Molecular Pharmacology Fast Forward. Published on February 16, 2006 as DOI: 10.1124/mol.105.019851
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MOL PHARM 019851

19nor-1,25-dihydroxyvitamin D_2 specifically induces CYP3A9 in rat intestine more strongly than 1,25-dihydroxyvitamin D_3 *in vivo* and *in vitro*.

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Running Title: 19nor-1,25$(OH)_2D_2$ is a stimulator of CYP3A9 mRNA in rat intestine

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No. of Pages: 33

Number of Tables: 0

Number of Figures: 10

Number of References: 24

Number of words in Abstract: 258

No. of words in Introduction: 634

No. of words in Discussion: 1215

ABBREVIATIONS: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 19nor-1,25$(OH)_2D_2$, 19nor-1,25-dihydroxyvitamin D_2; VDRE, vitamin D response element; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; EMSA, electrophoretic mobility shift assay;

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ABSTRACT

In the intestine, the vitamin D receptor is activated by 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) to perform its function in calcium homeostasis, or it is activated by lithocholic acid when its levels are elevated following a meal. Both ligands transcriptionally upregulate the mRNA of enzymes belonging to the CYP3A subfamily, increasing the metabolism of a wide variety of carcinogens, drugs and hormones. Of the cytochrome P450 enzymes, the CYP3A subfamily is the most abundant in liver and intestine, and has the widest range of substrate specificity. In addition to being a ligand for the vitamin D receptor, lithocholic acid is also a substrate for CYP3A enzymes. Lithocholic acid causes colon cancer, thus decreasing lithocholic acid levels in the intestine by upregulating CYP3A enzymes with $1,25(OH)_2D_3$ analogs may have therapeutic value in the prevention of colon cancer. We investigated the induction of CYP3A9 by $1,25(OH)_2D_3$ and 19nor-1α,25-dihydroxyvitamin D_2 (19nor-$1,25(OH)_2D_2$). We observed that the latter analog, currently used to treat renal osteodystrophy, is more efficacious than $1,25(OH)_2D_3$ in inducing CYP3A9 in rat intestines. CYP3A9 mRNA was maximally elevated 5-7 h after a single dose of $1,25(OH)_2D_3$ to rats, and then gradually returned to baseline. We performed promoter deletion analysis of the rat CYP3A9 promoter and identified one proximal vitamin D response element located at –119 to –133 from the transcriptional start site, which is responsible for a large part of the $1,25(OH)_2D_3$ response, and two other vitamin D response elements located at –726 to –744 and at –754 to -776 which together are responsible for the increased sensitivity of CYP3A9 to 19nor-$1,25(OH)_2D_2$.

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Maintenance of calcium and phosphate homeostasis is the principal function of the hormonal form of vitamin D, which acts through the vitamin D receptor to influence target gene expression by transcriptional regulation (Jones et al., 1998). Other roles for the active form of vitamin D have more recently been discovered in cell proliferation and differentiation, keratinocyte function, in osteoclastogenesis, and the immune system (Jones et al., 1998). A number of reports recently showed that the human CYP3A4 mRNA is stimulated by 1,25$(OH)_2D_3$ in cell culture (Schmiedlin-Ren et al., 1997; Thompson et al., 2002; Thummel et al., 2001). Members of the cytochrome P450 3A subfamily are the most abundant CYP enzymes in the human liver and intestine and display a broad substrate specificity (Kliewer et al., 2002), allowing detoxification of a wide variety of drugs, including cyclosporin A, and various carcinogens, certain antidepressants, and steroid hormones (Spicer et al., 1997).

A carcinogen of particular interest that is catabolizxed by CYP3A enzymes is the secondary bile acid lithocholic acid, which was shown to promote colon cancer in animals (Narisawa et al., 1974). In addition, patients with colorectal cancer were shown to have higher levels of lithocholic acid in the feces (Owen et al., 1987). Lithocholic acid is produced from chenodeoxycholic acid, a primary bile acid, in the intestine by bacteria (Jurutka et al., 2005). Lithocholic acid is not taken up into the entero-hepatic system, and remains in the intestine (Kozoni et al., 2000) where at high concentrations causes tumors by inducing DNA strand breaks and inhibiting DNA repair enzymes (Makishima et al., 2002). High concentrations of lithocholic acid in the intestine happen as a result of high fat diets (Jurutka et al., 2005). At high intestinal concentrations (10 μM), lithocholic acid can also activate the vitamin D receptor to induce CYP3A expression for

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detoxification and breakdown of lithocholic acid (Makishima et al., 2002). $1,25(OH)_2D_3$ can also induce CYP3A through the vitamin D receptor (Thompson et al., 2002; Thummel et al., 2001), a role that is not surprising since sequence analysis of receptors suggests that vitamin D receptor served a detoxification role early in evolution before it became crucial for calcium homeostasis (Whitfield et al., 2003). Figure 1 shows a simplified diagram of the putative relationship between CYP3A, vitamin D receptor, lithocholic acid and $1,25(OH)_2D_3$ analogs.

Analogs of $1,25(OH)_2D_3$ are wildly used in the treatment of secondary hyperparathyroidism in dialysis patients. In particular $1,25(OH)_2D_3$, the hormonal form of vitamin D (calcitriol) is used clinically for the treatment of renal osteodystrophy. 19nor-$1,25(OH)_2D_2$ or Zemplar^R is used for secondary hyperparathyroidism in dialysis patients (Brown, 2001). Identification of an analog that maximally induces intestinal CYP3A with minimal side-effects, such as elevating serum calcium levels, may have great pharmaceutical potential in colon cancer prevention.

The induction of human CYP3A4 by $1,25(OH)_2D_3$ has been well characterized in cell culture, and vitamin D responsive elements (VDRE) have been identified in the promoter of CYP3A4 and have been shown to be of two types DR3 (direct repeats spaced by 3 base pairs) and ER6 (everted repeats spaced by 6 base pairs) (Thompson et al., 2002; Thummel et al., 2001). Additional work showed that the vitamin D receptor itself is responsible for the activation of these elements and not PXR, an orphan nuclear receptor which also activates CYP3A4, especially in liver (Thompson et al., 2002; Thummel et al., 2001).

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In this report we compared the induction of CYP3A9 following the administration of 1,25$(OH)_2D_3$ and 19nor-1,25$(OH)_2D_2$ in rats. Our studies show that 19nor-1,25$(OH)_2D_2$ is a more efficacious inducer of CYP3A9 than 1,25$(OH)_2D_3$ in rat intestine. This presents an interesting finding because 19nor-1,25$(OH)_2D_2$ is less efficacious than 1,25$(OH)_2D_3$ in other functions such as the induction of intestinal calcium absorption. Following promoter analysis, we identified three VDREs, two of which are responsible for the increased effect of 19nor-1,25$(OH)_2D_2$.

