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**Mechanism of Vitamin D receptor inhibition of cholesterol 7 $\alpha$ -hydroxylase gene  
transcription in human hepatocytes**

Shuxin Han and John Y. L. Chiang

Department of Integrative Medical Sciences

Northeastern Ohio Universities Colleges of Medicine and Pharmacy, Rootstown, OH 44272

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*Running title:* VDR regulation of CYP7A1

*Correspondence address:* Dr. John Y. L. Chiang, Department of Integrative Medical Sciences, Northeastern Ohio Universities Colleges of Medicine and Pharmacy, 4209 State Route 44, Rootstown, OH 44272, Tel: 330-325-6694, Fax: 330-325-5910. e-mail: [jchiang@neoucom.edu](mailto:jchiang@neoucom.edu)

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*Abbreviations used:* BARE, bile acid response element; ChIP, chromatin immunoprecipitation; CYP7A1, cholesterol 7 $\alpha$ -hydroxylase; CYP27B1, sterol 1 $\alpha$ -hydroxylase; CYP24A1, sterol 24-hydroxylase; 1 $\alpha$ , 25-(OH)<sub>2</sub>-VD<sub>3</sub>: 1 $\alpha$ , 25-dihydroxy-vitamin D<sub>3</sub>; GRIP-1, glucocorticoid receptor interacting protein-1; HNF4 $\alpha$ , hepatocyte nuclear factor 4 $\alpha$ ; LCA, lithocholic acid; NCoR-1, nuclear receptor co-repressor-1; PGC-1 $\alpha$ , peroxisome proliferators activator receptor  $\gamma$  coactivator 1 $\alpha$ ; SMRT, silencing mediator of retinoid and thyroid receptors; SRC-1, steroid receptor coactivator-1; VDR, vitamin D receptor.

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## Abstract

Lithocholic acid (LCA) is a potent endogenous vitamin D receptor (VDR) ligand. In cholestasis LCA levels increase in the liver and intestine. The objective of this study is to test the hypothesis that VDR plays a role in inhibiting cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) gene expression and bile acid synthesis in human hepatocytes. Immunoblot analysis has detected VDR proteins in the nucleus of the human hepatoma cell line HepG2 and human primary hepatocytes. 1 $\alpha$ , 25-dihydroxy-vitamin D<sub>3</sub> (1 $\alpha$ , 25-(OH)<sub>2</sub>-VD<sub>3</sub>) or LCA-acetate activated VDR inhibited CYP7A1 mRNA expression and bile acid synthesis, whereas siRNA to VDR completely abrogated VDR inhibition of CYP7A1 mRNA expression in HepG2 cells. Electrophoretic mobility shift assay and mutagenesis analyses have identified the negative VDR response elements that bind VDR/RXR $\alpha$  in the human CYP7A1 promoter. Mammalian two-hybrid, co-immunoprecipitation, GST pull down, and chromatin immunoprecipitation (ChIP) assays show that ligand-activated VDR specifically interacts with hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) to block HNF4 $\alpha$  interaction with co-activators or to compete with HNF4 $\alpha$  for co-activators or to compete for binding to CYP7A1 chromatin, which results in the inhibition of CYP7A1 gene transcription. This study shows that VDR is expressed in human hepatocytes and may play a critical role in the inhibition of bile acid synthesis thus protecting liver cells during cholestasis.

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## Introduction

Cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) is the initial and rate-limiting enzyme in the bile acid synthesis pathway in the liver. Bile acids are metabolites of cholesterol and are required for intestinal absorption and transport of lipid soluble vitamins, fats and steroids and disposal of toxic metabolites, drugs, and xenobiotics. Recent studies have established the critical roles of bile acids in the regulation of lipid, glucose and drug metabolism (Chiang, 2003). Bile acids are highly toxic molecules that cause cholestasis and colon cancer if accumulated in high amounts. Bile acid synthesis is regulated by the bile acid feedback mechanism that inhibits CYP7A1 gene transcription (Chiang, 2003). Recent studies have identified three bile acid-activated nuclear receptors, farnesoid X receptor (FXR, NR1H4), pregnane X receptor (PXR, NR1I2), and vitamin D<sub>3</sub> receptor (VDR, NR1I1) (Chiang, 2005). Among all bile acids tested, chenodeoxycholic acid (CDCA) is the most efficacious FXR ligand that induces a negative nuclear receptor, small heterodimer partner (SHP, NR0B2) to inhibit CYP7A1 gene transcription (Goodwin et al., 2000). More recent studies suggest that FXR induces fibroblast growth factor 15 (FGF15) in intestine, which activates liver FGF receptor 4 (FGFR4) signaling to inhibit CYP7A1 and bile acid synthesis (Holt et al., 2003; Inagaki et al., 2005; Kim et al., 2007). The xenobiotic receptor PXR is activated by the secondary bile acid, lithocholic acid (LCA) in the liver and intestine to induce phase I drug-metabolizing cytochrome P450 enzymes, phase II drug conjugation enzymes, and phase III drug transporters (Staudinger et al., 2001; Sonoda et al., 2002; Stedman et al., 2004; Zollner et al., 2006). LCA is also an efficacious VDR ligand (Makishima et al., 2002), which activates VDR at lower concentrations than PXR. VDR induces CYP3A4 (Drocourt et al., 2002) and sulfotransferase 2A1 (SULT2A1) (Echchgadda et al., 2004) in human hepatocytes and intestine cells. LCA is relatively non-toxic in rats and mice as the livers of these

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species are able to efficiently hydroxylate LCA for renal excretion (Hofmann, 2004).

Detoxification of LCA in human livers is mainly through sulfo-conjugation for biliary excretion.

During cholestasis, sulfonation of LCA is impaired and hepatic LCA levels are increased and may contribute to liver injury (Fischer et al., 1996).

VDR is activated by  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$ , an active form of vitamin D<sub>3</sub>, and plays critical roles not only in calcium and phosphate homeostasis and bone metabolism but also other physiological functions including immunomodulation, cell growth and differentiation (Norman, 2006). VDR is located in the cytosol. Upon binding of a ligand, VDR is translocated from the cytosol into the nucleus (Michigami et al., 1999), where VDR forms a heterodimer with retinoid X receptor  $\alpha$  (RXR $\alpha$ ) and binds to the response elements consisting of AGGTCA-like direct repeat sequences spaced by 3 or 4 nucleotides (DR3, DR4) or everted repeats with 6 nucleotide spacing (ER6) in the CYP3A4 gene promoters (Drocourt et al., 2002). VDR is abundantly expressed in kidney, intestine and bone, but expressed at low levels in most other tissues. It has been reported that VDR mRNA and protein are expressed in rat livers (Segura et al., 1999). In rat livers, VDR mRNA and protein are expressed mostly in non-parenchymal (Kupffer and stellate cell) and biliary epithelial cells (Gascon-Barre et al., 2003). Several studies show that mouse livers do not express VDR mRNA (McCarthy et al., 2005; Bookout et al., 2006). Expression of VDR in human livers has been reported only in one study (Berger et al., 1988).

Activation of vitamin D<sub>3</sub> (cholecalciferol) is initiated in the liver where sterol 25-hydroxylase (CYP2R1) converts vitamin D<sub>3</sub> to 25-hydroxyvitamin D<sub>3</sub>, which is then converted to the active form  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  by sterol  $1\alpha$ -hydroxylase (CYP27B1) mainly in the kidney (Sakaki et al., 2005). Sterol 24-hydroxylase (CYP24A1) converts  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  to  $1\alpha, 24$ ,

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25-trihydroxy-vitamine D<sub>3</sub> in the kidney, which is inactive and is excreted into urine. VDR feedback inhibits CYP27B1 and feed forwardly activates CYP24A1 gene transcription to maintain vitamin D<sub>3</sub> homeostasis (Lechner et al., 2007). In this study we identified VDR mRNA and protein in human hepatocytes and explored the potential role and mechanism of LCA-activated VDR in mediating bile acid feedback inhibition of CYP7A1 and bile acid synthesis in human hepatocytes.

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## Materials and methods

**Cell Culture.** The human hepatoblastoma cell line HepG2, the human colon adenocarcinoma cell line Caco2, and the human embryonic kidney cell line HEK293 were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured as described previously (Li and Chiang, 2005). Primary human hepatocytes were isolated from human donors and were obtained through the Liver Tissue Procurement and Distribution System of the National Institutes of Health (Dr. S. Strom, University of Pittsburgh, Pittsburgh, PA). Cells were maintained in Hepatocyte Maintenance Medium as described previously (Li and Chiang, 2005).

**Reporter and Expression Plasmids.** Human CYP7A1-Luc reporters (phCYP7A1/-298, phCYP7A1/-185, phCYP7A1/-150, phCYP7A1/-135, and phCYP7A1/-80) were constructed as previously described (Crestani et al., 1995; Wang et al., 1996). The reporters with mutations in the BARE-I (mBARE-I) or BARE-II (mBARE-II) were constructed as previously described (Li and Chiang, 2005). The mutant reporter with both BARE-I and -II mutated (mBARE-I&-II) was constructed by introducing the mutated BARE-I sequence into the mutant reporter mBARE-II using PCR-based Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). For construction of p3XBARE-II-TK-Luc, three copies of synthetic double-stranded BARE-II oligonucleotide (TGTGGACTTGTTC AAGGCCAG) with Bam HI and Hind III restriction sites built in at the 5 and 3' end, respectively, was ligated upstream to a thymidine kinase (TK) minimum promoter-Luciferase plasmid (pTK-Luc, Promega, Madison, WI). The heterologous promoter reporter p5XUAS-TK-Luc, VP16-SMRT, VP16-NCOR-1 and VP16-SRC-1 were kindly provided by Dr. A. Takeshita (Toranomon Hospital, Tokyo, Japan), Gal4-VDR by P. McDonald (Case Western Reserve University, Cleveland, OH), VP16-HNF4 $\alpha$  by David Moore (Baylor College of Medicine, Houston, TX). Expression plasmids for human VDR

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(pcDNA3.1/VDR) were provided by Y.C. Li (University of Chicago, Chicago, IL), human PGC-1 $\alpha$  (pcDNA3/HA-PGC-1 $\alpha$ ) by A. Kralli (The Scripps Research Institute, La Jolla, CA), and pCMV-HNF4 $\alpha$  were previously described (Crestani et al., 1998).

