

Vitamin D₃ Derivatives with Adamantane or Lactone Ring Side Chains are Cell Type-Selective Vitamin D Receptor Modulators

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ABSTRACT

The vitamin D receptor (VDR) mediates the biological actions of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D, which regulates calcium homeostasis, immunity, cellular differentiation, and other physiological processes. We investigated the effects of three 1,25(OH)₂D₃ derivatives on VDR function. AD47 has an adamantane ring and LAC67a and LAC67b have lactone ring substituents at the side chain position. These vitamin D derivatives bind to VDR but do not stabilize an active cofactor conformation. In a VDR transfection assay, AD47 and LAC67b act as partial agonists and all three compounds inhibit VDR activation by 1,25(OH)₂D₃. The derivatives enhanced the heterodimerization of VDR with the retinoid X receptor, an effect unrelated to agonist/antagonist activity. AD47 and LAC67b weakly induced recruitment of the SRC-1

cofactor to VDR, and all three derivatives inhibited the recruitment of p160 family cofactors to VDR induced by 1,25(OH)₂D₃. It is noteworthy that AD47 induced DRIP205 recruitment as effectively as 1,25(OH)₂D₃, whereas LAC67a and LAC67b were not effective. We examined the expression of endogenous VDR target genes and the nuclear protein levels of VDR and cofactors in several cell lines, including cells derived from intestine, bone, and monocytes, and found that the vitamin D₃ derivatives act as cell type-selective VDR modulators. The data indicate that side chain modification is useful in the development of VDR antagonists and tissue-selective modulators. Further elucidation of the molecular mechanisms of action of selective VDR modulators will be essential for their clinical application.

The vitamin D receptor (VDR; NR1I1) is a member of the nuclear receptor superfamily that regulates physiological processes, including cell growth and differentiation, embryonic development, and metabolic homeostasis (Makishima, 2005). Nuclear receptor transcriptional activity is modulated by ligands such as steroids, retinoids, and other lipid-soluble compounds. Upon ligand binding, nuclear receptors undergo a conformational change in the cofactor binding site and activation function 2 (AF2) helices, which results in dynamic exchange of cofactor complexes, allowing nuclear receptors to modulate the transcription of specific target genes (Rosenfeld et al., 2006). In the absence of ligand, corepressors bind to the

AF2 surface composed of portions of helix (H) 3, loop 3–4, H4/5, and H11. Ligand binding reduces the receptor affinity for corepressors and recruits coactivators to the altered AF2 surface formed by repositioning of H12. Recent studies suggest that DNA sequence-specific effects of transcription factor activity are associated with site-specific interaction with cofactor complexes (Rosenfeld et al., 2006). Chemical modification of ligands may induce AF2 conformations and cofactor interactions distinct from those of natural ligands and can result in cell type-selective modulation of target gene expression.

The active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], regulates calcium and bone homeostasis, immunity, and cellular growth and differentiation through VDR activation (DeLuca, 2004; Haussler et al., 1998; Makishima and Yamada, 2005). VDR forms a heterodimer with the retinoid X receptor (RXR; NR2B) and binds preferentially

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ABBREVIATIONS: VDR, vitamin D receptor; AF2, activation function 2; H, helix; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; RXR, retinoid X receptor; ER6, everted repeat 6; LBD, ligand-binding domain; MR, mineralocorticoids; HEK, human embryonic kidney; FBS, fetal bovine serum; GST, glutathione transferase; LCA, lithocholic acid; PCR, polymerase chain reaction; ZK159222, (24R)-25-(butoxycarbonyl)-1 α ,24-dihydroxy-26,27-cyclo-22,23-didehydrovitamin D₃; ZK191732, (24R)-1 α ,24-dihydroxy-25-(4-methylthiazol-2-yl)-26,27-cyclo-22,23-didehydrovitamin D₃; ZK16289, (24R)-1 α ,24-dihydroxy-25-(1-oxohex-1-yl)-26,27-cyclo-22,23-didehydrovitamin D₃; ZK168281, (24R)-1 α ,24-dihydroxy-25-(ethoxyacrylo-3-yl)-26,27-cyclo-22,23-didehydrovitamin D₃; TEI-9647, (23S)-1 α -hydroxy-25,27-didehydrovitamin D₃-26,23-lactone.

to a DNA response element that consists of a direct repeat of a hexanucleotide (AGGTCA or related sequence) motif separated by a three-nucleotide spacer, or an everted repeat motif with a six nucleotides spacer (ER6) (Makishima and Yamada, 2005). VDR is highly expressed in target organs that mediate calcium homeostasis, such as intestine, bone, and kidney. The crystal structure of the 1,25(OH)₂D₃-bound human VDR ligand-binding domain (LBD) reveals that 1,25(OH)₂D₃ is anchored by hydrogen bonds formed between its three hydroxyl groups and three pairs of hydrophilic residues: Tyr143 (in H1) and Ser278 (in H5) interact with the 3β-hydroxyl group of 1,25(OH)₂D₃, Ser237 (in H3) and Arg274 (in H5) hydrogen bonds with the 1α-hydroxyl group, and His305 (in loop 6–7) and His397 (in H11) coordinates the 25-hydroxyl group (Rochel et al., 2000). Hydrophobic interactions between the ligand and ligand-binding pocket residues, such as Leu233 (in H3) and Trp286 (in β-sheet), play a crucial role in stabilizing the active conformation of VDR-LBD. Stabilization of an active AF2 surface conformation requires hydrophobic interactions between Val418 (in H12) and Phe422 (in H12) with the terminal methyl group of the ligand and results in recruitment of coactivators (Yamada et al., 2003; Makishima and Yamada, 2005). In the active conformation, two highly conserved charge clamp residues, Lys246 (in H3) and Glu420 (in H12), are exposed at opposite edges of a hydrophobic cleft in the AF2 surface. Positioning of H11 is also important for formation of the active conformation. His305 (in loop 6–7) coordinates with His397 (in H11) via the 25-hydroxyl group of 1,25(OH)₂D₃, and this interaction connects the LBP at H6 to loop 6–7 and extends to Phe422 (in H12) (Yamamoto et al., 2006).

A number of vitamin D analogs have been synthesized and evaluated for selective VDR ligand activity (Yamada et al., 2003; DeLuca, 2004). With an improved understanding of the mechanisms of VDR signaling and the availability of sensitive VDR assays, the possibility of identifying VDR ligands with selective action is emerging. The development of VDR antagonists will not only provide useful tools in elucidating molecular mechanisms of nuclear receptor activation but also may yield clinically useful drugs, as in case of the selective estrogen receptor modulators. Two types of nuclear receptor antagonists have been reported. The selective estrogen modulator 4-hydroxyl tamoxifen defines the first group, which has a bulky functional group that creates steric hindrance with residues of H12 of estrogen receptor α (NR3A1) and prevents formation of the active AF2 conformation (Brzozowski et al., 1997; Shiau et al., 1998). Members of a second group of antagonists lack bulky substituents. For example, progesterone is an antagonist of the mineralocorticoid receptor (MR; NR3C2) because it fails to interact with H3 of MR and to stabilize an interaction between H3, H5, and the AF2 surface (Geller et al., 2000; Bledsoe et al., 2005). In this study, we examined the effects of three vitamin D₃ derivatives on VDR activation, cofactor interactions, and induction of target gene expression. One has a bulky structural unit (adamantane ring) at the side chain, and two have a methylene lactone. These compounds act as selective VDR modulators and may be useful in the elucidation of cellular and in vivo functions of VDR.

Materials and Methods

Plasmids. The expression vectors pCMX-VDR, pCMX-GAL4-VDR, pCMX-VP16-VDR, pCMX-GAL4-RXRα, pCMX-GAL4-RXRβ, pCMX-GAL4-RXRγ, pCMX-VP16-RXRα, pCMX-VP16-RXRβ, pCMX-VP16-RXRγ, pCMX-GAL4-RXRα (H1), pCMX-VP16-RXRα (H3-H12), pCMX-GAL4-SRC-1, pCMX-GAL4-ACR, pCMX-GAL4-DRIP205, and pCMX-GAL4-N-CoR were reported previously (Kaneko et al., 2003; Morita et al., 2005; Nakano et al., 2005). The nuclear receptor-interacting domains of SRC-1 (amino acids 595–771; GenBank accession no. U90661), GRIP1 (amino acids 629–792; GenBank accession no. NM_008678), ACTR (amino acids 601–780; GenBank accession no. AF036892), DRIP205 (amino acids 578–728; GenBank accession no. Y13467), N-CoR (amino acids 1990–2416; GenBank accession no. U35312), and SMRT (amino acids 2003–2517; GenBank accession no. AF113003) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-SRC-1, pCMX-GAL4-GRIP1, pCMX-GAL4-ACTR, pCMX-GAL4-DRIP205, pCMX-GAL4-N-CoR, and pCMX-GAL4-SMRT, respectively. The CYP24 promoter-luciferase reporter was constructed by inserting 1270 base pairs of the human CYP24A1 promoter (GenBank accession no. U60669) into the luciferase reporter pGL3 (Promega, Madison, WI). VDR-responsive hCYP3A4-ER6×3-tk-LUC and GAL4-responsive MH100(UAS)×4-tk-LUC were used in luciferase reporter assay (Nakano et al., 2005). pGEX vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used to generate glutathione transferase (GST) fusions (Kaneko et al., 2003). Fragments from pCMX-GAL4-VDR, pCMX-GAL4-RXRα, pCMX-GAL4-SRC-1, and pCMX-GAL4-DRIP205 were inserted into pGEX vector to generate pGEX-VDR, pGEX-RXRα, pGEX-SRC-1, and pGEX-DRIP205, respectively.

Cell Culture and Cotransfection Assay. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle medium containing 5% fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Human colon carcinoma SW480, HCT116, and immortalized keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle medium containing 10% FBS, monoclonal leukemia THP-1 cells were in RPMI 1640 medium containing 10% FBS, and osteosarcoma MG63 cells were in minimum essential medium containing 10% FBS.

Transfections in HEK293 cells were performed by the calcium phosphate coprecipitation assay as described previously (Kaneko et al., 2003). Eight hours after transfection, compounds were added. Cells were harvested after 16 to 24 h and were assayed for luciferase and β-galactosidase activities using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). Cotransfection experiments used 50 ng of reporter plasmid, 10 ng of pCMX-β-galactosidase, and 15 ng of each expression plasmid in each well of a 96-well plate. Luciferase data were normalized to the internal β-galactosidase control and represent the mean ± S.D. of triplicate assays.

Competitive Ligand-Binding Assay. Bovine thymus VDR (Yamada, Chiba, Japan) and GST-RXRα fusion proteins were used for a competitive ligand-binding assay (Yamamoto et al., 1996). The LBD of bovine VDR (GenBank accession no. Q28037) is identical to that of human VDR (GenBank accession no. P11473). The proteins were dissolved in 0.05 M phosphate buffer, pH 7.4, containing 0.3 M KCl and 5 mM dithiothreitol and were incubated with [26,27-methyl-³H] 1,25(OH)₂D₃ or 9-*cis*-[³H]retinoic acid at 4°C in the presence or absence of nonradioactive competitor compounds. Bound and labeled 1,25(OH)₂D₃ or 9-*cis* retinoic acid was assessed using scintillation counting.