Materials and Methods

Materials. 1,25$(OH)_2D_3$ and 19nor-1,25$(OH)_2D_2$ were purchased from Tetrionics (Madison, WI).

Animals. For the time course experiment, male Holtzmann rats were maintained on purified diet containing 0.47% calcium/0.3% phosphorus (Suda et al., 1970). On the day of the experiment, the rats were dosed intrajugularly with 270 ng of 1,25$(OH)_2D_3$ in 100 µl ethanol (dosed) or 100 µl of ethanol (control). At sacrifice, duodenal mucosa was collected and homogenized in a guanidium thiocyanate-contatining buffer for RNA isolation (Chomczynski and Sacchi, 1987). For the dose response experiment, male Sprague-Dawley (Madison, WI) 6 week old rats were maintained on purified diet containing 0.47% calcium/0.3% phosphorus. On the day of the experiment, the rats were orally gavaged with 200 µl of vegetable oil containing the designated amount of analog. After 6.5 h the animals were sacrificed, and the duodenal mucosa was collected and homogenized in TRI reagent (Molecular Research Center, Cincinnati, OH) for RNA isolation.

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Male NOD LtJ mice were maintained on LabDiet Mouse Diet 5015 (PMI Nutritional International, St. Louis, MO). On the day of sacrifice the mice were dosed intraperitoneally with the specified amounts of vitamin D analog in 50 μl propylene glycol containing 5% ethanol. The mice were sacrificed 7 h following the dose and mucosa from the duodenum was collected for RNA analysis.

Though the routes of analog administration were different throughout this study, we verified that different dosing routes would cause the same physiological response (dose response and time of response).

RNA isolation and Northern analysis. RNA was extracted using the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) for the time course experiment, or using TRI reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol for all other experiments. 25 μg of total RNA was separated by electrophoresis in a 1% agarose gel containing 15% formaldehyde. RNA was transferred to nylon membranes (Micron Separations Inc., Westborough, MA) with 20 x SSC by capillary action. The membranes were prehybridized and hybridized at 68 C using Quikhyb (Stratagene, La Jolla, CA) according to manufacturer's instructions. The membranes were washed with 2x SSC, 0.1% SDS for 20 min at 68 C, with 1x SSC, 0.1% SDS for 20 min at 68 C, and finally with 0.1x SSC, 0.1% SDS for 20 min at 68 C, and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Radiolabeled probes were produced by excising the cDNA fragments from the vector, purifying the insert using the GeneClean II kit (Bio101, Vista, CA), and labeling the purified insert using the Prime-a-Gene labeling System from Promega (Madison, WI)

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and (α-^{32}P)dCTP from Amersham (Arlington Heights, IL). Probes used were rCYP3A9 (bp 1520-2037 of U46118, (Wang et al., 1996), and rat GAPDH.

RNA isolation, reverse transcription, and quantitative PCR. RNA was isolated from rat duodenum using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to manufacterer's instructions. Five μg of total RNA were reverse transcribed at 42 C using random hexamers and AMV transcriptase (Promega, Madison, WI) according to manufacturer's protocol. Following reverse transcription, the samples were diluted eight-fold with water to 200 μl final volume, heated to 90 C for 5 minutes and used for quantitative PCR. Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Indianapolis, IN) with 5μl of the reverse transcribed material per reaction on a LightCycler 2.0 Instrument (Roche, Indianapolis, IN).

The amplification primers and conditions used were as follows: 1) rCYP3A9 (1481-1920): 5' TTC ACA AAG ACC CGC ATT ACT 3' and 5' GAA AGC TCA AAG AAC ACC CAA AAC 3', denaturing at 95 C for 5 seconds, annealing at 57 C for 6 seconds, elongation at 72 C for 18 seconds, 2) rat GAPDH: 5' TGA AGG TCG GTG TGA ACG GAT TTG GC 3' and 5' CAT GTA GGC CAT GAG GTC CAC CAC 3', denaturing at 95 C for 15 seconds, annealing at 58 C for 5 seconds, elongation at 72 C for 38 seconds. All PCR reactions were preceded by an initial 10 min. denaturation step at 95 C.

Cloning of CYP3A9 promoter. The rat genome was searched for the promoter of the rat CYP3A9 gene by performing a BLAST search using the rat CYP3A9 sequence (U46118). The mouse genome was also searched for the promoter of the mouse homologue CYP3A13 (NM_007819). The two promoter sequences were

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aligned and primers were designed to PCR amplify a region of the promoter that was most highly conserved between the two species and that contained a putative vitamin D response element. The primer sequences used were the following: CYP3A9promF1 5'-CCA GGA GCA GCC AGG TTT -3' and 5'- CCA GGA AGA GGG AAT CAG AGT T -3'. PCR amplification was carried out using Elongase (Gibco, Carlsbad, CA) according to manufacturer's protocol with an annealing temperature of 56 C and using rat tail genomic DNA as a template. The PCR products were first cloned into pCR-BluntII-TOPO using the TOPO blunt PCR cloning kit (Invitrogen, Carlsbad, CA), then sequenced and excised by BamHI and XbaI digestion. The gel-purified fragment was further subcloned into BglII and NheI digested pGL3basic (Promega, Madison, WI) to obtain the reporter construct with –783/+129 of the rat CYP3A9 promoter in pGL3-basic. Sequencing confirmed the identity of the clone.

CYP3A9 promoter deletion constructs. The clone CYP3A9(–726/+129) in pGL3-basic was obtained by excising a KpnI fragment that resulted from a restriction site in the multiple cloning site of the plasmid and the internal CYP3A9 promoter KpnI site at –726 and religating the plasmid. Sequencing confirmed the identity of the clone

The clone CYP3A9(-440/+129) in pGL3-basic was obtained by excising a EcoRV/SnaBI fragment from the CYP3A9(-783/+129) in pGL3-basic clone and religating the plasmid.

The clone -726/-440 in pGL3-promoter was obtained by releasing a SnaBI/KpnI fragment from –786/+129 in pGL3-basic, gel-purifying the fragment, and subcloning it into SmaI/KpnI-digested pGL3-promoter (Promega, Madison, WI). Sequencing confirmed the identity of the clone.

