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Dissociation of Growth Arrest and CYP24 Induction by VDR Ligands in Mammary Tumor Cells

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Abstract Murine mammary tumor cells with differential vitamin D receptor (VDR) expression were used to study the mechanisms of growth inhibition by vitamin D steroids. In VDR-expressing WT145 cells, 1,25D and its synthetic analog EB1089 induce growth arrest and transcriptionally upregulate the well-characterized VDR target gene CYP24. 1,25D also induces apoptosis in WT145 cells through activation of initiator and executioner caspases and the calcium-dependent protease calpain. We also demonstrate that WT145 cells express CYP27B1, the enzyme that converts 25-hydroxyvitamin D_3 (25D) to 1,25D, and that 25D inhibits growth of these cells but does not trigger apoptosis or induce CYP24 expression. Comparative studies were conducted in KO240 cells, which were derived from VDR knockout mice and found to retain expression of CYP27B1. KO240 cells were not growth inhibited nor rendered apoptotic by any of the tested vitamin D compounds. These data conclusively demonstrate that VDR mediates the anti-proliferative and pro-apoptotic effects of vitamin D metabolites and analogs, but that the potency of a vitamin D compound to induce the VDR target gene CYP24 does not accurately predict its potency in mediating growth regulation. J. Cell. Biochem. 101: 1505–1519, 2007.

Key words: vitamin d receptor; breast cancer; 1α,25-dihydroxyvitamin D_3; apoptosis; mammary tumor cells

1α,25-dihydroxyvitamin D_3 (1,25D) is the biologically active form of vitamin D_3, a fat soluble steroid hormone. The best characterized biological action of 1,25D is maintenance of extracellular calcium homeostasis, which is mediated via genomic signaling through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. It is well accepted that the 1,25D–VDR complex acts as a transcription factor through direct contact of its DNA binding domain to vitamin D response elements (VDREs) in the promoter regions of target genes. Most 1,25D-inducible genes contain VDREs composed of a direct repeat of two separate six-base pair elements with a three nucleotide spacer (DR3) [Haussler et al., 1998]. The best example of this type of VDRE has been identified in the proximal promoter of the 24-hydroxylase (CYP24) gene, which contains two well-defined DR3-type VDREs. In other VDR target genes, functional VDREs composed of inverted palindromes with 9-base pair spacers (IP9), everted repeats with 6 or 9-base pair spacers (ER6, ER9), and direct repeats with 4 or 6-base pair spacers (DR4, DR6) have been described [Schrader et al., 1997; Xie and Bikle, 1997; Quack and Carlberg, 2000; Thummel et al., 2001; Thompson et al., 2002; Saramaki et al., 2006]. Mutations in the amino acids within the DNA binding domain that make direct contact with DNA abolish the transcriptional activity of VDR, causing end-organ resistance to 1,25D and hypocalcemic rickets [Malloy and Feldman, 2003]. Thus, the transcriptional activity of the VDR is crucial for mediating the calcemic effects of vitamin D.

In addition to its calcemic effects, 1,25D can induce growth arrest, differentiation, and apoptosis in a variety of normal and transformed cells in vitro, and synthetic vitamin D analogs can mediate tumor regression in vivo

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[Ordonez-Moran et al., 2005; Yee et al., 2005; Gombart et al., 2006]. Furthermore, studies with VDR null mice have demonstrated that VDR status modulates proliferation and carcinogenesis in normal tissues including mammary gland, lymphoid tissue, colon, and skin [Li et al., 1997; Kallay et al., 2001; Colston and Welsh, 2005; Meindl et al., 2005; Zinser et al., 2005]. Multiple pathways and effectors have been implicated in the cell regulatory effects of vitamin D compounds, including inhibition of mitogenic growth factors, upregulation of cell cycle inhibitors such as p21 and p27, and induction of oxidative stress [Lowe et al., 2003]. Effectors linked to 1,25D-mediated apoptosis include bcl-2/bax, cytochrome-c, reactive oxygen species, calcium signaling, and the caspase cascade [Narvaez and Welsh, 2001; Guzey et al., 2002; Mathiasen et al., 2002].

Although VDR is required for 1,25D-mediated growth regulation, the targets of genomic signaling and the possible contribution of non-genomic pathways in mediating growth arrest, differentiation, and apoptosis remain to be clarified. The development of VDR agonists, such as the vitamin D analog EB1089, with enhanced anti-proliferative properties and minimal calcemic effects in vivo, indicates that the cell regulatory and calcemic actions of VDR can be dissociated. At the genomic level, this dissociation could be achieved via distinct response elements in growth regulatory versus calcemic regulatory genes. In support of this concept, induction of IP9-type VDREs correlated better with 1,25D-mediated apoptosis than did induction of DR3-type VDREs [Danielsson et al., 1997], and VDR bound to EB1089 exhibited increased affinity for IP9 VDREs as compared to DR3 VDREs [Nayeri et al., 1995]. Another study identified specific mutations in VDR that impair 1,25D induction of relB, a gene required for dendritic cell maturation, without impairing CYP24 activation [Nguyen et al., 2006]. Alternatively, VDR modulation of growth regulatory genes may be mediated via novel mechanisms, as has been demonstrated for induction of the cyclin-dependent kinase inhibitor p27 by 1,25D. On this gene promoter, ligand-occupied VDR does not contact DNA directly, but interacts with the Sp1 transcription factor to enhance its binding at GC rich regions [Huang et al., 2004; Cheng et al., 2006]. Thus, VDR may target gene promoters involved in growth regulation through mechanisms distinct from those involved in calcemic regulation. It is also likely that non-transcriptional regulation of signal transduction pathways at the membrane or in the cytosol by 1,25D and/or the VDR contribute to growth regulation [Capiati et al., 2004; Huhtakangas et al., 2004; Zanello and Norman, 2004; Rochel and Moras, 2006].

To study the association between VDR genomic signaling and the anti-cancer effects of 1,25D, murine mammary tumor cell lines that differentially express the VDR were developed from VDR knockout mice and their wild-type littermates [Zinser et al., 2003]. In the current studies, these cell lines were adapted to steroid-free culture conditions to further examine the role of the VDR in mediating the effects of 1,25D on growth and apoptosis. We show that the anti-proliferative and pro-apoptotic effects of 1,25D and other vitamin D compounds require VDR protein expression, and that the induction of apoptosis by 1,25D in mammary tumor cells is through VDR-dependent activation of mitochondrial and endoplasmic reticular caspase pathways. We also present proof of principle that some vitamin D compounds exert growth inhibitory actions in the absence of VDR-mediated transcriptional activation of CYP24.

