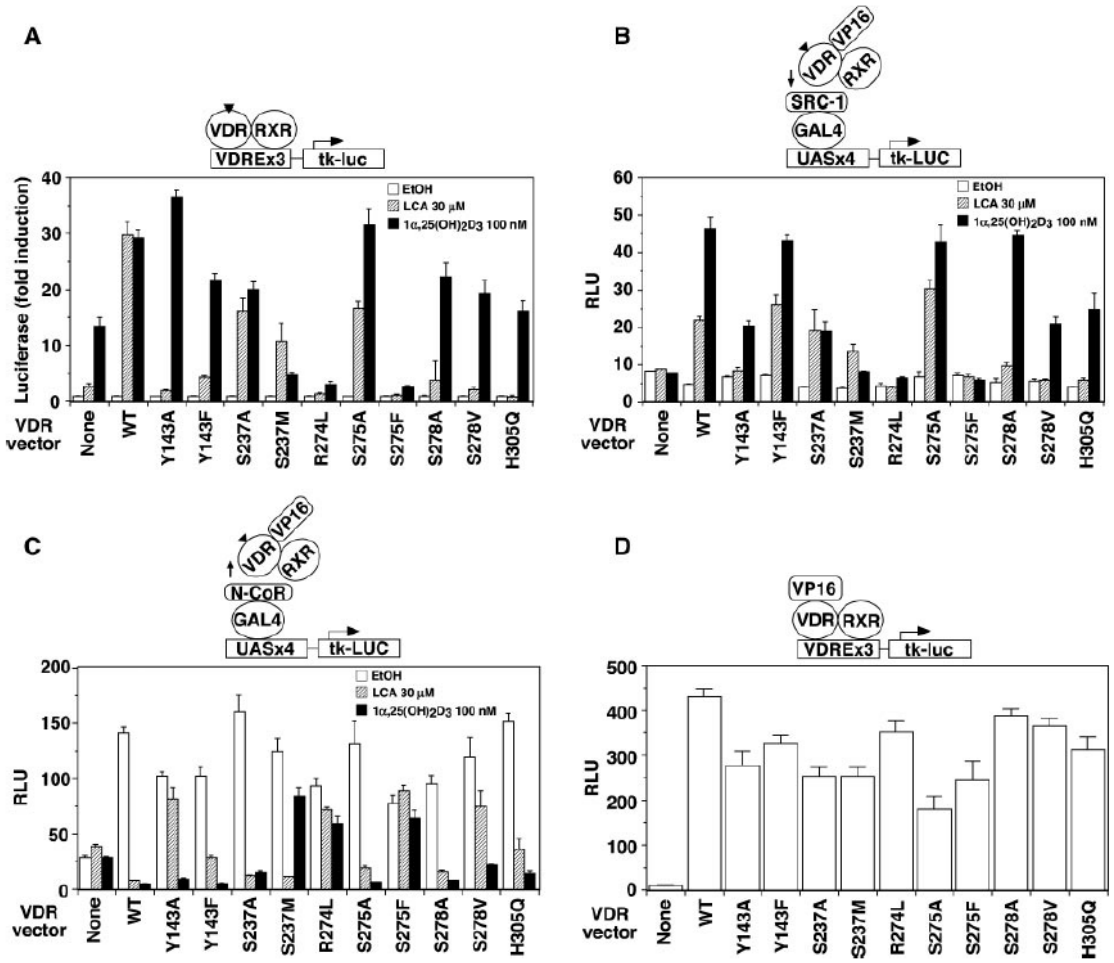

Fig. 5. Functional analyses of VDR mutants

A: Activation of VDR and its mutants by LCA and 1α, 25(OH)$_2$D$_3$. VDR expression vectors or a control vector and a CYP3A4-ER-6x3-tk-LUC reporter were transfected into HEK293 cells and treated with LCA and 1α, 25(OH)$_2$D$_3$. B: Association between SRC-1 and VDR and its mutants by LCA and1α, 25(OH)$_2$D$_3$. C: Dissociation of N-CoR from VDR and its mutants by LCA and 1α, 25(OH)$_2$D$_3$. Mammalian two-hybrid analysis using GAL4-SRC-1 or GAL4-N-CoR and VP16-VDR was performed in HEK293 cells. D: Evaluation of expression levels of functional VDR mutants in transfected cells. Cells were cotransfected with VP16-VDR

mutants or VP16 control vector and CYP3A4-ER-6x3-tk-LUC reporter. Fold induction by the ligands is relative to ethanol control (EtOH) vehicle. RLU, Relative light units. The values represent means ± SD of triplicate assays.