GST Pull-Down Assay. GST fusion proteins were expressed in BL21 DE3 cells (Promega), and purified using glutathione Sepharose 4B (GE Healthcare). [³⁵S]Labeled proteins were generated using the TNT Quick-Coupled Transcription/Translation System (Promega). GST pull-down was performed as reported previously (Kaneko et al., 2003). Approximately 5 μg of GST chimera protein was bound to glutathione Sepharose 4B and equilibrated in binding buffer (20 mM

Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40, and 2 mg/ml bovine serum albumin). Bound GST proteins were incubated with 10 μ l of reticulocyte lysate containing 35 S-labeled proteins and were treated with test compounds for 2 h at 4°C. After binding, beads were washed and resuspended in SDS sample buffer, and loaded on an 8% or 12% SDS-polyacrylamide gel. After electrophoresis, bound isotope-labeled proteins were visualized with the BAS2000 system (Fujifilm, Tokyo, Japan).

Quantitative Real-Time Reverse Transcription-PCR Analysis. Total RNAs from samples were prepared with RNagents Total RNA Isolation system (Promega), and cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega). Real-time PCR was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) (Nakano et al., 2005). Primers were as follows: CYP24A1, 5'-TGA ACG TTG GCT TCA GGA GAA-3' and 5'-AGG GTG CCT GAG TGT AGC ATC T-3'; CYP3A4, 5'-AGT GTG GGG CTT TTA TGA TG-3' and 5'-ATA CTG GGC AAT GAT AGG GA-3'; E-cadherin, 5'-GAA GGT GAC AGA GCC TCT GGA TAG-3' and 5'-CTG GAA GAG CAC CTT CCA TGA-3'; CaT1, 5'-GCT ACT TCA GGA AGC CTA CAT G-3' and 5'-TGT CCA AAG AAG CGA GTG ACC-3'; actin, 5'-GAC AGG ATG CAG AAG GAG AT-3' and 5'-GAA GCA TTT GCG GTG GAC GAT-3'. The RNA values were normalized to the level of actin mRNA and represent the mean \pm S.D. of triplicate assays.

Immunoblotting. Nuclear extracts were prepared as described previously (Schreiber et al., 1989). The proteins were separated by SDS-polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane, probed with anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-RXR α antibody (Santa Cruz Biotechnology), anti-SRC-1 antibody (Upstate, Lake Placid, NY), anti-DRIP205 antibody (Santa Cruz Biotechnology), or anti-Lamin B1 antibody (Santa Cruz Biotechnology), and visualized with an alkaline phosphatase conjugate substrate system (Uno et al., 2006).

Molecular Modeling. Ligand docking was performed using Sybyl 7.1 (Tripos, St. Louis, MO) (Yamamoto et al., 2006). The atomic coordinates of the human VDR-LBD (Δ 165–215) crystal structure were retrieved from the Protein Data Bank (code 1DB1). Ligands were docked into the ligand-binding pocket manually by superposition with 1,25(OH) $_2$ D $_3$ at the A- to D-ring.

Results

Vitamin D $_3$ Derivatives with an Adamantane or Lactone Ring Were VDR Antagonists. AD47 is a 19-nor-1,25(OH) $_2$ D $_3$ derivative that has a bulky adamantane ring at the terminal side chain, and LAC67b is a 2-methylene-19-nor-1,25(OH) $_2$ D $_3$ derivative with a methylene lactone side chain structure (Fig. 1). We previously reported that these compounds inhibited VDR transactivation induced by 1,25(OH) $_2$ D $_3$ in COS-7 cells (Yamamoto et al., 2006). We newly synthesized another 2-methylene-19-nor-1,25(OH) $_2$ D $_3$ derivative, LAC67a, a diastereoisomer of LAC67b (Fig. 1), and compared the effects of these vitamin D derivatives on VDR function. To examine VDR agonist activity, we transiently transfected HEK293 cells with a VDR expression vector and a human CYP24-luciferase reporter containing the proximal 1270 base pairs of the promoter (Pascucci et al., 2005). Treatment with 1,25(OH) $_2$ D $_3$ increased luciferase activity in a concentration-dependent manner (Fig. 2A). AD47 and LAC67b had maximal activation that was 12% and 70% of 1,25(OH) $_2$ D $_3$ induction, respectively. AD47 and LAC67b also induced a luciferase reporter containing the VDR-responsive ER6 element derived from the CYP3A4 promoter (Adachi et al., 2005), and their maximal activation was 39%

and 76% of 1,25(OH) $_2$ D $_3$ induction, respectively (Fig. 2B). AD47 showed more agonistic potency on this reporter. These findings indicate that these compounds are partial agonists. LAC67a did not effectively induce VDR activation on either reporter (Fig. 2, A and B). To examine VDR antagonist activity, we treated cells with these derivatives in combination with 10 nM 1,25(OH) $_2$ D $_3$. As reported previously (Yamamoto et al., 2006), AD47 and LAC67b inhibited 1,25(OH) $_2$ D $_3$ transactivation in a concentration-dependent manner (Fig. 2C). VDR inhibition by LAC67b was weaker than that of AD47 at 0.3 and 1 μ M. This may be due to the partial agonist activity of LAC67b observed in HEK293 cells. LAC67a also exhibited VDR antagonist activity but did so less effectively than AD47. Next, a mammalian one-hybrid assay was used to assess VDR activity without the potentially confounding effect of endogenous VDR. The LBD of VDR was fused to the DNA-binding domain of the yeast transcription factor GAL4 (Kaneko et al., 2003). The GAL4-VDR expression plasmid was cotransfected with a GAL4-responsive luciferase reporter. AD47 and LAC67b, and not LAC67a, induced the activation of GAL4-VDR at 1 μ M concentration (Fig. 2D). AD47 and LAC67a inhibited the GAL4-VDR activity induced by 10 nM 1,25(OH) $_2$ D $_3$. Because 1 μ M LAC67b induced GAL4-VDR transactivation as efficiently as 10 nM 1,25(OH) $_2$ D $_3$, LAC67b at this concentration did not inhibit 1,25(OH) $_2$ D $_3$ activity. The bile acid lithocholic acid (LCA) was recently identified as an additional physiological VDR ligand (Makishima et al., 2002). LCA at 10 μ M induced GAL4-VDR activation as reported previously (Makishima et al., 2002). AD47 and LAC67a inhibited LCA-induced VDR activity in a concentration-dependent manner (Fig. 2E). LAC67b was not effective in inhibiting VDR activity induced by LCA, a finding that may be explained by its function as a partial agonist.

The VDR binding affinity of 1,25(OH) $_2$ D $_3$ and its deriva-

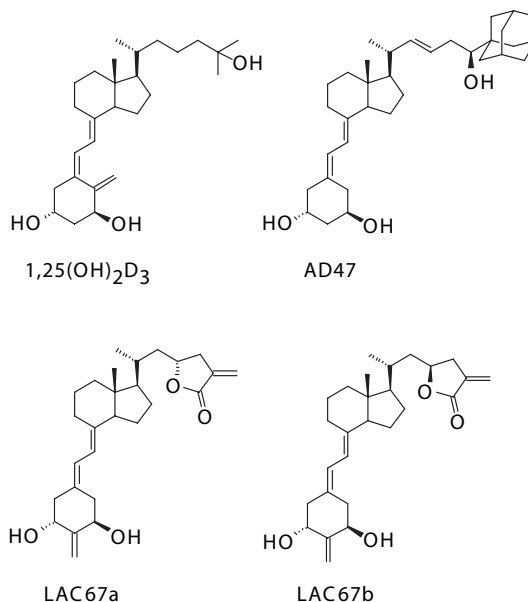


Fig. 1. Structures of 1,25(OH) $_2$ D $_3$, AD47, LAC67a, and LAC67b. According to the IUPAC nomenclature, AD47 is (1*R*,3*R*,7*E*)-17-[(1*R*,2*E*,5*S*)-5-(1-adamantyl)-5-hydroxy-1-methyl-2-pentenyl]-9,10-secoestra-5,7-diene-1,3-diol, LAC67a is (5*S*)-5-[(2*R*)-2-[(1*R*,3*R*,7*E*)-1,3-dihydroxy-2-methylene-9,10-secoestra-5,7-dien-17-yl]propyl]-3-methylenedihydro-2(3*H*)-furanone, and LAC67b is (5*S*)-5-[(2*R*)-2-[(1*R*,3*R*,7*E*)-1,3-dihydroxy-2-methylene-9,10-secoestra-5,7-dien-17-yl]propyl]-3-methylenedihydro-2(3*H*)-furanone.

tives was examined by a competitive binding assay. Isotopically labeled $1,25(\text{OH})_2\text{D}_3$ was incubated with VDR protein in the absence or presence of test compounds. The binding affinities of AD47, LAC67a, and LAC67b to VDR protein were 2%, 3.3%, and 10% of that of $1,25(\text{OH})_2\text{D}_3$, respectively (Fig. 3A). The low affinity of LAC67a binding to VDR may contribute to its diminished antagonist activity (Fig. 2C).

Effects of Vitamin D₃ Derivatives on VDR-RXR Heterodimerization. $1,25(\text{OH})_2\text{D}_3$ and its derivatives have been reported to promote the heterodimerization of VDR with RXR (Ma et al., 2006). To examine the effect of AD47, LAC67a, and LAC67b on VDR-RXR heterodimerization, mammalian two-hybrid experiments were performed using VP16-VDR and GAL4-RXR α . AD47, LAC67a, and LAC67b induced the heterodimerization of GAL4-RXR α with VP16-VDR with an EC₅₀ of 10, 20, and 3 nM, respectively, whereas the EC₅₀ for $1,25(\text{OH})_2\text{D}_3$ was 0.7 nM (Fig. 4A). These derivatives induced RXR α heterodimerization with nearly the same maximal efficiency as $1,25(\text{OH})_2\text{D}_3$. We examined the possibility that AD47, LAC67a, and LAC67b might directly bind to RXR α using a competitive ligand-binding assay. The

addition of $1,25(\text{OH})_2\text{D}_3$ or its derivatives did not inhibit the binding of labeled 9-*cis* retinoic acid, a potent RXR ligand, whereas unlabeled 9-*cis* retinoic acid competed with the labeled ligand as expected, indicating that these vitamin D₃ derivatives are not RXR α ligands (Fig. 3B). The interaction of VDR and RXR was examined in vitro using a GST pull-down assay. We generated a GST-VDR fusion protein and evaluated its interaction with isotope-labeled RXR α in the presence or absence of 1 μM test compound. $1,25(\text{OH})_2\text{D}_3$ induced the interaction of RXR α with GST-VDR, but not with GST control protein (Fig. 4B). LAC67b induced the binding of GST-VDR and isotope-labeled RXR α as effectively as $1,25(\text{OH})_2\text{D}_3$, whereas AD47 and LAC67a were less effective. $1,25(\text{OH})_2\text{D}_3$ and LAC67b also induced the reciprocal interaction between GST-RXR α and isotope-labeled VDR. Although the effect of AD47 on this interaction was modest, induction mediated by LAC67a was marginal.