MATERIALS AND METHODS

Cell Culture

WT145 and KO240 cells [Zinser et al., 2003] were maintained in DMEM/F12 medium containing 5% charcoal-stripped fetal bovine serum (CSS). Cells were routinely passaged twice weekly with trypsin/EDTA.

Western Blotting

Cells were seeded in 150-mm dishes (Corning, Corning, NY) at a density of 500,000 cells per dish, and treated 24 h later with indicated doses of test compounds (gift of Leo Pharmaceuticals, Ballerup, Denmark) or vehicle control. For whole cell lysates, cells were harvested 48 h post-treatment by scraping into 2× Laemmli buffer containing protease and phosphatase inhibitors. Subcellular fractions were prepared as previously described [Narvaez and Welsh, 2001]. Lysates and subcellular fractions were separated via SDS–PAGE, transferred to nitrocellulose filters and incubated with primary antibodies including rat monoclonal VDR Clone

9A7 (NeoMarkers, Fremont, CA), mouse monoclonal VDR Clone D-6 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse specific Apoptosis Antibody Sampler Kit (Cell Signaling Technology, Danvers, MA), rabbit polyclonal caspase 7 (Cell Signaling Technology), rabbit polyclonal mu-calpain (Affinity Bioreagents, Golden, CO), mouse monoclonal CYP24 (Cytochroma, Markham, ON, Canada), and sheep polyclonal CYP27B1 (The Binding Site, San Diego, CA). Appropriate secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ), and specific bands were detected via chemiluminescence (Pierce, Rockford, IL). Blots were performed in triplicate with separate cell lysates and representative images are shown.

Immunofluorescence

Cells were seeded in 2-well chamber slides (Nalge Nunc, Naperville, IL) at a density of 10,000 cells per chamber, and treated 24 h later with 100 nM 1,25D or vehicle control. Twenty-four hours post-treatment, cells were fixed, permeabilized with ice cold methanol, and incubated with VDR antibody clone D-6 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA) secondary antibody. DNA was counterstained using Hoechst 33342 (Invitrogen). Coverslips were mounted with anti-fade mounting medium, and slides viewed on an Olympus Provis AX70 microscope with a Spot RT Slider digital camera.

VDR Transactivation Assay

Cells were seeded in 12-well plates (Corning) at a density of 30,000 cells per well. After 24 h, cells were transfected with the pGL3-24 hydroxylase luciferase reporter vector which contains approximately 300 bp of the human CYP24 gene promoter with its two DR3 VDRE regions (gift of the late Dr. Omdahl). A pRL-TK-driven luciferase plasmid (Promega, Madison, WI) was co-transfected to normalize for transfection efficiency. TransFast transfection reagent (Promega) was used. Twenty-four hours post-transfection, cells were treated with the indicated doses of test compounds. After 24 h of treatment, cells were harvested with 1× Passive Lysis Buffer and fluorescence was read via the Dual Luciferase system (Promega). Assays were performed in triplicate with three different vector preparations. Representative data are shown.

Cell Growth

Cells were seeded in 24-well plates (ICN Biomedicals, Aurora, OH) at a density of 2,000 cells per well and treated 24 h later with the indicated doses of test compounds. Ninety-six hours post-treatment, cells were fixed using 2% glutaraldehyde in PBS and stained with crystal violet dye. Dye was resuspended in 0.1% Triton-X100 and absorbance was measured at 590 nm. For doubling time, cells were fixed and stained for 5 consecutive days, and doubling time calculated according to measured absorbance. Assays were performed at least three times; representative results are shown.

Caspase Activity

Cells were seeded in 150-mm dishes (Corning) at a density of 300,000 cells per dish, and treated 24 h later with the indicated doses of test compounds or vehicle controls. Forty-eight hours post-treatment, cells were harvested via trypsinization. One million cells per treatment were assayed using a commercially available caspase 3 activity assay (BD Biosciences, San Jose, CA), according to manufacturer's recommendations. Assays were performed at least three times with three separate cell preparations. Representative results are shown.

Quantitative RT-PCR

Cells were seeded in 100-mm dishes (BD Biosciences) at a density of 500,000 cells per dish, and treated 24 h later with indicated doses of test compounds. Cells were pelleted via centrifugation and RNA was harvested using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. Three cDNA replicates were made for each RNA, using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). Gene expression analysis was performed using SYBR green (ABGene, Rochester, NY), and values were normalized against 18S RNA. Primer sequences are as follows: *Mus CYP24 Forward*—AAGTCATGGACTTGGCCTTCA; *Mus CYP24 Reverse*—GCTCCGCCTTCTCGTTGA; *Mus CYP27B1 Forward*—CAGAGCGCTGTAGTTTCTCATCA; *Mus CYP27B1 Reverse*—CGTTAGCAATCCGCAAGCA; *18S rRNA Forward*—AGTCCCTGCCCTTTGTACACA; *18S rRNA Reverse*—GTTCCGAGGGCCTCACTAAAC. Plates were run in triplicate on the ABI-Prism 7700 (Applied Biosystems), and mean values of representative results are shown.

Statistical Analysis

Data are expressed as mean ± standard error. ANOVA was performed using GraphPad software, and means were considered statistically significant when *P*-values less than 0.05 were obtained with the Tukey post-test. Statistical significance is indicated on all data figures as letters above bars; bars are labeled with different letters for means that are significantly different.

RESULTS

VDR Protein Expression and Transcriptional Activity in WT and VDRKO Cells

The cells used in these studies were isolated from mammary tumors that developed in WT and VDRKO mice [Zinser et al., 2003] and were adapted to steroid-free medium containing stripped serum. Western blotting was used to confirm the differential expression of VDR in these cells under these conditions. As shown in Figure 1a, the 50-kDa VDR protein was expressed in WT145 cells but was undetectable in KO240 cells. The level of VDR protein in WT145 cells was increased following treatment with 1,25D, but was not induced in KO240 cells. Subcellular fractionation of WT145 cells indicated that the VDR protein was detected in both cytosolic and nuclear fractions under control conditions, and was markedly increased in both compartments following treatment with 1,25D (Fig. 1b). By immunofluorescence microscopy (Fig. 1c), VDR expression was low in WT145 cells in the absence of 1,25D, and accumulated in the nucleus upon hormone treatment. In KO240 cells, negligible VDR staining was detected even in the presence of 1,25D, a finding that was confirmed with several distinct antibodies to VDR (data not shown). Thus, these VDRKO cells, derived from the colony generated by Demay's group [Li et al., 1997], do not express a truncated VDR protein that retains ligand binding ability as reported for another strain of VDRKO mice [Bula et al., 2005].

