Molecular Pharmacology Fast Forward. Published on February 26, 2007 as doi:10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 32318

Vitamin D_3 Derivatives with Adamantane or Lactone Ring Side Chains are Cell Type-Selective Vitamin D Receptor Modulators

Yuka Inaba, Keiko Yamamoto, Nobuko Yoshimoto, Manabu Matsunawa, Shigeyuki Uno, Sachiko Yamada, Makoto Makishima

Institute of Biomaterials and Bioengineering (Y.I., K.Y., N.Y., S.Y.), Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan; Department of Biochemistry (Y.I., M.M., S.U., S.Y., M.M.), Nihon University School of Medicine, Itabashi-ku, Tokyo 173-8610, Japan

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Cell Type-Selective VDR Modulators

Address correspondence to: Makoto Makishima, Department of Biochemistry, Nihon University School of Medicine, 30-1 Oyaguchi-kamicho, Itabashi-ku, Tokyo 173-8610, Japan. E-mail: maxima@med.nihon-u.ac.jp.

Number of text pages: 56

Number of tables: 0

Number of figures: 11

Number of references: 42

Number of words in Abstract: 237

Number of words in Introduction: 747

Number of words in Discussion: 1488

Abbreviations: VDR, vitamin D receptor; AF2, activation function 2; H, helix; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; RXR, retinoid X receptor; ER6, everted repeat 6; LBD, ligand-binding domain; ER, estrogen receptor; MR, mineralocorticoids; HEK, human embryonic kidney; FBS, fetal bovine serum; GST, glutathione *S*-transferase; LCA, lithocholic acid

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The vitamin D receptor (VDR) mediates the biological actions of the active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], which regulates calcium homeostasis, immunity, cellular differentiation, and other physiological processes. We investigated the effects of three 1,25$(OH)_2D_3$ 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$(OH)_2D_3$. 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$(OH)_2D_3$. Interestingly, AD47 induced DRIP205 recruitment as effectively as 1,25$(OH)_2D_3$, while 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 D_3 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.

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

result in cell type-selective modulation of target gene expression.

The active form vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], 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 to a DNA response element that consists of a direct repeat of a hexanucleotide (AGGTCA or related sequence) motif separated by a three nucleotides 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 $1,25(OH)_2D_3$-bound human VDR ligand-binding domain (LBD) reveals that $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$, Ser237 (in H3) and Arg274 (in H5) hydrogen bonds with the 1α-hydroxyl group, His305 (in loop 6-7) and His397 (in H11) coordinates the 25-hydroxyl group (Rochel et al., 2000). Hydrophobic interactions between the ligand

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Makishima and Yamada, 2005; Yamada et al., 2003). 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 $1,25(OH)_2D_3$, and this interaction connects the LBP at H6 to loop 6-7 and extends to Phe 422 (in H12) (Yamamoto et al., 2006).

A number of vitamin D analogues have been synthesized and evaluated for selective VDR ligand activity (DeLuca, 2004; Yamada et al., 2003). 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 Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

molecular mechanisms of nuclear receptor activation, but also may yield clinically useful drugs as in case of the selective estrogen receptor (ER) 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 resides of H12 of ERα (NR3A1) and prevents formation of the active AF2 conformation (Brzozowski et al., 1997; Shiau et al., 1998). 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 (Bledsoe et al., 2005; Geller et al., 2000). In this study, we examined the effects of three vitamin D_3 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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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-ACTR, 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 bp of the human CYP24A1 promoter (GenBank accession no. **U60669**) into the luciferase reporter pGL3

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

(Promega, Madison, WI). VDR-responsive hCYP3A4-ER6x3-tk-LUC and GAL4-responsive MH100(UAS)x4-tk-LUC were utilized in luciferase reporter assay (Nakano et al., 2005). pGEX vector (GE Healthcare Bio-Sciences, Piscataway, NJ) was utilized to generate glutathione *S*-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 ^{o}C in a humidified atmosphere containing 5% CO_2. Human colon carcinoma SW480, HCT116 and immortalized keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle medium containing 10% FBS, monoblastic leukemia THP-1 cells were in RPMI1640 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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

coprecipitation assay as described previously (Kaneko et al., 2003). Eight hours after transfection, compounds were added. Cells were harvested after 16-24 hours 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 per 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 (Yamasa, Chiba, Japan) and GST-RXRα fusion proteins were utilized 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-^{3}H] 1,25$(OH)_2D_3$ or 9-*cis*-[^{3}H] retinoic acid at 4 ^oC in the presence or absence of non-radioactive competitor compounds. Bound and labeled 1,25$(OH)_2D_3$ or 9-*cis* retinoic acid was assessed using scintillation counting.

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

GST Pull-Down Assay. GST fusion proteins were expressed in BL21 DE3 cells (Promega), and purified using Glutathione Sepharose 4B (GE Healthcare Bio-Sciences). [^{35}S]-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). About 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, 2 mg/ml bovine serum albumin). Bound GST proteins were incubated with 10 μl of reticulocyte lysate containing [^{35}S]-labeled proteins and were treated with test compounds for 2 hours at 4 ^{o}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 RT-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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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'; 5-lipoxygenase, 5'-TCC TCC CTT CGG ATG CAA AA-3' and 5'-CAG ACA CCA GAT GTG TTC GCA G-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 ± S.D. of triplicate assays.

Immunoblotting. Nuclear extracts were prepared as described previously (Schreiber et al., 1989). The proteins were separated by SDS-PAGE and were transferred to a nitrocellulose membrane, probed with anti-VDR antibody (sc-1008, Santa Cruz Biotechnology, Santa Cruz, CA), anti-RXRα antibody (sc-774, Santa Cruz Biotechnology), anti-SRC-1 antibody (clone 1135, Upstate, Lake Placid, NY),

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

anti-DRIP205 antibody (sc-8998, Santa Cruz Biotechnology), or anti-Lamin B1 antibody (sc-6216, 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 (Δ165-215) crystal structure were retrieved from the Protein Data Bank (PDB #1DB1). Ligands were docked into the ligand-binding pocket manually by superposition with $1,25(OH)_2D_3$ at the A- to D-ring.

Results

Vitamin D_3 Derivatives with an Adamantane or Lactone Ring are VDR Antagonists. AD47 is a 19-nor-$1,25(OH)_2D_3$ derivative that has a bulky adamantane ring at the terminal side chain and LAC67b is a 2-methylene-19-nor-$1,25(OH)_2D_3$ derivative with a methylene lactone side chain structure (Fig. 1). We previously reported that these

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

compounds inhibited VDR transactivation induced by $1,25(OH)_2D_3$ in COS7 cells (Yamamoto et al., 2006). We newly synthesized another 2-methylene-19-nor-$1,25(OH)_2D_3$ 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 bp of the promoter (Pascussi et al., 2005). Treatment with $1,25(OH)_2D_3$ increased luciferase activity in a concentration-dependent manner (Fig. 2A). AD47 and LAC67b had maximal activation that was 12% and 70% of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 the both reporters (Fig. 2A and B). To examine VDR antagonist activity, we treated cells with these derivatives in combination with 10 nM $1,25(OH)_2D_3$. As reported previously

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

(Yamamoto et al., 2006), AD47 and LAC67b inhibited 1,25$(OH)_2D_3$ transactivation in a concentration-dependent manner (Fig. 2C). VDR inhibition by LAC67b was weaker than that of AD47 at 0.3 μM and 1 μM. This may be due to the partial agonist activity of LAC67b observed in HEK293 cells. LAC67a also exhibited VDR antagonist activity but less effectively than AD47. Next, a mammalian one-hybrid assay was utilized 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 μM concentration (Fig. 2D). AD47 and LAC67a inhibited the GAL4-VDR activity induced by 10 nM 1,25$(OH)_2D_3$. Since 1 μM LAC67b induced GAL4-VDR transactivation as efficiently as 10 nM 1,25$(OH)_2D_3$, LAC67b at this concentration did not inhibit 1,25$(OH)_2D_3$ activity. The bile acid lithocholic acid (LCA) was recently identified as an additional physiological VDR ligand (Makishima et al., 2002). LCA at 10 μM induced GAL4-VDR activation as reported previously (Makishima et al., 2002). AD47 and LAC67a inhibited LCA-induced VDR activity in a

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 $1,25(OH)_2D_3$ and its derivatives was examined by a competitive binding assay. Isotopically labeled $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$, respectively (Fig. 3A). The low affinity of LAC67a binding to VDR may contribute to its diminished antagonist activity (Fig. 2C).