The effects of vitamin D₃ derivatives on the interaction of VDR with RXR β and RXR γ were examined in a mammalian two-hybrid assay. Although AD47 and LAC67a at 1 μM induced the interaction of VDR and RXR β as effectively as 10

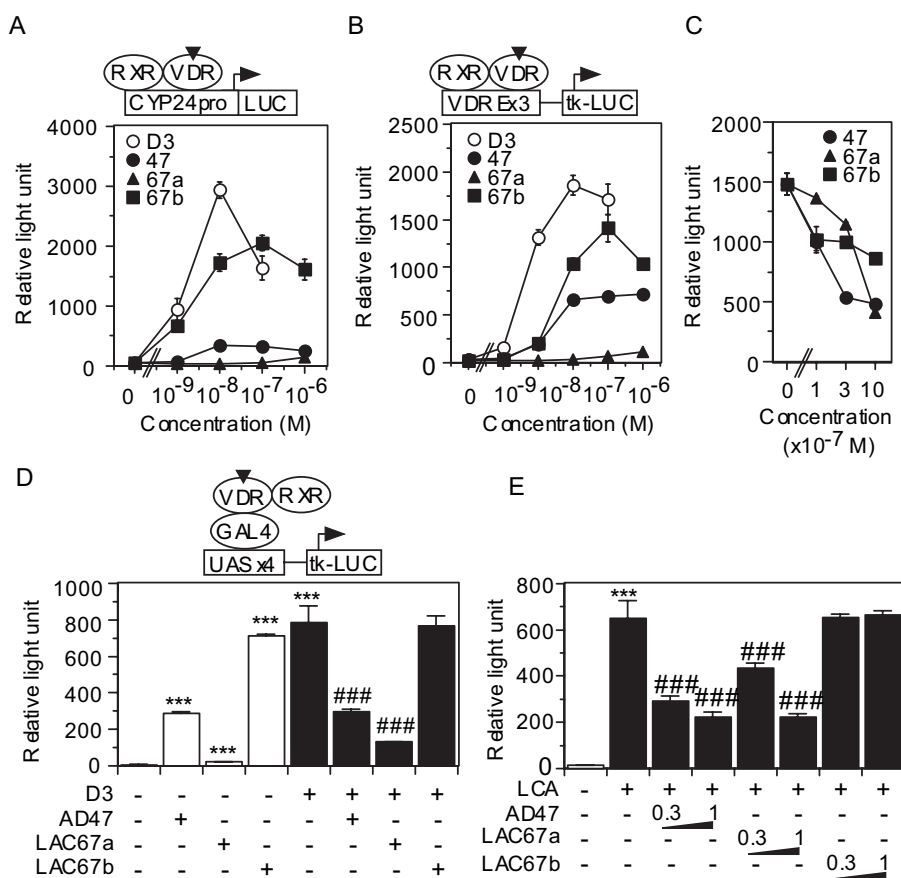


Fig. 2. Vitamin D₃ derivatives antagonize VDR activation induced by $1,25(\text{OH})_2\text{D}_3$. A, concentration-dependent activation of VDR on CYP24 promoter by vitamin D₃ derivatives. HEK293 cells were cotransfected with CMX-VDR and the human CYP24 promoter-luciferase reporter and treated with several concentrations of $1,25(\text{OH})_2\text{D}_3$ (D3), AD47 (47), LAC67a (67a), and LAC67b (67b). B, concentration-dependent activation of VDR on a luciferase reporter having a VDR-responsive ER6 by vitamin D₃ derivatives. HEK293 cells were cotransfected with CMX-VDR and the CYP3A4-ER6 \times 3-tk-LUC reporter and treated with several concentrations of $1,25(\text{OH})_2\text{D}_3$, AD47, LAC67a, and LAC67b. C, antagonistic effect of vitamin D₃ derivatives on VDR activated by $1,25(\text{OH})_2\text{D}_3$. HEK293 cells were cotransfected as in B and were treated with AD47, LAC67a, and LAC67b at a range of concentrations in combination with 10 nM $1,25(\text{OH})_2\text{D}_3$. D, effect of vitamin D₃ derivatives on the GAL4-VDR chimeric receptor. HEK293 cells were cotransfected with CMX-GAL4-VDR and the MH100(UAS) \times 4-tk-LUC reporter, and were treated with 1 μM AD47, LAC67a, or LAC67b in the absence or presence of 10 nM $1,25(\text{OH})_2\text{D}_3$. ***, $p < 0.001$ compared with untreated control. ###, $p < 0.001$ compared with the activity induced by $1,25(\text{OH})_2\text{D}_3$ alone. E, effect of vitamin D₃ derivatives on GAL4-VDR activated by lithocholic acid. HEK293 cells were cotransfected as in D and treated with 0.3 or 1 μM AD47, LAC67a, or LAC67b in the presence of 30 μM lithocholic acid (LCA). ***, $p < 0.001$ compared with untreated control; ###, $p < 0.001$ compared with the activity induced by lithocholic acid alone.

nM $1,25(\text{OH})_2\text{D}_3$, LAC67b was more effective than these derivatives (Fig. 4C). The effect on VDR-RXR β heterodimerization was comparable with that on VDR-RXR α heterodimerization. It is noteworthy that GAL4-RXR γ bound to VP16-VDR in the absence of ligand (Fig. 4C), and the addition of neither $1,25(\text{OH})_2\text{D}_3$ (10 nM) nor AD47 (1 μM) enhanced this interaction. LAC67a and LAC67b were more potent inducers of the VDR-RXR γ interaction. We further examined the ligand-induced interaction of VDR with RXR using GAL4-VDR and VP16-RXR vectors. In the absence of ligand, cotransfection of VP16-RXR α , VP16-RXR β , and VP16-RXR γ increased luciferase activity, indicating that RXR proteins were bound to VDR in cells (Fig. 4D). $1,25(\text{OH})_2\text{D}_3$ at 10 nM induced GAL4-VDR activation in the presence of control VP16 protein, and GAL4-VDR activity induced by $1,25(\text{OH})_2\text{D}_3$ was augmented by cotransfection of VP16-RXR α , VP16-RXR β , and VP16-RXR γ , indicating that $1,25(\text{OH})_2\text{D}_3$ enhanced the interaction between VDR and all three RXR isomers. With cotransfection of VP16 control vector, 1 μM AD47 and LAC67b, but much less LAC67a, induced the activation of GAL4-VDR (Fig. 4D). With VP16-RXR α , VP16-RXR β , or VP16-RXR γ cotransfection, the effect of AD47 and LAC67b on GAL4-VDR activation was enhanced and LAC67a effectively induced luciferase activity (Fig. 4D). These results show that the effect of vitamin D $_3$ derivatives on VDR-RXR heterodimerization is independent of their agonistic/antagonistic activity.

Ligand binding induces a conformational change that results in stabilization of the nuclear receptor LBD (Pissios et al., 2000). This ligand-dependent structural rearrangement can be detected by assaying the interaction between the isolated H1 domain and an engineered receptor lacking H1. RXR ligand induced the assembly of RXR α H1 domain and the H3–H12 fragment (Pissios et al., 2000). We previously applied the assembly assay to characterize the conformation of the nuclear receptor NGFI-B (NR4A1) in complex with RXR and reported that the dibenzodiazepine RXR ligand HX600 induced the assembly in NGFI-B-LBD in the presence of RXR α cotransfection (Morita et al., 2005). The H1 fragment of RXR α was fused to the GAL4 DNA-binding domain and was cotransfected with a LBD fragment lacking the H1 fused to the transactivation domain of the herpesvirus VP16 protein. As reported previously (Pissios et al., 2000; Morita et al., 2005), the RXR ligand 9-*cis* retinoic acid induced the assembly of the H1 and H3–H12 fragments of RXR α -LBD, but $1,25(\text{OH})_2\text{D}_3$ and its derivatives did not promote RXR α

assembly (Fig. 4E). In the presence of VDR cotransfection, $1,25(\text{OH})_2\text{D}_3$ induced the interaction of GAL4-RXR α (H1) with VP16-RXR α (H3–H12). Although LAC67a and LAC67b effectively induced RXR α assembly through heterodimerization with VDR, the effect of AD47 was only slight. These results show that AD47, LAC67a, and LAC67b mediate distinct allosteric effects on VDR-RXR heterodimers.

Vitamin D $_3$ Derivatives Induced Unique Cofactor Interactions. Upon ligand binding, nuclear receptors undergo conformational changes in the cofactor binding site and AF2 helix that result in the dissociation of corepressors and recruitment of coactivators (Rosenfeld et al., 2006). The p160 family proteins SRC-1, GRIP1 (SRC-2/TIF2), and ACTR (SRC-3) are well characterized coactivators that bind to the AF2 surface and transmit the allosteric signal of ligand binding to the chromatin remodeling system. We examined the effect of vitamin D $_3$ derivatives on the interaction of VDR with SRC-1 using a mammalian two-hybrid assay. $1,25(\text{OH})_2\text{D}_3$ induced a concentration-dependent association of VDR with SRC-1, as reported previously (Herdick et al., 2000a; Adachi et al., 2004). AD47 and LAC67b induced SRC-1 recruitment to VDR less effectively than $1,25(\text{OH})_2\text{D}_3$ (Fig. 5A). It is noteworthy that although LAC67b induced VDR transactivation more efficiently than AD47 (Fig. 2), AD47 more effectively promoted the interaction of VDR and SRC-1. LAC67a did not recruit SRC-1 to VDR. We compared the effect of AD47 and LAC67b on the association of VDR and SRC-1 in *in vitro* GST pull-down experiments. AD47 induced the interaction between GST-SRC-1 and isotope-labeled VDR proteins more effectively than LAC67b (Fig. 5B). AD47 also recruited isotope-labeled full-length SRC-1 to GST-VDR, but LAC67a and LAC67b did not induce this interaction. Next, we examined the inhibitory effects of vitamin D $_3$ derivatives on the coactivator interaction induced by $1,25(\text{OH})_2\text{D}_3$ in mammalian two-hybrid experiments. In cells transfected with GAL4-SRC-1 and VP16-VDR, AD47 (1 μM) reduced the luciferase activity induced by $1,25(\text{OH})_2\text{D}_3$ (10 nM) to the level observed in the absence of vitamin D (Fig. 5C). LAC67a and LAC67b modestly repressed this interaction. The effect of AD47, LAC67a, and LAC67b at 1 μM on the interaction of VDR with GRIP1 was marginal (Fig. 5D). These vitamin D $_3$ derivatives reduced the GRIP1 interaction induced by $1,25(\text{OH})_2\text{D}_3$ (10 nM) to basal levels. AD47, LAC67a, and LAC67b at 1 μM did not effectively recruit ACTR to VDR, but these three derivatives effectively repressed the interaction induced by $1,25(\text{OH})_2\text{D}_3$ (10 nM) (Fig. 5E).