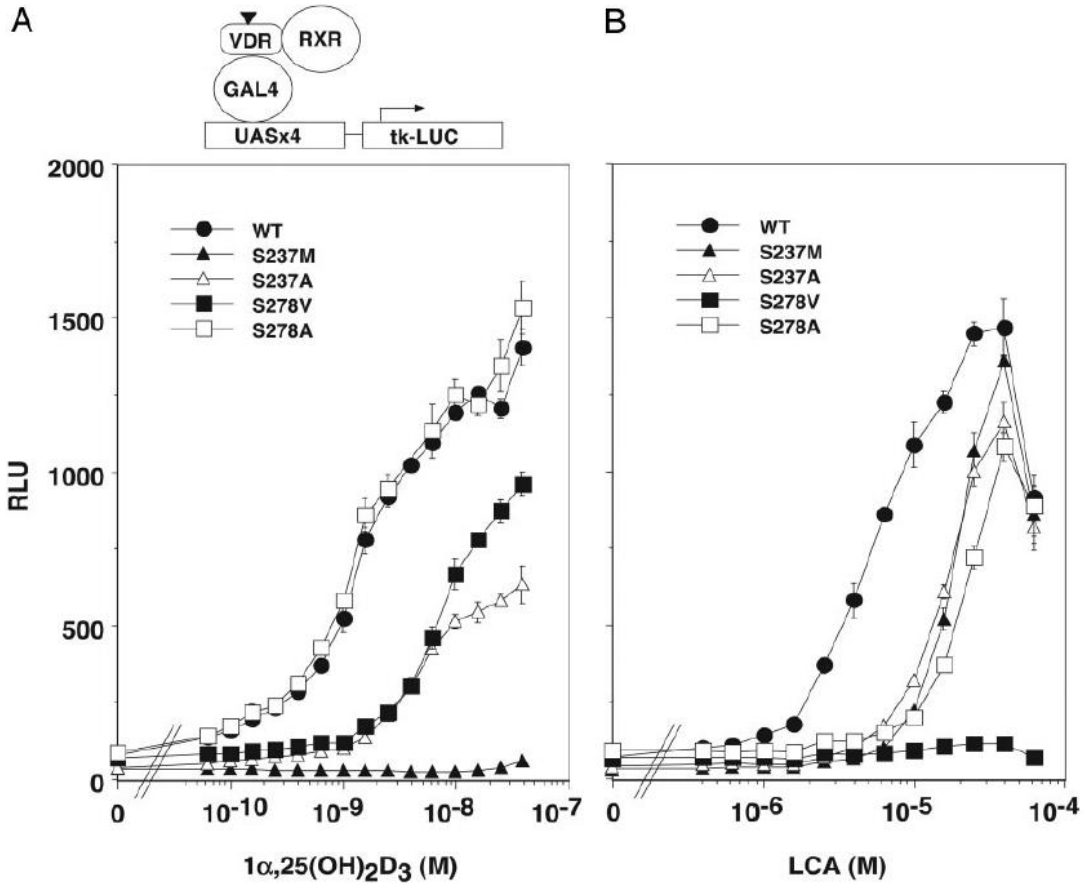

Fig. 6. Dose response of wild-type VDR, S237M, S237A, S278V, and S278A mutants to 1α, $25(OH)_2D_3$ and LCA using aGAL4-receptor luciferase assay

Cells were cotransfected with GAL4-VDRs and MH100(UAS)x4-tk-LUC reporter, followed by treatment with the various concentrations of 1α, $25(OH)_2D_3$ (A) and LCA(B). RLU, Relative light units. The values represent means ± SD of triplicate assays.

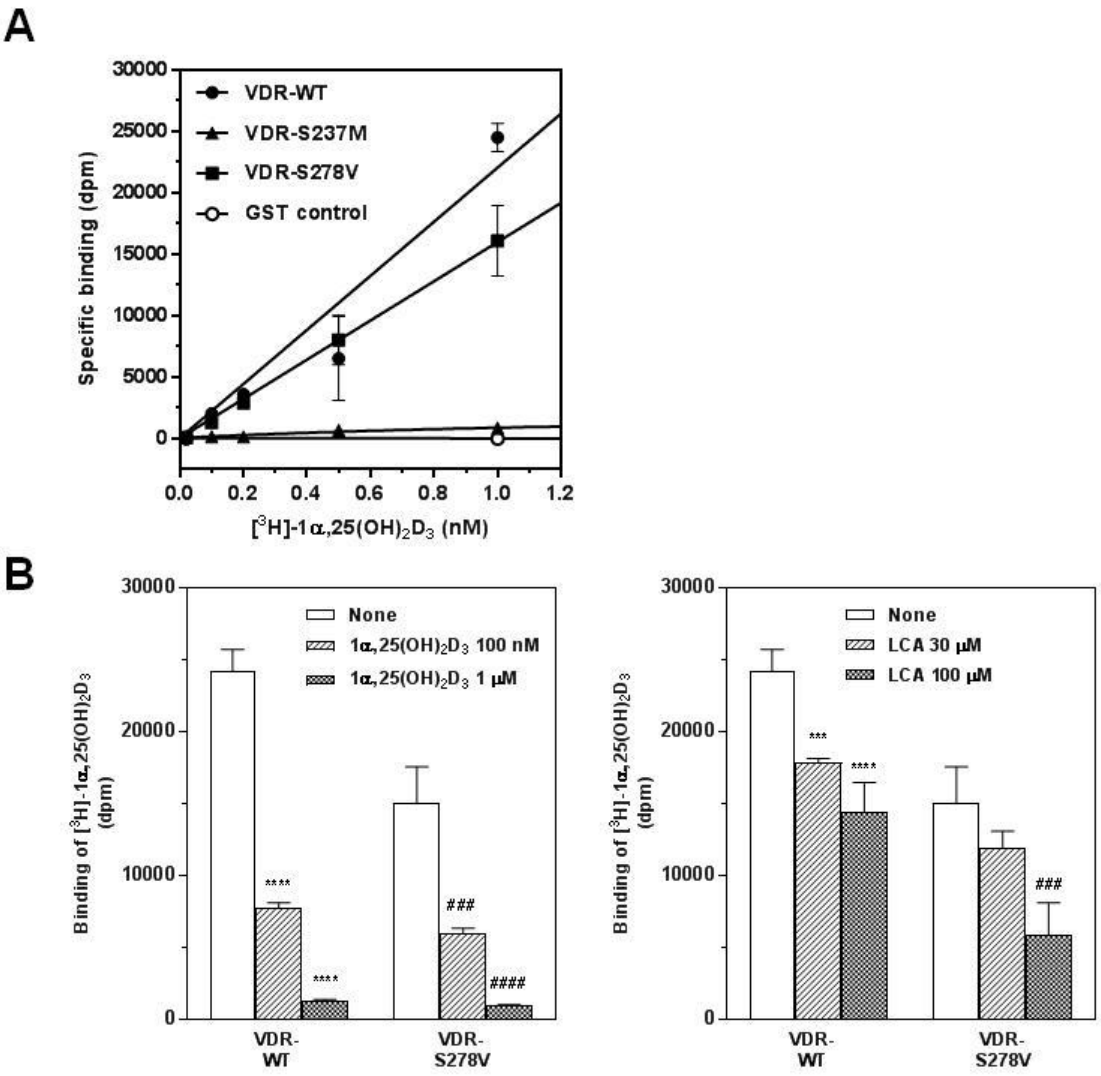

Fig. 7. Ligand binding specificity of wild-type VDR, S237M, and S278V mutants

A: Direct binding of 1α, $25(OH)_2D_3$ to VDR. GST-fusion VDR proteins or GST control protein were incubated with increasing concentrations of $[^3H]$1α, $25(OH)_2D_3$ in the presence or absence of 400-fold excess nonradioactive 1α, $25(OH)_2D_3$ to calculate the specific binding. B: Competitive binding of 1α, $25(OH)_2D_3$ and LCA to wild-type VDR and S278V mutants. GST-fusion VDR proteins were incubated with 1 nM $[^3H]$1α, $25(OH)_2D_3$ in the presence or