**Transient Transfection Assay.** HepG2 cells were grown to about 80% confluence in 24-well tissue culture plates, and treated with LCA-acetate (Steraloids, Newport, RI) or 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  (Cayman Chemicals, Ann Arbor, MI). LCA-acetate is a non-toxic LCA derivative, which activates VDR with 30-fold higher efficacy than LCA and does not activate PXR or FXR (Adachi et al., 2005). In this study we used LCA-acetate instead of LCA to activate VDR. Luciferase reporters and expression plasmids were transfected into HepG2 cells using Tfx-20 reagent (Promega) following manufacturer's instructions. Luciferase reporter assays were performed as previously described (Crestani et al., 1995). Assays were performed in duplicates and each experiment was repeated at least four times. Data were plotted as means  $\pm$  standard deviation (SD).

**Bile Acid Assay.** Primary human hepatocytes or HepG2 cells were maintained in serum-free media overnight followed by the treatment with vehicle (EtOH), or 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  (100 nM) for 24 hours. Cell culture media were collected and slowly passed through a Sep-Pak C18 reversed-phase cartridge (Waters Associates, Inc., Milford, MA), which were then washed with 8 ml of water and 2 ml of 1.5% EtOH. Bile acids were eluted from the Sep-Pak C18 with 4 ml of methanol. The solutions were evaporated to dryness at 37 °C. Total bile acids were analyzed by 3-hydroxysteroid dehydrogenase method using the Total Bile Acid Assay kit (Bio-quant Inc., San Diego, CA) according to the manufacturer's instruction.



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***Mammalian two-hybrid assays.*** The reporter plasmid p5xUAS-TK-Luc containing five copies of the upstream activating sequence (UAS) fused to the upstream of the TK promoter and the luciferase reporter gene was used for mammalian two-hybrid assays. GAL4-VDR was co-transfected with VP16-SRC-1, VP16-HNF4 $\alpha$ , VP16-SMRT, or VP16-NCoR-1 into HEK293 cells, and reporter activity was assayed as described above.

***RNA Isolation and Quantitative Real-time PCR (Q-PCR).*** Primary human hepatocytes were maintained in serum-free media overnight. Cells were treated with 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  or LCA-acetate in the amounts and times indicated. Total RNA was isolated using Tri-Reagent (Sigma, St. Louis, MO). Reverse-transcription (RT) reactions were performed using RETROscript kit (Ambion, Austin, TX). Q-PCR assays of relative mRNA expression were performed as previously described (Li and Chiang, 2005) using an ABI PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA). TaqMan PCR primers and probes were ordered from TaqMan Gene Expression Assays (Applied Biosystems): CYP7A1 (Hs00167982\_m1), CYP27A1 (Hs00168003\_m1), CYP24A1 (Hs00167999\_m1), VDR (Hs00172113\_m1) and UBC (Hs00824723\_m1), and mouse VDR (Mm00437297\_m1) and UBC (Mm01201237\_m1). Data were analyzed by the Sequence Detector version 1.7 (Applied Biosystems). Relative mRNA expression levels were calculated by the  $\Delta\Delta$ Ct method recommended by Applied Biosystems (User Bulletin no. 2, 1997). All PCR reactions were done in duplicate and each reaction was repeated at least four times.

***Protein Extraction and Immunoblot Assay.*** Caco2, HepG2 cells and primary human hepatocytes in T75 flasks were lysed in modified RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25%-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (1 mM PMSF,

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1  $\mu\text{g/ml}$  aprotinin, 1  $\mu\text{g/ml}$  pepstatin, Sigma, St Louis, MO) for 30 min. Nuclear fractions were isolated and lysed using a Nuclear Extraction kit (Millipore, Temecula, CA). Total cell lysates or nucleus fractions were centrifuged at 10,000 g at 4 °C for 10 min and the supernatants were then pre-cleared with Protein A agarose beads. VDR was *in vitro* synthesized using a transcription and translation (TNT) lysate system as a positive control for VDR (Promega, Madison, WI). Nuclear extracts were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and antibodies against VDR,  $\beta$ -actin and lamin B (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoblotting and detected by ECL detection kit (Amersham Biosciences, UK).

***Co-immunoprecipitation (Co-IP) Assay.*** Primary human hepatocytes or HepG2 cells in T75 flasks were maintained in serum-free media overnight followed by the treatment with vehicle (EtOH), or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) for 24 hours. Cells were incubated in modified RIPA buffer containing protease inhibitors as described above for 30 min. Total cell lysates were centrifuged at 10,000 g at 4°C for 10 min and pre-cleared with protein A agarose beads. One mg of cell protein extract was incubated with 20  $\mu\text{g}$  of goat anti-HNF4 $\alpha$  antibody (Santa Cruz Biotechnology) at 4°C with rotation overnight, followed by an additional incubation for 2 h with protein G agarose beads. The beads were then washed three times with cold 1XPBS, boiled in 2 X protein loading buffer for 5 min, and then loaded on SDS-PAGE gels for immunoblot analysis using rabbit antibody against VDR (Santa Cruz Biotechnology). Thirty-five  $\mu\text{g}$  of cell protein extracts were loaded as input. Goat non-immune IgG was used as a negative control.

***Electrophoretic Mobility Shift Assay (EMSA).*** VDR, PXR, HNF4 $\alpha$  and RXR $\alpha$  were *in vitro* synthesized using the TNT lysate system (Promega). Double-stranded synthetic oligonucleotide

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probes (sequences in Fig. 4A), a VDR binding site in human Cyp3A4 gene (ER6), and bile acid response element-I (BARE-I) and BARE-II of human CYP7A1, and mutant BARE-I (M-I), and mutant BARE-II (M-II), were labeled with [ $\alpha$ <sup>32</sup>P]- dCTP for EMSA as described previously (Li and Chiang, 2005).

**GST Pull-down Assay.** GST-HNF4 $\alpha$  was expressed in *E. coli* BL21 cells. Cell extracts containing GST-HNF4 $\alpha$  fusion proteins were immobilized with glutathione beads and incubated with HepG2 cell extracts. Rabbit anti-VDR was used to detect VDR by Immunoblot analysis.

**Small Interfering RNA (siRNA).** The Accell SMART pool siRNAs for knockdown of VDR mRNA and control SMART pool were purchased from Thermo Scientific Dharmacon (Lafayette, Co) and transfected into HepG2 cells using Accell siRNA delivery media for 27 hours according to the manufacturer's instructions. Cells were treated with vehicle, 1 $\alpha$ , 25(OH)<sub>2</sub>-VD<sub>3</sub> (100 nM) or LCA-acetate (20  $\mu$ M) for 24 hours, and mRNA and proteins were extracted for analysis.

**Chromatin Immunoprecipitation (ChIP) Assay.** Primary human hepatocytes in T75 flasks or HepG2 cells in a 100 mm tissue culture dishes were maintained in serum-free media overnight followed with the treatment of vehicle (EtOH), 1 $\alpha$ , 25-(OH)<sub>2</sub>-VD<sub>3</sub> (100 nM) or LCA-acetate (20  $\mu$ M). CHIP assays were performed using ChIP assay kit (Millipore, Chicago, IL) following the manufacturer's protocol. Cells were cross-linked in 1% formaldehyde and sonicated. Protein-DNA complexes were precipitated using rabbit anti-HNF4 $\alpha$ , rabbit anti-VDR, goat anti-PGC-1 $\alpha$ , rabbit anti-GRIP-1, goat anti-NCoR-1, or rabbit anti-SMRT (Santa Cruz Biotechnology). Rabbit non-immune IgG was added as a control. The immunoprecipitated-CYP7A1 chromatin (nt-432 to -41 containing BARE-I and BARE-II) and intron 2 (nt 2485 to 2879, as a background) were

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amplified by PCR as described previously (Li and Chiang, 2007). The PCR primers used for CYP7A1 chromatin were: forward primer: 5'- ATCACCGTCTCTCTGGCAAAGCAC; reverse primer: 5'-CCATTA ACTTGAGCTTGGTTGACAAAG. The PCR primers used for intron 2 were: forward primer: 5'- GCTGGACACAATGGAACACAC; reverse primer: 5'- CTTGGTAAACACGGGAAATTGG.