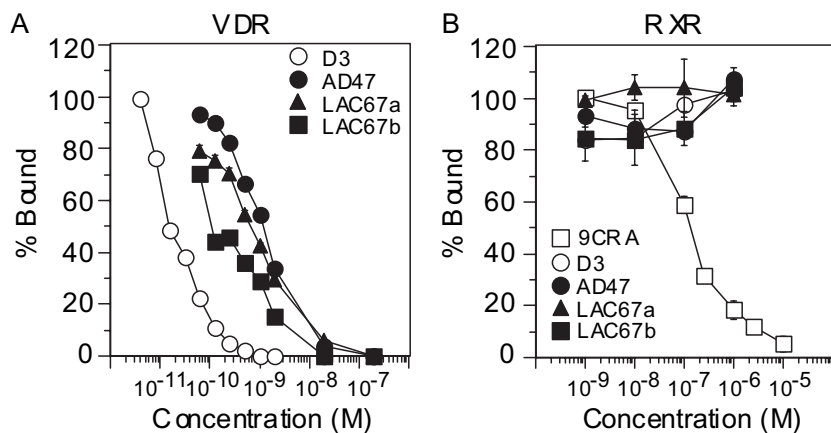


Fig. 3. Effect of vitamin D $_3$ derivatives on direct binding of $1,25(\text{OH})_2\text{D}_3$ to VDR (A) and that of 9-*cis* retinoic acid to RXR α (B). A, VDR proteins were incubated with [^3H] $1,25(\text{OH})_2\text{D}_3$ in the presence of nonradioactive $1,25(\text{OH})_2\text{D}_3$, AD47, LAC67a, and LAC67b at a range of concentrations. B, GST-RXR α proteins were incubated with [^3H]9-*cis*-retinoic acid in the presence of nonradioactive 9-*cis*-retinoic acid, $1,25(\text{OH})_2\text{D}_3$, AD47, LAC67a, and LAC67b at a range of concentrations.

The mediator complex is an important coactivator assembly that bridges nuclear receptors and the basal transcriptional machinery (Malik and Roeder, 2005). DRIP205 (MED1), a component of the mediator complex, interacts with the AF2 domain of nuclear receptors in a ligand-dependent manner through its LXXLL-containing nuclear receptor-interacting motif. The interaction of DRIP205 with VDR

was examined in a mammalian two-hybrid assay. AD47 induced a concentration-dependent association of DRIP205 and VDR (Fig. 6A). We were surprised to find that AD47 promoted DRIP recruitment with an efficacy nearly equivalent to that of $1,25(\text{OH})_2\text{D}_3$. LAC67a and LAC67b, even at concentrations of $1\ \mu\text{M}$, did not induce the association of DRIP205 and VDR. In a GST pull-down assay, AD47 induced

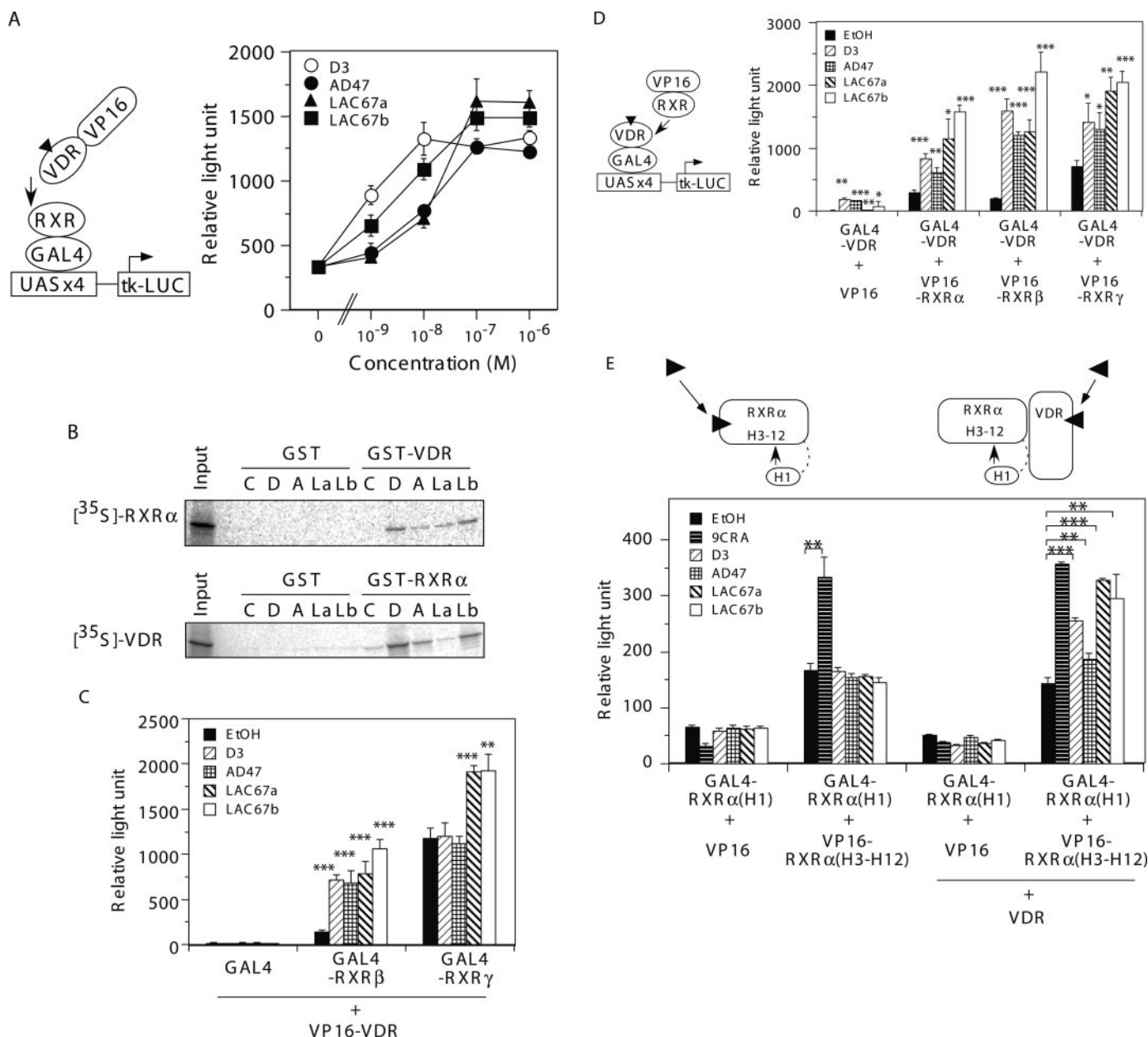


Fig. 4. Vitamin D derivatives induce VDR-RXR heterodimerization. **A**, effect of vitamin D₃ derivatives on the interaction between VP16-VDR and GAL4-RXRα. HEK293 cells were cotransfected with CMX-GAL4-RXRα, CMX-VP16-VDR, and MH100(UAS)x4-tk-LUC, and were treated with $1,25(\text{OH})_2\text{D}_3$, AD47, LAC67a, or LAC67b at a range of concentrations. **B**, effect of vitamin D₃ derivatives on in vitro interaction between VDR and RXRα. GST pull-down assays were performed to evaluate ligand-dependent interactions between GST or GST-VDR and [³⁵S]RXRα (top) and between GST or GST-RXRα and [³⁵S]VDR (bottom) in the presence of ethanol control (C), $1\ \mu\text{M}$ $1,25(\text{OH})_2\text{D}_3$ (D), AD47 (A), LAC67a (La), or LAC67b (Lb). **C**, effect of vitamin D₃ derivatives on the interaction of VP16-VDR with GAL4-RXRβ and GAL4-RXRγ. HEK293 cells were cotransfected with CMX-GAL4 control, CMX-GAL4-RXRβ, or CMX-GAL4-RXRγ in combination with CMX-VP16-VDR and MH100(UAS)x4-tk-LUC and were treated with ethanol control (EtOH), $10\ \text{nM}$ $1,25(\text{OH})_2\text{D}_3$, $1\ \mu\text{M}$ AD47, LAC67a or LAC67b. **D**, vitamin D₃ derivatives enhance the interaction between GAL4-VDR and VP16-RXRα. HEK293 cells were cotransfected with CMX-GAL4-VDR in combination with CMX-VP16 control, CMX-VP16-RXRα, CMX-VP16-RXRβ, or CMX-VP16-RXRγ, and MH100(UAS)x4-tk-LUC, and were treated with ethanol control (EtOH), $10\ \text{nM}$ $1,25(\text{OH})_2\text{D}_3$, $1\ \mu\text{M}$ AD47, LAC67a, or LAC67b. **E**, effect of vitamin D₃ derivatives on RXRα-LBD H1/H3-12 assembly through VDR interaction. HEK293 cells were cotransfected with CMX-GAL4-RXRα (H1) in combination with CMX-VP16 or CMX-VP16-RXRα (H3-H12), in the absence or presence of cotransfection with CMX-VDR, and MH100(UAS)x4-tk-LUC, and were treated with ethanol (EtOH), $100\ \text{nM}$ 9-*cis*-retinoic acid (9CRA), $10\ \text{nM}$ $1,25(\text{OH})_2\text{D}_3$, $1\ \mu\text{M}$ AD47, LAC67a, or LAC67b. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with ethanol control.

the interaction of GST-DRIP205 and isotope-labeled VDR to an extent weaker than that of 1,25(OH)₂D₃ (Fig. 6B), suggesting that AD47 can induce a stable ligand-VDR-DRIP205 complex in cells more effectively than in the *in vitro* context. AD47 at 1 μM did not reduce the interaction of DRIP205 induced by 1,25(OH)₂D₃ (10 nM) (Fig. 6C). LAC67a and LAC67b, which alone did not induce DRIP205 recruitment, antagonized the 1,25(OH)₂D₃-induced interaction of VDR and DRIP205.

The interaction of VDR with the corepressors N-CoR and SMRT was evaluated. In the absence of ligand, N-CoR and SMRT bound to VDR, and treatment with 1,25(OH)₂D₃ reduced these interactions (Adachi et al., 2004). AD47, LAC67a, and LAC67b induced the dissociation of N-CoR from VDR in a concentration-dependent manner, whereas the effect of LAC67a was weak (Fig. 7A). Neither AD47, LAC67a, nor LAC67b diminished 1,25(OH)₂D₃-mediated dissociation of N-CoR from VDR (Fig. 7B). 1,25(OH)₂D₃ and the three

vitamin D₃ derivatives also promoted the concentration-dependent dissociation of SMRT from VDR (Fig. 7C). Combined administration of these vitamin D₃ derivatives did not alter SMRT dissociation induced by 1,25(OH)₂D₃ (Fig. 7D). Thus, AD47, LAC67a, and LAC67b exhibit distinct coactivator recruitment activities while similarly inducing corepressor dissociation.