Effects of Vitamin D_3 Derivatives on VDR-RXR Heterodimerization. $1,25(OH)_2D_3$ 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-RXRα. AD47, LAC67a and LAC67b induced the heterodimerization of GAL4-RXRα with VP16-VDR with an EC_{50} of 10 nM, 20 nM and 3 nM, respectively, while the EC_{50} for $1,25(OH)_2D_3$ was 0.7 nM (Fig. 4A). These

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

derivatives induced RXRα heterodimerization with nearly the same maximal efficiency as 1,25$(OH)_2D_3$. We examined the possibility that AD47, LAC67a and LAC67b might directly bind to RXRα using a competitive ligand-binding assay. The addition of 1,25$(OH)_2D_3$ or its derivatives did not inhibit the binding of labeled 9-*cis* retinoic acid, a potent RXR ligand, while unlabeled 9-*cis* retinoic acid competed with the labeled ligand as expected, indicating that these vitamin D_3 derivatives are not RXRα 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 RXRα in the presence or absence of 1 μM test compound. 1,25$(OH)_2D_3$ induced the interaction of RXRα with GST-VDR, but not with GST control protein (Fig. 4B). LAC67b induced the binding of GST-VDR and isotope-labeled RXRα as effectively as 1,25$(OH)_2D_3$, while AD47 and LAC67a were less effective. 1,25$(OH)_2D_3$ and LAC67b also induced the reciprocal interaction between GST-RXRα and isotope-labeled VDR. While the effect of AD47 on this interaction was modest, induction mediated by LAC67a was marginal.

The effects of vitamin D_3 derivatives on the interaction of VDR with RXRβ and

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

RXRγ were examined in a mammalian two-hybrid assay. While AD47 and LAC67a at 1 μM induced the interaction of VDR and RXRβ as effectively as 10 nM $1,25(OH)_2D_3$, LAC67b was more effective than these derivatives (Fig. 4C). The effect on VDR-RXRβ heterodimerization was comparable to that on VDR-RXRα heterodimerization. Interestingly, GAL4-RXRγ bound to VP16-VDR in the absence of ligand (Fig. 4C) and the addition of $1,25(OH)_2D_3$ (10 nM) and AD47 (1 μM) did not enhance this interaction. LAC67a and LAC67b were more potent inducers of the VDR-RXRγ 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-RXRα, VP16-RXRβ and VP16-RXRγ increased luciferase activity, indicating that RXR proteins were bound to VDR in cells (Fig. 4D). $1,25(OH)_2D_3$ at 10 nM induced GAL4-VDR activation in the presence of control VP16 protein, and GAL4-VDR activity induced by $1,25(OH)_2D_3$ was augmented by cotransfection of VP16-RXRα, VP16-RXRβ and VP16-RXRγ, indicating that $1,25(OH)_2D_3$ 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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

VP16-RXRα, VP16-RXRβ or VP16-RXRγ 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 RXRα 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 RXRα cotransfection (Morita et al., 2005). The H1 fragment of RXRα 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 previously reported (Morita et al., 2005; Pissios et al., 2000), the RXR ligand 9-*cis* retinoic acid induced the assembly

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

of the H1 and H3-12 fragments of RXRα-LBD, but $1,25(OH)_2D_3$ and its derivatives did not promote RXRα assembly (Fig. 4E). In the presence of VDR cotransfection, $1,25(OH)_2D_3$ induced the interaction of GAL4-RXRα (H1) with VP16-RXRα (H3-H12). While LAC67a and LAC67b effectively induced RXRα 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 Induce Unique Cofactor Interactions. Upon ligand binding, nuclear receptors undergo conformational changes in the cofactor binding site and AF2 helix that results 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 utilizing a mammalian two-hybrid assay. $1,25(OH)_2D_3$ induced a concentration-dependent association of VDR with SRC-1, as reported previously

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

(Adachi et al., 2004; Herdick et al., 2000a). AD47 and LAC67b induced SRC-1 recruitment to VDR less effectively than $1,25(OH)_2D_3$ (Fig. 5A). Interestingly, while 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 $1,25(OH)_2D_3$ in mammalian two-hybrid experiments. In cells transfected with GAL4-SRC-1 and VP16-VDR, AD47 (1 μM) reduced the luciferase activity induced by $1,25(OH)_2D_3$ (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 $1,25(OH)_2D_3$ (10 nM) to basal

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 $1,25(OH)_2D_3$ (10 nM) (Fig. 5E).

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). Surprisingly, AD47 promoted DRIP recruitment with an efficacy nearly equivalent to that of $1,25(OH)_2D_3$. LAC67a and LAC67b, even at concentrations of 1 μM, did not induce the association of DRIP205 and VDR. In a GST pull-down assay, AD47 induced the interaction of GST-DRIP205 and isotope-labeled VDR to an extent weaker than that of $1,25(OH)_2D_3$ (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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

$1,25(OH)_2D_3$ (10 nM) (Fig. 6C). LAC67a and LAC67b, which alone did not induce DRIP205 recruitment, antagonized the $1,25(OH)_2D_3$-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 $1,25(OH)_2D_3$ reduced these interactions (Adachi et al., 2004). AD47, LAC67a and LAC67b induced the dissociation of N-CoR from VDR in a concentration-dependent manner, while the effect of LAC67a was weak (Fig. 7A). AD47, LAC67a and LAC67b did not diminish $1,25(OH)_2D_3$-mediated dissociation of N-CoR from VDR (Fig. 7B). $1,25(OH)_2D_3$ and the three vitamin D_3 derivatives also promoted the concentration-dependent disassociation of SMRT from VDR (Fig. 7C). Combined administration of these vitamin D_3 derivatives did not alter SMRT dissociation induced by $1,25(OH)_2D_3$ (Fig. 7D). Thus, AD47, LAC67a and LAC67b exhibit distinct coactivator recruitment activities while similarly inducing corepressor dissociation.

Effect of Vitamin D_3 Derivatives on Endogenous Gene Expression in Cells.

VDR is expressed in the vitamin D_3 target organs that mediate calcium homeostasis, such

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

as intestine, bone and kidney (DeLuca, 2004; Haussler et al., 1998). The non-calcemic actions of vitamin D_3 are associated with VDR expression in other tissues, including blood cells and skin (Nagpal et al., 2005). We examined the effects of the vitamin D_3 derivatives on the expression of endogenous VDR target genes. $1,25(OH)_2D_3$ induces its own metabolism through VDR-dependent activation of the enzyme CYP24A1 in many tissues (Haussler et al., 1998). We treated intestinal 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 μM AD47, LAC67a and LAC67b in the absence or presence of 10 nM $1,25(OH)_2D_3$, and evaluated CYP24A1 mRNA expression by quantitative real time PCR. In intestinal SW480 cells, AD47 (1 μM) induced the expression of CYP24A1 29% as effectively as $1,25(OH)_2D_3$ (10 nM), while LAC67a and LAC67b had no effect (Fig. 8A). AD47 and LAC67a suppressed CYP24A1 expression induced by $1,25(OH)_2D_3$ and LAC67b antagonized $1,25(OH)_2D_3$ more effectively than AD47 and LAC67a in SW480 cells. AD47 (1μM) induced CYP24A1 expression 77% of the levels observed with $1,25(OH)_2D_3$ (10nM) in intestinal HCT116 cells (Fig. 8B). Since AD47 is an effective

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

agonist, this derivative did not suppress CYP24A1 expression induced by $1,25(OH)_2D_3$. LAC67a did not induce CYP24A1 expression and weakly antagonized the effect of $1,25(OH)_2D_3$. LAC67b suppressed CYP24A1 induction by $1,25(OH)_2D_3$ more effectively than LAC67a. AD47 and LAC67a did not induce CYP24A1 expression in kidney-derived HEK293 cells (Fig. 8C). LAC67b weakly induced its expression. Interestingly, while 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 1μM suppressed CYP24A1 induction by $1,25(OH)_2D_3$ (10 nM) in HEK293 cells (Fig. 8C). AD47 and LAC67b acted as partial agonists in osteoblast MG63 cells and suppressed CYP24A1 expression induced by $1,25(OH)_2D_3$ (Fig. 8D). LAC67a, a pure antagonist in MG63 cells, inhibited the induction of CYP24A1 expression by $1,25(OH)_2D_3$ more effectively than AD47 and LAC67b. In monoblastic leukemia THP-1 cells, AD47, LAC67a and LAC67b (1 μM) induced CYP24A1 expression less than 6% as efficiently as $1,25(OH)_2D_3$ (10 nM), and these derivatives acted as strong inhibitors of CYP24A1 expression induced by $1,25(OH)_2D_3$ (Fig. 8E). The effect of the vitamin D_3 derivatives in keratinocyte HaCaT cells was