absence of the indicated concentrations of nonradioactive 1α, 25$(OH)_2D_3$ or LCA. *** $P <$ 0.0005, **** $P < 0.0001$ compared with control for wild-type VDR; ### $P < 0.0005$, #### $P <$ 0.0001 compared with control for S278V. The values represent means ± SD of triplicate assays.

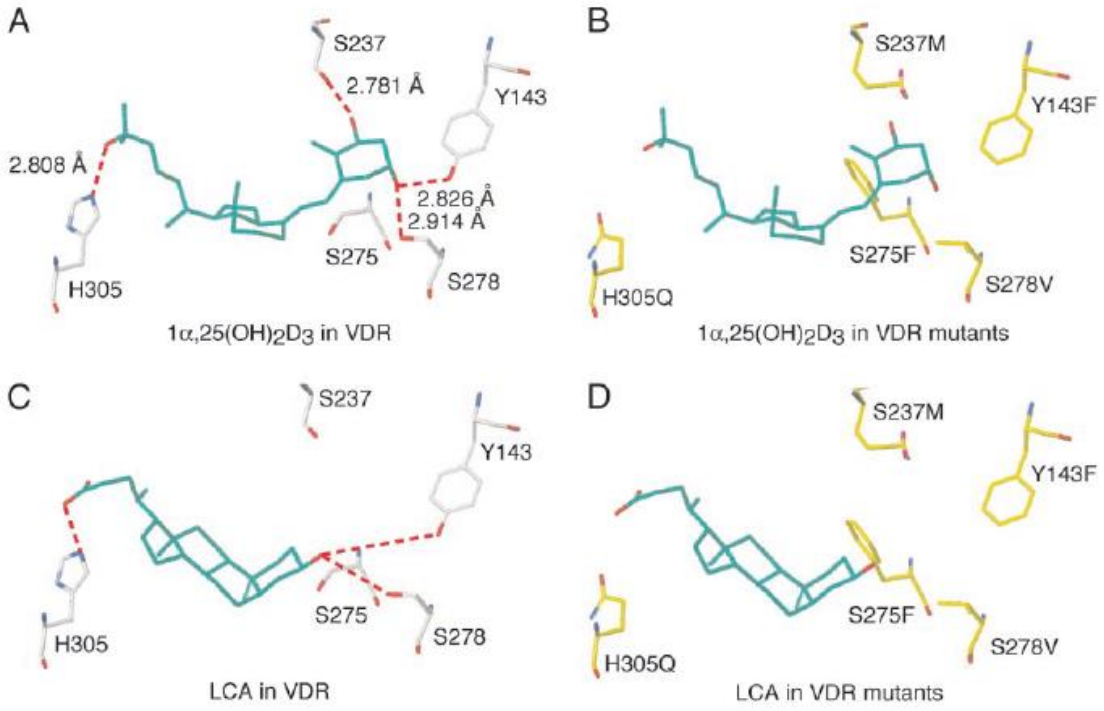

Fig. 8. Molecular modeling of VDR interaction with ligands

A: 25-Hydroxyl group, 1-hydroxyl group, and 3-hydroxyl group of 1α,25(OH)$_2$D$_3$ make hydrogen bonds with H305, S237, and Y143/S278 of VDR, respectively. B: Docking model of VDR point mutants with 1α,25(OH)$_2$D$_3$. S237M and S275F mutations are predicted to weaken interaction with 1α,25(OH)$_2$D$_3$, whereas the effects of H305Q, Y143F, and S278V on the modeled interaction are minor. C: Docking model of LCA and wild-type VDR. Y143 and S278 weakly interact with the 3-hydroxyl group of LCA. These interactions are critical because S237 cannot make a hydrogen bond with LCA. H305 is suitably positioned to form a hydrogen bond with the carboxyl group of LCA. D: Docking model of LCA and VDR mutants. Mutation of

S278V, H305Q, and S275F would be expected to destabilize interaction with LCA. Because S237 does not interact with LCA, S237M would be expected to have no effect on LCA interaction.