Effect of Vitamin D₃ Derivatives on Endogenous Gene Expression in Cells. VDR is expressed in the vitamin D₃ target organs that mediate calcium homeostasis, such as intestine, bone and kidney (DeLuca, 2004; Haussler et al., 1998). The noncalcemic actions of vitamin D₃ are associated with VDR expression in other tissues, including blood cells and skin (Nagpal et al., 2005). We examined the effects of the vitamin D₃ derivatives on the expression of endogenous VDR target genes. 1,25(OH)₂D₃ induces its own metabolism through VDR-dependent activation of the enzyme CYP24A1 in many tissues (Haussler et al., 1998). We treated intestinal

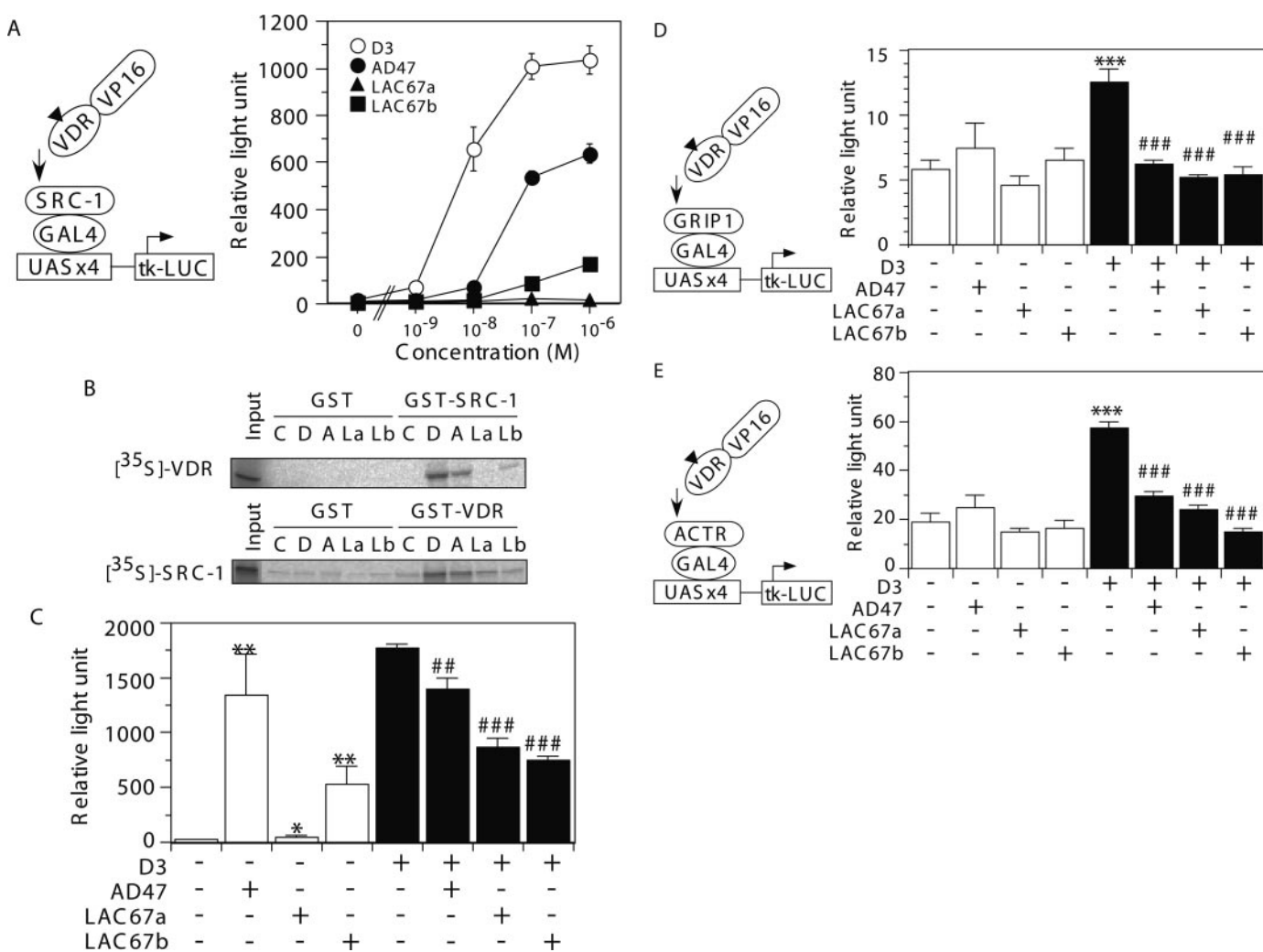


Fig. 5. Effect of vitamin D₃ derivatives on p160 family coactivator recruitment. AD47 and LAC67b induce the interaction of VDR and SRC-1 in a mammalian two-hybrid assay (A) and *in vitro* GST pull-down assay (B). A, HEK293 cells were cotransfected with CMX-GAL4-SRC-1, CMX-VP16-VDR and MH100(UAS)×4-tk-LUC, and were treated with 1,25(OH)₂D₃, AD47, LAC67a, or LAC67b at a range of concentrations. B, GST pull-down assays were performed to evaluate ligand-dependent interactions between GST or GST-SRC-1 and [³⁵S]VDR (top) and between GST or GST-VDR and [³⁵S]-labeled full-length SRC-1 (bottom) in the presence of ethanol control (C), 100 nM 1,25(OH)₂D₃ (D), 10 μM AD47 (A), LAC67a (La), or LAC67b (Lb). Effects of vitamin D₃ derivatives on interaction of VDR with SRC-1 (C), GRIP1 (D), or ACTR (E) induced by 1,25(OH)₂D₃. HEK293 cells were cotransfected with CMX-GAL4-SRC-1 (C), CMX-GAL4-GRIP1 (D), or CMX-GAL4-ACTR (E) in combination with CMX-VP16-VDR and were treated with 1 μM AD47, LAC67a, or LAC67b in the absence or presence of 10 nM 1,25(OH)₂D₃. **, *p* < 0.01; ***, *p* < 0.001 compared with untreated control. ##, *p* < 0.01; ###, *p* < 0.001 compared with the activity induced by 1,25(OH)₂D₃ alone.

mucosa-derived SW480, HCT116 cells, kidney epithelium-derived HEK293 cells, osteoblast-derived MG63 cells, monoblast-derived THP-1 cells, and skin keratinocyte-derived HaCaT cells with 1 μ M AD47, LAC67a, and LAC67b in the absence or presence of 10 nM 1,25(OH) $_2$ D $_3$, and evaluated CYP24A1 mRNA expression by quantitative real-time PCR. In intestinal SW480 cells, AD47 (1 μ M) induced the expression of CYP24A1 29% as effectively as 1,25(OH) $_2$ D $_3$ (10 nM), whereas LAC67a and LAC67b had no effect (Fig. 8A). AD47 and LAC67a suppressed CYP24A1 expression induced by 1,25(OH) $_2$ D $_3$ and LAC67b antagonized 1,25(OH) $_2$ D $_3$ more effectively than AD47 and LAC67a in SW480 cells. AD47 (1 μ M) induced CYP24A1 expression 77% of the levels observed with 1,25(OH) $_2$ D $_3$ (10 nM) in intestinal HCT116 cells (Fig. 8B). Because AD47 is an effective agonist, this derivative did not suppress CYP24A1 expression induced by 1,25(OH) $_2$ D $_3$. LAC67a did not induce CYP24A1 expression and weakly antagonized the effect of 1,25(OH) $_2$ D $_3$. LAC67b suppressed

CYP24A1 induction by 1,25(OH) $_2$ D $_3$ more effectively than LAC67a. AD47 and LAC67a did not induce CYP24A1 expression in kidney-derived HEK293 cells (Fig. 8C). LAC67b weakly induced its expression. It is noteworthy that although AD47 is a more potent agonist than LAC67b in SW480 and HCT116 cells, LAC67b induced CYP24A1 expression more effectively than AD47 in HEK293 cells. AD47, LAC67a, and LAC67b at 1 μ M suppressed CYP24A1 induction by 1,25(OH) $_2$ D $_3$ (10 nM) in HEK293 cells (Fig. 8C). AD47 and LAC67b acted as partial agonists in osteoblast MG63 cells and suppressed CYP24A1 expression induced by 1,25(OH) $_2$ D $_3$ (Fig. 8D). LAC67a, a pure antagonist in MG63 cells, inhibited the induction of CYP24A1 expression by 1,25(OH) $_2$ D $_3$ more effectively than AD47 and LAC67b. In monoblastic leukemia THP-1 cells, AD47, LAC67a, and LAC67b (1 μ M) induced CYP24A1 expression less than 6% as efficiently as 1,25(OH) $_2$ D $_3$ (10 nM), and these derivatives acted as strong inhibitors of CYP24A1 expression induced by 1,25(OH) $_2$ D $_3$ (Fig. 8E). The effect of the vitamin D $_3$ derivatives in keratinocyte HaCaT cells was similar to that in SW480 cells (Fig. 8F). AD47 acted as a weak agonist, and LAC67a and LAC67b induced CYP24A1 expression less efficiently than AD47. All three derivatives antagonized the effect of 1,25(OH) $_2$ D $_3$, and LAC67b was the most potent inhibitor. These findings indicate that agonistic and antagonistic actions of AD47, LAC67a, and LAC67b differed in vitamin D-responsive cell types.

We examined expression of VDR, RXR α , SRC-1, and DRIP205 proteins in nuclear extracts by immunoblotting. VDR and RXR α proteins were detected in SW480, HCT116, HEK293, MG63, THP-1, and HaCaT cells (Fig. 9A). SRC-1 was highly expressed in HEK293 cells, whereas SW480, HCT116, MG63, and THP-1 cells had modest expression. SRC-1 was not detected in HaCaT cells. DRIP205 expression was observed in HCT116 and HaCaT cells, and slightly in SW480 cells, but not in HEK293, MG63, or THP-1 cells. Thus, the expression level of cofactor proteins is different among cells. 1,25(OH) $_2$ D $_3$ treatment induces nuclear accumulation of VDR (Michigami et al., 1999). We next examined the effect of vitamin D $_3$ derivatives on nuclear VDR expression in HCT116, THP-1, and HaCaT cells (Fig. 9B). 1,25(OH) $_2$ D $_3$ (10 nM) increased nuclear VDR in HCT116, THP-1, and HaCaT cells. It is noteworthy that 10 μ M AD47, LAC67a, and LAC67b increased nuclear VDR proteins in HCT116 and HaCaT cells in which these compounds exhibited some agonistic activity (Fig. 8, B and F). On the other hand, these derivatives were not effective on nuclear VDR localization in THP-1 cells, in which they are effective antagonists (Fig. 8E).

The administration of VDR agonists results in hypercalcemia by increasing calcium absorption of the intestine (Bouillon et al., 2003). VDR activation in enterocytes is associated with expression of calcium metabolism-related genes, such as the calcium channel CaT1 (Van Cromphaut et al., 2001). We examined the effect of vitamin D $_3$ derivatives on induction of other VDR target genes in intestinal SW480 cells. AD47 (1 μ M) weakly induced CaT1 expression and suppressed its activation by 1,25(OH) $_2$ D $_3$ (10 nM) (Fig. 10A). LAC67a and LAC67b did not induce CaT1 expression and antagonized the effect of 1,25(OH) $_2$ D $_3$ more strongly than AD47. As was observed for CYP24A1 and CaT1, AD47 also induced weak expression of CYP3A4 and E-cadherin (Fig. 10, B and C).