Q-PCR was used to quantify ChIP assays of the CYP7A1 chromatin (nt -180 to -111, BARE-II, HNF4 $\alpha$  binding site) was described previously (Li and Chiang, 2007). The standard curves of Ct versus Log<sub>2</sub> (ng of chromatin) for both CYP7A1 and intron 5 chromatins were established using sonicated and purified chromatin from the same ChIP assay sample. The amount of chromatin immunoprecipitated with each antibody was determined from the standard curves after subtracting the background (intron 5 chromatin) and expressed as arbitrary units with vehicle-treated control as "1". TaqMan real-time PCR primers/probe sets were ordered from Applied Biosystems: BARE-II primer set (nt -180 to -111), forward primer, 5'- GGTCTCTGATTGCTTTGGAACC; reverse primer, 5'- AAAAGTGGTAGTAACTGGCCTTGAA, and the TaqMan probe: TTCTGATACCTGTGGACTTA; intron 5 primer set (nt 8127 to 8195), forward primer, 5'- TTTCTTCTGGGAACCCTTCTCTC, reverse primer, 5'- TCCTATCCTGCTTGAACGATTAGTT, and the Taqman probe: CTAGCTCTGCCTGACTAA.

**Statistical Analysis.** All results were expressed as mean  $\pm$  SD. Data were analyzed with student's t-test. The *p* values of  $< 0.05$  were considered as statistically significant difference between treated and un-treated control.

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## Results

***VDR is expressed in human hepatocytes.*** We first used an antibody against human VDR to detect VDR protein expression in primary human hepatocytes and HepG2 cells. Fig. 1A shows that immunoblot analysis detected VDR proteins in whole cell lysates isolated from these hepatocytes. Fig. 1B shows that VDR proteins were detected in the nuclear extracts after treating primary human hepatocytes with  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (50 nM). In HepG2 cells, VDR proteins were detected in the nuclei with or without  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  treatment. These data support the conclusion that VDR proteins are expressed in human hepatoma cells and in primary human hepatocytes.

***Analysis of VDR mRNA expression levels in human hepatocytes.*** We used Q-PCR assays to identify and determine VDR mRNA expression levels in primary human hepatocytes. The comparative Ct method ( $\Delta\Delta$  Ct) is widely used to assay relative mRNA expression levels in the same cells treated with different reagents. However, this method cannot be used to compare mRNA expression levels in different types of cells or species. We thus used the threshold cycle number (Ct) as an indication of relative mRNA expression levels in different cells and species. The Ct values vary from  $28.1 \pm 0.11$  to  $32.2 \pm 0.08$  in seven donor hepatocytes (Table I). The average Ct value of  $30.2 \pm 1.79$  in primary human hepatocytes is much lower than that in HepG2 cells ( $34.8 \pm 0.05$ ) indicating much higher VDR mRNA expression levels in primary human hepatocytes than in HepG2 cells (Table I). The Ct value for VDR mRNA expression is  $27.9 \pm 0.13$  in a human colon carcinoma cell line Caco2, and  $34.7 \pm 0.17$  in human embryonic kidney HEK293 cells. The Ct value for VDR in mouse livers is  $37.4 \pm 1.56$  indicating extremely low levels of VDR mRNA in mouse livers as reported (Bookout et al., 2006). Table I also shows

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the  $\Delta C_t$  values ( $C_t$  of VDR –  $C_t$  of internal standard UBC), commonly used for calculation of relative mRNA expression levels by the  $\Delta\Delta C_t$  method. The  $\Delta C_t$  value for VDR mRNA expression in human primary hepatocytes is  $10.2 \pm 1.79$ , about 16-fold higher than that in HepG2 ( $\Delta C_t = 13.9 \pm 0.06$ ), and 250-fold higher than in mouse livers ( $\Delta C_t = 18.6 \pm 0.64$ ). It should be noted that comparison of relative mRNA expression levels in different types of cells or species is valid if amplification of the amplicons is linear in different cells.

***VDR ligand inhibits bile acid synthesis in human hepatocytes.*** We then assayed the effect of  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  on total bile acids synthesized in primary human hepatocytes and HepG2 cells. Table 2 shows that  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) inhibited the amount of bile acids synthesized in primary human hepatocytes and HepG2 cells by about 47% and 33%, respectively.

***LCA-acetate and  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  inhibit human CYP7A1 mRNA expression in human hepatocytes.*** We used Q-PCR to analyze the effects of LCA-acetate on CYP7A1 mRNA expression in primary human hepatocytes. LCA-acetate is non-toxic and is 30-fold more efficacious in activation of VDR than LCA. LCA-acetate is specific in activation of VDR but not FXR and PXR (Adachi et al., 2005). Fig. 2A shows that LCA-acetate at 20  $\mu\text{M}$  markedly reduced CYP7A1 mRNA expression levels in a time-dependent manner. Fig. 2B shows that LCA-acetate inhibits the relative CYP7A1 mRNA expression levels in a dose-dependent manner. Similarly  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  also inhibited CYP7A1 mRNA expression in a dose-dependent manner (Supplemental Fig. S1). However, LCA-acetate did not affect sterol 27-hydroxylase (CYP27A1) mRNA expression levels (Fig. 2A and Fig. 2B). We also assayed the effect of  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  on mRNA expression of sterol 24-hydroxylase (CYP24A1), which is a VDR up-

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regulated gene. Fig. 2C shows that CYP24A1 mRNA expression levels in primary human hepatocytes were markedly induced by LCA-acetate (Fig. 3C) or  $1\alpha, 25(\text{OH})_2\text{-VD}_3$  (supplemental Fig. S1) by 300- to 400- fold in 12 hours. These data suggest that VDR specifically inhibits CYP7A1 expression in human hepatocytes and that CYP24A1 is highly induced by LCA-acetate and  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  in human hepatocytes.

***Knockdown of VDR by siRNA increased CYP7A1 mRNA expression.*** To further confirm that VDR plays a role in inhibiting CYP7A1 expression, SMART pool siRNA to VDR was used to knockdown VDR expression to assay its effect on CYP7A1 mRNA expression. The SMART pool siRNA to VDR completely abolished VDR protein (Fig 3A) and mRNA expression (Fig 3B) in HepG2 cells. The SMART pool siRNA to VDR prevented  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  and LCA-acetate inhibition of CYP7A1 mRNA expression (Fig 3C). The SMART pool siRNA to VDR inhibited  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  and LCA-acetate induction of CYP24A1 (Fig 3D) and had no effect on CYP27A1 (Fig 3E) mRNA expression. These data further support the finding that ligand-activated VDR specifically inhibited CYP7A1 expression in hepatocytes.

***The negative VDR response elements are localized in the BARE-I and BARE-II of the human CYP7A1 gene promoter.*** Previous studies from our laboratory have identified two bile acid response elements, BARE-I and BARE-II that are essential for basal transcriptional activity and also for conferring bile acid feedback inhibition (Chiang, 2002). These elements contain several AGGTCA-like repeating sequences, which are potential binding sites for nuclear receptors. The BARE-II is a conserved 18 bp sequence in all species, which contains a direct repeat with one-base spacing (DR1) sequence for HNF4 $\alpha$  binding. We performed transient transfection assay using human CYP7A1 promoter/Luciferase (luc) reporter constructs. A series of 5' deletion mutant constructs of CYP7A1/Luc reporter were co-transfected with VDR

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expression plasmid into HepG2 cells and treated with LCA-acetate (5  $\mu$ M) or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (5 nM). Fig. 4 shows that these two VDR ligands strongly inhibited the luciferase reporter activity of ph-298-Luc, ph-185 and ph-150 that contain both the BARE-I and BARE-II sequences. We also mutated either the BARE-I or BARE-II sequence in the ph-298 reporter plasmid. These two mutant reporters had much lower reporter activities than the wild type ph-298Luc, and VDR ligands inhibited mutant reporter activities by about 50%. The reporter with both BARE-I and BARE-II mutated was not inhibited significantly by VDR ligands. These results suggest that both BARE-I and BARE-II are responsive to VDR inhibition.

***VDR binds to human CYP7A1 promoter.*** To test if VDR binds to the BARE-I and BARE-II sequences, we performed EMSA using oligonucleotide probes designed based on the BARE-I and BARE-II sequences of the human CYP7A1 gene (Fig. 5A). Fig. 5B shows that TNT lysates programmed with both VDR and RXR $\alpha$  expression vectors shifted the BARE-I probe. When TNT lysates programmed with either VDR or RXR $\alpha$  were used for EMSA, no band shift was observed. A mutant BARE-I probe (M-I) did not bind VDR/RXR $\alpha$ . To study the specificity of VDR binding, an unlabeled BARE-I probe (B-I), but not mutant BARE-I probe (M-I) was able to compete out VDR/RXR $\alpha$  binding. An anti-VDR antibody partially shifted the VDR/RXR $\alpha$ /DNA complex. The PXR/RXR $\alpha$  heterodimers bound to the BARE-I probe as we reported previously (Li and Chiang, 2005). As a positive control for VDR binding, an ER6 probe designed according to a well-characterized VDR response element in the CYP3A4 gene strongly bound VDR/RXR $\alpha$ . Fig. 5C shows EMSA using the BARE-II sequence as a probe. VDR/RXR $\alpha$  was able to bind to the BARE-II probe, but not the mutant BARE-II (M-II) probe. Addition of 50-fold excess of un-labeled ER6 probe could compete out VDR/RXR $\alpha$  binding to



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the BARE-II probe. These assays indicate that the VDR/RXR $\alpha$  heterodimer binds to both BARE-I and BARE-II of the human CYP7A1 promoter.