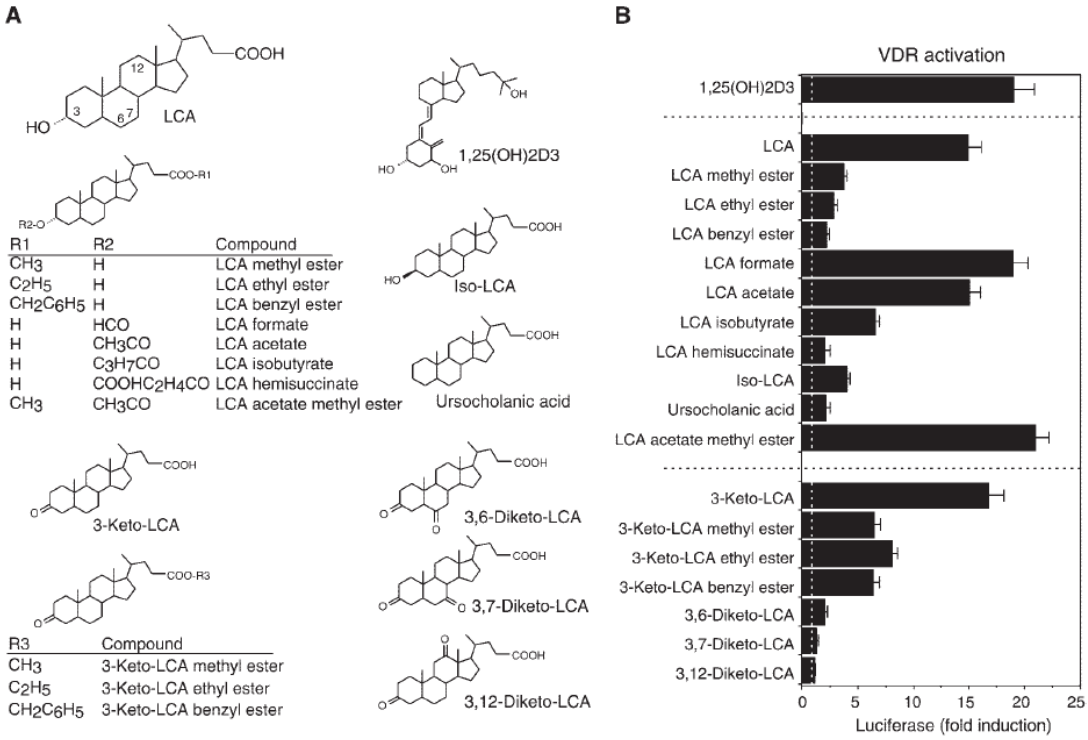

Fig. 9. LCA derivatives activate VDR

A: Structures of LCA, its derivatives, and 1α,25$(OH)_2D_3$ 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), 1α, 25$(OH)_2D_3$ (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 of triplicate assays.

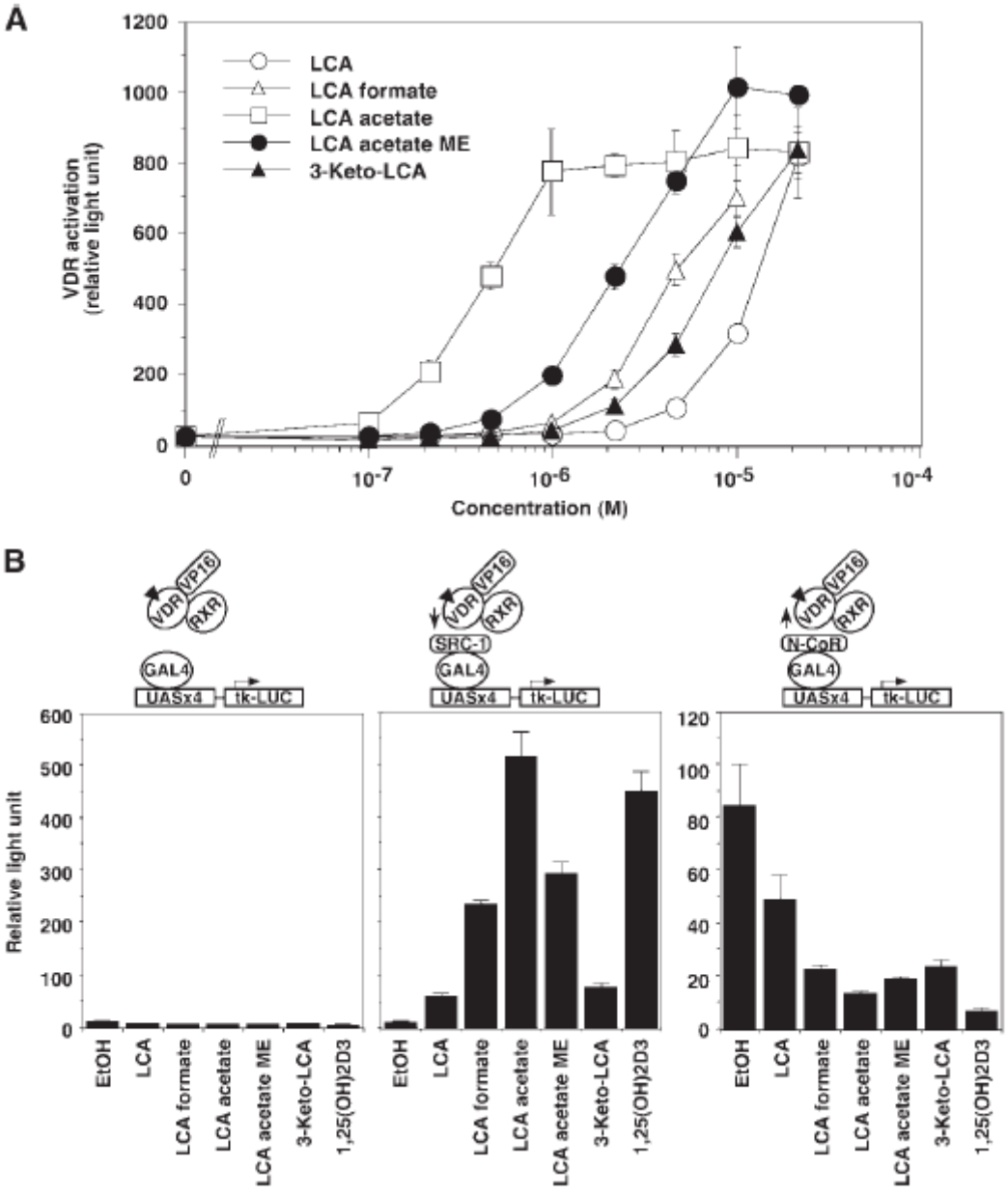
A
VDR activation (relative light unit)
Concentration (M)
LCA
LCA formate
LCA acetate
LCA acetate ME
3-Keto-LCA
B
Relative light unit
VDR
VP16
RXR
GAL4
UASx4
tk-LUC
SRC-1
N-CoR
EtOH
LCA
LCA formate
LCA acetate
LCA acetate ME
3-Keto-LCA
1,25(OH)2D3

Fig. 10. 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. Activation of VDR was monitored by measuring the luciferase activities. B: Interactions of VDR with SRC-1 and 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. The luciferase activities were measured. The values represent means ± SD of triplicate assays.