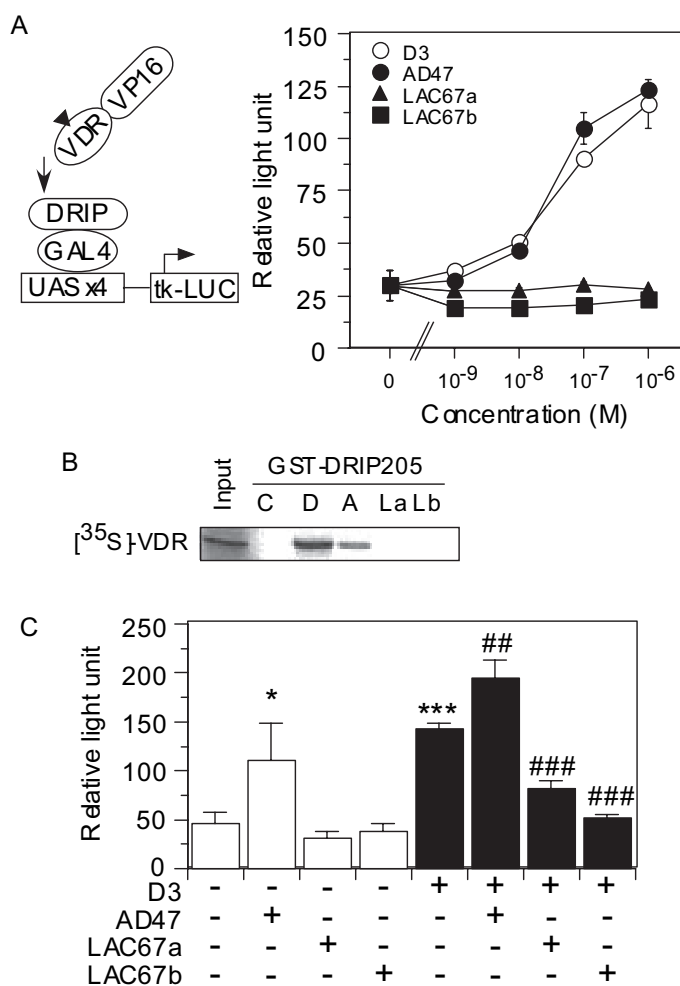


Fig. 6. Effect of vitamin D $_3$ derivatives on DRIP205 coactivator recruitment. **A**, AD47 induces interaction of VDR and DRIP205 as efficiently as 1,25(OH) $_2$ D $_3$. HEK293 cells were cotransfected with CMX-GAL4-DRIP205 and CMX-VP16-VDR. **B**, AD47, but not LAC67a or LAC67b, induces in vitro interaction between VDR and DRIP205. GST pull-down assay using GST-DRIP205 and [35 S]VDR proteins was performed as in Fig. 4B. **C**, effects of vitamin D $_3$ derivatives on the interaction of VDR with DRIP205 induced by 1,25(OH) $_2$ D $_3$. HEK293 cells were cotransfected as in **A** and were treated with compounds as in Fig. 4C. *, $p < 0.05$; ***, $p < 0.001$ compared with untreated control. ##, $p < 0.01$; ###, $p < 0.001$ compared with the activity induced by 1,25(OH) $_2$ D $_3$ alone.

AD47, LAC67a, and LAC67b suppressed vitamin D induction of CYP3A4 and E-cadherin, and LAC67b was the most potent inhibitor. Thus, AD47 acts as a partial agonist/antagonist, whereas LAC67a and LAC67b are VDR antagonists in SW480 cells.

Docking Models of the Vitamin D₃ Derivatives in the VDR-LBD. We previously reported docking models of AD47 and LAC67b in the VDR ligand-binding pocket that were supported by alanine scanning mutational analysis combined with functional assays in COS-7 cells (Yamamoto et al., 2006). The docking model was further refined to account for the partial agonist function of AD47. AD47 has an adamantane ring functional group at the vitamin D₃ side chain (Fig. 1). As shown in Fig. 11A, middle, this bulky side chain substituent specially interferes with residues on H12 of the VDR. This interaction would be expected to destabilize the transcriptionally active conformation of VDR that is supported by 1,25(OH)₂D₃. The AD47-VDR complex is in equilibrium between transcriptionally active and inactive conformations, which results in AD47 functioning as a partial agonist. Figure 11A, right, shows a docking model of AD47-VDR complex in an agonist conformation. The 25-hydroxyl group of AD47 could form a hydrogen bond only with His305 but not with His397, indicating that AD47-VDR complex in this conformation is less stable than 1,25(OH)₂D₃-VDR complex (Fig. 11A, left). Figure 11B shows the complexes of

1,25(OH)₂D₃, LAC67a, and LAC67b with the VDR-LBD. All three compounds are accommodated by the VDR ligand-binding pocket and form hydrogen bonds with His305 and His397. Differences are found in the interactions between H11/H12 and the ligand. Whereas the terminal methyl group at carbon-26 of 1,25(OH)₂D₃ directly interacts with the aromatic residues Phe422 on H12 and Tyr401 on H11, the terminal methylene group of LAC67a is too distant to interact. As a result, LAC67a can bind to VDR but cannot form a transcriptionally active conformation in which H12 folds against the remainder of the LBD to form the AF2 surface. Therefore, LAC67a acts as a VDR antagonist. In LAC67b, the Phe422 and Tyr401 residues are closer than is seen in the LAC67a-VDR complex, resulting in partial agonist activity.

Discussion

Vitamin D₃ derivatives with adamantane or lactone ring side chain substituents act as selective VDR modulators. In a transfection assay in HEK293 cells, AD47 and LAC67b exhibited partial agonist activity on VDR, and LAC67b activated VDR more effectively than AD47, whereas LAC67a did not activate VDR (Fig. 2). In the previous study, although agonist activity was weak, AD47 was more potent than LAC67b in mediating VDR activation in COS-7 cells (Yamamoto et al., 2006). The effect of LAC67b in the HEK293

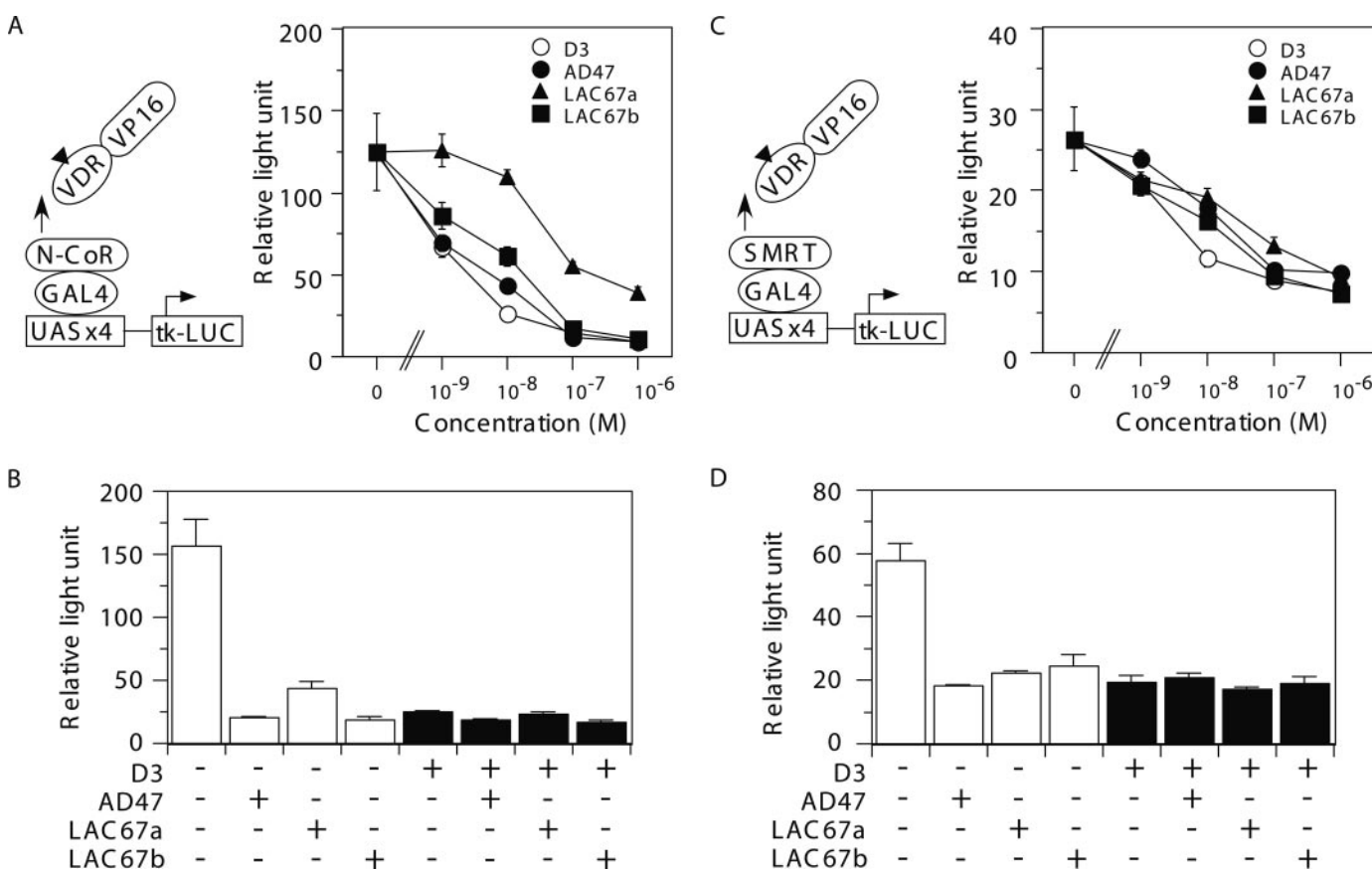


Fig. 7. Effect of vitamin D₃ derivatives on corepressor interaction. A, AD47, LAC67a, and LAC67b induce dissociation of N-CoR from VDR. B, vitamin D₃ derivatives do not suppress the dissociation of N-CoR from VDR induced by 1,25(OH)₂D₃. C, vitamin D₃ derivatives also induce dissociation of SMRT from VDR. D, vitamin D₃ derivatives do not suppress the dissociation of SMRT from VDR induced by 1,25(OH)₂D₃. HEK293 cells were cotransfected with CMX-GAL4-N-CoR (A and B) or CMX-GAL4-SMRT (C and D) in combination with CMX-VP16-VDR and were treated with 1,25(OH)₂D₃, AD47, LAC67a, or LAC67b at a range of concentrations (A and C) or with 1 μ M AD47, LAC67a, or LAC67b in the absence or presence of 10 nM 1,25(OH)₂D₃.

transient transfection assay was comparable with that on endogenous VDR target gene expression in HEK293 cells (Fig. 8). The agonist activity of LAC67b may be related with high expression of SRC-1 in HEK293 cells (Fig. 9A), although other mechanisms cannot be ruled out. The agonist activity of AD47 is cell type-dependent, and AD47 induced endogenous VDR target genes more effectively than LAC67b in HCT116, SW480, and HaCaT cells. Interestingly LAC67b was a strong antagonist of vitamin D-induced VDR activity in SW480, HaCaT, and THP-1 cells, where it has only marginal agonist activity (Fig. 8). These data indicate that AD47 and LAC67b act as cell type-selective VDR modulators. For example, AD47 acts as a partial agonist and LAC67b acts as an antagonist in SW480 and HaCaT cells, whereas AD47 is an effective antagonist, and LAC67b functions as a partial

agonist in HEK293 cells. LAC67a, a diastereoisomer of LAC67b, does not exhibit agonist activity and has different antagonist activity in the cell types examined in this study. It effectively repressed VDR target gene expression induced by $1,25(\text{OH})_2\text{D}_3$ in SW480, MG63, and THP-1 cells but not in HCT116, HaCaT, and HEK293 cells (Fig. 8). Thus, LAC67a is a cell type-selective antagonist.