***VDR inhibits HNF4 $\alpha$  and PGC-1 $\alpha$  co-activation of the human CYP7A1 gene.*** Previous studies have established that HNF4 $\alpha$  binds to the BARE-II sequence and regulates CYP7A1 gene transcription and a co-activator PGC-1 $\alpha$  stimulates HNF4 $\alpha$  activity (Stroup and Chiang, 2000). Fig. 6A shows that co-transfection with VDR/RXR $\alpha$  strongly inhibited CYP7A1 reporter activity in HepG2 cells only when 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  was added. We constructed a heterologous promoter luciferase construct (p3XBARE-II-TK-Luc) that contains three copies of the human CYP7A1 BARE-II inserted upstream of the TK promoter for reporter assay. Fig. 6B shows that the 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$ -activated VDR strongly inhibited the heterologous reporter activity stimulated by HNF4 $\alpha$  and PGC-1 $\alpha$  in HepG2 cells. These results suggest that the ligand-activated VDR may either compete with HNF4 $\alpha$  for PGC-1 $\alpha$  (squenching effect), or interact with HNF4 $\alpha$  to block HNF4 $\alpha$  interaction with PGC-1 $\alpha$ , or compete with HNF4 $\alpha$  for binding to the BARE-II, which results in inhibition of CYP7A1 gene transcription.

***VDR interacts with HNF4 $\alpha$ .*** To test the possibility that VDR may directly interact with HNF4 $\alpha$ , we performed mammalian two-hybrid assay to study the interaction of VDR with HNF4 $\alpha$ . Fig. 7A shows that VP16-HNF4 $\alpha$  interacts with GAL4-VDR and stimulates GAL4 reporter activity in HEK293 cells when 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  or LCA-acetate was added. As a positive control, VP-16-SRC-1 strongly interacts with GAL4-VDR and stimulates GAL4 reporter activity in the presence of 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  or LCA-acetate. The interaction of VP-16-NCOR-1 or VP16-SMRT fusion protein with GAL4-VDR was weak as compared with VP16. We then performed a cell-based co-immunoprecipitation (Co-IP) assay for the protein-protein

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interaction between HNF4 $\alpha$  and VDR. The antibody against human HNF4 $\alpha$  was added to the protein extracts from HepG2 or primary human hepatocytes treated with vehicle (ethanol) or 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  (100 nM). Fig. 7B shows that VDR was co-immunoprecipitated from HepG2 and primary human hepatocytes with anti-HNF4 $\alpha$ . We also performed non-cell based GST pull-down assays for protein-protein interaction. Fig. 7C shows that the GST-HNF4 $\alpha$  fusion protein was able to pull-down VDR from HepG2 cell extracts. All three protein-protein interaction assays support the specific interaction between VDR and HNF4 $\alpha$ . The mammalian two-hybrid assay is a cell-based functional assay that shows a ligand-dependent interaction between VDR and HNF4 $\alpha$ , whereas the physical interaction assays of Co-IP and GST pull-down showed a ligand-independent interaction.

***1 $\alpha$ , 25 (OH) $_2$ -VD $_3$  increases VDR and co-repressors, and decreases co-activators recruitment to human CYP7A1 chromatin.*** We performed ChIP assays to study the effects of 1 $\alpha$ , 25 (OH) $_2$ -VD $_3$  on the association of VDR, HNF4 $\alpha$ , PGC-1 $\alpha$ , GRIP-1, NCoR-1 and SMRT to a human CYP7A1 chromatin containing both the BARE-I and BARE-II sequences (nt -432 to -41). Specific antibodies were used to immunoprecipitate chromatins from primary human hepatocytes for PCR amplification of DNA fragments. Fig. 8A (left panel) shows that HNF4 $\alpha$ , PGC-1 $\alpha$  and GRIP-1 were associated with CYP7A1 chromatin in vehicle-treated primary human hepatocytes. Upon treatment with 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$ , VDR binding was increased, whereas HNF4 $\alpha$ , PGC-1 $\alpha$  and GRIP-1 binding to chromatin was strongly reduced. A negative control of intron 2 shows no binding of these factors (Fig 8A, right panel).

We also used HepG2 cells for quantitative ChIP assays of a CYP7A1 chromatin containing the HNF4 $\alpha$  binding site (nt -180 to -111, BARE-II). Results show that LCA-acetate or

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$1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  treatment reduced HNF4 $\alpha$ , PGC-1 $\alpha$  and GRIP-1 binding by 30 to 80%, while increased NCoR-1 and SMRT binding to CYP7A1 chromatin by 2 to 3-fold (Fig. 8B). These results suggest that activation of VDR increased the recruitment of VDR and co-repressors to replace co-activators, thus resulting in inhibition of CYP7A1 gene transcription.

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## Discussion

In this study we identified VDR protein and mRNA in primary human hepatocytes. LCA-acetate or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$ -activated VDR strongly inhibited CYP7A1 mRNA expression and reduced bile acid synthesis in human hepatocytes. Furthermore, siRNA knockdown of VDR completely abrogated VDR inhibition of CYP7A1 gene expression. Our results show that VDR specifically interacts with HNF4 $\alpha$ , and the VDR/RXR $\alpha$  heterodimer binds to both the BARE-I and BARE-II sequences in the human CYP7A1 promoter. LCA and  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  increased VDR/RXR $\alpha$ , SMRT and NCoR-1 binding, and reduced HNF4 $\alpha$ , PGC-1 $\alpha$  and GRIP-1 binding to CYP7A1 chromatin. The ligand-dependent recruitment of co-repressors SMRT and NCoR-1 to CYP7A1 chromatin is consistent with a recent report that VDR ligands unmask the co-repressor interaction surface of RXR $\alpha$  to allow SMRT and NCoR-1 binding to VDR/RXR $\alpha$  (Sanchez-Martinez et al., 2008). Based on these results, we propose three possible mechanisms for VDR inhibition of CYP7A1 gene transcription. First, the VDR bound to the BARE-I may interact with the HNF4 $\alpha$  bound to the BARE-II and result in blocking SRC-1, GRIP-1 and PGC-1 $\alpha$  interaction with these two receptors. Second, VDR may compete with HNF4 $\alpha$  for binding to the BARE-II and result in inactivating the CYP7A1 gene. Third, VDR may compete with HNF4 $\alpha$  for interacting with common co-activators (squenching effect). All these mechanisms may result in recruitment of co-repressors to CYP7A1 chromatin to inhibit gene transcription.

VDR has been shown to inhibit LXR induction of the rat CYP7A1 gene (Jiang et al., 2006). VDR also interacts with FXR and inhibits the FXR target genes, SHP, bile salt export pump, and ileum bile acid binding protein (Honjo et al., 2006). These two mechanisms cannot be

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involved in regulating the human CYP7A1 gene, since human CYP7A1 is not activated by LXR (Chiang et al., 2001) and FXR indirectly inhibits CYP7A1 via SHP or FGF15 mechanisms.

A recent study shows that VDR, rather than PXR, is activated by LCA to induce CYP3A4 in the liver and intestinal cells and suggests that LCA selectively activates VDR to induce human and mouse CYP3A4 in vivo (Matsubara et al., 2008). A recent evolutionary and functional study of NR1I family receptors (VDR, PXR and constitutive androstane receptor) has found that PXR and VDR are co-expressed in diverse vertebrates from fish to mammals, and suggests that these two xenobiotic receptors may arise from duplication of an ancestral gene (Reschly and Krasowski, 2006). Interestingly, these investigators find that sea lamprey hepatocytes only express VDR, but not PXR suggesting that VDR may be the original NR1I gene (Reschly et al., 2007). It is likely that VDR may play a role in detoxification of steroids and bile acids in addition to calcium metabolism.

In cholestasis, hepatic LCA concentrations may increase to a level that activates VDR to inhibit bile acid synthesis, and to induce SULT2A1 to conjugate LCA for biliary excretion. Primary biliary cirrhosis patients have a high prevalence for bone metabolic diseases (Pares et al., 2001). VDR polymorphisms have been linked to primary biliary cirrhosis and autoimmune hepatitis (Vogel et al., 2002). VDR may play a protective role in the hepatobiliary system. It was reported recently that LCA might substitute vitamin D in increasing serum calcium and mobilizing calcium from bone in vitamin D-deficient rats (Nehring et al., 2007). LCA derivatives that specifically activate VDR without activating PXR and inducing hypercalcemia (Ishizawa et al., 2008) may be used for treating intrahepatic cholestasis and primary biliary cirrhosis.

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## Figure legends

**Fig. 1.** Immunoblot analysis of the VDR protein expression and cellular distribution in HepG2 and primary human hepatocytes. (A) Immunoblot of VDR protein expression in total cell lysates of HepG2 and primary human hepatocytes (#HH1320). In vitro synthesized human VDR protein was loaded as the molecular marker. Human primary hepatocytes were treated with vehicle (EtOH) or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (50 nM) for 6 hours.  $\beta$ -actin protein expression was monitored as the internal loading control. (B) Immunoblot of VDR in the nuclear fractions of primary human hepatocytes (#HH1391) and HepG2. Cells were treated with vehicle (EtOH) or 50 nM  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  for 6 hours. Lamin B protein levels in nuclei were monitored as the internal loading control.

**Fig. 2.** Real-time PCR assays of the effects of LCA-acetate on CYP7A1 mRNA expression in primary human hepatocytes. Data were pooled from assays using three donor hepatocytes (#HH1403, #HH1410, #HH1412). (A) Time-dependent effects of LCA-acetate (20  $\mu\text{M}$ ) on CYP7A1 mRNA expression. (B) Dose-dependent effect of LCA-acetate on CYP7A1 mRNA expression. (C) Time-dependent effect of LCA-acetate on CYP24A1 mRNA expression. (D) Dose-dependent induction of CYP24A1 mRNA expression by LCA-acetate. In time course study, primary human hepatocytes were treated with 20  $\mu\text{M}$  LCA-acetate for the time indicated. In dose-dependent study, primary human hepatocytes were treated with increasing doses of LCA-acetate for 24 hours. CYP27A1 mRNA expression was assayed as a negative control (A and B). CYP24A1 mRNA expression was assayed as a positive control of VDR induced gene (C and D). Each experiment was done in duplicate and same experiments were repeated at least five times in each donor hepatocytes.