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The clone CYP3A9(-727/-783)x3 in pGL3-promoter was obtained by excising a KpnI fragment from –783/+129 in pGL3-basic, gel-purifying it, and subcloning it into KpnI-digested pGL3-promoter to obtain the reporter construct with multiple KpnI fragments

The clone named CYP3A9(-119/-133)x3 in pGL3-promoter containing three copies of the proximal VDRE was obtained by phosphorylating the synthetic double stranded oligonucleotide with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA), followed by incubation with T4 DNA ligase to form concatamers of different length. The ligation product was shot-gun cloned into XbaI digested pGL3-promoter and sequenced. The oligonucleotide sequences were 5' CTA GAG TGT ACT CTC TAA CCT AGT 3' and 5' CTA GAC TAG GTT AGA GAG TAC ACT 3', and were designed to have XbaI overhangs.

The clone named CYP3A9(-137/-151)x4 in pGL3-promoter was obtained using the procedure mentioned above (clone CYP3A9(-119/-133)x3 in pGL3-promoter). The oligonucleotide sequences were 5' CTA GAG CAG TTG GCA AAG GAT CAT 3' and 5' CTA GAT GAT CCT TTG CCA ACT GCT 3', and were designed to have XbaI overhangs.

The clone named CYP3A9(-726/-750)x3 in pGL3-promoter was obtained using the procedure mentioned above (clone CYP3A9(-119/-133)x3 in pGL3-promoter). The oligonucleotide sequences were 5' CTA GAG GTA CCT ACA GGC CAT TCT GGT GGA T 3' and 5' CTA GAT CCA CCA GAA TGG CCT GTA GGT ACC T 3', and were designed to have XbaI overhangs.

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The clone named CYP3A9(-754/-775)x3 in pGL3-promoter was obtained using the procedure mentioned above (clone CYP3A9(-119/-133)x3 in pGL3-promoter). The oligonucleotide sequences were 5' CTA GAA TTT CTT AAC TCT GAT TCC CTC TGG T 3' and 5' CTA GAC CAG AGG GAA TCA GAG TTA AGA AAT T 3', and were designed to have XbaI overhangs.

Site-directed mutagenesis. Mutants of the wild-type CYP3A9(-783/+129) promoter construct were obtained using the Quik Change II Site directed mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's protocol. The oligonucleotides were designed to introduce the following mutations:
Mutant 726/740: 5' GTT TCC ACC AGA Aac gtg Agt AGG TAa Atc AGT GGG GGA ATA CTC 3', to yield the construct named CYP3A9(-783/+129)mut740 and
Mutant 761/774: 5' CGC CCT TCC AGG AAG Aac ctA TCA tgt aat AGA AAT TGT TTC CAC 3' to yield the construct named CYP3A9(-783/+129)mut774.

Cell Culture. Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) from Gibco BRL (Grand Island, NY) plus 20% fetal bovine serum from Hyclone Laboratories, Inc. (Logan, UT) at 37 C in an atmosphere of 6.0% CO_2.

Transient transfections. Caco-2 cells were transfected in 24-well plates containing 70-90% confluent cells. Each well received 0.33 μg DNA, 3 μl of Plus reagent, and 2 μl of Lipofectamine (Invitrogen, Carlsbad, CA) in 1 ml of DMEM for 3 h. After 3 h, 0.5 ml of DMEM containing 30% FCS were added per well and the cells were dosed with vitamin D analogs at the designated concentrations for 18-24 h. Each μg of DNA contained 0.80 μg of rCYP3A9prom, 0.045 μg of pCMVβ, and 0.155 μg of vitamin D receptor expression vector or 0.015 μg of vitamin D receptor expression vector with

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0.139 μg of pCMV500 ("low" vitamin D receptor levels). Cells were washed with PBS and collected in 100μl reporter lysis buffer (Promega, Madison, WI). Luciferase activity was assayed using a MLX Microtiter Plate Luminometer (Dynex technologies, Chantilly, VA). β-Galactosidase activity was assessed by colorimetric assay using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate (Sambrook et al., 1989).

Electrophoretic mobility shift assay. Both strands of "CYP3A9(-119/-133)" (CTA GAG TGT ACT CTC TAA CCT AGT), "CYP3A9(-726/-750)" (CTA GAG GTA CCT ACA GGC CAT TCT GGT GGA T), and "CYP3A9(-754/-775)" (CTA GAA TTT CTT AAC TCT GAT TCC CTC TGG T) oligonucleotides were synthesized with overhanging XbaI sites and annealed to form double-stranded probes. These probes were labeled by filling in recessed ends using [^{32}P]dCTP (Perkin Elmer/NEN, Boston, MA) and the Klenow fragment of DNA polymerase (Promega, Madison, WI). Gel purification of the radiolabelled probes was performed in a 6% polyacrylamide gel in 1xTBE. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (Darwish and DeLuca, 1992) using porcine intestinal nuclear extract. Monoclonal antibodies XVIE10 and IVG8 against the vitamin D receptor were used to show specificity of the complexes. The oligonucleotide sequences used for specific competition was the distal rat 24-hydroxylase VDRE (CTA GAG AGC GCA CCC GCT GAA CCC TGG GCT).

Sequencing. Sequencing was performed at the University of Wisconsin Biotech Center (Madison, WI). Samples were electrophoresed on an Applied Biosystems (Foster City, CA) 3730xl automated DNA sequencing instrument, using 50 cm capillary arrays and POP-7 polymer. Data were analyzed using PE-Biosystems version 3.7 of

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Sequencing Analysis. Typical sequencing reactions consisted of 2 ul of BigDye Terminator v. 3.1 mix (Applied Biosystems), 3 ul of dilution buffer (Applied Biosystems), 5-20 pmol of primer, and 0.2 μg of template DNA in a final reaction volume of 20 μl.

Statistics. All data were standardized and expressed as fold-induction over vehicle-treated controls. Averages and standard errors of the mean (SEM) of multiple experiments were calculated and Student's t-tests were performed.

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Results

CYP3A9 mRNA response to 1,25(OH)$_2$D$_3$ and 19nor-1,25(OH)$_2$D$_2$ in rats. The analog 19nor-1,25(OH)$_2$D$_2$ (Zemplar^R) is a more efficacious inducer of CYP3A9 mRNA than 1,25(OH)$_2$D$_3$ in rats. A dose response experiment showed that 19nor-1,25(OH)$_2$D$_2$ has a significantly higher fold induction than 1,25(OH)$_2$D$_3$ at a dose of 16μg/kg ($p<0.001$, Figure 2A). The results were corroborated in mice, which were more sensitive than rats: the CYP3A9 mRNA induction was 4-fold at levels of analog as low as 2μg/kg while rats need almost 8μg/kg to attain the same levels of induction (Figure 2B).