To assess VDR transcriptional activity, transient transfection assays were conducted with a VDR-responsive luciferase construct that contains the promoter region of the human CYP24 gene. In the WT145 cell line, CYP24 promoter activity was low under basal conditions, and was significantly increased after 1,25D treatment. In the KO240 cell line, basal CYP24 promoter activity level was low and was not induced by 1,25D treatment (Fig. 2a). Dose-response experiments (Fig. 2b) indicated that CYP24 promoter activity was significantly increased in WT145 cells following treatment with concentrations of 1,25D as low as 1 nM. Real-time PCR analysis (Fig. 2c) indicated that 1,25D also induced the endogenous CYP24 gene and protein (Fig. 2d) in WT145 cells, but not KO240 cells. These data confirm that VDR remains functional in WT145 cells selected for growth in stripped serum devoid of 1,25D, and that murine mammary cells to not express any proteins other than VDR that can mediate transcription of the CYP24 gene in response to 1,25D.

Differential Growth Inhibition by 1,25D in WT and VDRKO Cells

Growth assays were conducted to determine whether the VDR present in WT145 cells could mediate growth inhibitory effects of 1,25D. Similar to data obtained in VDR-positive human breast cancer cells [Simboli-Campbell et al., 1997], 96-h treatment with 1,25D at concentrations as low as 1 nM inhibited growth of WT145 cultures (Fig. 3A). Under the same conditions, 1,25D had no effect on growth of KO240 cells. Thus, although selection for growth in CSS increased the doubling time of both cell lines (Fig. 3b) and abolished expression of the estrogen receptor (data not shown), WT145 cells remained sensitive, and KO240 cells remained insensitive, to 1,25D-mediated growth inhibition.

1,25D Activates Apoptosis-Related Proteases in Tumor Cells That Express VDR

To assess whether apoptosis is regulated by vitamin D signaling in this model system, we first examined whether expression and/or activation of caspase 3 was altered by VDR ablation or 1,25D treatment. Western blotting was used to monitor expression of the inactive caspase 3 zymogen as well as the cleaved, catalytically active mature form (Fig. 4a). Although VDR ablation per se did not alter basal caspase 3 expression, treatment with 100 nM 1,25D-induced caspase 3 cleavage in WT145 cells but not in KO240 cells. Measurement of caspase 3 activity (Fig. 4b) indicated that the low basal level of caspase 3 activity was significantly increased in WT145 cells treated with 100 nM 1,25D. In KO240 cells, basal caspase 3 activity

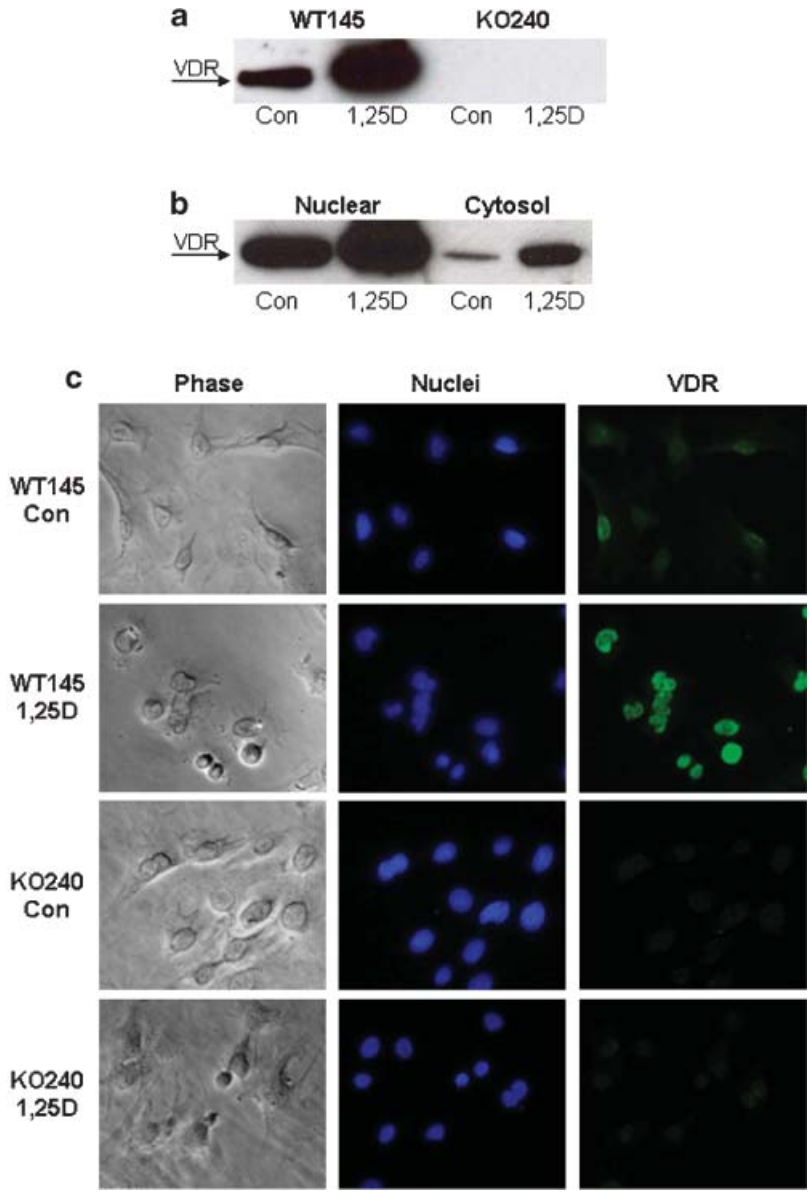


Fig. 1. VDR is present only in WT145 cells, and concentrates in the nucleus following treatment with 1,25D. **a**: Western blot for VDR in WT145 and KO240 whole cell lysates treated for 48 h with 100 nM 1,25D or vehicle control. **b**: Western blot for VDR in subcellular fractions from WT145 cells treated for 48 h with 100 nM 1,25D or vehicle control. **c**: VDR immunofluorescence in WT145 and KO240 cells treated for 24 h with 100 nM 1,25D or vehicle control.

was lower than that of WT145 cells and was unaffected by 1,25D.