**Fig. 3.** The siRNA knock down of VDR prevents ligand-dependent VDR inhibition of CYP7A1 expression in HepG2 cells. HepG2 cells were transfected with the SMART pool siRNA to VDR or control siRNA and incubated for 72 hours. Cells were then treated with vehicle (EtOH),  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) or LCA-acetate (20  $\mu\text{M}$ ) for 24 hours. Cells extracts were isolated for immunoblot analysis of VDR protein expression (A). Total RNA was isolated for real time PCR analysis of VDR (B), CYP7A1 (C), CYP24A1 (D), CYP27A1 (E) mRNA levels. Data show

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relative mRNA expression of VDR siRNA treated to the control siRNA treated samples. Data represent the mean  $\pm$  SD of at least three individual experiments.

**Fig. 4.** Reporter assays of the effects of  $1\alpha$ ,  $25\text{-(OH)}_2\text{-VD}_3$  and LCA-acetate on CYP7A1 luciferase reporter activities. CYP7A1 promoter deletion reporter constructs (0.2  $\mu\text{g}$ ) or empty reporter vector pGL3-basic was cotransfected with VDR (0.1  $\mu\text{g}$ ) into HepG2 cells. CYP7A1 wild type promoter reporter construct phCYP7A1/-298, reporter with mutations in the BARE-I (mBARE-I), or BARE-II (mBARE-II), or the BARE-I&II double mutation (mBARE-I&II) (0.2  $\mu\text{g}$ ) was co-transfected with VDR expression plasmid (0.1  $\mu\text{g}$ ) into HepG2 cells. The mutant sequences are shown. After transfecting for 24 hours, cells were treated with vehicle (EtOH),  $1\alpha$ ,  $25\text{-(OH)}_2\text{-VD}_3$  (5 nM) or LCA-acetate (5  $\mu\text{M}$ ) for 16 h before harvesting. The luciferase activity was normalized by  $\beta$ -gal activity. Each experiment was done in duplicate and the same experiment was repeated five times. An “\*” indicates statistically significant difference,  $p < 0.05$ ,  $1\alpha$ ,  $25\text{-(OH)}_2\text{-VD}_3$ - or LCA-acetate-treated vs. vehicle control.

**Fig. 5.** EMSA of VDR/RXR $\alpha$  binding to human CYP7A1. (A) Nucleotide sequences of the probes used in the EMSA. Arrows above sequences indicate hormone response element half-sites. Lower case letters indicate mutations. I, BARE-I; M-I, mutant BARE-I; M-II, mutant BARE-II; DR, direct repeat; ER, everted repeat. (B) EMSA of VDR/RXR $\alpha$  binding to human BARE-I probes. VDR/RXR $\alpha$  binding to CYP3A4 ER6 probe and PXR/RXR $\alpha$  binding to BARE-I were used as controls. Excess (50-fold) of unlabeled BARE-I (B-I) and mutant (M-I) probes were used as cold competitor. VDR antibody was used to form a super shift band with VDR/RXR $\alpha$  and BARE-I complex as indicated by an arrow. (C) EMSA of VDR/RXR $\alpha$  binding to human BARE II probe. VDR/RXR $\alpha$  binding to CYP3A4 ER6 probe and HNF4 $\alpha$  binding to BARE-II were used as controls. Excess (50-fold) of unlabeled ER6 probes were used as cold competitor. Each EMSA binding reaction contained 2  $\mu\text{l}$   $\alpha$ - $\text{P}^{32}$ -labeled probes ( $2 \times 10^4$  cpm) incubated with in vitro synthesized proteins (TNT lysate) for 20 min before loaded into the gel.

**Fig. 6.** Reporter assays of the effects of VDR on CYP7A1 gene transcription in HepG2 cells. (A) The human CYP7A1 promoter luciferase reporter, phCYP7A1/-298-Luc was used to assay the effect of HNF4 $\alpha$ , PGC-1 $\alpha$  and VDR/RXR $\alpha$  on reporter activity with or without  $1\alpha$ ,  $25\text{-(OH)}_2\text{-VD}_3$ . (B) Reporter assay of a heterologous reporter (p3XBARE II-TK-Luc) containing 3 copies

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of the BARE-II sequence of human CYP7A1 linked to TK-Luc promoter. After transfecting for 24 hours, cells were treated with EtOH or 5 nM  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  for 16 hours before harvesting. The luciferase activity was normalized by  $\beta$ -gal activity. Each experiment was done in duplicate and the same experiment was repeated four times. An “\*\*” indicated statistically significant difference,  $p < 0.05$ ,  $1\alpha, 25\text{-(OH)}_2\text{-D}_3$ -treated vs. control.

**Fig. 7.** VDR interacts with HNF4 $\alpha$ . A. Mammalian two-hybrid assay of VDR and HNF4 $\alpha$  interaction. The reporter construct p5XUAS-TK-Luc (0.2  $\mu\text{g}$ ) was transfected into HEK 293 cells. 0.1  $\mu\text{g}$  GAL4-VDR was cotransfected with 0.1  $\mu\text{g}$  VP16 empty vector, VP16-SRC-1, VP16-NCOR-1, VP16-SMRT, or VP16-HNF4 $\alpha$  into HEK293 cells. After 24 hours, cells were treated with vehicle (EtOH),  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (10 nM) or LCA-acetate (5  $\mu\text{M}$ ) for 16 hours before harvesting. The experiment was done in duplicate and the same experiment was repeated four times. An “\*\*” indicates statistically significant difference,  $p < 0.05$   $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$ - or LCA-acetate-treated vs. control. B. Co-immunoprecipitation (Co-IP) assay of HNF4 $\alpha$  and VDR interaction. Primary human hepatocytes (HH1393) and HepG2 cells were treated with vehicle (EtOH) or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) for 6 hours. Whole cell extracts were incubated with an HNF4 $\alpha$  antibody or non-immune IgG. Co-precipitated VDR was detected by a VDR antibody. C. Bacterium protein extracts containing GST-HNF4 $\alpha$  or GST were immobilized to glutathione beads for 16 hours at 4°C. GST-HNF4 $\alpha$ -bound beads were incubated with 1 mg protein extracts isolated from HepG2 cells treated with vehicle (EtOH) or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) for 24 hours at 4°C. VDR protein was detected by immunoblotting. Thirty five  $\mu\text{g}$  of HepG2 protein extract was loaded as input.

**Fig. 8.** ChIP assay of the effect of  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  on CYP7A1 chromatin structure in primary human hepatocytes and HepG2. (A) ChIP assay of CYP7A1 chromatin containing the BARE-I and BARE-II (nt -432 to -41). Primary human hepatocytes (HH1432) were treated with vehicle (EtOH) or  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) for 16 hours. An antibody against VDR, HNF4 $\alpha$ , PGC-1 $\alpha$ , GRIP-1, or non-immune IgG (IgG, as a negative control) was used to precipitate chromatin fragments. Five % cell extracts were set aside as input. PCR primers were used to amplify the



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region from -432 to -41 of *CYP7A1* promoter, and the intron 2 region of *CYP7A1* from nt 2485 to 2879 as a negative control. (B) Q-PCR assay of *CYP7A1* chromatin containing the BARE-II (nt -180 to -111, the HNF4 $\alpha$  binding site) HepG2 cells were treated with vehicle (EtOH), 1 $\alpha$ , 25-(OH) $_2$ -VD $_3$  (100 nM) or LCA-acetate (20  $\mu$ M) for 24 hours. An antibody against VDR, HNF4 $\alpha$ , PGC-1 $\alpha$ , or GRIP-1, NCoR-1, SMRT or non-immune IgG was used to precipitate chromatin fragments. TaqMan primers/probe sets were used for Q-PCR assay of the amount of transcription factor binding to the *CYP7A1* chromatin containing the HNF4 $\alpha$  binding site (nt -180 to -111) as described in the Materials and Methods section.

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Table 1. Real-time PCR assays of VDR mRNA expression in Caco2, HEK293, HepG2, primary human hepatocytes and mouse livers.

mRNA sample	VDR	
	Ct value $\pm$ SD	$\Delta$ Ct Value $\pm$ SD
Caco2	27.5 $\pm$ 0.13	7.8 $\pm$ 0.13
HEK293	34.7 $\pm$ 0.17	13.2 $\pm$ 0.17
HepG2	34.8 $\pm$ 0.05	13.9 $\pm$ 0.06
HH1318	31.5 $\pm$ 0.14	10.9 $\pm$ 0.18
HH1320	31.7 $\pm$ 0.13	12.3 $\pm$ 0.13
HH1358	30.1 $\pm$ 0.07	10.8 $\pm$ 0.09
HH1361	27.6 $\pm$ 0.06	7.8 $\pm$ 0.07
HH1363	28.1 $\pm$ 0.11	9.0 $\pm$ 0.11
HH1367	30.5 $\pm$ 0.06	8.4 $\pm$ 0.17
HH1393	32.2 $\pm$ 0.01	11.9 $\pm$ 0.01
Average human hepatocytes	30.2 $\pm$ 1.79	10.2 $\pm$ 1.76
Mouse 1	38.5 $\pm$ 0.08	19.0 $\pm$ 0.09
Mouse 2	36.3 $\pm$ 0.1	18.1 $\pm$ 0.1
Average mouse livers	37.4 $\pm$ 1.56	18.6 $\pm$ 0.64

Primary human hepatocytes were isolated from seven human donors (indicated by HH#). Two  $\mu$ g mRNA of Caco2, HEK293, HepG2, human hepatocytes or mouse livers were used for the reverse transcription (RT) reaction. Ubiquitin C (UBC) mRNA expression was monitored as the internal control. Ct values of VDR and UBC were measured by quantitative real-time PCR. Assays were done in triplicates.  $\Delta$ Ct was calculated by subtracting Ct of UBC from Ct of VDR. The standard deviation (SD) of  $\Delta$ Ct = square root [(SD of Ct of UBC)<sup>2</sup> + (SD of VDR)<sup>2</sup>].