The effect of 19nor-1,25(OH)$_2$D$_2$ was specific to the regulation of CYP3A9. 24-Hydroxylase mRNA was clearly more responsive to 1,25(OH)$_2$D$_3$ than 19nor-1,25(OH)$_2$D$_2$ (Figure 2C), and intestinal calcium transport data showed that 2400 pmoles/day of 19nor-1,25(OH)$_2$D$_2$ and 240 pmoles/day of 1,25(OH)$_2$D$_3$ were required to obtain similar intestinal calcium transport ratios (5.3 $\pm$ 0.3 and 6.7 $\pm$ 0.9 serosal Ca/mucosal Ca, respectively) by the everted gut sac method (Perlman et al., 1990).

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Previous work on the induction of the human CYP3A4 by 1,25$(OH)_2D_3$ in cell culture showed that the mRNA for this enzyme is up-regulated in cells treated for 24 h or longer (Thompson et al., 2002; Thummel et al., 2001). A time course study in rats administered a single dose of 1,25$(OH)_2D_3$ intrajugularly showed that CYP3A9 mRNA was up-regulated by 5 h, remained at peak levels for about 2 h, then gradually returned to baseline (Figure 3).

Identification of the proximal VDRE in the CYP3A9 promoter. We cloned the rat CYP3A9 promoter as described in Materials and Methods to perform promoter deletion analysis for the identification of VDREs. When different size fragments of the promoter controlling the luciferase gene were transfected into Caco-2 cells, the promoter fragment containing the sequence –440 to +129 remained responsive to 1,25$(OH)_2D_3$ (Figure 4). Analyis of the sequence contained in the fragment from –440 to +1 to locate VDREs using the consensus sequence RGKKSW NNN VRKNYV (R = A or G, K = G or T, S = G or C, W = A or T, Y = C or T, V = A, C, or G) revealed three putative VDREs at positions –119 to –133 from the transcription start site (antisense strand), at position -137 to –151, and at position –348 to -374 (antisense strand). EMSA showed that a synthetic oligonucleotide containing the sequence from –119 to –133 formed two complexes with pig intestinal nuclear extract: these complexes formed a supershift with the anti- vitamin D receptor antibody XVIE10, while the antibody IVG8 diminished complex formation. In addition, excess radioinert oligonucleotide of the previously established 24-hydroxylase VDRE (Zierold et al., 1994) competed for complex formation, while nonspecific oligonucleotide did not (Figure 5). The sequences -137 to –151 and –348 to -374 did not form specific complexes that would interact with

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either the vitamin D receptor antibodies or nonradioactive distal 24-hydroxylase (data not shown).

We tested both the sequences from –119 to –133 and from –137 to –151 for responsiveness to 1,25$(OH)_2D_3$ in cells by inserting them in front of the SV40 promoter controlling a luciferase reporter gene. We found that the fragment from –119 to –133 clearly induced luciferase activity in response to 1,25$(OH)_2D_3$, while the fragment from –137 to –151 was not responsive to 1,25$(OH)_2D_3$ (Figure 6). Both the EMSA data and the *in vivo* data identify the region between –119 and –133 as a VDRE that has similar binding pattern to previously identified VDREs (Zierold et al., 1995).

A cell culture model that responds to 19nor-1,25$(OH)_2D_2$ with higher efficacy than 1,25$(OH)_2D_3$. From a mechanistic point of view, we were intrigued by the fact that 19nor-1,25$(OH)_2D_2$ is a more efficacious inducer of the CYP3A9 mRNA than 1,25$(OH)_2D_3$. To investigate the mechanism of action we identified a cell culture model that also responded to 19nor-1,25$(OH)_2D_2$ more strongly than 1,25$(OH)_2D_3$. We tested various transfection conditions and found that when Caco-2 cells were cotransfected with low levels of vitamin D receptor expression vector in addition to reporter vectors, they more strongly responded to 19nor-1,25$(OH)_2D_2$ as shown by the dose-response curve in Figure 7A. When 10-fold higher levels of vitamin D receptor expression vector were contransfected (“normal” amounts) both analogs had similar response levels (Figure 7B).

To explain this observation we determined the levels of vitamin D receptor in Caco-2 cells transfected with either “low” or “normal” levels of vitamin D receptor and compared them to the levels found in vitamin D sufficient rat intestines. The levels of

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vitamin D receptor were considerably higher in rat intestines (2399 ± 340 fmoles/mg protein) than in either low or high vitamin D receptor Caco-2 cells (92 ± 7 and 529 ± 7 respectively). However, the vitamin D receptor levels in the transfected cells reflected an average over all cells and did not take into consideration that individual cells will transfect and express vitamin D receptor unevenly. We hypothesize that available vitamin D receptor for the CYP3A9 VDREs may be low, because VDR may be in use on other genes in the intestine, and thus CYP3A9 would be more responsive to 19nor-1,25$(OH)_2D_2$.

Identification of distal VDREs in the CYP3A9 promoter with increased 19nor-1,25$(OH)_2D_2$ sensitivity. Analysis of the reporter constructs expressing low vitamin D receptor levels dosed with 1,25$(OH)_2D_3$ or 19nor-1,25$(OH)_2D_2$ showed that eliminating a 58 base pair piece of promoter (-727 to –783) eliminated the response to 19nor-1,25$(OH)_2D_2$ (Figure 8A). When the 58 base pair fragment was placed in front of the SV40 promoter controlling luciferase, the reporter activity became much more responsive to 19nor-1,25$(OH)_2D_2$ than 1,25$(OH)_2D_3$, though to a more limited extent than when kept in front of the native promoter, probably because of the fact that some of the native promoter context is required for optimal activity (Figure 8B).

Sequence analysis for putative VDREs as described in Material and Methods resulted in three possible candidate sequences: -745 to –759, -726 to –750, and –754 to –776 (antisense strand). EMSA showed that the latter two sequences were able to form a complex that reacted with vitamin D receptor antibodies (Figure 9A & 9B), while the sequence –745 to –759 formed a complex that did not interact with vitamin D receptor antibodies or excess nonradioactive distal 24-hydroxylase VDRE (data not

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shown). The binding pattern for these distal VDREs was different when compared to the proximal VDRE or other VDREs such as those of the 24-hydroxylase promoter. These differences include: 1) much less complex was formed when similar amounts of radiolabelled probe were used, suggesting that the affinity of the vitamin D receptor for these sequences is much lower, 2) only the larger complex interacts with vitamin D receptor antibodies or excess radioinert 24-hydroxylase distal VDRE, 3) the sequence –754 to –776, when incubated with XVIE10 antibody, does not supershift the complex as previously observed, instead the antibody seems to compete for the formation of the upper complex suggesting that the vitamin D receptor is in the complex but in a different arrangement that is structurally inhibited by XVIE10. We compared the effect of 19nor-1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ on complex formation in EMSA using pig intestinal nuclear extract or recombinant vitamin D receptor and RXR, but quantification of the complexes incubated with 19nor-1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ did not show any differences (data not shown).