To further characterize cell death induction by 1,25D, we examined caspases 9 and 12, which act as initiator caspases in the mitochondrial and endoplasmic reticular pathways, respectively. Activation of both caspase 9 and 12 (monitored as disappearance of the pro-caspase forms due to lack of antibodies that recognize the cleaved fragments) was observed in WT145 cells treated with 100 nM 1,25D, but not in KO240 cells. We next assessed the downstream effector caspases that propagate apoptotic signals, and found that procaspases 7 and 10, but not procaspase 6, were activated by 1,25D in WT145 cells but not KO240 cells. Consistent with evidence of caspase activation in WT145 cells, cleavage of its substrate poly (ADP-ribose) polymerase (PARP) was enhanced in WT145 cells but not KO240 cells following 1,25D

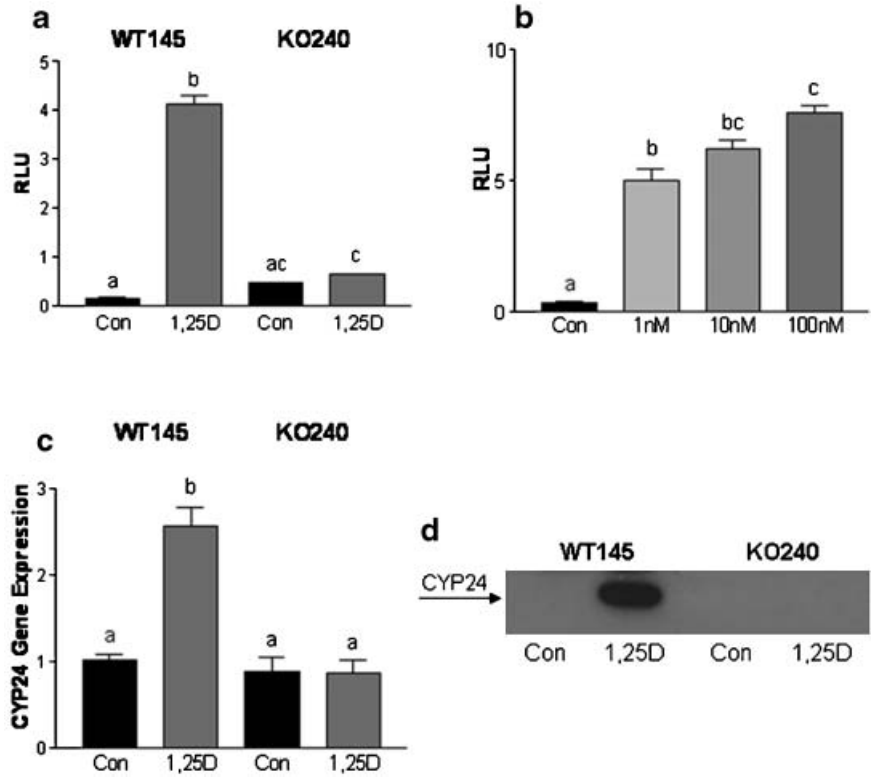
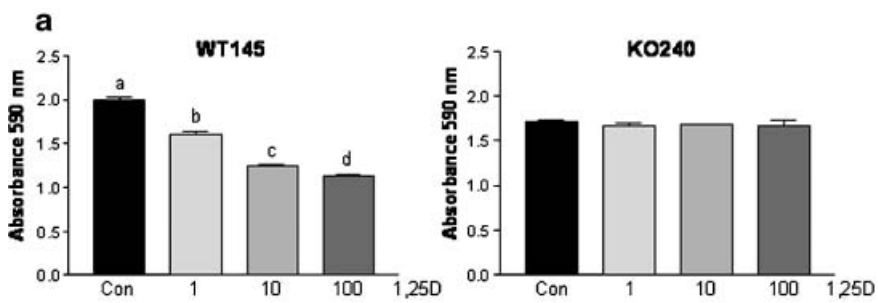


Fig. 2. WT145 cells express transcriptionally active VDR. **a**: CYP24 reporter gene activity in WT145 and KO240 cells treated for 24 h with 100 nM 1,25D or vehicle control. Data presented as relative luciferase units (RLU). **b**: CYP24 promoter induction in WT145 cells treated for 24 h with indicated doses of 1,25D. **c**: Real time PCR for endogenous CYP24 mRNA in WT145 and KO240 cells after 6 h of treatment with 100 nM 1,25D or vehicle control. **d**: Western blot of CYP24 in WT145 and KO240 cells after 48 h of treatment with 100 nM 1,25D or vehicle control.



b

	WT145		KO240	
	FBS	CSS	FBS	CSS
Con	15.8	40.0	15.8	30.5
1,25D	45.9	132.0	16.1	34.0
Fold Change	(-2.9)	(-3.3)	1.0	1.1

Fig. 3. WT145 cells, but not KO240 cells, are growth inhibited by 1,25D. **a**: Crystal violet growth assay in WT145 and KO240 cells treated for 96 h with 1,25D at indicated concentrations (nM) or vehicle control. **b**: Doubling time, in hours, of WT145 and KO240 cells under different media conditions, following 96 h of treatment with 100 nM 1,25D or vehicle control.

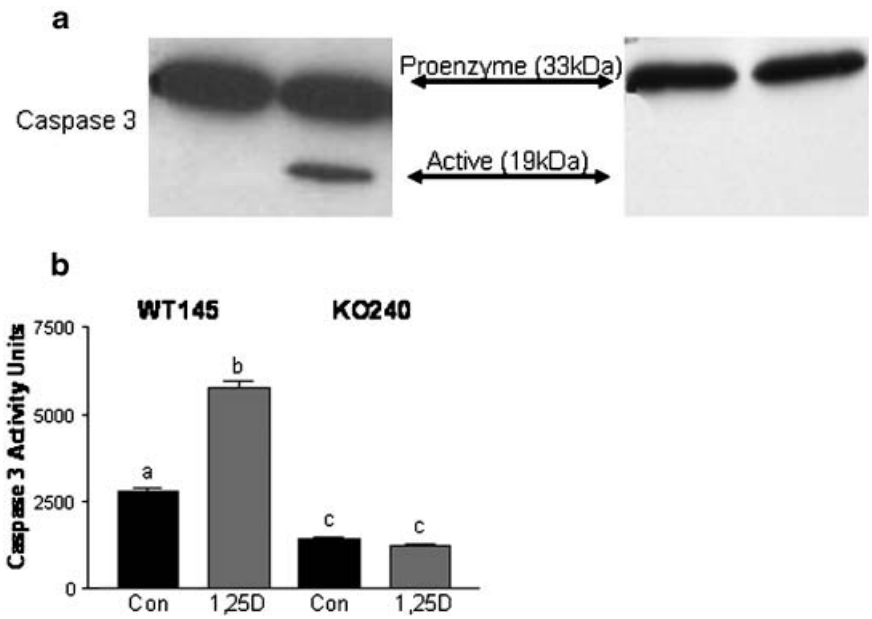


Fig. 4. 1,25D induces apoptosis in WT145 cells, but not KO240 cells. **a**: Western blot for caspase 3 in WT145 and KO240 cells after 48 h of treatment with 100 nM 1,25D or vehicle control. **b**: Caspase 3 activity in WT145 and KO240 cells following 48 h of treatment with 100 nM 1,25D or vehicle control.