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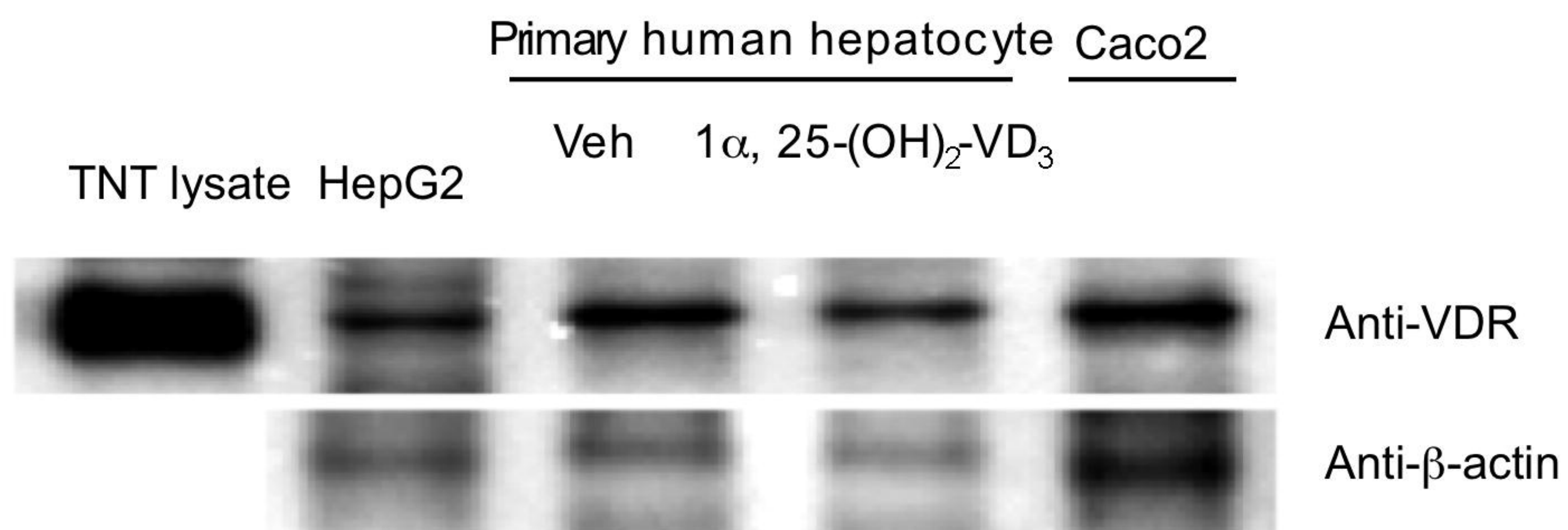
Table 2. Effect of  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  on total bile acid synthesis in HepG2 and primary human hepatocytes.

Cells	Total bile acid synthesis (% of control)
HepG2	$67.3 \pm 1.5$
HH1393	51
HH1408	48
HH1393	59
Average human hepatocytes	$52.7 \pm 5.7$

Primary human hepatocytes (#HH1393, #HH1408, #HH1410) or HepG2 cells were treated with  $1\alpha, 25\text{-(OH)}_2\text{-VD}_3$  (100 nM) for 24 hours, and total bile acids synthesized in hepatocytes were assayed and expressed as the percentage of vehicle-treated control.

Fig. 1

A



B

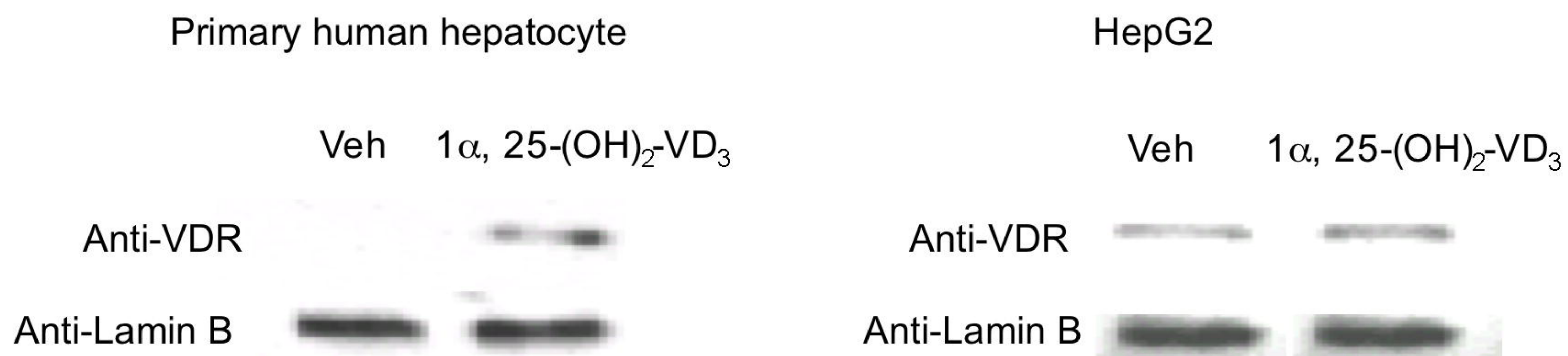
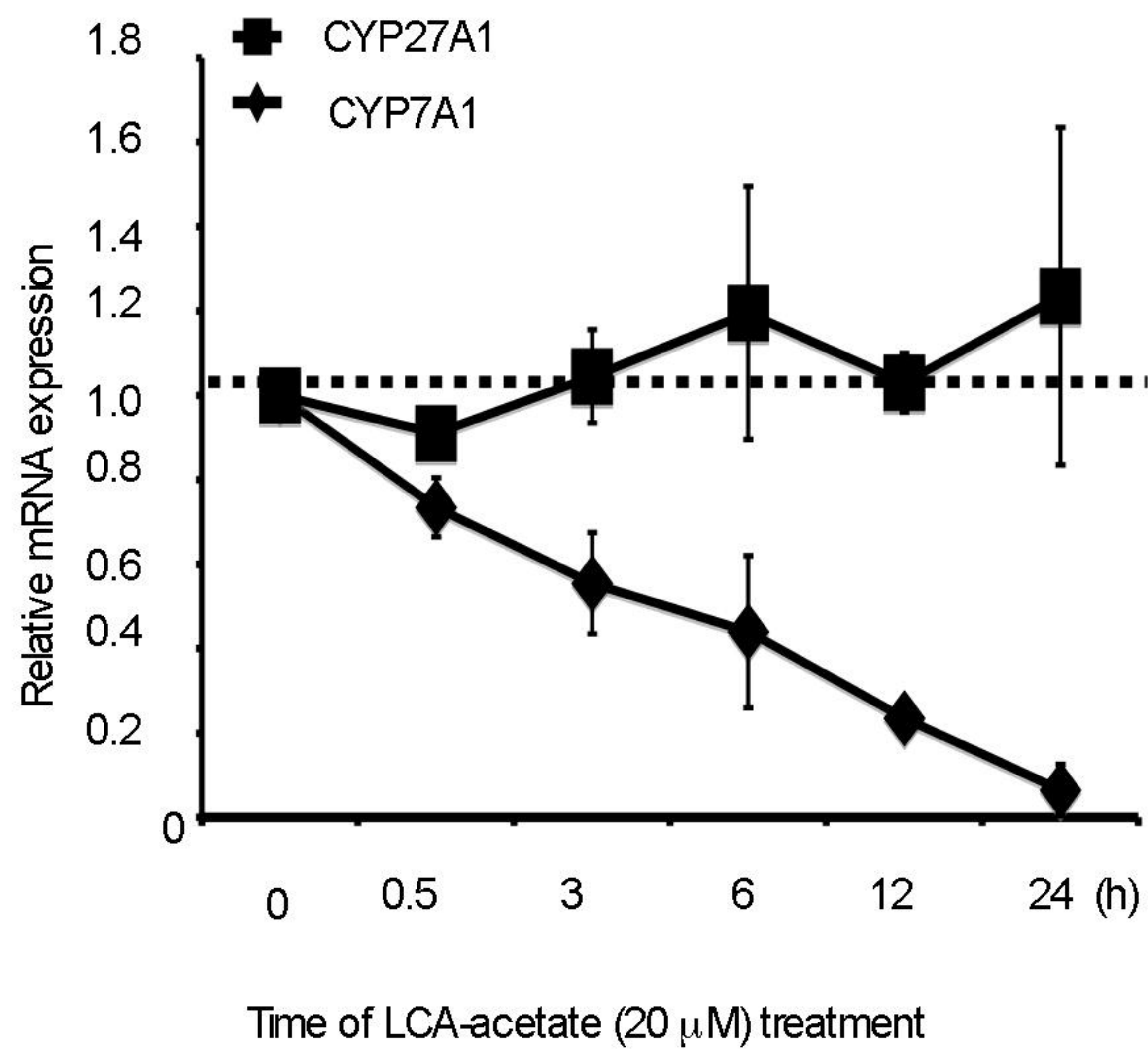
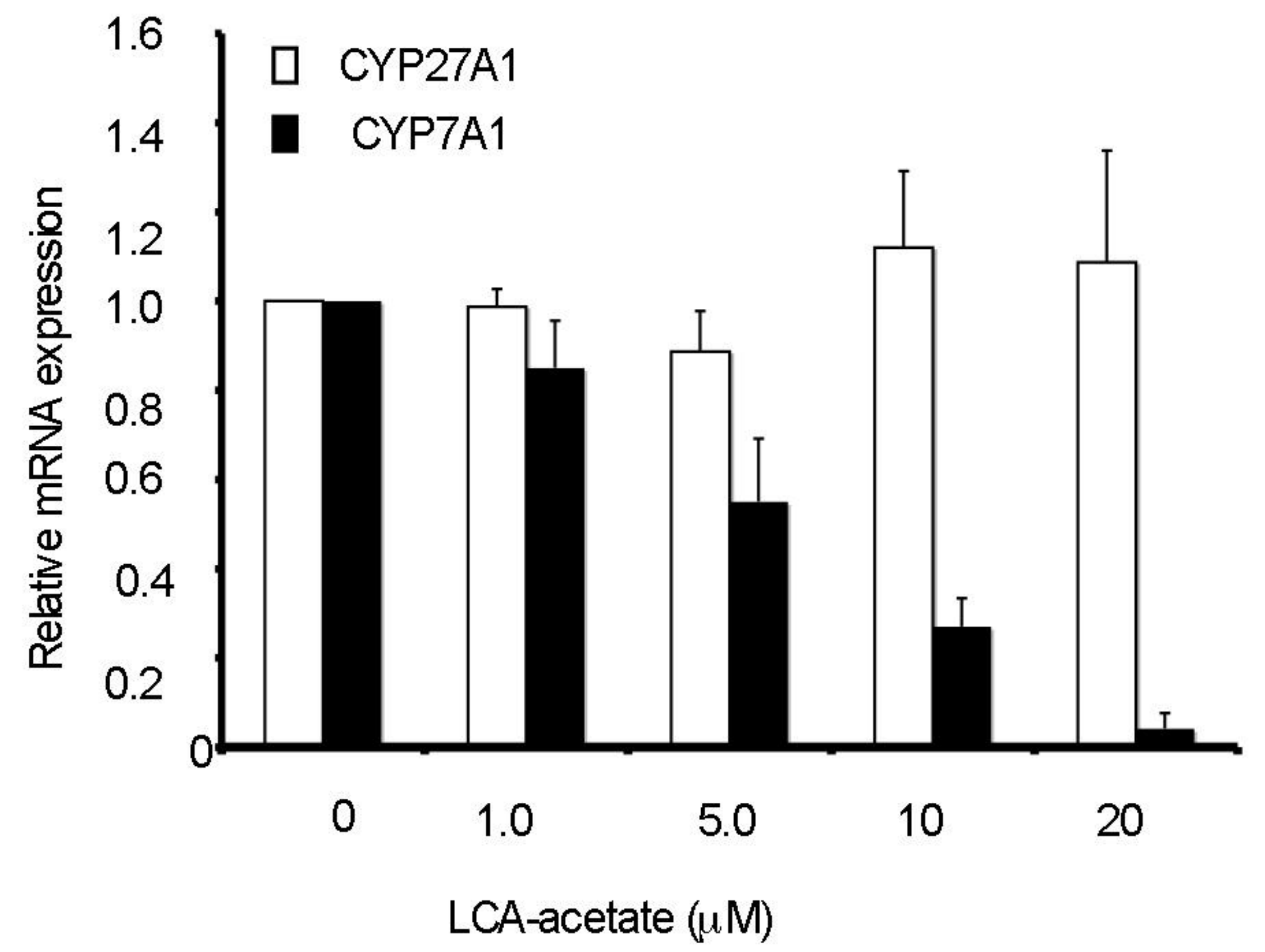