Distinct patterns of cofactor recruitment may contribute to the cell type-selective actions of vitamin D_3 derivatives. AD47 induced recruitment of the SRC-1 coactivator to VDR less efficiently than $1,25(\text{OH})_2\text{D}_3$, whereas LAC67b did only weakly and LAC67a did not (Fig. 5, A and B). The effect of these derivatives is similar to that of TEI-9647, a VDR antagonist that has a methylene lactone side chain like LAC67b (Ozono et al., 1999). TEI-9647 did not induce effective SRC-1

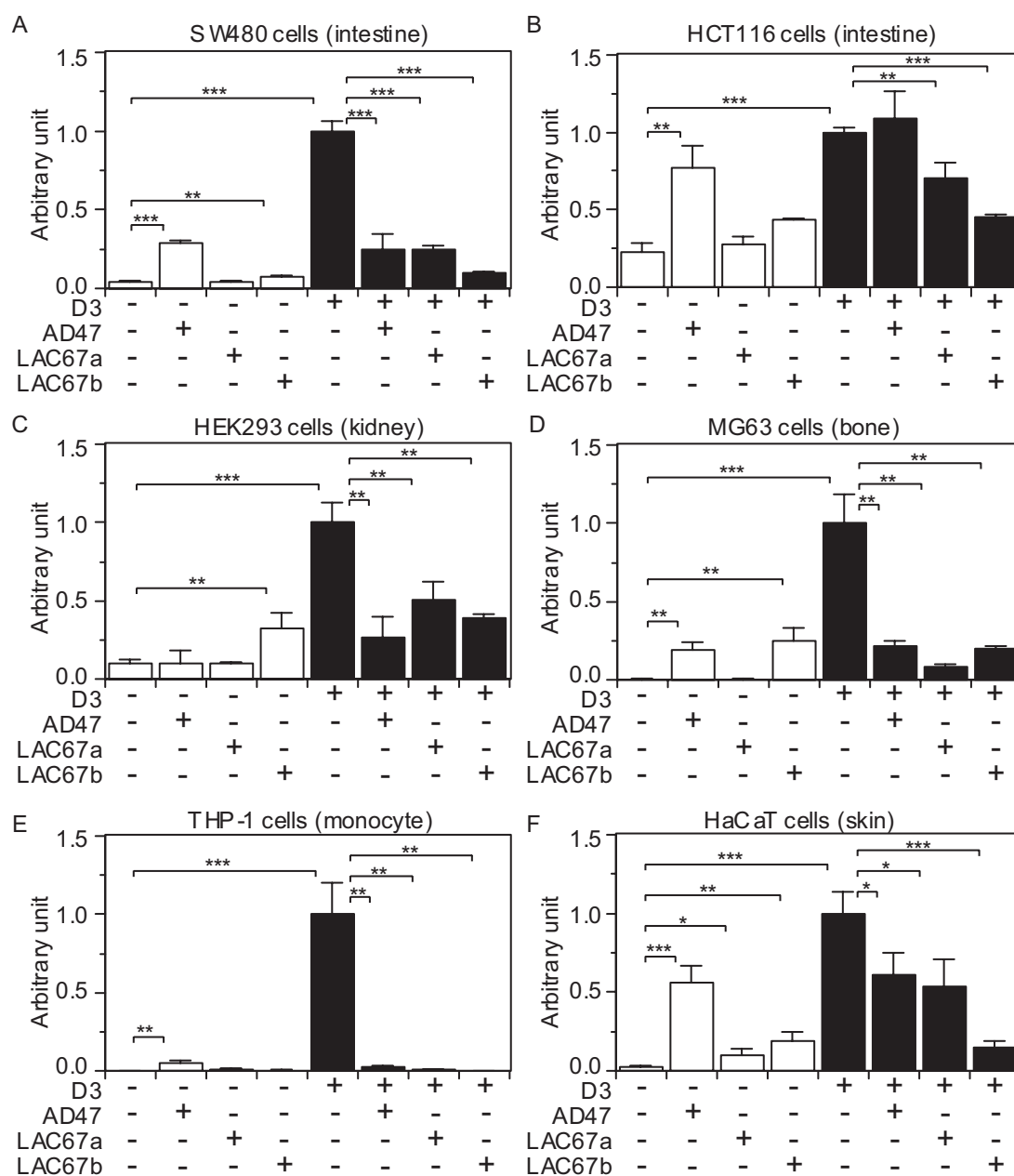


Fig. 8. Effect of vitamin D_3 derivatives on expression of CYP24A1 gene in intestinal SW480 cells (A), HCT116 cells (B), kidney-derived HEK293 cells (C), osteoblastic MG63 cells (D), monocyte-derived THP-1 cells (E), and keratinocyte HaCaT cells (F). Cells were treated with $1 \mu\text{M}$ AD47, LAC67a, or LAC67b in the absence or presence of 10 nM $1,25(\text{OH})_2\text{D}_3$ for 24 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

recruitment to VDR but inhibited the SRC-1 interaction mediated by $1,25(\text{OH})_2\text{D}_3$. AD47, LAC67a, and LAC67b did not effectively recruit GRIP1 and ACTR to VDR and inhibited the interaction of these coactivators with VDR induced by $1,25(\text{OH})_2\text{D}_3$ (Fig. 5, D and E). The VDR antagonist ZK159222 did not promote interaction of VDR with the p160 family coactivators SRC-1, GRIP1, and ACTR and suppressed their interaction induced by $1,25(\text{OH})_2\text{D}_3$ (Herdick et al., 2000a). These data suggest that antagonism is linked to a lack of ligand-induced interaction of VDR with coactivators. It is noteworthy that AD47 induced DRIP205 recruitment as efficiently as $1,25(\text{OH})_2\text{D}_3$ (Fig. 6A), although it is a weaker agonist than $1,25(\text{OH})_2\text{D}_3$ (Fig. 2B). The AD47-induced interaction of VDR and DRIP205 was relatively unstable in the *in vitro* condition (Fig. 6B). The methylene lactone derivatives LAC67a and LAC67b did not stabilize the DRIP205 interaction. LAC67a and LAC67b, but not AD47, suppressed the VDR-DRIP205 interaction induced by $1,25(\text{OH})_2\text{D}_3$ (Fig. 6C). These findings suggest that chemical modification of $1,25(\text{OH})_2\text{D}_3$ induces a different pattern of cofactor recruitment and contributes to cell type-selective action. We examined the expression of SRC-1 and DRIP205 proteins in the cells shown in Fig. 8. It is noteworthy that DRIP205 expression was high in HaCaT and HCT116 cells, in which AD47 acts as an agonist. Although DRIP205 expression level was not reported to be associated with its coactivator activity on VDR in keratinocytes (Oda et al., 2003), the agonistic activity of AD47 in HaCaT and HCT116 cells may be due to high DRIP205 expression.

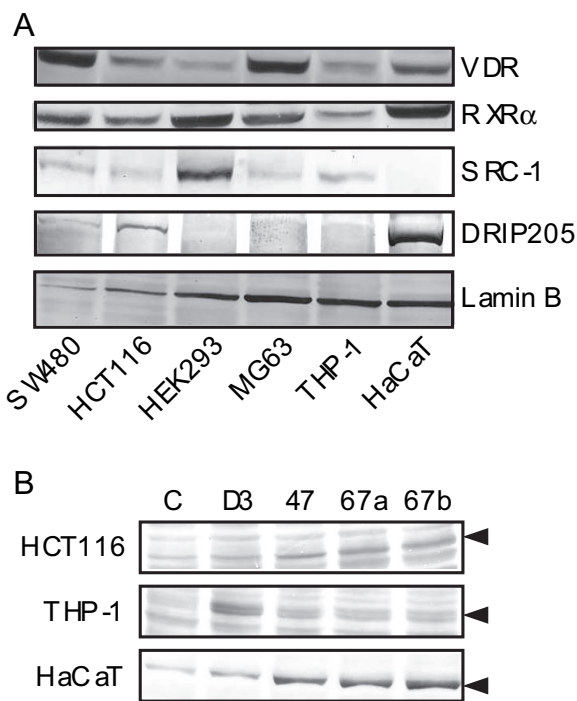


Fig. 9. Immunoblotting analysis of VDR, RXR α , SRC-1, and DRIP205. A, nuclear expression of VDR, RXR α , SRC-1, DRIP205, and Lamin B in SW480 cells, HCT116 cells, HEK293 cells, MG63 cells, THP-1 cells, and HaCaT cells. B, ligand-inducible nuclear localization of VDR in HCT116 cells, THP-1 cells, and HaCaT cells. Cells were treated with ethanol control (C), 10 nM $1,25(\text{OH})_2\text{D}_3$ (D3), 1 μM AD47 (47), LAC67a (67a), or LAC67b (67b) for 24 h as in Fig. 8. After preparation of nuclear extracts, 10 μg was subjected to SDS-polyacrylamide gel electrophoresis for VDR and RXR α , 30 μg for SRC-1 and DRIP205, and 5 μg for Lamin B.

Promoter-selective effects were also reported. $1,25(\text{OH})_2\text{D}_3$ induces a dynamic recruitment of cofactor complexes on Cyp24 promoter distinctly from that on osteopontin gene in osteoblasts (Kim et al., 2005). The vitamin D₃ derivatives 25-hydroxyvitamin D₃, ZK191732, and ZK168289 were reported to exhibit promoter-selective agonistic potency in HeLa cells (Castillo et al., 2006). AD47 was more effective on a reporter containing ER6 from CYP3A4 promoter than on a CYP24 promoter reporter (Fig. 2, A and B). The transcriptional activity of nuclear receptors is regulated by dynamic recruitment of multisubunit coactivator complexes (Kitagawa et al., 2003; Rosenfeld et al., 2006). Involvement of other transcription factors may contribute the promoter-selective effects (Vaisanen et al., 2005). In addition, metabolism of ligands should affect cell type-selective activities of ligands. $1,25(\text{OH})_2\text{D}_3$ induces the expression of CYP24, an