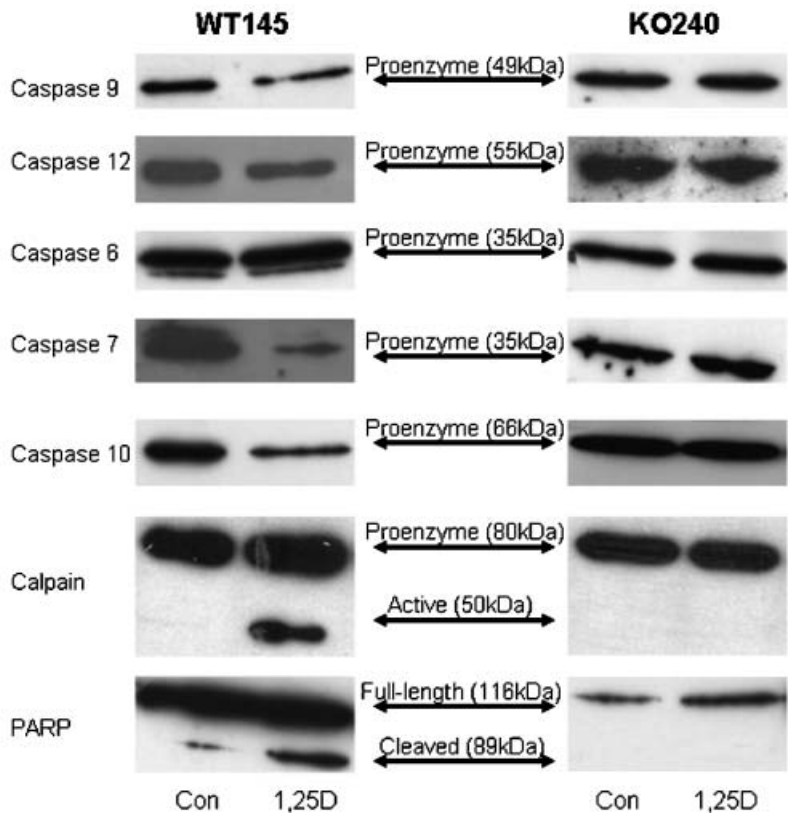


Fig. 5. Protease activation in WT145 cells following 1,25D treatment. Western blots for various caspases, calpain, and poly(ADP-ribose)polymerase (PARP) in WT145 and KO240 cells after 48 h of treatment with 100 nM 1,25D or vehicle control.

treatment. The calcium-dependent protease calpain was also cleaved to its active form in 1,25D-treated WT145 cells but not in KO240 cells.

Of note, VDR ablation did not alter cellular sensitivity to apoptosis in general, as both WT145 and KO240 cells were comparably growth inhibited by increasing concentrations of the DNA-damaging agent etoposide (Fig. 6a). Although caspase 3 activation occurred at lower etoposide doses in WT145 cells than KO240 cells, both cell lines exhibited comparable responses to concentrations of 1 μM and higher (Fig. 6b,c). Thus, cells lacking VDR retain a functional DNA damage response pathway leading to activation of caspase 3 and cell death. Collectively, these data indicate that 1,25D induces apoptosis in murine mammary cells through VDR dependent pathways that disrupt intracellular organelles and trigger activation of multiple proteases.

EB1089 Mediates Growth Arrest Through VDR-Dependent Pathways

We next examined whether growth of these murine mammary cells was affected by EB1089, a synthetic vitamin D analog that is known to exert less calcemic activity than 1,25D in vivo. As shown in Figure 7, EB1089 at concentration of 1 nM inhibited growth more potently than 1,25D (Fig. 3). In contrast, the potency of EB1089 to upregulate transcription of the human CYP24 promoter was significantly less than that of 1,25D (Fig. 7b). Similarly, EB1089 induced the endogenous murine CYP24 gene in WT145 cells, but the time course of induction was slower than that of 1,25D (Fig. 7c). As expected, EB1089 did not inhibit growth, activate the CYP24 promoter, or induce the endogenous CYP24 gene in KO240 cells.

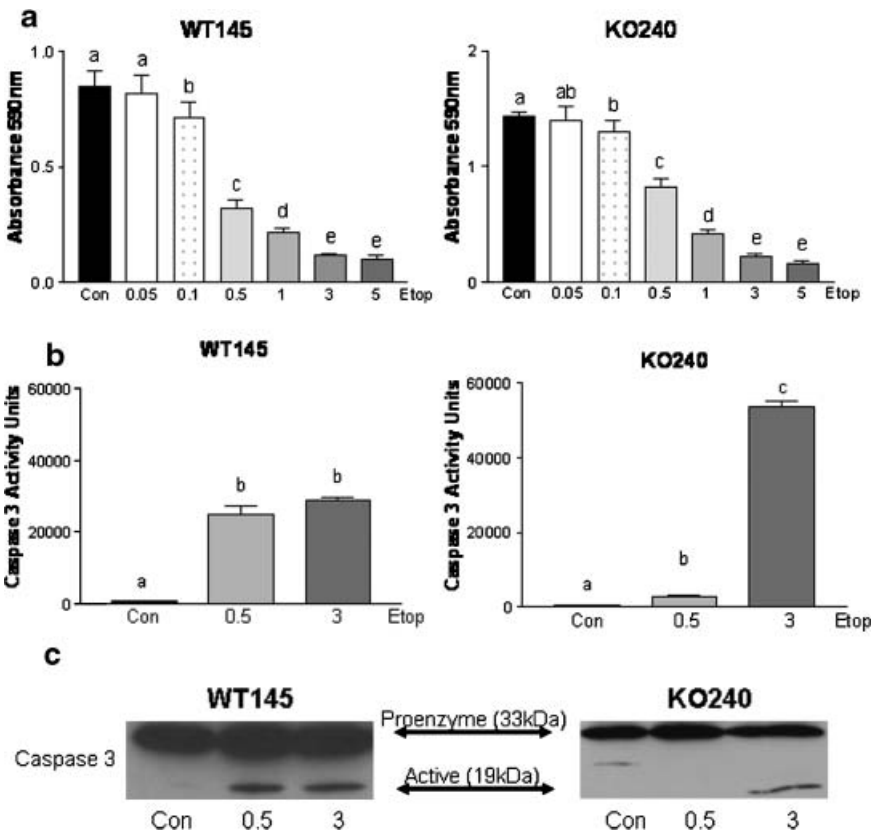


Fig. 6. WT145 and KO240 cells are equally sensitive to etoposide-mediated apoptosis. **a**: Crystal violet growth assay in WT145 and KO240 cells following 96 hours of treatment with indicated concentrations of etoposide (μM) or vehicle control. **b**: Caspase 3 activity in WT145 and KO240 cells following 48 h of treatment with indicated concentrations of etoposide (μM) or vehicle control. **c**: Western blot for caspase 3 in WT145 and KO240 cells after 48 h of treatment with indicated concentrations of etoposide (μM) or vehicle control.