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 $1,25(OH)_2D_3$, 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, RXRα, SRC-1 and DRIP205 proteins in nuclear extracts by immunoblotting. VDR and RXRα proteins were detected in SW480, HCT116, HEK293, MG63, THP-1 and HaCaT cells (Fig. 9A). SRC-1 was highly expressed in HEK293 cells, while 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. $1,25(OH)_2D_3$ 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)2D3 (10 nM) increased nuclear VDR in HCT116, THP-1 and HaCaT cells. Interestingly, 10 μM AD47, LAC67a and

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

LAC67b increased nuclear VDR proteins in HCT116 and HaCaT cells, in which these compounds exhibited some agonistic activity (Fig. 8B 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 μM) weakly induced CaT1 expression and suppressed its activation by $1,25(OH)_2D_3$ (10 nM) (Fig. 10A). LAC67a and LAC67b did not induce CaT1 expression and antagonized the effect of $1,25(OH)_2D_3$ more strongly than AD47. As was observed for CYP24A1 and CaT1, AD47 also induced weak expression of CYP3A4 and E-cadherin (Fig. 10B and C). 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, while LAC67a and LAC67b are VDR antagonists in SW480

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

cells.

Docking Models of the Vitamin D_3 Derivatives in the VDR-LBD. We previously reported docking models of AD47 and LAC67b in the VDR ligand-binding pocket that was supported by alanine scanning mutational analysis combined with functional assays in COS7 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 D_3 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 $1,25(OH)_2D_3$. The AD47-VDR complex is in equilibrium between transcriptionally active and inactive conformations, which results in AD47 functioning as a partial agonist. Fig. 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 $1,25(OH)_2D_3$-VDR complex (Fig. 11A, left). Fig. 11B shows the complexes of $1,25(OH)_2D_3$, LAC67a and LAC67b with the

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 H11/12 and the ligand. While the terminal methyl group at C(26) of 1,25$(OH)_2D_3$ 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 D_3 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, while LAC67a did not activate VDR (Fig. 2). In the previous study, although agonist activity was weak, AD47 was more potent than LAC67b in

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

mediating VDR activation in COS7 cells (Yamamoto et al., 2006). The effect of LAC67b in the HEK293 transient transfection assay was comparable to 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, while 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 differing antagonist activity in the cell types examined in this study. It effectively repressed VDR target gene expression induced by $1,25(OH)_2D_3$ in SW480, MG63 and THP-1 cells, but not in HCT116, HaCaT and HEK293 cells (Fig. 8). Thus, LAC67a is a cell type-selective

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 $1,25(OH)_2D_3$, while LAC67b did only weakly and LAC67a did not (Fig. 5A 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 recruitment to VDR, but inhibited the SRC-1 interaction mediated by $1,25(OH)_2D_3$. AD47, LAC67a and LAC67b did not effectively recruit GRIP1 and ACTR to VDR and inhibited the interaction of these coactivators with VDR induced by $1,25(OH)_2D_3$ (Fig. 5D 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 $1,25(OH)_2D_3$ (Herdick et al., 2000a). These data suggest that antagonism is linked to a lack of ligand-induced interaction of VDR with coactivators. Interestingly, AD47 induced DRIP205 recruitment as efficiently as $1,25(OH)_2D_3$ (Fig. 6A), although it is a weaker agonist than $1,25(OH)_2D_3$ (Fig. 2B). The AD47-induced interaction of VDR and DRIP205 was relatively unstable

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 $1,25(OH)_2D_3$ (Fig. 6C). These findings suggest that chemical modification of $1,25(OH)_2D_3$ 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. Interestingly, 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.

Promoter-selective effects were also reported. $1,25(OH)_2D_3$ induces a dynamic recruitment of cofactor complexes on Cyp24 promoter distinctly from that on osteopontin gene in osteoblasts (Kim et al., 2005). The vitamin D_3 derivatives ZK136607, 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. 2A and B). The

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

transcriptional activity of nuclear receptors is regulated by dynamic recruitment of multi-subunit 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. $1,25(OH)_2D_3$ induces the expression of CYP24, an enzyme inactivating vitamin D_3, including $1,25(OH)_2D_3$, 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 (Miyauchi 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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 $1,25(OH)_2D_3$, 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 re-positioning 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 H11/12 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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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 RXRα H1 and H3-12 through VDR binding (Fig. 4). On the other hand, TEI-9647 was reported to reduce the interaction between VDR and RXRα 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 (H10/11) and the AF2 surface (Yamamoto et al., 2006). Vitamin D_3 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). $1,25(OH)_2D_3$ and its derivatives are effective drugs in

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

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. Recently, nonsecosteroidal VDR ligands were 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. Fig. 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 $1,25(OH)_2D_3$ in bone marrow cells derived from Paget's disease patients. AD47,

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

LAC67a and LAC67b strongly inhibited VDR target gene expression induced by $1,25(OH)_2D_3$ in monoblast THP-1 cells (Fig. 8). Since 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.

Acknowledgements

The authors thank Dr. David J. Mangelsdorf of the Howard Hughes Medical Institute, University of Texas Southwestern Medical Center (Dallas, TX) for providing plasmids, Katsuyoshi Kawana and Michiyasu Ishizawa for technical assistance, Dr. Yuko Oda of University of California San Francisco and Veterans Affairs Medical Center (San Francisco, CA), Dr. Ken-ichi Takeyama of Tokyo University (Tokyo, Japan), Dr. Riki Kurokawa of Saitama Medical School (Saitama, Japan), Dr. Mitsuhiro Ito of Kobe University School of Medicine (Kobe, Japan), and Dr. Shigeru Nishida and other members of the Makishima laboratory for helpful comments.

References

Adachi R, Honma Y, Masuno H, Kawana K, Shimomura I, Yamada S and Makishima M (2005) Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative. *J Lipid Res* **46**:46-57.

Adachi R, Shulman AI, Yamamoto K, Shimomura I, Yamada S, Mangelsdorf DJ and Makishima M (2004) Structural determinants for vitamin D receptor response to endocrine and xenobiotic signals. *Mol Endocrinol* **18**:43-52.

Bledsoe RK, Madauss KP, Holt JA, Apolito CJ, Lambert MH, Pearce KH, Stanley TB, Stewart EL, Trump RP, Willson TM and Williams SP (2005) A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. *J Biol Chem* **280**:31283-31293.

Bouillon R, Van Cromphaut S and Carmeliet G (2003) Intestinal calcium absorption: molecular vitamin D mediated mechanisms. *J Cell Biochem* **88**:332-339.

Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson J-A and Carlquist M (1997) Molecular basis of agonism

and antagonism in the oestrogen receptor. *Nature* **389**:753-758.

Castillo AI, Sanchez-Martinez R, Jimenez-Lara AM, Steinmeyer A, Zugel U and Aranda A (2006) Characterization of vitamin D receptor ligands with cell-specific and dissociated activity. *Mol Endocrinol* **20**:3093-3104.

Chen W, Chen G, Head DL, Mangelsdorf DJ and Russell DW (2007) Enzymatic reduction of oxysterols impairs LXR signaling in cultured cells and the livers of mice. *Cell Metab* **5**:73-79.

DeLuca HF (2004) Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr* **80**:1689S-1696.

Geller DS, Farhi A, Pinkerton N, Fradley M, Moritz M, Spitzer A, Meinke G, Tsai FTF, Sigler PB and Lifton RP (2000) Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. *Science* **289**:119-123.

Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE and Jurutka PW (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res* **13**:325-349.

Herdick M, Steinmeyer A and Carlberg C (2000a) Antagonistic action of a 25-carboxylic ester analogue of 1α,25-dihydroxyvitamin D_3 is mediated by a lack of ligand-induced vitamin D receptor interaction with coactivators. *J Biol Chem* **275**:16506-16512.

Herdick M, Steinmeyer A and Carlberg C (2000b) Carboxylic ester antagonists of 1α,25-dihydroxyvitamin D_3 show cell-specific actions. *Chem Biol* **7**:885-894.