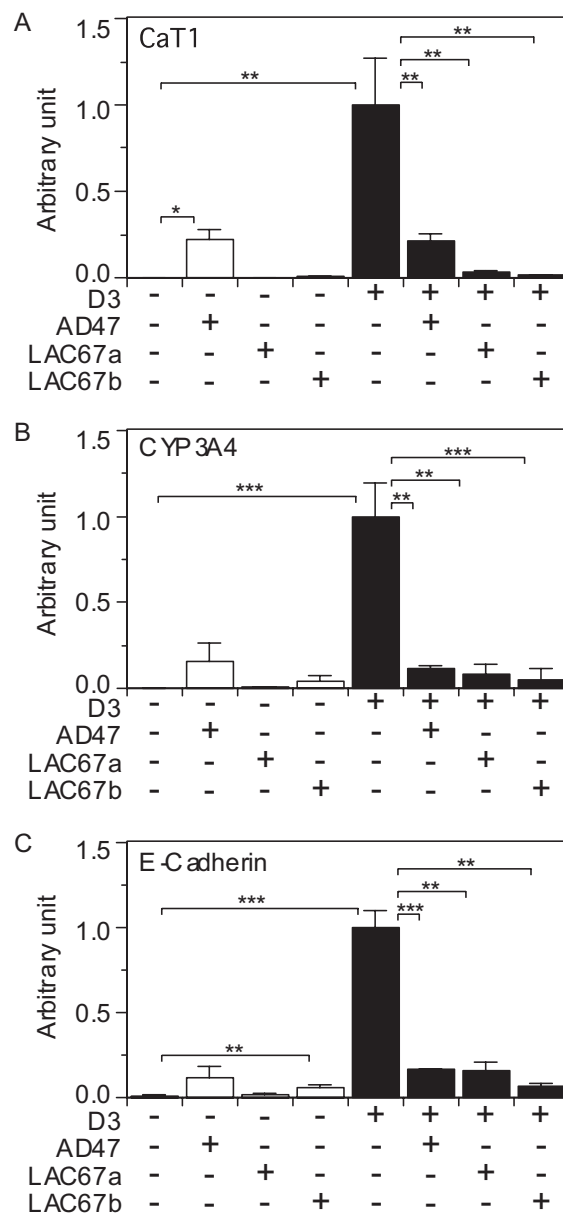


Fig. 10. Effect of vitamin D₃ derivatives on expression of CaT1 (A), CYP3A4 (B), and E-cadherin (C) in SW480 cells. Cells were treated with 1 μM AD47, LAC67a, or LAC67b in the absence or presence of 10 nM $1,25(\text{OH})_2\text{D}_3$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

enzyme inactivating vitamin D₃, including 1,25(OH)₂D₃, but metabolism of synthetic VDR ligands remain to be determined. An understanding of the *in vivo* metabolism of endogenous ligands is beginning to emerge (Chen et al., 2007), and pharmacokinetics of natural and synthetic ligands is an important factor influencing cell type-selectivity. Ligand-inducible nuclear localization of VDR is different among cells (Fig. 9B). Inability to localize VDR in nucleus may be associated with antagonistic activity, although the mechanism of ligand-inducible VDR transport has not been elucidated (Miyachi et al., 2005). Further studies are required to elucidate the molecular mechanism of cell type-selective modulation of VDR activity and its linkage with ligand-specific cofactor complexes.

The docking model reveals that an adamantane ring structure in the side chain of AD47 makes the AF2 conformation relatively unstable, inducing equilibrium between agonistic and antagonistic conformations (Fig. 11A). For the AD47-VDR complex, the interaction with SRC-1 was partially suppressed, and the association with GRIP1 and ACTR did not occur, indicating the adoption of the antagonistic conformation. AD47 transactivated VDR, although less efficiently

than 1,25(OH)₂D₃, and induced efficient DRIP205 recruitment and the dissociation of corepressors, a characteristic of the agonist conformation. On the other hand, ZK168281, which has a long ester group at the side chain terminal, induces a drastic displacement of H12 from the active conformation and acts as a pure antagonist (Vaisanen et al., 2002). ZK168281 was also reported to stabilize VDR-RXR heterodimers (Herdick et al., 2000b). AD47 enhanced the heterodimerization of VDR with RXRs (Fig. 4). These findings suggest that a repositioning of H12 from the active AF2 conformation does not suppress VDR-RXR heterodimerization. The presence of methylene lactone rings in LAC67a and LAC67b results in insufficient positioning of H11/H12 to adopt the active AF2 conformation (Fig. 11B). Differences in the distance from the methylene group to Tyr401 in H11 and Phe422 in H12 may contribute to the agonistic/antagonistic actions of these derivatives. The VDR antagonist TEI-9647, which also has a methylene lactone structure in the side chain, has been suggested to act like LAC67b. LAC67a and LAC67b promoted heterodimerization of VDR with RXRs and allosterically promoted assembly between RXR α H1 and H3–H12 through VDR binding (Fig. 4). On the other hand,

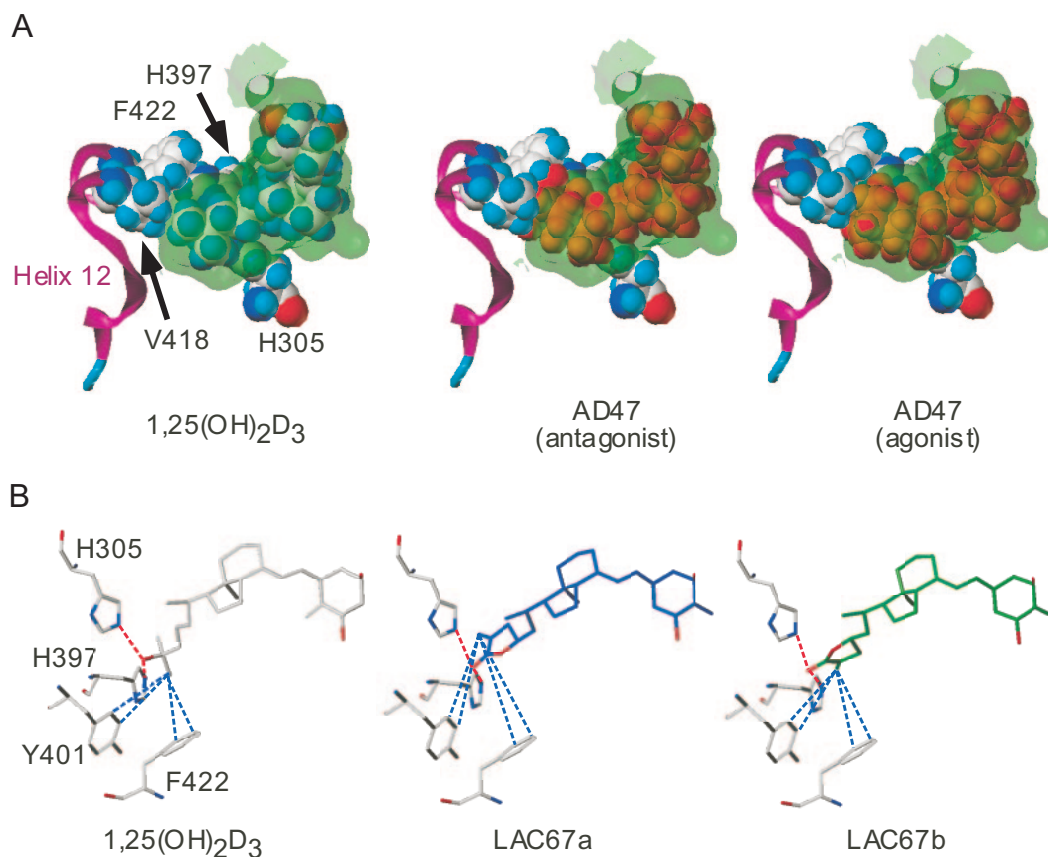


Fig. 11. Docking models of VDR with AD47, LAC67a, and LAC67b. A, space-filling models of 1,25(OH)₂D₃ and AD47 in the VDR ligand-binding pocket. His305 (H305), His397 (H397), Phe422 (F422), Val418 (V418), and 1,25(OH)₂D₃ are represented in atom-type color and AD47 is in red. AD47 has steric repulsion with F422 of H12 due to its bulky side chain (middle), making the AD47-VDR complex more unstable than the 1,25(OH)₂D₃-VDR complex (left). In a docking model of AD47-VDR complex in an agonist conformation (right), the 25-hydroxyl group of AD47 forms a hydrogen bond with His305 but not with His397, indicating that this complex is rather unstable. Because H12 in VDR LBD is in equilibrium between the inactive antagonist-bound conformation and the active agonist-bound conformation, AD47 acts as a partial agonist. B, interactions between amino acid residues and the side chains of 1,25(OH)₂D₃, LAC67a, and LAC67b. 1,25(OH)₂D₃, LAC67a, and LAC67b are drawn in atom-type, blue and green color, respectively. 26-Methyl group of 1,25(OH)₂D₃ makes van der Waals contacts with Tyr401 (Y401) (4.4 Å) and Phe422 (F422) (4.3–4.4 Å) of the VDR-LBD, and this 1,25(OH)₂D₃-VDR complex shows transcriptionally active conformation. Methylene of LAC67a is outside of the proximity required to make appropriate van der Waals contacts with both of these residues (5.6–6.0 Å from Y401 and 7.5–7.6 Å from F422). Therefore, the LAC67a-VDR complex does not form transcriptionally active conformation, and LAC67a behaves as an antagonist. In LAC67b, 26-methylene is closer to the residues, Y401 (4.6–4.7 Å), and F422 (5.7 Å), so LAC67b might act as a partial agonist.

TEI-9647 was reported to reduce the interaction between VDR and RXR α in osteoblastic Saos-2 cells (Ozono et al., 1999). These differences in VDR-RXR heterodimerization may be due to the existence of an allosteric network of residues that link the ligand binding pocket and the heterodimer interface (Shulman et al., 2004). Phe422 (in H12) is located in the network that transmits the effect of the ligands to the dimer interface (H10/H11) and the AF2 surface (Yamamoto et al., 2006). Vitamin D₃ derivatives exhibit differences in cofactor recruitment and VDR-RXR heterodimerization, and this may be due to subtle differences in the interaction of these compounds with residues in the VDR ligand-binding pocket. Further studies are required to elucidate the allosteric and functional linkage from the ligand-binding pocket to the transcriptional activity of VDR-RXR heterodimer.

Vitamin D derivatives are important therapeutics in osteoporosis, psoriasis and rickets (Nagpal et al., 2005). 1,25(OH)₂D₃ and its derivatives are effective drugs in experimental models of immune disorders and malignancies, such as breast cancer, prostate cancer, and leukemia (Makishima and Yamada, 2005). However, the adverse effects, including hypercalcemia, limit the clinical application of VDR agonists in the management of immune disorders and malignancies. To overcome these adverse effects, the development of tissue-selective VDR modulators that act as agonists in some cell types and lack activity in others has pharmaceutical significance. Noncosteroidal VDR ligands have been reported to function as potent agonists in keratinocytes, osteoblasts, and peripheral blood mononuclear cells but show weak activity in intestinal cells (Ma et al., 2006). The hypercalcemia-inducing effect of VDR ligands is considered to be due to the activation of these compounds in the intestine. Figure 8 shows that LAC67b acts as an agonist in osteoblast MG63 and keratinocyte HaCaT cells, but not in enterocytes SW480 and HCT116 cells, suggesting that LAC67b may modulate VDR activity without inducing hypercalcemia. VDR antagonists hold promise in the treatment of diseases with increased VDR activity, including Paget's disease (Ishizuka et al., 2004). TEI-9647 was reported to inhibit the osteoclast formation induced by 1,25(OH)₂D₃ in bone marrow cells derived from patients with Paget's disease. AD47, LAC67a, and LAC67b strongly inhibited VDR target gene expression induced by 1,25(OH)₂D₃ in monoblast THP-1 cells (Fig. 8). Because osteoclasts are derived from bone marrow monocytes, these VDR derivatives may be useful in the treatment of VDR activation diseases, such as Paget's disease.

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