We tested the response of luciferase when under the control of the SV40 promoter and the sequences from -726 to –750, or –754 to –776, and observed that separately these elements show very small induction by 19nor-1,25$(OH)_2D_2$ or 1,25$(OH)_2D_3$ over vehicle, as well as considerable amount of variability (data not shown). Because these two elements have an enhanced response to 19nor-1,25$(OH)_2D_2$ when together (Figure 8B) but not when separated, it is likely that these two elements interact with vitamin D receptor together in a way that is more accessible to 19nor-1,25$(OH)_2D_2$ than 1,25$(OH)_2D_3$.

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Finally, we mutated the distal VDRE sequences individually in the native promoter as described in Material and methods. As shown in Figure 10, the construct CYP3A9(-783/+129)mut740 with mutated nucleotides between –726 and –740 did not respond more strongly to 19nor-1,25$(OH)_2D_2$ as did the wild type construct, providing additional proof that this distal region is important for the19nor-1,25$(OH)_2D_2$ response. Surprisingly, the construct CYP3A9(-783/+129)mut774 with mutated nucleotides between –761 and –774 was almost completely inactive resulting in luciferase activity less than 4% of wild-type (data not shown). Because the reporter activity was so low, we were not able to accurately determine how the mutations influenced the effects of the19nor-1,25$(OH)_2D_2$. Binding of vitamin D receptor to the mutated sequences was not observed when tested by EMSA (data not shown).

Discussion

We report that 19nor-1,25$(OH)_2D_2$ is more efficacious in the induction of the rat CYP3A9 than 1,25$(OH)_2D_3$. This finding was unexpected because in calcium absorption and other target gene regulations such as 24-hydroxylase, 1,25$(OH)_2D_3$ is more efficacious than 19nor-1,25$(OH)_2D_2$. Physiologically, patients treated with 19nor-1,25$(OH)_2D_2$ would have a higher rate of xenobiotic transformations that may be beneficial if molecules such as carcinogens are cleared. Specifically, the breakdown of the CYP3A9 substrate lithocholic acid, would lead to a decreased incidence of colon cancer in patients treated with 19nor-1,25$(OH)_2D_2$ (Zemplar). No data exist on the incidence of colon cancer in 1,25$(OH)_2D_3$ or 19nor-1,25$(OH)_2D_2$-treated patients versus

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placebo treated patients, however a long-term study by Teng *et al.* showed that dialysis patients administered 19nor-1,25$(OH)_2D_2$ had a much higher survival rate than patients administered 1,25$(OH)_2D_3$ or placebo (Teng et al., 2003). The higher survival rate may in part be related to the up-regulation of the P4503A4 enzyme by 19nor-1,25$(OH)_2D_2$ in the intestine.

The use of 19nor-1,25$(OH)_2D_2$ may also reduce the efficacy of certain drugs that are metabolized by this enzyme. One of the major substrates for CYP3A is cyclosporine A, which has been used to prevent organ transplant rejection. *Hullet et al.* observed that mice administered 500 or 1000 ng of 1,25$(OH)_2D_3$ per mouse per day with cyclosporine A (1.5 mg/kg/day) had much lower levels of cyclosporine A in the serum than mice given cyclosporine A alone (237$\pm$ 108, 721 $\pm$ 402, and 1152 $\pm$ 452 ng/ml, respectively) (Hullett et al., 1998), which suggests that 1,25$(OH)_2D_3$ induces CYP3A enzymes in the intestines with pharmacological repercussions. These data combined with those presented here illustrate the importance of carefully assessing the dosage of drugs that are substrates for P4503A4 in people treated with vitamin D analogs.

Xenobiotic transforming enzymes were shown to be regulated by other steroid hormones such us estrogen, pregnelone, and dexamethasone, but the regulation is most commonly demonstrated in the liver (Quattrochi and Guzelian, 2001). Previous work showed that the effect of 1,25$(OH)_2D_3$ analogs on the CYP3A9 occurs through the vitamin D receptor, which is not present in liver cells. Thus, in liver CYP3A9 cannot be regulated by 1,25$(OH)_2D_3$ and its analogs. However, detoxification occurring in the intestine may be much a more efficient site of action in protecting the organism by

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metabolizing toxins before their systemic absorption. In the case of enteric carcinogens such as lithocholic acid, $1,25(OH)_2D_3$ analogs can induce xenobiotic enzymes to protect the colon.

The rat CYP3A9 is believed to be the homolog of the human CYP3A4, but there are several differences between the two enzymes. As described by Xue *et al.,* the activities of the two enzymes are similar but not identical due to four different amino acids in the substrate recognition site. These changes alter the activities in such a way that certain substrates are differentially metabolized (Xue et al., 2003). Further differences lie in the promoter region. Though the mouse and the rat promoter sequences are very similar for the first 1150 base pairs, the human promoter sequence does not align with either the rat or the mouse sequence, even at important transcription regulatory element binding sites. In addition, while the rat CYP3A9 promoter has three VDREs within the first 783 base pairs from the transcription start site, the CYP3A4 promoter has two more distal elements one being a DR3 and the other an ER6 (Thompson et al., 2002; Thummel et al., 2001).

This is the first report of VDREs that shows preferential activity for one $1,25(OH)_2D_3$ analog over another. By lowering the levels of vitamin D receptor expression in transfection experiments, we were able to create a model in cell culture to study the variation in efficacy between 19nor-$1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ observed *in vivo*. To explain why limiting levels of vitamin D receptor are responsible for increased response to 19nor-$1,25(OH)_2D_2$ the local subcellular levels of vitamin D receptor would have to be determined; the vitamin D receptor may be sequestered to some sites away from others. In EMSA the affinity of the “19nor-sensitive” VDREs seemed much lower

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than ordinary VDREs, but to be certain the Kd values would need to be determined. To have receptor binding with lower affinity in a situation where low vitamin D receptor levels are preferred seems contradictory, however, the proximity of the "19nor-sensitive" VDREs may allow strong interactions that greatly stabilize the complexes. This was previously shown for the two VDREs in the 24-hydroxylase promoter (Zierold et al., 1995). In that instance, the VDREs had stronger affinity but were spaced by 93 base pairs. Here the VDREs have weaker affinity, yet separated by only 10 base pairs, thus they may interact together with 19nor-1,25$(OH)_2D_2$ to form a much more stable complex than with 1,25$(OH)_2D_3$ *in vivo*. These two elements must interact because when separated and placed in front of a hetereologous promoter, they barely respond to 19nor-1,25$(OH)_2D_2$ or1,25$(OH)_2D_3$ at any level of vitamin D receptor (data not shown). Sequence analysis of the distal VDREs shows that the element between -726 to –750 has two mismatches, and the element between –754 to –776 has one when compared with the consensus described in Material and methods. These mismatches may account for the decrease in binding affinity and lack of vitamin D-dependent reporter activity when tested separately. Interestingly, the sequence that separates the two elements (–745 to –759) contains a "perfect" VDRE, which does not bind vitamin D receptor in EMSA (data not shown).