Ishizuka S, Kurihara N, Miura D, Takenouchi K, Cornish J, Cundy T, Reddy SV and Roodman GD (2004) Vitamin D antagonist, TEI-9647, inhibits osteoclast formation induced by 1α,25-dihydroxyvitamin D_3 from pagetic bone marrow cells. *J Steroid Biochem Mol Biol* **89-90**:331-334.

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

Kim S, Shevde NK and Pike JW (2005) 1,25-Dihydroxyvitamin D3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. *J Bone Miner Res* **20**:305-317.

Kitagawa H, Fujiki R, Yoshimura K, Mezaki Y, Uematsu Y, Matsui D, Ogawa S, Unno K, Okubo M, Tokita A, Nakagawa T, Ito T, Ishimi Y, Nagasawa H, Matsumoto T, Yanagisawa J and Kato S (2003) The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* **113**:905-917.

Ma Y, Khalifa B, Yee YK, Lu J, Memezawa A, Savkur RS, Yamamoto Y, Chintalacharuvu SR, Yamaoka K, Stayrook KR, Bramlett KS, Zeng QQ, Chandrasekhar S, Yu X-P, Linebarger JH, Iturria SJ, Burris TP, Kato S, Chin WW and Nagpal S (2006) Identification and characterization of noncalcemic, tissue-selective, nonsecosteroidal vitamin D receptor modulators. *J Clin Invest* **116**:892-904.

Makishima M (2005) Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors. *J Pharmacol Sci* **97**:177-183.

Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR and Mangelsdorf DJ (2002) Vitamin D receptor as an intestinal bile acid sensor.

Science **296**:1313-1316.

Makishima M and Yamada S (2005) Targeting the vitamin D receptor: advances in drug discovery. *Expert Opin Ther Pat* **15**:1133-1145.

Malik S and Roeder RG (2005) Dynamic regulation of pol II transcription by the mammalian Mediator complex. *Trends Biochem Sci* **30**:256-263.

Michigami T, Suga A, Yamazaki M, Shimizu C, Cai G, Okada S and Ozono K (1999) Identification of amino acid sequence in the hinge region of human vitamin D receptor that transfers a cytosolic protein to the nucleus. *J Biol Chem* **274**:33531-33538.

Miyauchi Y, Michigami T, Sakaguchi N, Sekimoto T, Yoneda Y, Pike JW, Yamagata M and Ozono K (2005) Importin 4 is responsible for ligand-independent nuclear translocation of vitamin D receptor. *J Biol Chem* **280**:40901-40908.

Morita K, Kawana K, Sodeyama M, Shimomura I, Kagechika H and Makishima M (2005) Selective allosteric ligand activation of the retinoid X receptor heterodimers of NGFI-B and Nurr1. *Biochem Pharmacol* **71**:98-107.

Nagpal S, Na S and Rathnachalam R (2005) Noncalcemic actions of vitamin D receptor

ligands. *Endocr Rev* **26**:662-687.

Nakano H, Matsunawa M, Yasui A, Adachi R, Kawana K, Shimomura I and Makishima M (2005) Enhancement of ligand-dependent Vitamin D receptor transactivation by the cardiotonic steroid bufalin. *Biochem Pharmacol* **70**:1479-1486.

Oda Y, Sihlbom C, Chalkley RJ, Huang L, Rachez C, Chang C-PB, Burlingame AL, Freedman LP and Bikle DD (2003) Two distinct coactivators, DRIP/Mediator and SRC/p160, are differentially involved in vitamin D receptor transactivation during keratinocyte differentiation. *Mol Endocrinol* **17**:2329-2339.

Ozono K, Saito M, Miura D, Michigami T, Nakajima S and Ishizuka S (1999) Analysis of the molecular mechanism for the antagonistic action of a novel 1α,25-dihydroxyvitamin D_3 analogue toward vitamin D receptor function. *J Biol Chem* **274**:32376-32381.

Pascussi JM, Robert A, Nguyen M, Walrant-Debray O, Garabedian M, Martin P, Pineau T, Saric J, Navarro F, Maurel P and Vilarem MJ (2005) Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia. *J Clin Invest* **115**:177-186.

Pissios P, Tzameli I, Kushner P and Moore DD (2000) Dynamic stabilization of nuclear receptor ligand binding domains by hormone or corepressor binding. *Mol Cell* **6**:245-253.

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

Rosenfeld MG, Lunyak VV and Glass CK (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* **20**:1405-1428.

Schreiber E, Matthias P, Muller MM and Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**:6419.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA and Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**:927-937.

Shulman AI, Larson C, Mangelsdorf DJ and Ranganathan R (2004) Structural determinants of allosteric ligand activation in RXR heterodimers. *Cell*

116:417-429.

Uno S, Dalton TP, Dragin N, Curran CP, Derkenne S, Miller ML, Shertzer HG, Gonzalez FJ and Nebert DW (2006) Oral benzo[a]pyrene in Cyp1 knockout mouse lines: CYP1A1 important in detoxication, CYP1B1 metabolism required for immune damage independent of total-body burden and clearance rate. *Mol Pharmacol* **69**:1103-1114.

Vaisanen S, Perakyla M, Karkkainen JI, Steinmeyer A and Carlberg C (2002) Critical role of helix 12 of the vitamin D(3) receptor for the partial agonism of carboxylic ester antagonists. *J Mol Biol* **315**:229-238.

Vaisanen S, Dunlop TW, Sinkkonen L, Frank C and Carlberg C (2005) Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1α,25-dihydroxyvitamin D_3. *J Mol Biol* **350**:65-77.

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

Yamada S, Shimizu M and Yamamoto K (2003) Structure-function relationships of vitamin D including ligand recognition by the vitamin D receptor. *Med Res Rev* **23**:89-115.

Yamamoto K, Sun WY, Ohta M, Hamada K, DeLuca HF and Yamada S (1996) Conformationally restricted analogs of 1α,25-dihydroxyvitamin D_3 and its 20-epimer: compounds for study of the three-dimensional structure of vitamin D responsible for binding to the receptor. *J Med Chem* **39**:2727-2737.

Yamamoto K, Abe D, Yoshimoto N, Choi M, Yamagishi K, Tokiwa H, Shimizu M, Makishima M and Yamada S (2006) Vitamin D receptor: ligand recognition and allosteric network. *J Med Chem* **49**:1313-1324.

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (M.M.), and the Ministry of Health, Labor, and Welfare, Japan (M.M.).

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1. Structures of 1,25$(OH)_2D_3$, 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-seco estra-5,7-diene-1,3-diol, LAC67a is (5*R*)-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(3H)-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(3H)-furanone.

Fig. 2. Vitamin D_3 derivatives antagonize VDR activation induced by 1,25$(OH)_2D_3$. (A) Concentration-dependent activation of VDR on CYP24 promoter by vitamin D_3 derivatives. HEK293 cells were cotransfected with CMX-VDR and the human CYP24 promoter-luciferase reporter and treated with several concentrations of 1,25$(OH)_2D_3$ (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 D_3 derivatives.

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

HEK293 cells were cotransfected with CMX-VDR and the CYP3A4-ER6x3-tk-LUC reporter and treated with several concentrations of $1,25(OH)_2D_3$, AD47, LAC67a and LAC67b. (C) Antagonistic effect of vitamin D_3 derivatives on VDR activated by $1,25(OH)_2D_3$. 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 $1,25(OH)_2D_3$. (D) Effect of vitamin D_3 derivatives on the GAL4-VDR chimeric receptor. HEK293 cells were cotransfected with CMX-GAL4-VDR and the MH100(UAS)x4-tk-LUC reporter, and were treated with 1 μM AD47, LAC67a or LAC67b in the absence or presence of 10 nM $1,25(OH)_2D_3$. ***, $p<0.001$ compared with untreated control. ###, $p<0.001$ compared with the activity induced by $1,25(OH)_2D_3$ alone. (E) Effect of vitamin D_3 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.

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3. Effect of vitamin D_3 derivatives on direct binding of $1,25(OH)_2D_3$ to VDR (A) and that of 9-*cis* retinoic acid to RXRα (B). (A) VDR proteins were incubated with [^{3}H] $1,25(OH)_2D_3$ in the presence of non-radioactive $1,25(OH)_2D_3$, AD47, LAC67a and LAC67b at a range of concentrations. (B) GST-RXRα proteins were incubated with [^{3}H] 9-*cis* retinoic acid in the presence of non-radioactive 9-*cis* retinoic acid, $1,25(OH)_2D_3$, AD47, LAC67a and LAC67b at a range of concentrations.