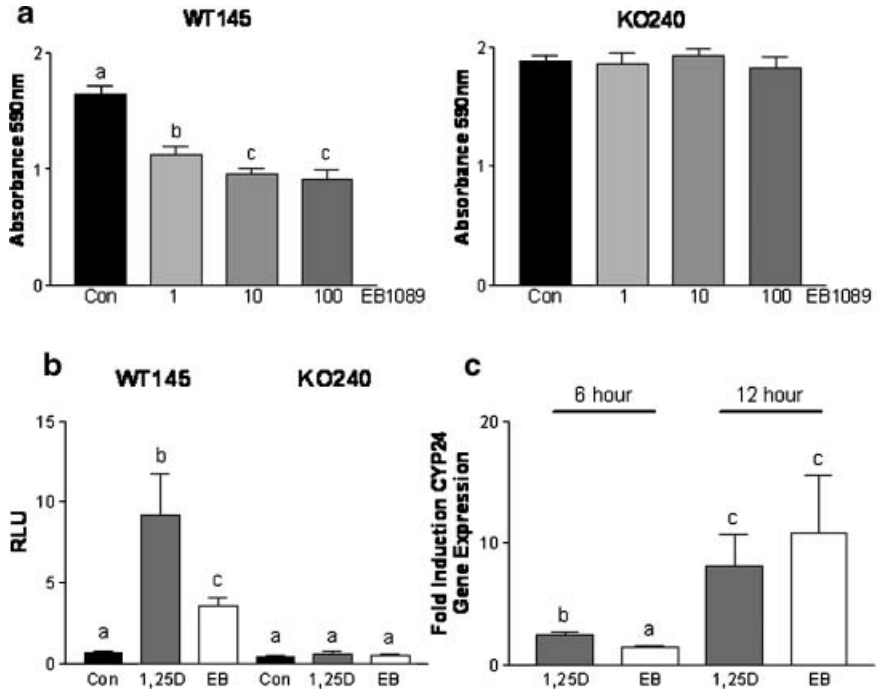


Fig. 7. EB1089 induces growth arrest and CYP24 in WT145 cells. **a**: Crystal violet growth assay in WT145 and KO240 cells following treatment with indicated concentrations of EB1089 (nM) or vehicle control. **b**: CYP24 reporter gene assay in WT145 and KO240 cells, after 24 h treatment with 100 nM 1,25D, 100 nM EB1089, or vehicle control. **c**: RT-PCR for endogenous CYP24 mRNA levels in WT145 and KO240 cells treated for 6 or 12 h with 100 nM 1,25D, EB1089, or vehicle control. Data are expressed as mean fold increase over control values.

WT145 Cells Express CYP27B1 and Respond to 25-Hydroxyvitamin D_3

CYP27B1, the cytochrome P450 enzyme that generates 1,25D from 25-hydroxyvitamin D_3 (25D), has been localized to human mammary cells as well as mouse mammary gland [Zinser and Welsh, 2004; Townsend et al., 2005]. Western blotting indicated that the WT145 and KO240 cells, which were derived from carcinogen-induced mouse mammary tumors, retain expression of CYP27B1 protein (Fig. 8a) and RNA (real time PCR data not shown). In vitro, CYP27B1 expression was not consistently altered by exposure to 25D or 1,25D in either cell line, after correction for protein loading determined by blotting for actin. To test whether the presence of CYP27B1 sensitized cells to growth inhibition by 25D, as reported in human mammary cells [Friedrich et al., 2000; Kemmis et al., 2006], growth was assessed in WT145 and KO240 cells treated with increasing doses of 25D for 96 h. As shown in Figure 8b, 25D inhibited growth of WT145 cells at the physiologically relevant concentration of 100 nM, but had no effect on growth of KO240 cells. In contrast to 1,25D, which increased caspase 3 activity in WT145 cells, 25D did not activate caspase 3 (Fig. 8c), suggesting that 25D inhibited growth without induction of apoptosis.

Reporter gene assays were conducted to determine whether the concentration of 25D that inhibited growth of WT145 cells was sufficient to activate VDR transcription. In contrast to 1,25D, 25D did not activate the heterologous CYP24 promoter (Fig. 9a), and did not induce the endogenous murine CYP24 protein (Fig. 9b), or mRNA (Fig. 9c) in WT145 cells. These experiments were repeated in media, which was not charcoal stripped, and no activation of either the endogenous or heterologous CYP24 promoters was observed (data not shown). A detailed quantitative real time PCR time course showed no elevation of CYP24 mRNA in WT145 cells treated with 25D for up to 96 h (Fig. 9d). Collectively, these data suggest that the presence of both CYP27B1 and VDR are permissive for growth inhibition by 25D, but that activation of the VDR target gene CYP24 is not required.