Fig. 2

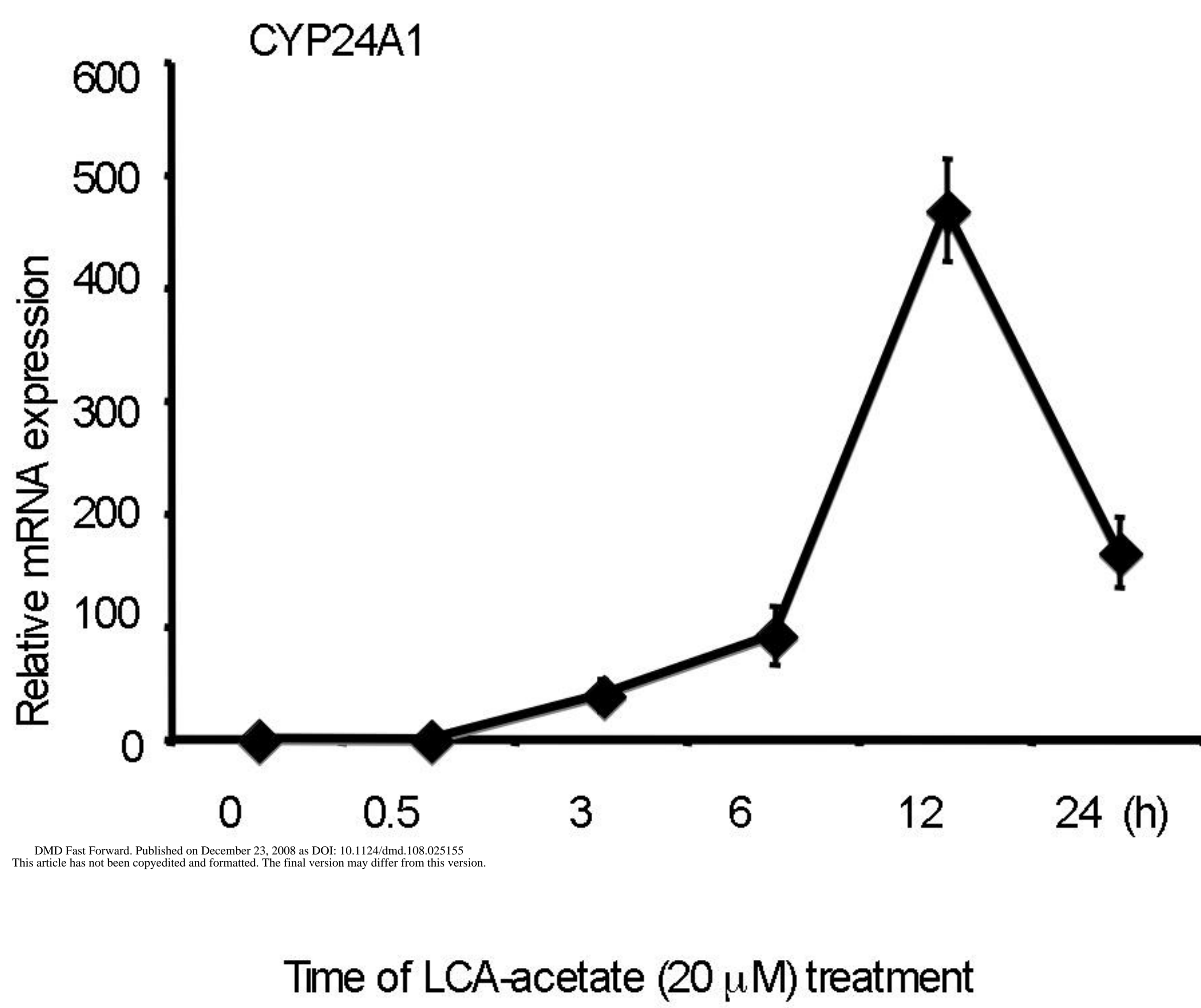
A



B



C



D

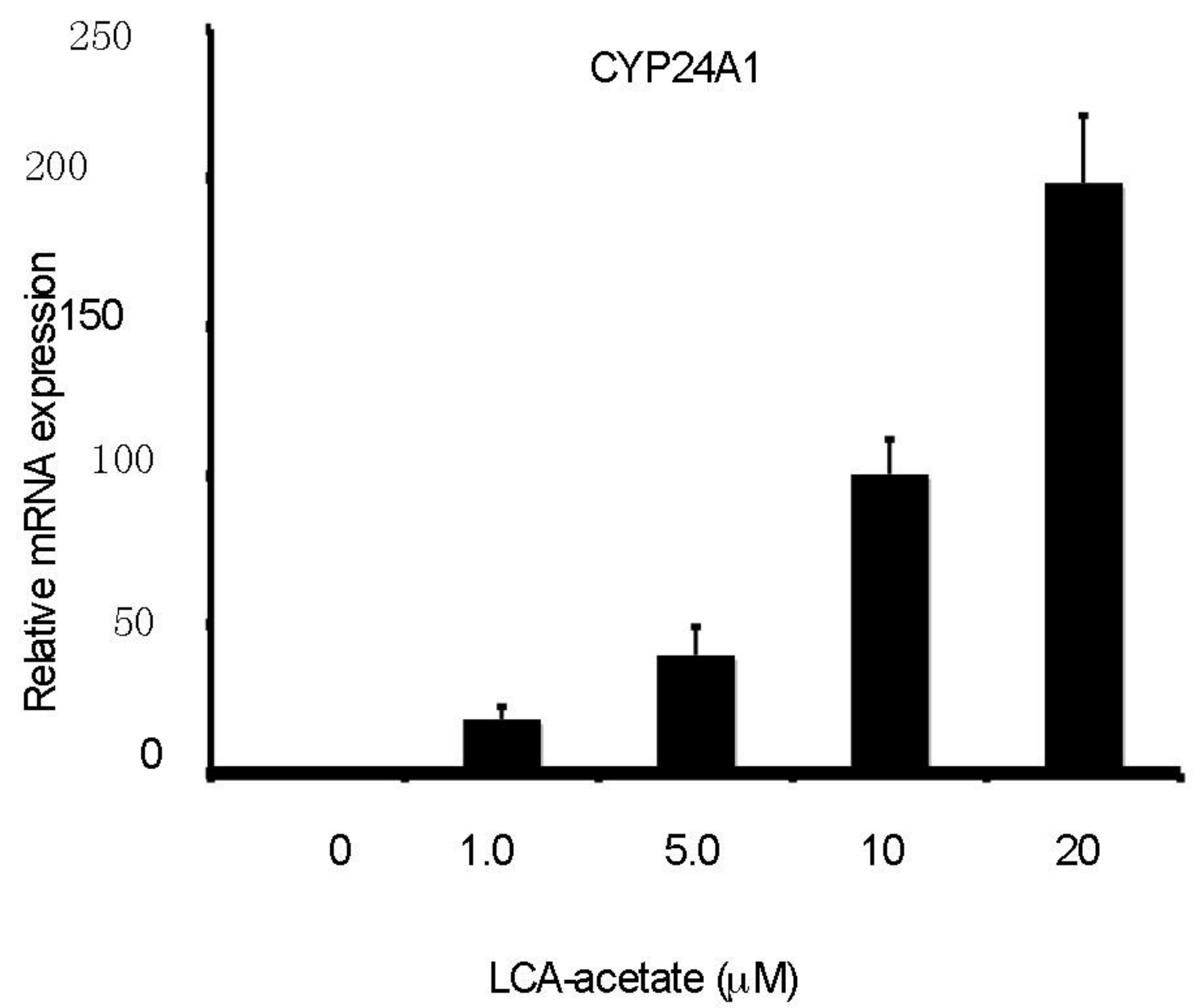