Fig. 4. Vitamin D derivatives induce VDR-RXR heterodimerization. (A) Effect of vitamin D_3 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 $1,25(OH)_2D_3$, AD47, LAC67a or LAC67b at a range of concentrations. (B) Effect of vitamin D_3 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 [^{35}S]-RXRα (upper panel) and between GST or GST-RXRα and [^{35}S]-VDR (lower panel) in the presence of ethanol control (C), 1 μM $1,25(OH)_2D_3$ (D), AD47 (A), LAC67a (La) or LAC67b (Lb). (C) Effect

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

of vitamin D_3 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 nM $1,25(OH)_2D_3$, 1 μM AD47, LAC67a or LAC67b. (D) Vitamin D_3 derivatives enhance the interaction between GAL4-VDR and VP16-RXRs. 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 nM $1,25(OH)_2D_3$, 1 μM AD47, LAC67a or LAC67b. (E) Effect of vitamin D_3 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 (H3-12), in the absence or presence of cotransfection with CMX-VDR, and MH100(UAS)x4-tk-LUC, and were treated with ethanol (EtOH), 100 nM 9-*cis* retinoic acid (9CRA), 10 nM $1,25(OH)_2D_3$, 1 μM AD47, LAC67a or LAC67b. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with ethanol control.

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 5. Effect of vitamin D_3 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)x4-tk-LUC, and were treated with 1,25$(OH)_2D_3$, 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 [^{35}S]-VDR (upper panel) and between GST or GST-VDR and [^{35}S]-labeled full-length SRC-1 (lower panel) in the presence of ethanol control (C), 100 nM 1,25$(OH)_2D_3$ (D), 10 μM AD47 (A), LAC67a (La) or LAC67b (Lb). Effects of vitamin D_3 derivatives on interaction of VDR with SRC-1 (C), GRIP1 (D) or ACTR (E) induced by 1,25$(OH)_2D_3$. 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$(OH)_2D_3$. **, $p<0.01$; ***, $p<0.001$ compared with untreated control. ##, $p<0.01$; ###, $p<0.001$ compared with the activity induced by

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

$1,25(OH)_2D_3$ alone.

Fig. 6. Effect of vitamin D_3 derivatives on DRIP205 coactivator recruitment. (A) AD47 induces interaction of VDR and DRIP205 as efficiently as $1,25(OH)_2D_3$. 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 $1,25(OH)_2D_3$. 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 $1,25(OH)_2D_3$ alone.

Fig. 7. Effect of vitamin D_3 derivatives on corepressor interaction. (A) AD47, LAC67a and LAC67b induce dissociation of N-CoR from VDR. (B) Vitamin D_3 derivatives do not suppress the dissociation of N-CoR from VDR induced by $1,25(OH)_2D_3$. (C) Vitamin D_3 derivatives also induce dissociation of SMRT from VDR. (D) Vitamin D_3 derivatives do

not suppress the dissociation of SMRT from VDR induced by $1,25(OH)_2D_3$. HEK293 cells were cotransfected with CMX-GAL4-N-CoR (A, B) or CMX-GAL4-SMRT (C, D) in combination with CMX-VP16-VDR, and were treated with $1,25(OH)_2D_3$, AD47, LAC67a or LAC67b at a range of concentrations (A, C), or with 1 μM AD47, LAC67a or LAC67b in the absence or presence of 10 nM $1,25(OH)_2D_3$.

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 μM AD47, LAC67a, or LAC67b in the absence or presence of 10 nM $1,25(OH)_2D_3$ for 24 hours. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig.9. Immunoblotting analysis of VDR, RXRα, SRC-1 and DRIP205. (A) Nuclear expression of VDR, RXRα, 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

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

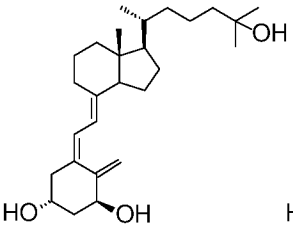
treated with ethanol control (C), 10 nM 1,25$(OH)_2D_3$ (D3), 1 μM AD47 (47), LAC67a (67a), or LAC67b (67b) for 24 hours as in Fig. 8. After preparation of nuclear extracts, 10 μg was subjected to SDS-PAGE for VDR and RXRα, 30 μg for SRC-1 and DRIP205, and 5 μg for Lamin B.

Fig. 10. Effect of vitamin D_3 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$(OH)_2D_3$. *, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$.

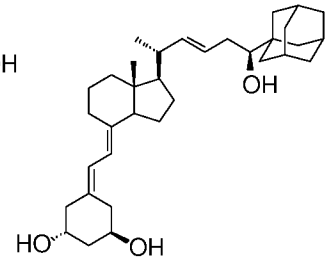
Fig. 11. Docking models of VDR with AD47, LAC67a and LAC67b. (A) Space filling models of 1,25$(OH)_2D_3$ and AD47 in the VDR ligand-binding pocket. His305 (H305), His397 (H397), Phe422 (F422), Val418 (V418) and 1,25$(OH)_2D_3$ are represented in atom–type color and AD47 is in red. AD47 has steric repulsion with F422 of H12 due to its bulky side chain (middle), making the AD47-VDR complex more unstable than the 1,25$(OH)_2D_3$-VDR complex (left). In a docking model of AD47-VDR complex in an

Molecular Pharmacology Fast Forward. Published on February 26, 2007 as DOI: 10.1124/mol.106.032318
This article has not been copyedited and formatted. The final version may differ from this version.

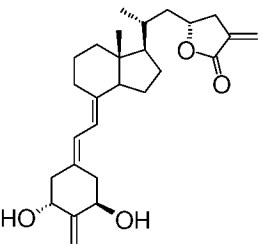
agonist conformation (right), the 25-hydroxyl group of AD47 forms a hydrogen bond with His305 but not with His397, indicating this complex is rather unstable. Since H12 in VDR LBD is in equilibrium between the inactive antagonist-bound conformation and the active agonist-bound conformation, AD47 acts as a partial agonist. (B) Interactions between amino acid residues and the side chains of $1,25(OH)_2D_3$, LAC67a and LAC67b. $1,25(OH)_2D_3$, LAC67a and LAC67b are drawn in atom-type, blue and green color, respectively. 26-Methyl group of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$-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 LAC67a might act as a partial agonist.



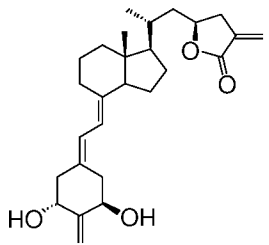
$1,25(OH)_2D_3$



AD47



LAC67a



LAC67b

Fig. 1

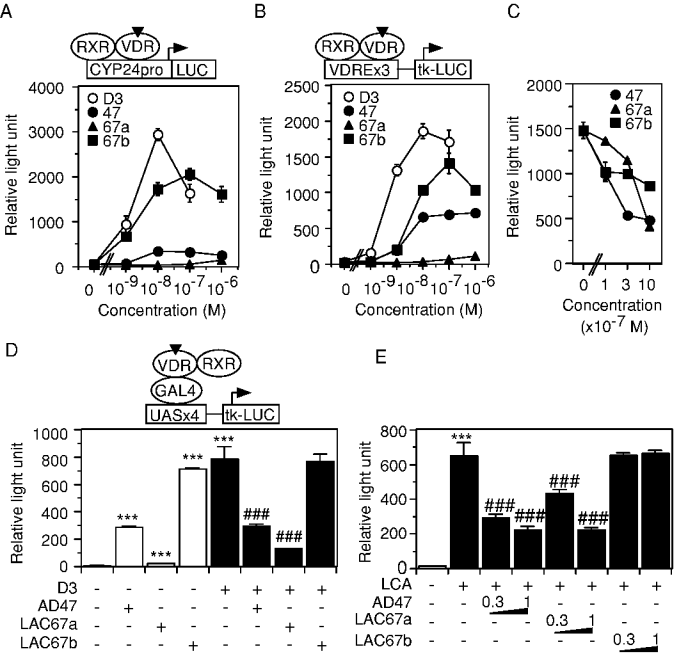

A
RXR
VDR
CYP24pro
LUC
D3
47
67a
67b
Relative light unit
Concentration (M)
B
VDREx3
tk-LUC
C
Concentration
(x10-7 M)
D
GAL4
UASx4
D3
AD47
LAC67a
LAC67b
E
LCA
0.3
1
0.3
1
0.3
1