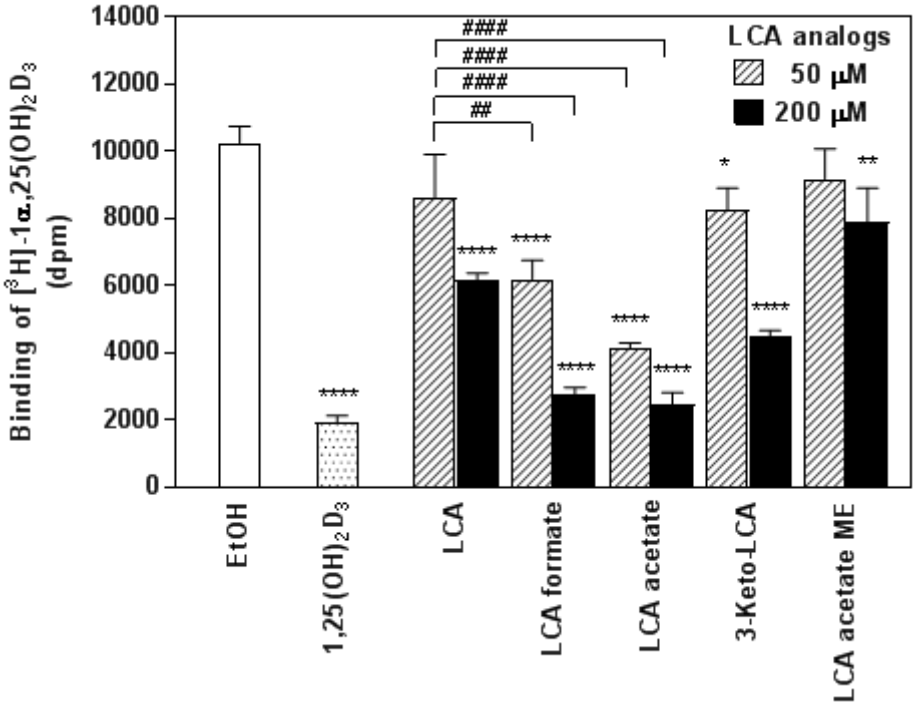

Fig. 11. LCA acetate is a potent VDR ligand

Direct binding of LCA acetate to VDR. *In vitro* translated VDR proteins were incubated with 1nM [^{3}H] 1α, 25(OH)$_2$D$_3$ in the presence or absence of nonradioactive 10 nM 1α, 25(OH)$_2$D$_3$ or 50 μM or 200 μM bile acid derivatives. Bound [^{3}H] 1α, 25(OH)$_2$D$_3$ was measured. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared with EtOH control; ## $P < 0.01$, #### $P < 0.0001$ compared with 50 μM LCA. The values represent means ± SD of triplicate assays.

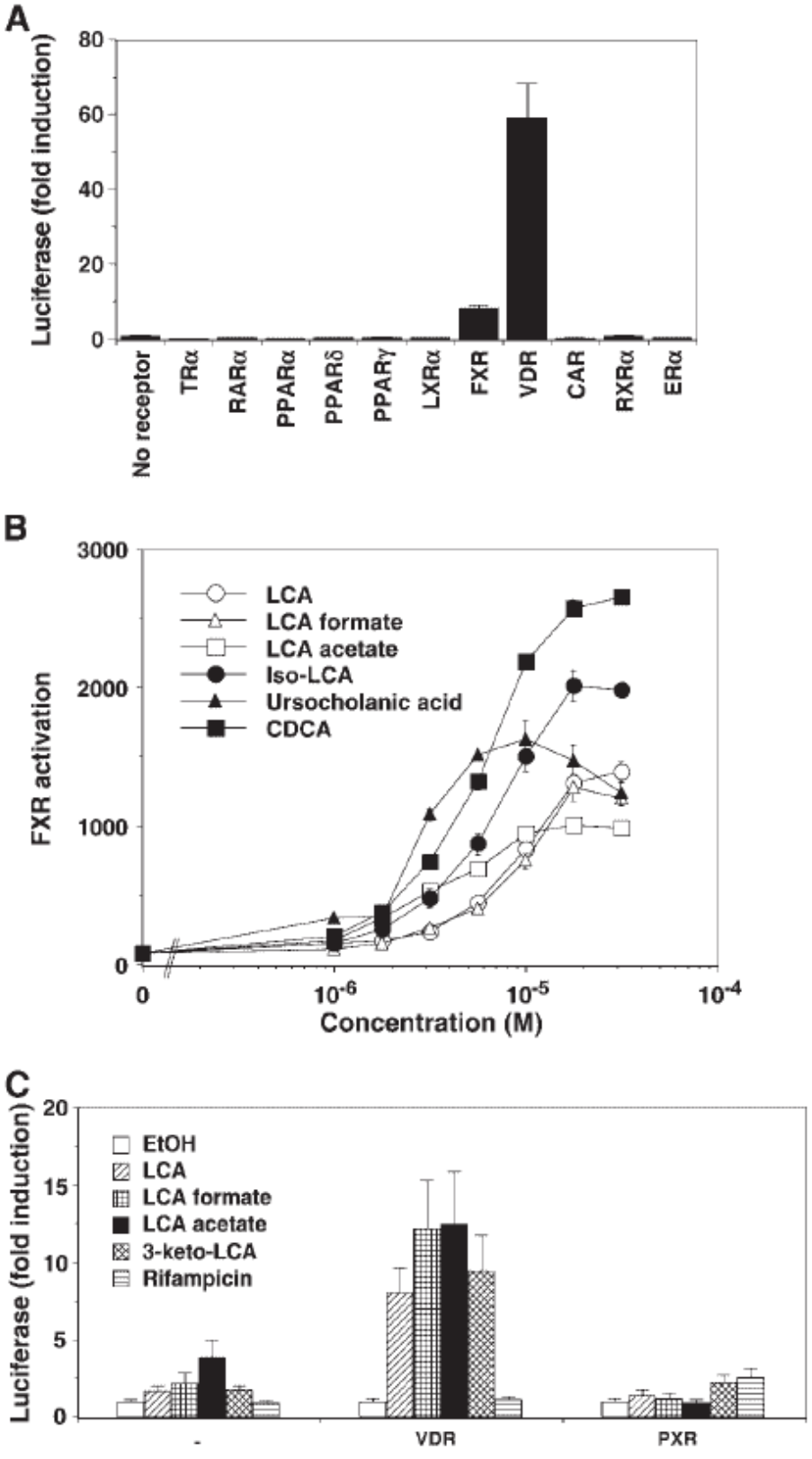
A
Luciferase (fold induction)
80
60
40
20
0
No receptor
TRα
RARα
PPARα
PPARδ
PPARγ
LXRα
FXR
VDR
CAR
RXRα
ERα
B
FXR activation
3000
2000
1000
0
LCA
LCA formate
LCA acetate
Iso-LCA
Ursocholanic acid
CDCA
0
10-6
10-5
10-4
Concentration (M)
C
Luciferase (fold induction)
20
15
10
5
0
EtOH
LCA
LCA formate
LCA acetate
3-keto-LCA
Rifampicin
-
VDR
PXR