We have provided some insight as to the mechanism of action of 19nor-1,25$(OH)_2D_2$ versus 1,25$(OH)_2D_3$ in this promoter by locating VDREs that interact with vitamin D receptor in an unusual way and that respond to 19nor-1,25$(OH)_2D_2$ much more strongly than 1,25$(OH)_2D_3$ when low levels of vitamin D receptor are present. Further research will have to explore the binding complexes and interactions of the

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distal VDREs in more detail to elucidate the molecular mechanism behind the analog specificity of these elements. The drastic reduction in reporter activity observed when the sequence –761 to –774 was mutated is a very interesting finding that will have to be further pursued. Elimination of this element as in the constructs used in Figure 4 & 8 did not reduce luciferase activity, indicating that this element is not indispensable. However, mutation of just a few base pairs, while maintaining everything else intact, almost completely eliminated reporter activity. The transfection efficiency checked by β-galactosidase production was normal for this construct, and the integrity of the plasmid was confirmed by sequencing and restriction digest.

In conclusion, our data also suggest that 19nor-1,25$(OH)_2D_2$, a vitamin D analog already commercially available, may be a good candidate to test for the prevention of colon cancer. Therapeutic use of 19nor-1,25$(OH)_2D_2$ to increase the levels of CYP3A in the intestine, to catabolize lithocholic acid and other enteric carcinogens that accumulate in the intestine shows promise as a preventive mechanism. Compared to 1,25$(OH)_2D_3$, 19nor-1,25$(OH)_2D_2$ also has less calcemic activity, thus the undesirable side-effect of hypercalcemia is reduced.

These data show that the efficacy of a vitamin D analogs on intestinal calcium absorption is not correlated with the efficacy of induction of the CYP3A enzymes: future work should be directed at testing more vitamin analogs for maximum induction of CYP3A and minimal effect on serum calcium levels to find the optimal compound to be used as chemopreventive agent for colon cancer.

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Acknoledgements

We would like to thank the media laboratory at the University of Wisconsin-Biochemistry Department for assistance with figures, and Pat Mings for assistance with manuscript preparation.

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Footnote

This work was supported in part by a fund from the Wisconsin Alumni Research Foundation.

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Legends for figures

Figure 1. Diagram of interactions between CYP3A9, lithocholic acid, vitamin D receptor and 1,25$(OH)_2D_3$ analogs.

Figure 2. (A) Dose response of CYP3A9 mRNA in the intestine of rats following administration of 1,25$(OH)_2D_3$ and 19nor-1,25$(OH)_2D_2$ by oral gavage. The figure shows the fold-induction of CYP3A9 mRNA normalized to GAPDH mRNA from1,25$(OH)_2D_3$-dosed rats over vehicle-dosed rats sacrificed 6.5-7 h post-dose. Quantitations were performed using real-time PCR as described in Materials and Methods. The difference between the two analogs was significant at $p<0.001$ (***). **(B)** Effects on CYP3A9 mRNA in the intestine of mice following administration of two dose levels of 1,25$(OH)_2D_3$ and 19nor-1,25$(OH)_2D_2$ by intraperitoneal injection. The figure shows the fold-induction of CYP3A9 mRNA normalized to GAPDH mRNA from1,25$(OH)_2D_3$-dosed mice over vehicle-dosed mice sacrificed 6.5-7 h post-dose Northern analysis and quantitations were performed as described in Materials and Methods. The data are expressed as mean $\pm$ S.E.M. **(C)** Dose response of 24-hydroxylase mRNA in the intestine of rats following administration of 1,25$(OH)_2D_3$ and 19nor-1,25$(OH)_2D_2$ by oral gavage. The figure shows the fold-induction of 24-hydroxylase mRNA normalized to GAPDH mRNA from1,25$(OH)_2D_3$-dosed rats over vehicle-dosed rats sacrificed 6.5-7 h post-dose. Quantitations were performed using real-time PCR as described in Materials and Methods. The difference between the two analogs was significant at $p<0.05$ (*).

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Figure 3. Time course of induction of CYP3A9 in the intestine of rats following an intrajugular injection of 1,25$(OH)_2D_3$ or ethanol. The figure shows the fold-induction of CYP3A9 mRNA normalized to GAPDH mRNA from1,25$(OH)_2D_3$-dosed rats over vehicle-dosed rats sacrificed at 0, 2.5, 5, 7.5, 12, 24, 31, and 48 h following the vehicle or 1,25$(OH)_2D_3$-dose. Northern analysis was performed as described in Materials and Methods. The data are expressed as mean $\pm$ S.E.M.

Figure 4. CYP3A9 promoter deletion analysis. Caco-2 cells were transiently transfected with luciferase constructs containing the indicated fragments of the rat CYP3A9 promoter, and a mammalian expression vector for vitamin D receptor. The cells were dosed with vehicle or $4x10^{-9}$ M 1,25$(OH)_2D_3$. Cells were collected in Reporter Lysis Buffer and assayed for luciferase activity, and normalized for β-galactosidase activity. The data are shown as fold induction of 1,25$(OH)_2D_3$ over vehicle-treated cells (mean $\pm$ S.E.M.). Asterisks indicate significant differences from the fold induction of the –783/+129 construct: $p<0.05$ (*) and $p<0.01$ (**).

Figure 5. Electrophoretic mobility shift assay of the proximal VDRE. A double stranded oligonucleotide of the sequence –119 to –133 of the CYP3A9 promoter was radiolabelled and incubated with pig intestinal nuclear extract as described in Material and Methods. Competition with excess unlabelled oligonucleotides (50 ng) was used to identify the specificity of the complexes. Antibodies to the vitamin D receptor (0.5 μg) were used to show the presence of the receptor in the complexes.