Fig.2

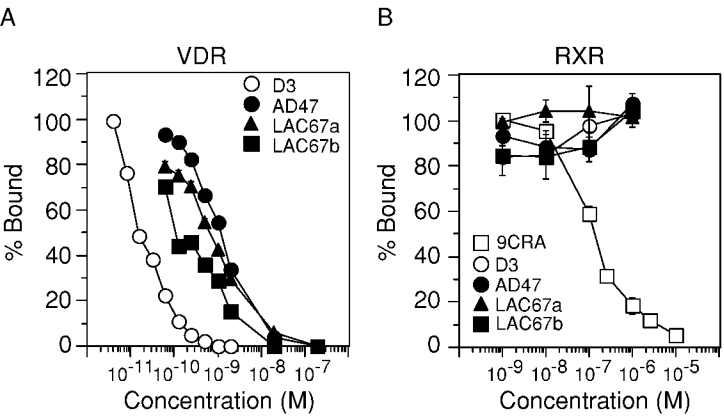
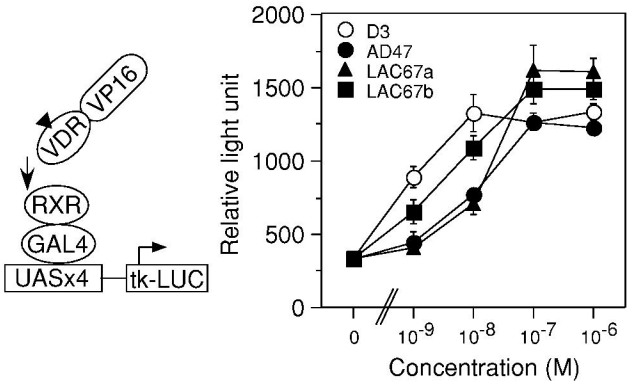

A
VDR
D3
AD47
LAC67a
LAC67b
% Bound
120
100
80
60
40
20
0
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
Concentration (M)
B
RXR
9CRA
D3
AD47
LAC67a
LAC67b
% Bound
120
100
80
60
40
20
0
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}
Concentration (M)

Fig.3

A


VP16
VDR
RXR
GAL4
UASx4
tk-LUC
Relative light unit
2000
1500
1000
500
0
D3
AD47
LAC67a
LAC67b
0
10-9
10-8
10-7
10-6
Concentration (M)

B

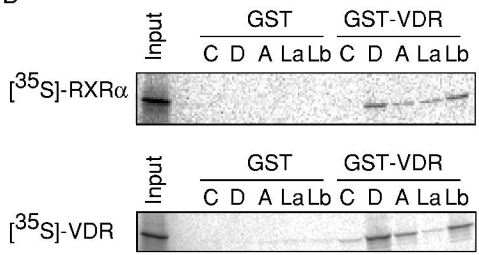

Input
GST
GST-VDR
C D A La Lb C D A La Lb
[35S]-RXRα
Input
GST
GST-VDR
C D A La Lb C D A La Lb
[35S]-VDR

Fig. 4AB

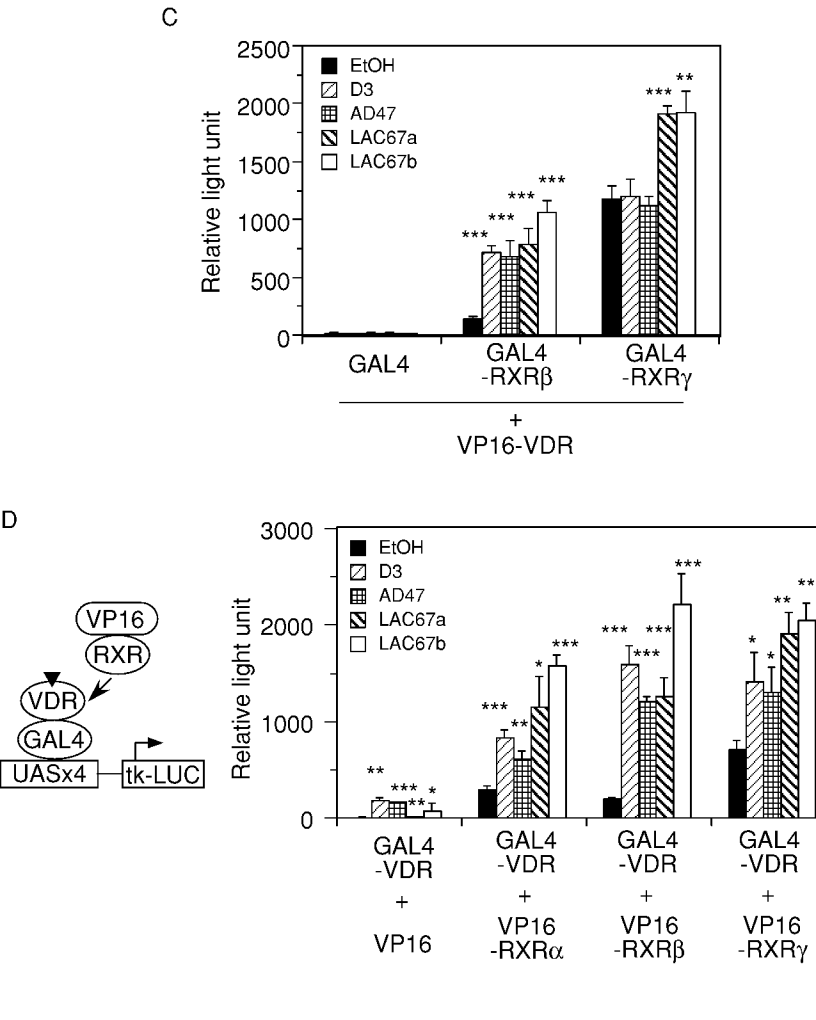

C
Relative light unit
2500
2000
1500
1000
500
0
EtOH
D3
AD47
LAC67a
LAC67b
GAL4
GAL4 -RXRβ
GAL4 -RXRγ
+ VP16-VDR
D
VP16
RXR
VDR
GAL4
UASx4
tk-LUC
Relative light unit
3000
2000
1000
0
EtOH
D3
AD47
LAC67a
LAC67b
GAL4 -VDR + VP16
GAL4 -VDR + VP16 -RXRα
GAL4 -VDR + VP16 -RXRβ
GAL4 -VDR + VP16 -RXRγ

Fig. 4CD

E

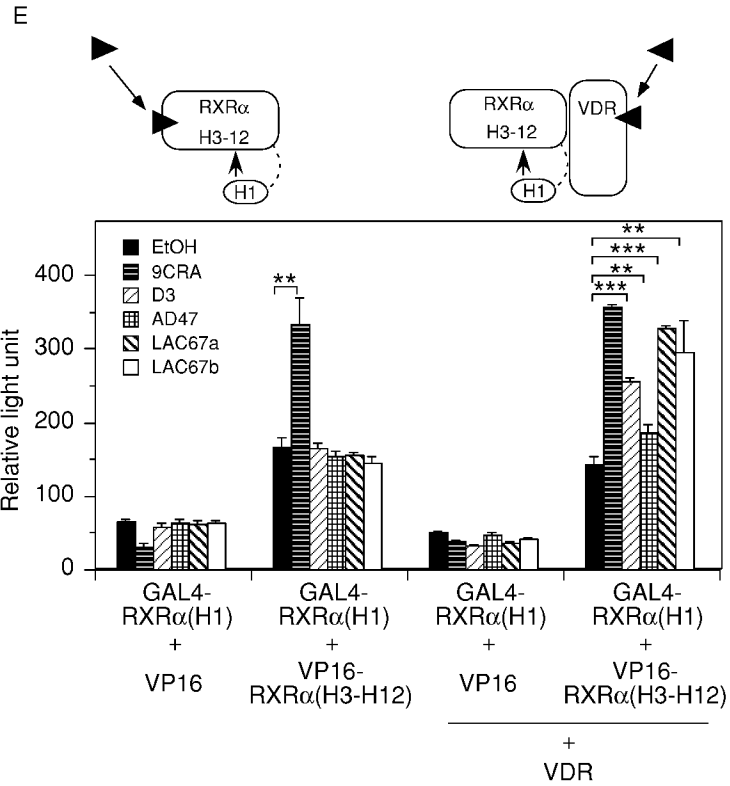

RXRα
H3-12
H1
RXRα
H3-12
VDR
H1
EtOH
9CRA
D3
AD47
LAC67a
LAC67b
Relative light unit
0
100
200
300
400
**
**
***
**
***
GAL4-
RXRα(H1)
+
VP16
GAL4-
RXRα(H1)
+
VP16-
RXRα(H3-H12)
GAL4-
RXRα(H1)
+
VP16
GAL4-
RXRα(H1)
+
VP16-
RXRα(H3-H12)
+
VDR