Fig. 12. LCA acetate is a selective agonist for VDR

A: Receptor-specific activation by LCA acetate. GAL4-chimera receptors for various NRs 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. B: Concentration-dependent activation of 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 CDCA. The luciferase activities were measured. C: Comparative response of VDR and 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 (EtOH), 30 μM LCA, LCA formate, LCA acetate, 3-keto-LCA, or rifampicin. The luciferase activities were measured. The values represent means ± SD of triplicate assays.

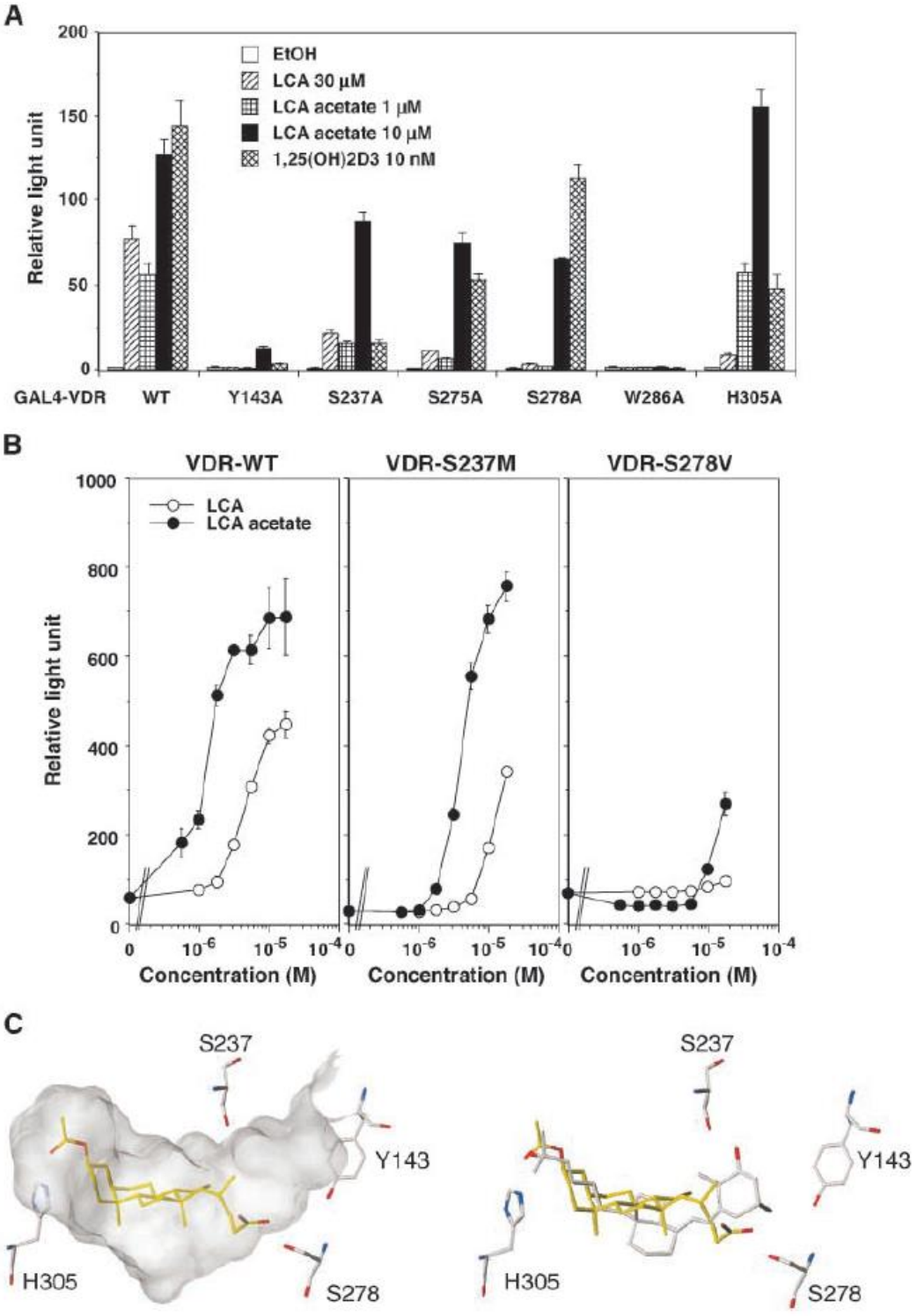
A
Relative light unit
200
150
100
50
0
EtOH
LCA 30 μM
LCA acetate 1 μM
LCA acetate 10 μM
1,25(OH)2D3 10 nM
GAL4-VDR
WT
Y143A
S237A
S275A
S278A
W286A
H305A
B
VDR-WT
VDR-S237M
VDR-S278V
LCA
LCA acetate
Relative light unit
1000
800
600
400
200
0
10-6
10-5
10-4
Concentration (M)
C
S237
Y143
H305
S278

Fig. 13. Structure-function analysis of LCA acetate and VDR

A: Activation of VDR or its mutants by LCA acetate. GAL4-VDR and its 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: Does 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 of triplicate assays. 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-LBP is shown in translucent gray. Right panel: Overlay of LCA acetate (yellow) and 1α, 25$(OH)_2D_3$ (gray) accommodated in the VDR-LBP.