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Figure 6. The sequence from –119 to –133 of the CYP3A9 promoter imparts $1,25(OH)_2D_3$-dependence to the SV40 promoter. Caco-2 cells were transiently transfected with luciferase constructs containing the SV40 promoter under the control of the indicated sequences. The cells were dosed with vehicle or $4x10^{-9}$ M $1,25(OH)_2D_3$. Cells were collected in Reporter Lysis Buffer and assayed for luciferase activity, and normalized for β-galactosidase activity. The data are shown as fold induction of $1,25(OH)_2D_3$ over vehicle-treated cells (mean ± SEM). The induction by $1,25(OH)_2D_3$ was significant at $p<0.01$ (**).

Figure 7. Dose-response curve of $1,25(OH)_2D_3$ and 19nor-$1,25(OH)_2D_2$ in the presence of (**A**) low levels of vitamin D receptor expression vector (**B**) normal levels of vitamin D receptor expression vector. Caco-2 cells were transiently transfected with the luciferase construct containing the –783/+129 fragment of the rat CYP3A9 promoter, and a mammalian expression vector for vitamin D receptor. The cells were dosed with vehicle or the indicated concentrations of $1,25(OH)_2D_3$ or 19nor-$1,25(OH)_2D_2$. Cells were collected in Reporter Lysis Buffer, assayed for luciferase activity, and normalized for β-galactosidase activity. The data are shown as fold induction of analog- over vehicle-treated cells (mean ± S.E.M.). The differences between the two analogs were significant at $p<0.05$ (*) and $p<0.01$ (**).

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Figure 8. The promoter fragment from –726 to –783 responds better to 19nor-1,25$(OH)_2D_2$ than 1,25$(OH)_2D_3$ when Caco-2 cells are transfected with low levels of vitamin D receptor expression vector. (**A**) Caco-2 cells were transiently transfected with the indicated CYP3A9 promoter constructs, and a mammalian expression vector for vitamin D receptor at low levels (see Material and Methods). The cells were dosed with vehicle or the indicated concentrations of 1,25$(OH)_2D_3$ or 19nor-1,25$(OH)_2D_2$. (**B**) Caco-2 cells were transiently transfected with the –727 to –783 fragment of the CYP3A9 promoter controlling the SV40 promoter, and a mammalian expression vector for vitamin D receptor at low levels (see Material and Methods). The cells were dosed with vehicle or $5x10^{-9}$ M of 1,25$(OH)_2D_3$ or 19nor-1,25$(OH)_2D_2$. Cells were collected in Reporter Lysis Buffer, assayed for luciferase activity, and normalized for β-galactosidase activity. The data are shown as fold induction of analog- over vehicle-treated cells (mean ± S.E.M.). The difference in fold induction between the two analogs was significant at $p<0.05$ (*) or $p<0.01$ (**).

Figure 9. Electrophoretic mobility shift assays of the distal VDREs. Double stranded oligonucleotides of the sequence (**A**) –726 to –750 and (**B**) –754 to –775 of the CYP3A9 promoter were radiolabelled and incubated with pig intestinal nuclear extract as described in Material and Methods. Competition with excess unlabelled oligonucleotides (50ng) was used to identify the specificity of the complexes. Antibodies to the vitamin D receptor (0.5 μg) were used to show the presence of the receptor in the complexes. MOPC21 antibody was used as a nonspecific control.

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Figure 10. Mutations in the native promoter at the site of the distal VDRES eliminate the increased response to 19nor-1,25$(OH)_2D_2$ when Caco-2 cells are transfected with low levels of vitamin D receptor expression vector. Caco-2 cells were transiently transfected with the indicated CYP3A9 promoter constructs, and a mammalian expression vector for vitamin D receptor at low levels (see Material and Methods). The cells were dosed with vehicle or $4x10^{-9}$ M of 1,25$(OH)_2D_3$ or 19nor-1,25$(OH)_2D_2$. Cells were collected in Reporter Lysis Buffer, assayed for luciferase activity, and normalized for β-galactosidase activity. The data are shown as fold induction of analog- over vehicle-treated cells (mean ± S.E.M.). The difference in fold induction between the two analogs was significant at $p<0.01$ (**).

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Figure 1

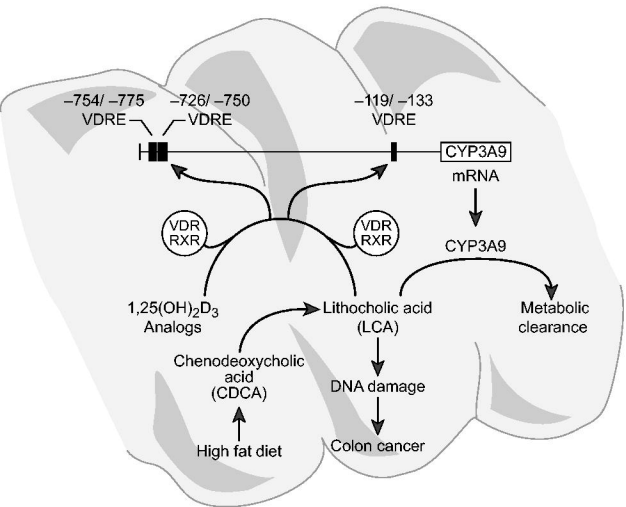
–754/ –775
VDRE
–726/ –750
VDRE
–119/ –133
VDRE
CYP3A9
mRNA
VDR
RXR
VDR
RXR
CYP3A9
1,25(OH)2D3
Analogs
Lithocholic acid
(LCA)
Metabolic
clearance
Chenodeoxycholic
acid
(CDCA)
DNA damage
High fat diet
Colon cancer

Figure 2
Zierold et al.

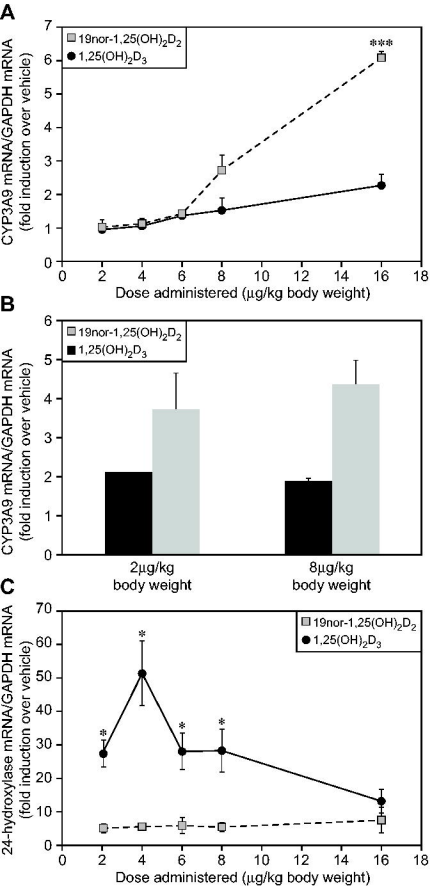

Figure 3
Zierold et al.