Fig. 4E

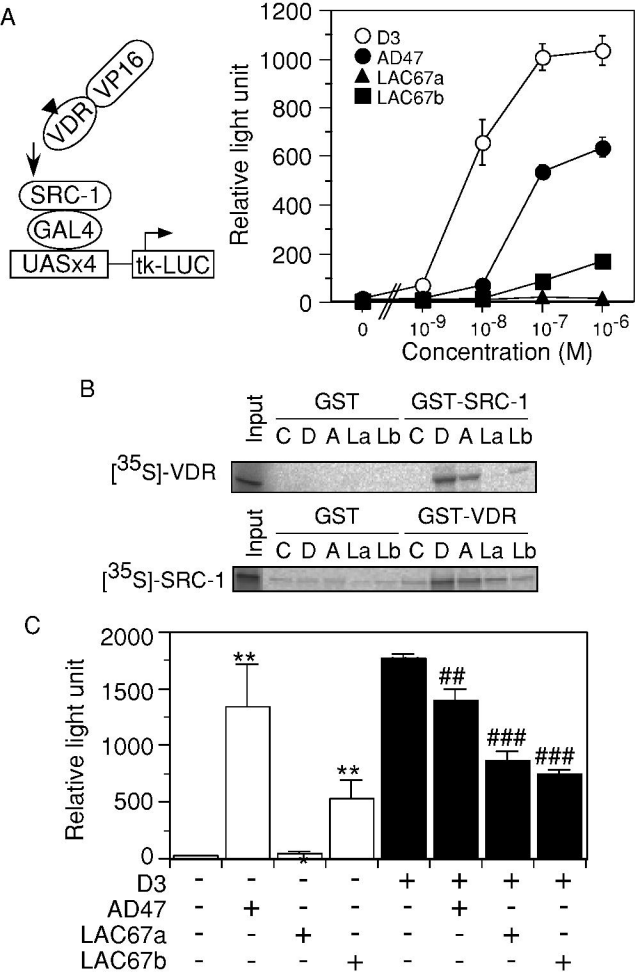
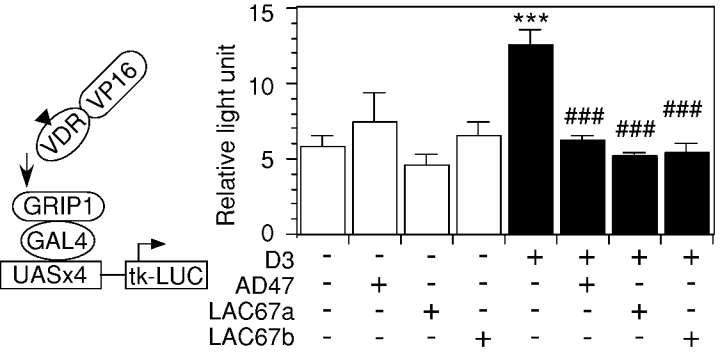

A
VP16
VDR
SRC-1
GAL4
UASx4
tk-LUC
Relative light unit
1200
1000
800
600
400
200
0
D3
AD47
LAC67a
LAC67b
0
10-9
10-8
10-7
10-6
Concentration (M)
B
Input
GST
GST-SRC-1
C D A La Lb C D A La Lb
[35S]-VDR
Input
GST
GST-VDR
C D A La Lb C D A La Lb
[35S]-SRC-1
C
Relative light unit
2000
1500
1000
500
0
**
**
##
###
###
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

Fig. 5ABC

D


VDR
VP16
GRIP1
GAL4
UASx4
tk-LUC
Relative light unit
15
10
5
0
***
###
###
###
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

E

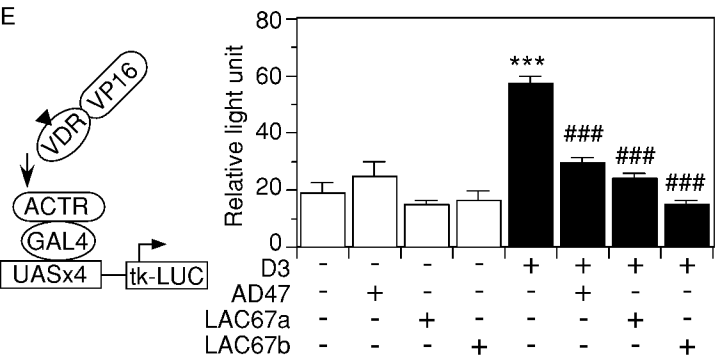
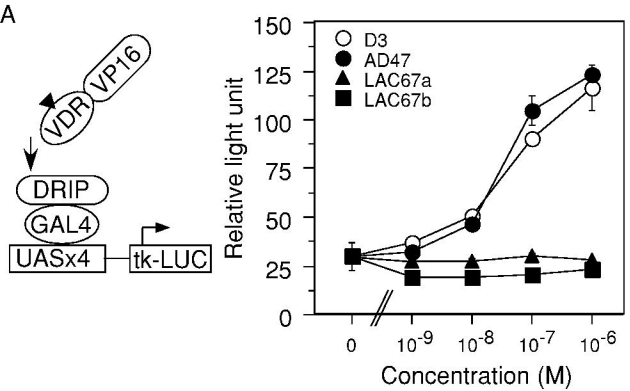
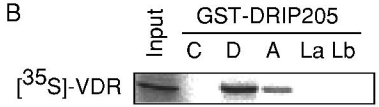

VDR
VP16
ACTR
GAL4
UASx4
tk-LUC
Relative light unit
80
60
40
20
0
***
###
###
###
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

Fig. 5DE


A
VP16
VDR
DRIP
GAL4
UASx4
tk-LUC
Relative light unit
150
125
100
75
50
25
0
D3
AD47
LAC67a
LAC67b
0
10-9
10-8
10-7
10-6
Concentration (M)


B
Input
GST-DRIP205
C D A La Lb
[35S]-VDR

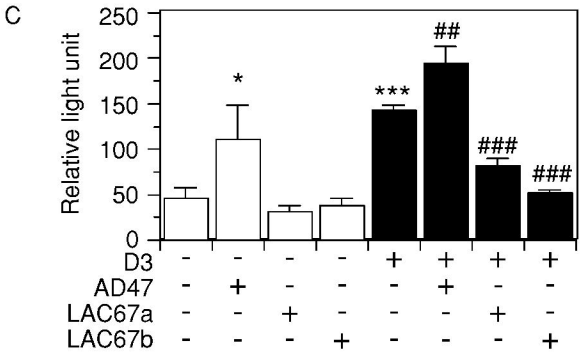
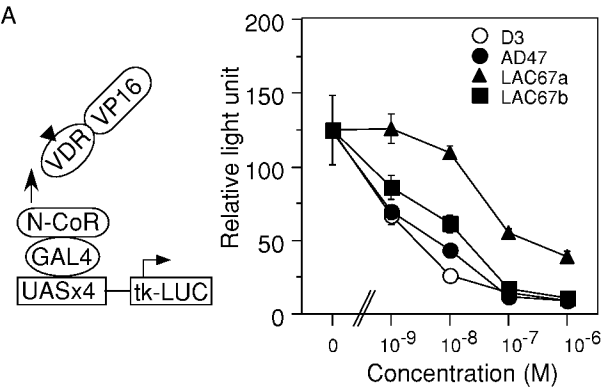

C
Relative light unit
250
200
150
100
50
0
*
***
##
###
###
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

Fig. 6


A
VDR
VP16
N-CoR
GAL4
UASx4
tk-LUC
Relative light unit
200
150
100
50
0
D3
AD47
LAC67a
LAC67b
0
10-9
10-8
10-7
10-6
Concentration (M)