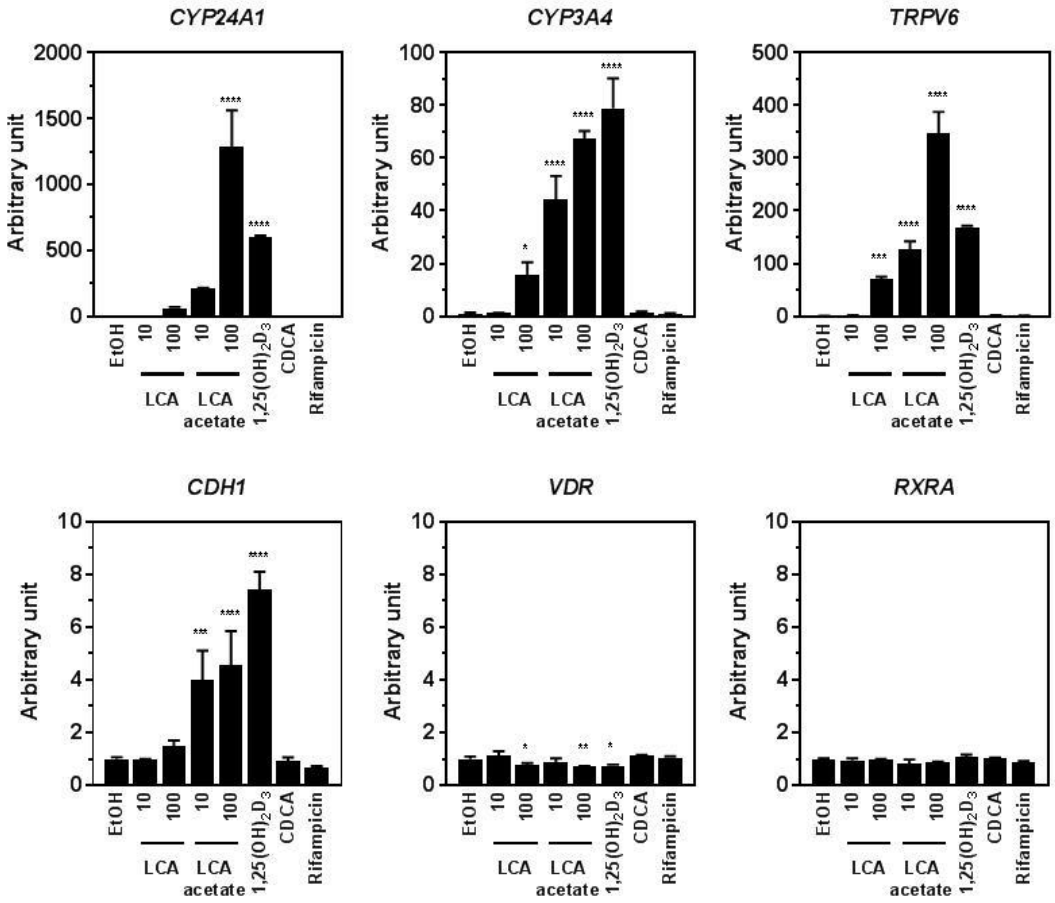

Fig. 14. Induction of VDR target genes by LCA acetate in intestinal cells

LCA acetate induced VDR target genes more effectively than LCA in colon cancer-derived SW480 cells. Cells were treated with vehicle control (EtOH), 10 μM or 100 μM of LCA or LCA acetate, 100 nM 1α, 25(OH)$_2$D$_3$, 100 μM CDCA, or 30 μM rifampicin for 24 h. Quantitative real-time PCR from mRNA for *CYP24A1*, *CYP3A4*, *TRPV6*, CDH1 (E-cadherin), *VDR*, and *RXRA*(RXRα) was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0005$, **** $P < 0.0001$ compared with EtOH control. The values represent means ± SD of triplicate assays.

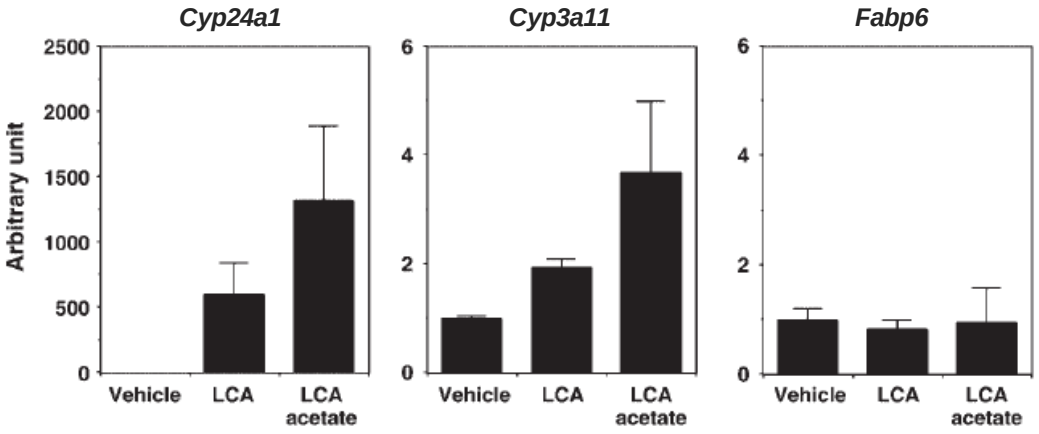

Fig. 15. Induction of VDR target genes by LCA acetate in the intestine

Mice were orally administrated with vehicle (n=3), 200 mg/kg LCA (n=3), or 200 mg/kg LCA acetate (n=3). Twelve hours after administration, total RNA was extracted from intestinal mucosa and quantitative real-time PCR from mRNA for *Cyp24a1*, *Cyp3a11*, and *Fabp6* (ileal bile acid binding protein) was performed. The values represent means ± SD.