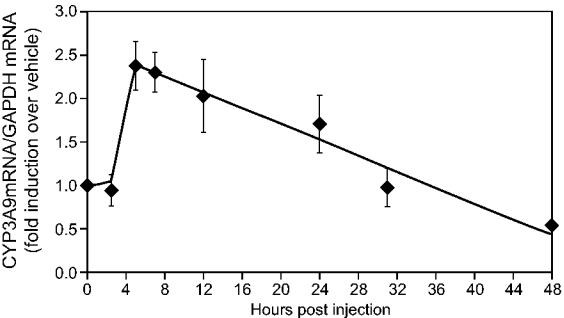

Figure 4
Zierold et al.

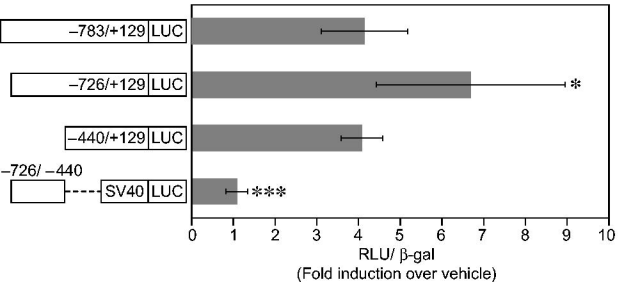

Figure 5
Zierold et al.

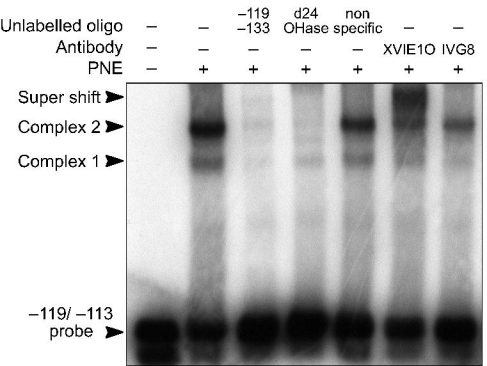

Figure 6
Zierold et al.

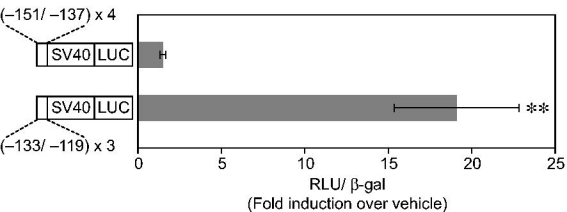

Figure 7
Zierold et al.

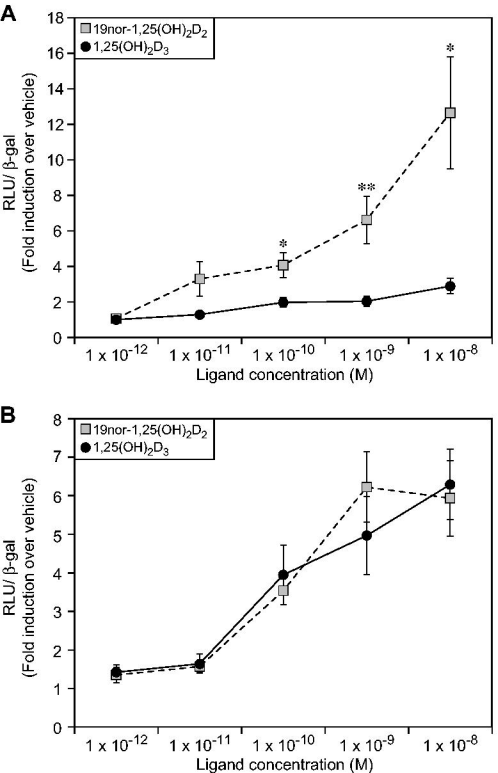

Figure 8
Zierold et al.

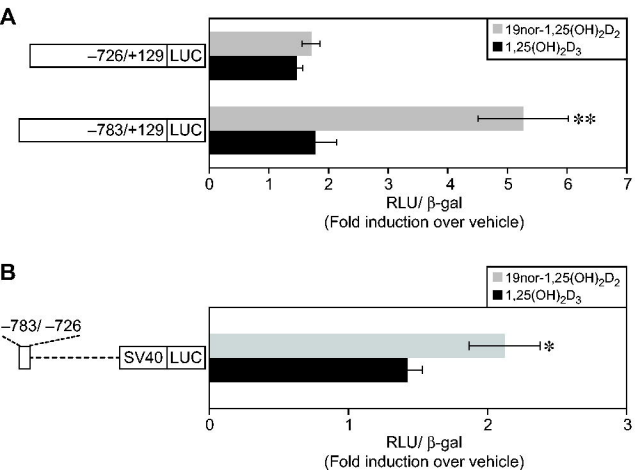

Figure 9
Zierold et al.

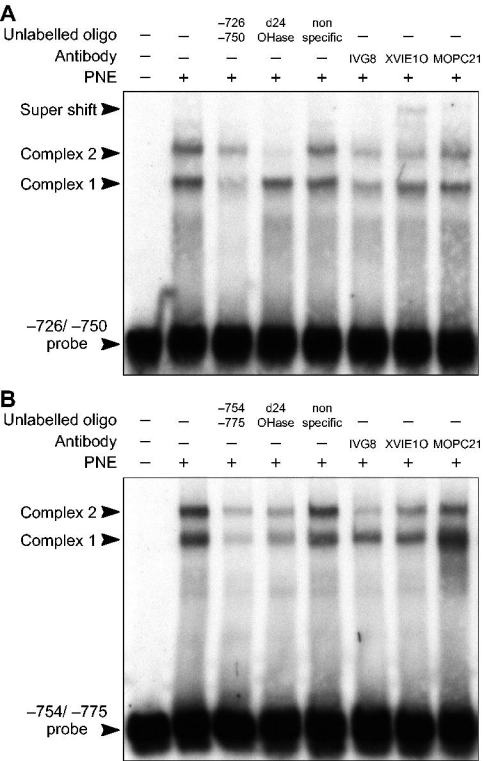
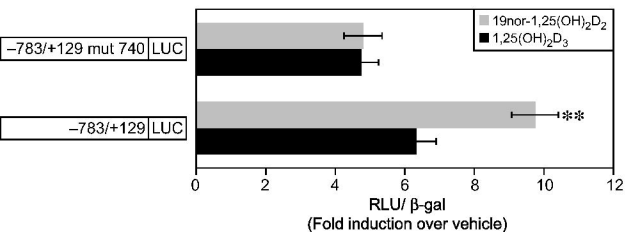
Figure 10
Zierold et al.