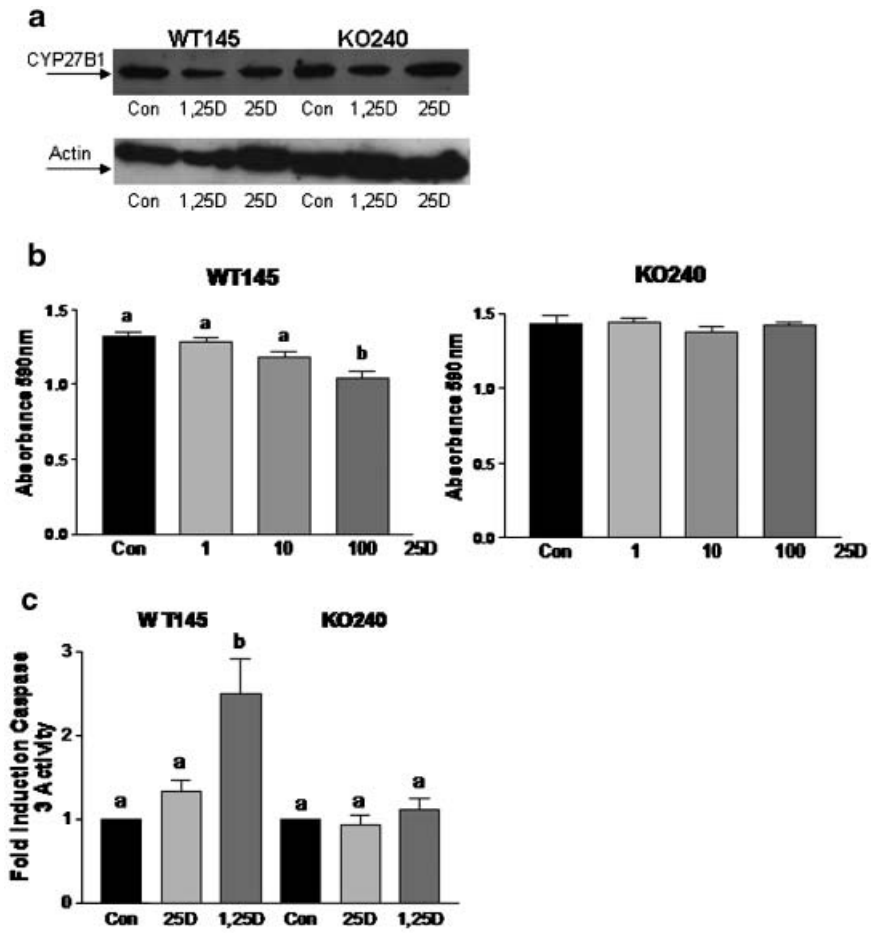


Fig. 8. 25D induces growth arrest, but not apoptosis, in WT145 cells. **a**: Western blot for CYP27B1 in WT145 and KO240 cells following treatment with 100 nM 25D, 100 nM 1,25D, or vehicle control. Western blot for actin is shown as a loading control. **b**: Crystal violet growth assay in WT145 and KO240 cells following treatment with indicated concentrations of 25D (nM) or vehicle control. **c**: Caspase 3 activity in WT145 and KO240 cells following 48 h of treatment with 100 nM 25D or 1,25D. Values are presented relative to control, which was set to one.

DISCUSSION

In these studies, we have utilized mammary tumor cell lines from mice with targeted deletion of VDR and their wild type littermates to examine cellular mechanisms involved in vitamin D-mediated growth inhibition. We specifically compared the efficacy of selected vitamin D compounds to inhibit growth and to activate the CYP24 gene in these cells. The CYP24 promoter contains several DR3-type VDREs [Vaisanen et al., 2005], is highly induced by 1,25D, and is commonly used as an indicator of VDR transcriptional activity. However, the suitability of CYP24 as a measure of sensitivity to other cellular processes mediated by vitamin D compounds has not been established. In these studies, we demonstrate that although VDR is absolutely required for growth arrest and apoptosis, the potency of a vitamin D metabolite or analog to induce the VDR target gene CYP24 does not predict its potency in mediating growth regulation.

It is worth noting that the exogenous CYP24 reporter used in these studies contains the human CYP24 promoter (rather than the murine sequence, which to our knowledge, has yet to be published). However, both murine and human VDR comparably activate the human CYP24 promoter in response to 1,25D and EB1089 in these cells (data not shown). Furthermore, there was complete concordance between regulation (or lack of) the endogenous murine CYP24 gene and the exogenous human

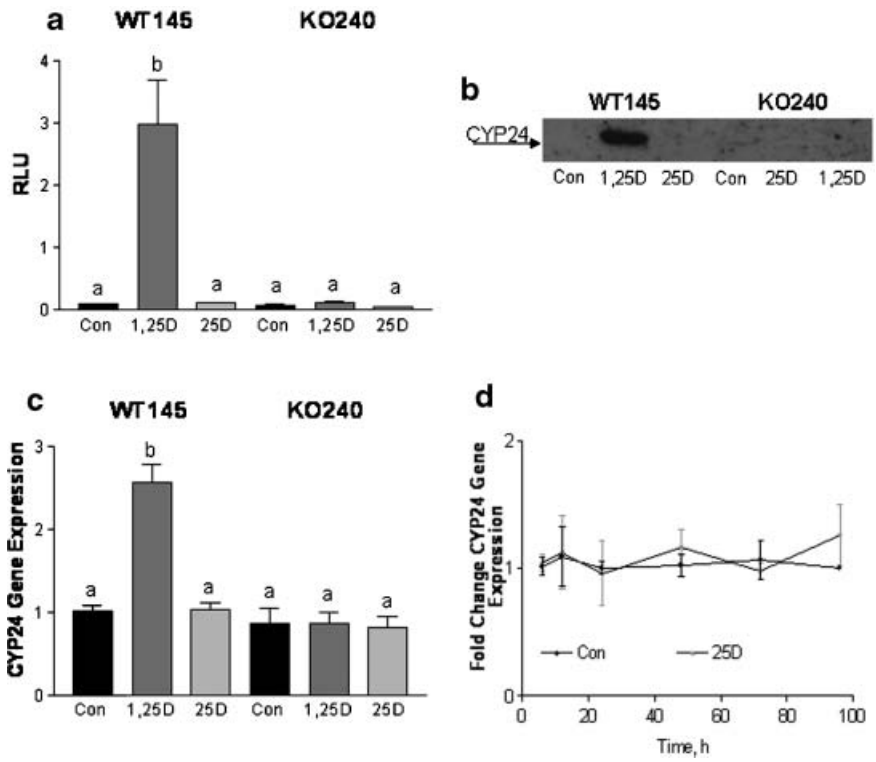


Fig. 9. 25D does not induce CYP24 in WT145 cells. **a**: CYP24 reporter gene assay in WT145 and KO240 cells, treated for 24 h with 100 nM 1,25D, 100 nM 25D, or vehicle control. **b**: Western blot for CYP24 in WT145 and KO240 cells following 48 h treatment with 100 nM 25D, 100 nM 1,25D, or vehicle control. **c**: Real time PCR of CYP24 mRNA in WT145 and KO240 cells treated for 6 h with 100 nM 1,25D, 25D, or vehicle control. **d**: Real time PCR time course of CYP24 mRNA in WT145 and KO240 cells treated with 100 nM 25D or vehicle control for up to 96 h.

CYP24 promoter in all of our experiments. Therefore, the human CYP24 promoter construct appears to be an appropriate research tool for use in this model system.

The biggest discrepancy between growth inhibition and CYP24 induction was observed when cells were treated with physiological concentrations of the natural vitamin D metabolite, 25D. We and others have demonstrated that human mammary cells express CYP27B1 and are growth inhibited by 25D [Townsend et al., 2005; Kemmis et al., 2006] and in the present study, we detected CYP27B1 in both WT145 and KO240 murine mammary cell lines. The presence of CYP27B1 conferred sensitivity to 25D-mediated growth arrest in WT145, but not KO240 cells, indicating that growth inhibition by 25D was VDR-dependent. We therefore predicted that 25D-mediated growth arrest in WT145 cells would be associated with CYP24 induction. Surprisingly, three different assays (human CYP24 luciferase reporter, real time PCR for the endogenous murine gene, and Western blot for CYP24 protein) failed to detect induction of CYP24 in WT cells treated with 100 nM 25D. Assays conducted in parallel demonstrated that 1,25D activated the CYP24 reporter gene and increased both CYP24 mRNA and protein in WT145 cells. In fact, a fivefold induction of the CYP24 promoter was consistently observed at 1 nM 1,25D, a dose which induced growth inhibition of WT145 cells comparable to that achieved with 100 nM 25D.