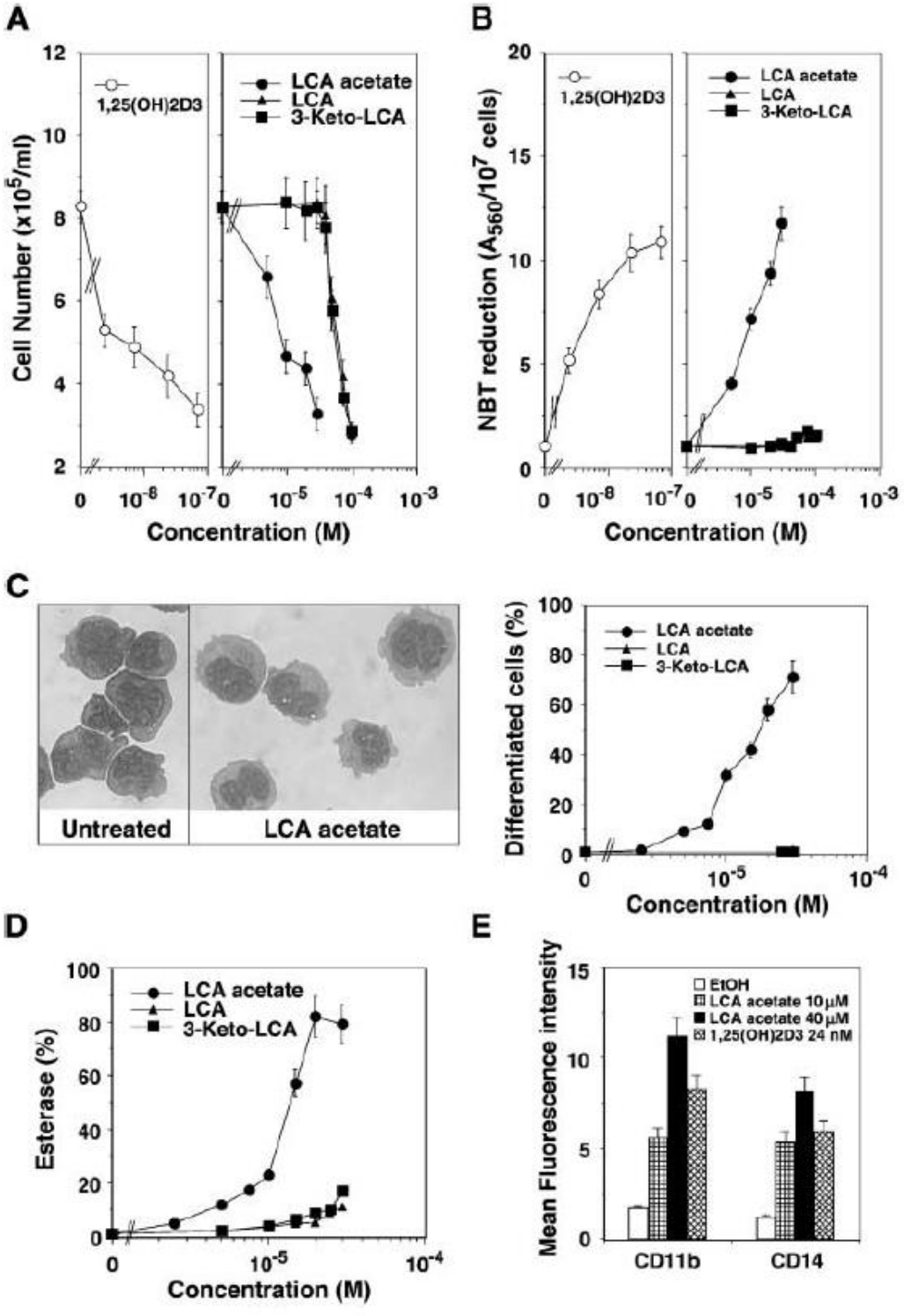
A
Cell Number (x10^5/ml)
1,25(OH)2D3
LCA acetate
LCA
3-Keto-LCA
Concentration (M)
B
NBT reduction (A560/10^7 cells)
1,25(OH)2D3
LCA acetate
LCA
3-Keto-LCA
Concentration (M)
C
Untreated
LCA acetate
Differentiated cells (%)
LCA acetate
LCA
3-Keto-LCA
Concentration (M)
D
Esterase (%)
LCA acetate
LCA
3-Keto-LCA
Concentration (M)
E
Mean Fluorescence intensity
EtOH
LCA acetate 10 μM
LCA acetate 40 μM
1,25(OH)2D3 24 nM
CD11b
CD14

Fig. 16. Effects of LCA acetate on growth and differentiation of human myeloid leukemia THP-1 cells

A: Growth inhibition of THP-1 cells by 1α, $25(OH)_2D_3$ and LCA acetate. B: Induction of NBT-reducing activity in THP-1 cells by 1α, $25(OH)_2D_3$ and LCA acetate. THP-1 cells were treated with 1α, $25(OH)_2D_3$, 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 as shown in the left panel were counted. Percentage of the differentiated cells are plotted in the right graph in response to the compounds. D: LCA acetate induces monocyte-specific esterase activity. Cells were treated with LCA acetate, LCA, or 3-keto-LCA for 6 days. Esterase activities were measured for monitoring differentiation of the cells. E: LCA acetate increases the expression of CD11b and CD14 surface antigens. Cells were treated with LCA or 1α, $25(OH)_2D_3$ for 4 days and CD11b and CD14 expression was examined using monoclonal antibodies and flow cytometry. EtOH, ethanol. The values represent means ± SD of triplicate assays.