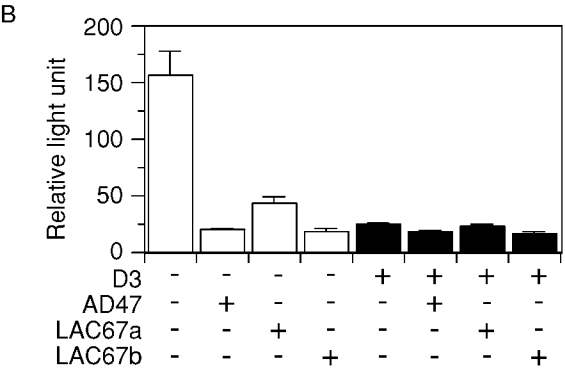
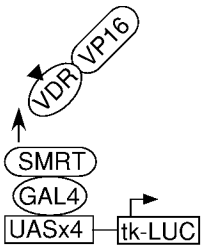
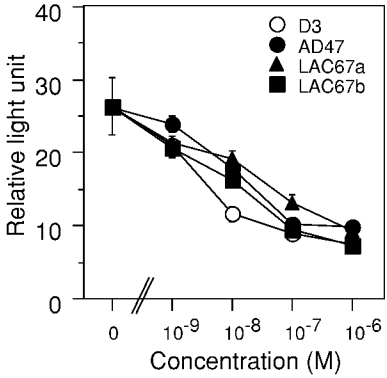

B
Relative light unit
200
150
100
50
0
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

Fig. 7AB

C


VDR
VP16
SMRT
GAL4
UASx4
tk-LUC


D3
AD47
LAC67a
LAC67b
Relative light unit
40
30
20
10
0
0
10-9
10-8
10-7
10-6
Concentration (M)

D

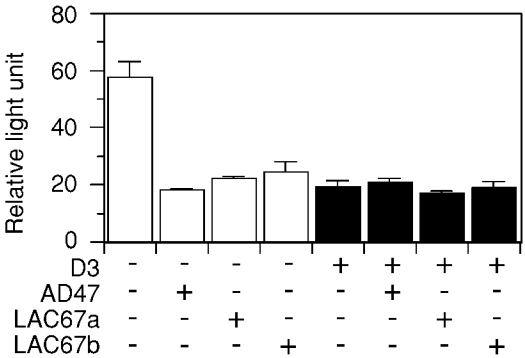

Relative light unit
80
60
40
20
0
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

Fig. 7CD

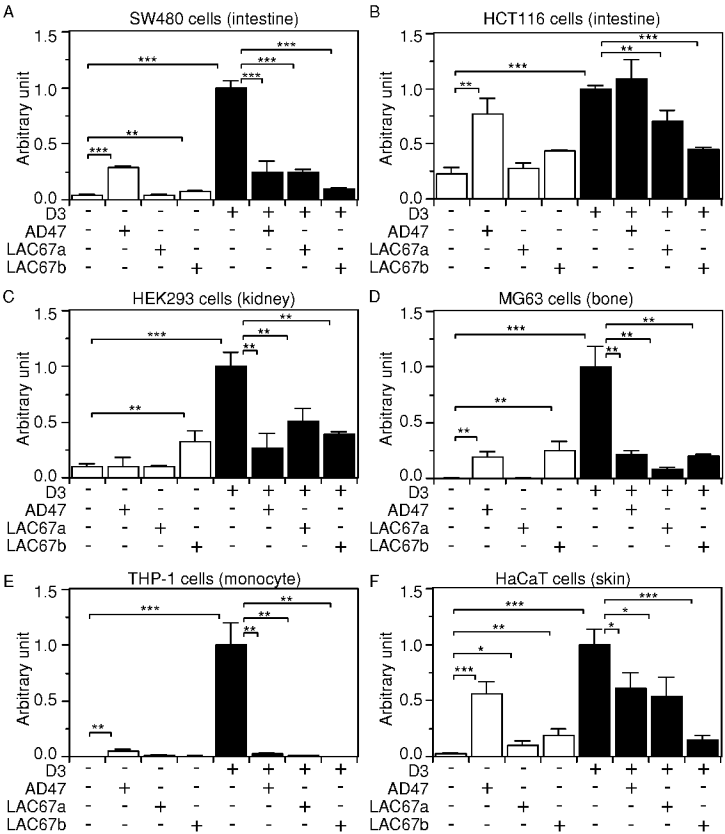

A
SW480 cells (intestine)
B
HCT116 cells (intestine)
C
HEK293 cells (kidney)
D
MG63 cells (bone)
E
THP-1 cells (monocyte)
F
HaCaT cells (skin)
Arbitrary unit
1.5
1.0
0.5
0.0
D3
AD47
LAC67a
LAC67b

Fig.8

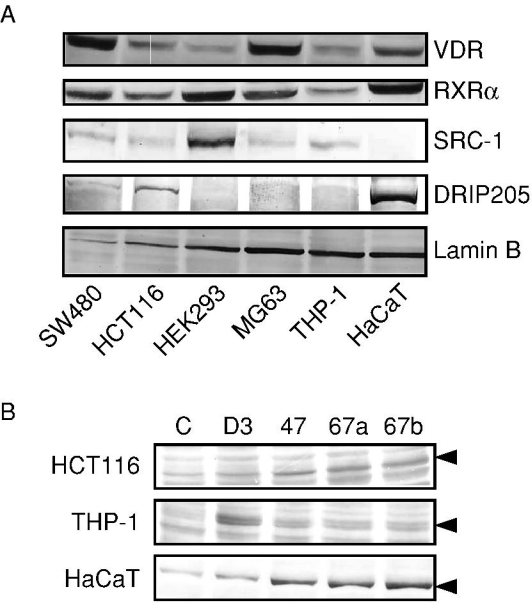

A
VDR
RXRα
SRC-1
DRIP205
Lamin B
SW480
HCT116
HEK293
MG63
THP-1
HaCaT
B
C
D3
47
67a
67b
HCT116
THP-1
HaCaT

Fig.9

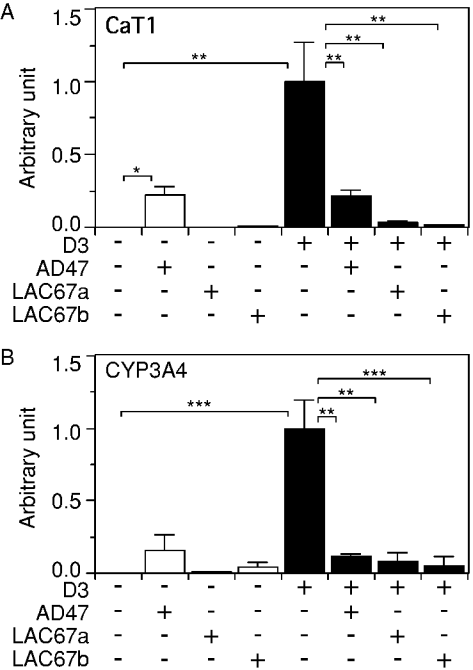

A
CaT1
Arbitrary unit
1.5
1.0
0.5
0.0
*
**
**
**
**
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +
B
CYP3A4
Arbitrary unit
1.5
1.0
0.5
0.0
***
**
**
***
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +
C
E-Cadherin
Arbitrary unit
1.5
1.0
0.5
0.0
**
***
**
**
***
D3 - - - - + + + +
AD47 - + - - - + - -
LAC67a - - + - - - + -
LAC67b - - - + - - - +

Fig.10

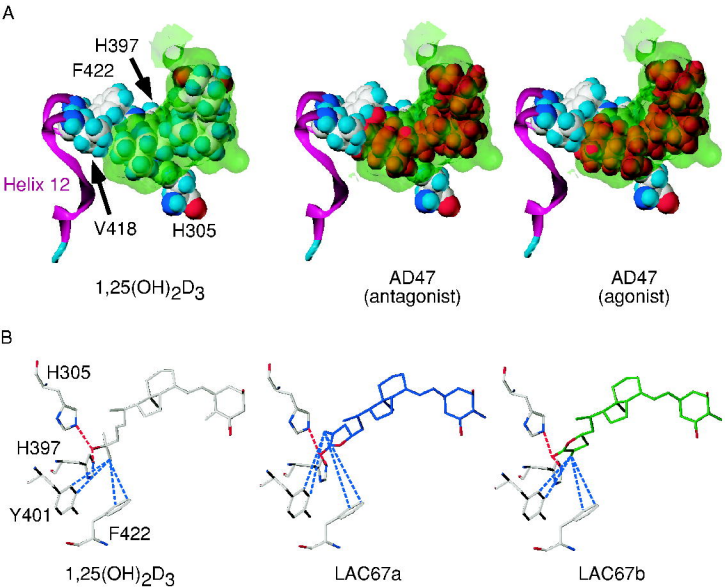

A
H397
F422
Helix 12
V418
H305
1,25(OH)2D3
AD47
(antagonist)
AD47
(agonist)
B
H305
H397
Y401
F422
1,25(OH)2D3
LAC67a
LAC67b

Fig. 11