The observed dissociation between growth inhibition and CYP24 induction by 25D was unexpected, and clarification of the mechanisms and significance of this finding will require additional study. It is possible that the amount of 1,25D generated from 25D in WT145 cells is sufficient to inhibit growth but is too low to activate CYP24 transcription. Mechanistically, this would presume that growth arrest of cells treated with 25D is mediated by VDR target genes that are responsive to much lower 1,25D concentrations than CYP24 and/or that low concentrations of 1,25D impact on growth via

novel mechanisms. Our data is also consistent with the possibility that 25D mediates growth inhibition of WT145 cells by directly binding VDR, independent of its conversion to 1,25D. Indeed, precedent for VDR activation by ligands other than 1,25D exists, as bile acids have been identified as a new class of VDR ligands that participate in regulation of metabolic gene expression in the intestine [Thompson et al., 2002; Jurutka et al., 2005; McCarthy et al., 2005]. Although the affinity of 25D for VDR is approximately 60-fold less than that of 1,25D [Skowronski et al., 1995], it is conceivable that the intracellular concentrations of 25D are sufficient for VDR binding. If so, the 25D–VDR complex could trigger either genomic or non-genomic effects. However, recent modeling studies have suggested the existence of an alternate binding pocket in VDR that binds 25D and generates a receptor conformation that does not support genomic signaling but could likely support non-genomic effects [Mizwicki et al., 2005]. Examples of non-genomic effects of VDR ligands implicated in cell growth and differentiation include modulation of phosphorylation cascades in leukemic cells [Bhatia et al., 1995; Berry et al., 1996], activation of protein phosphatases in colon carcinoma cells [Bettoun et al., 2002; Bettoun et al., 2004] and rapid effects on multiple kinase pathways in breast and squamous carcinoma cells [Capiati et al., 2004; Ma et al., 2006]. Further studies will be necessary to determine whether 25D directly binds VDR in WT145 cells and if so, whether the resulting 25D–VDR complex exerts genomic or non-genomic effects that can be linked to growth arrest.

We also observed dissociation between growth inhibition and CYP24 induction in WT145 cells treated with the synthetic vitamin D analog EB1089, which was more potent than 1,25D in triggering growth inhibition, but less potent than 1,25D in CYP24 activation. These observations mirror those obtained in MCF-7 human breast cancer cells [Danielsson et al., 1997], where EB1089 more potently induced growth arrest and apoptosis than 1,25D but was approximately 10-fold less potent than 1,25D in activation of the DR3 VDRE of the osteopontin gene. In our study, no effects of EB1089 were observed in KO240 cells, indicating that VDR is required for growth regulation by EB1089. Collectively, these data are consistent with the concept that synthetic vitamin D analogs exert unique effects by promoting VDR conformations that differentially activate target gene expression. Notably, in MCF-7 cells EB1089 preferentially activated IP9-type VDREs compared to DR3-type VDREs [Nayeri et al., 1995]. Since EB1089 displayed an activity profile intermediate between 25D and 1,25D, further comparison of the potency of these three vitamin D compounds to activate additional VDR responsive promoters in WT145 cells will be of particular interest.

In contrast to growth arrest, which was induced by all VDR ligands tested, apoptosis of WT145 cells was induced by 1,25D (Figs. 4–5) and EB1089 [Valrance and Welsh, 2004] but not by 25D. The pro-apoptotic effect of 1,25D was associated with activation of caspases 3, 9, and 12, but no evidence for activation of caspase 8, which transduces membrane death receptor signals, was obtained (data not shown). These observations suggest that in murine mammary cells, 1,25D signals through intracellular organelles to activate caspase 9 (mitochondria) and caspase 12 (endoplasmic reticular), leading to activation of caspase 3 and PARP cleavage. These three caspases have also been implicated in 1,25D-mediated apoptosis of prostate cancer cells [Guzey et al., 2002]. A variety of effector caspases, including 7 and 10, as well as calpain, were also activated during 1,25D-mediated apoptosis of WT145 cells. Activation of calpain, a ubiquitously expressed protease linked to disruptions in cellular calcium homeostasis, has previously been correlated with 1,25D-mediated apoptosis of human breast cancer cells [Mathiasen et al., 2002], and may propagate the apoptotic signal via activation of caspase 12 [Nakagawa and Yuan, 2000]. Further studies will be required to determine whether 1,25D activates caspase 12 solely through calpain-mediated cleavage or whether calcium release secondary to endoplasmic reticular stress is also involved. Regardless of the specific mechanisms, it is clear that protease activation during 1,25D-mediated apoptosis requires the VDR, since neither caspases nor calpain were activated by 1,25D in KO240 cells. However, VDR ablation did not alter sensitivity of KO240 cells to apoptosis induction by the DNA-damaging agent etoposide, indicating that VDR transmits apoptotic signals but its absence does not significantly impact on common apoptotic pathways. Since 1,25D and EB1089, but not 25D, induced CYP24, it is

tempting to speculate that induction of CYP24 by a VDR ligand may correlate with its ability to induce apoptosis, but additional studies are necessary to test this possibility.

In summary, we report that the growth inhibitory effects of vitamin D compounds depend upon the presence of VDR protein, but do not necessarily correlate with transcriptional activation of CYP24, a classical VDR target gene that contains DR3-type VDREs. This suggests that the CYP24 gene, widely considered an indicator of VDR activation, is not an accurate predictor of the anti-proliferative efficacy of VDR ligands. Clearly, occupancy of VDR with different ligands may subtly alter its ability to activate different classes of VDREs, leading to selectivity in outcome. The newly discovered dual-pocket actions of the VDR [Mizwicki et al., 2005; Rochel and Moras, 2006], in which ligand binding in one orientation mediates gene transcription, and ligand binding in an alternative orientation mediates non-classical effects, provides another mechanism to explain the dissociation between growth inhibition and CYP24 gene activation by vitamin D compounds. These and other possibilities can be tested in our model system by examining the effects of structurally different VDR ligands in KO240 cells stably expressing VDRs containing point mutations that selectively abolish binding to the alternate pocket and/or interaction with specific co-factors or response elements.

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