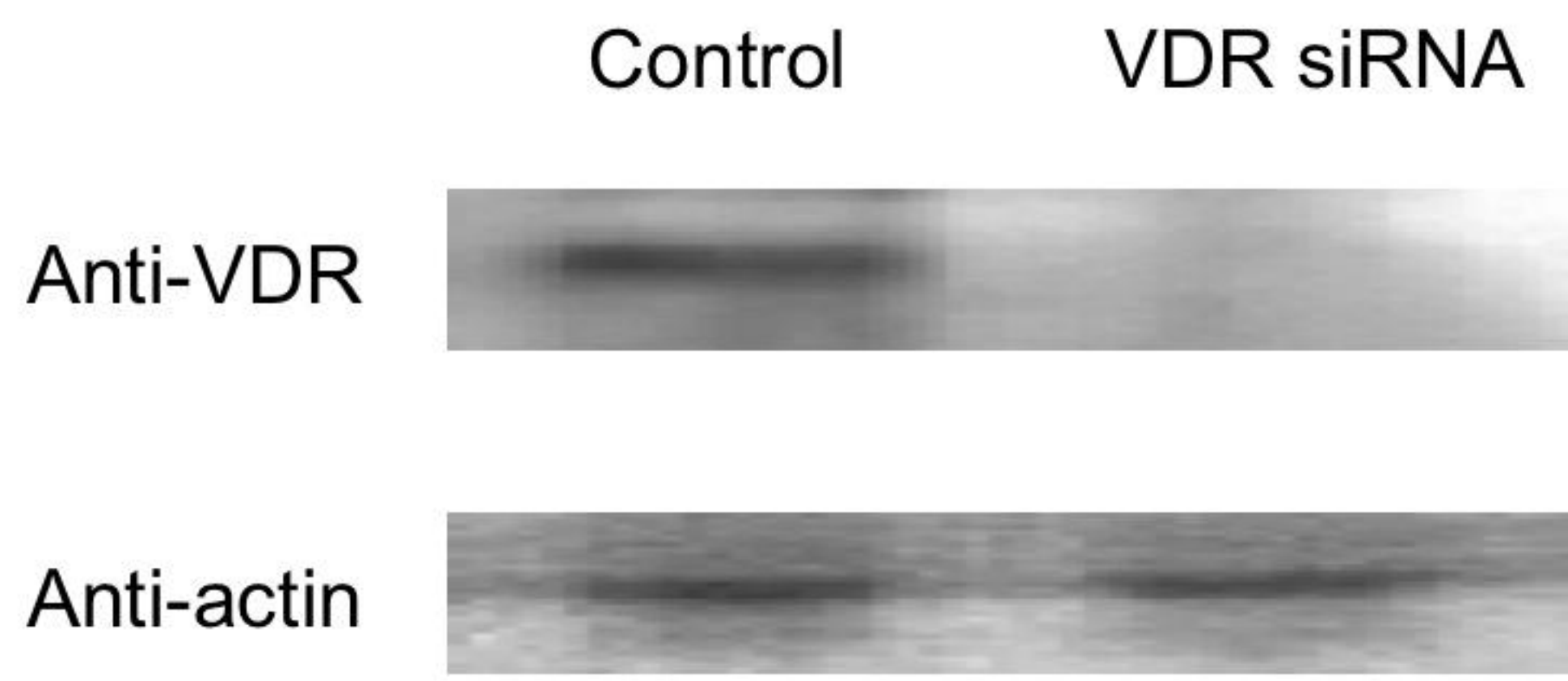
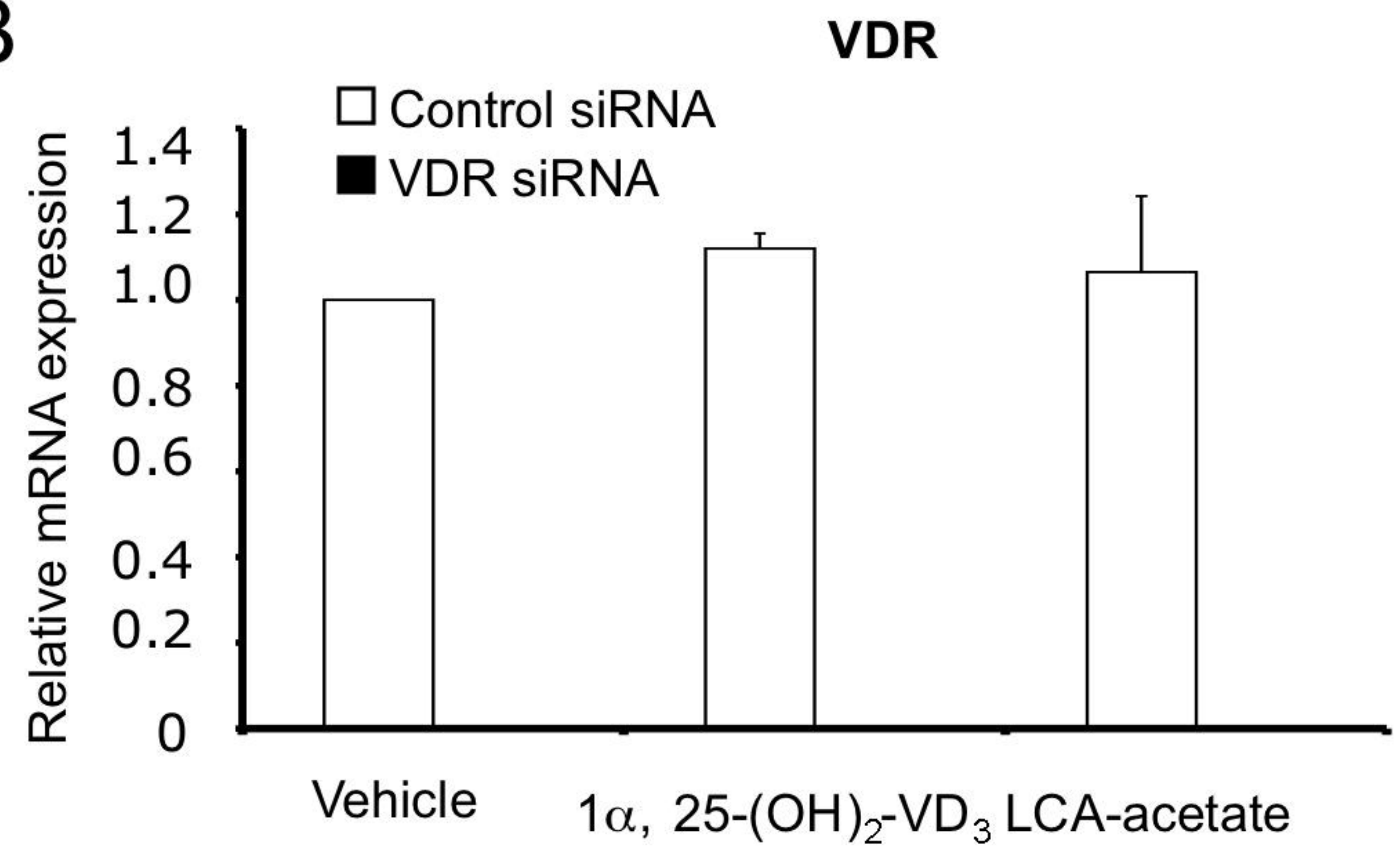


Fig. 3

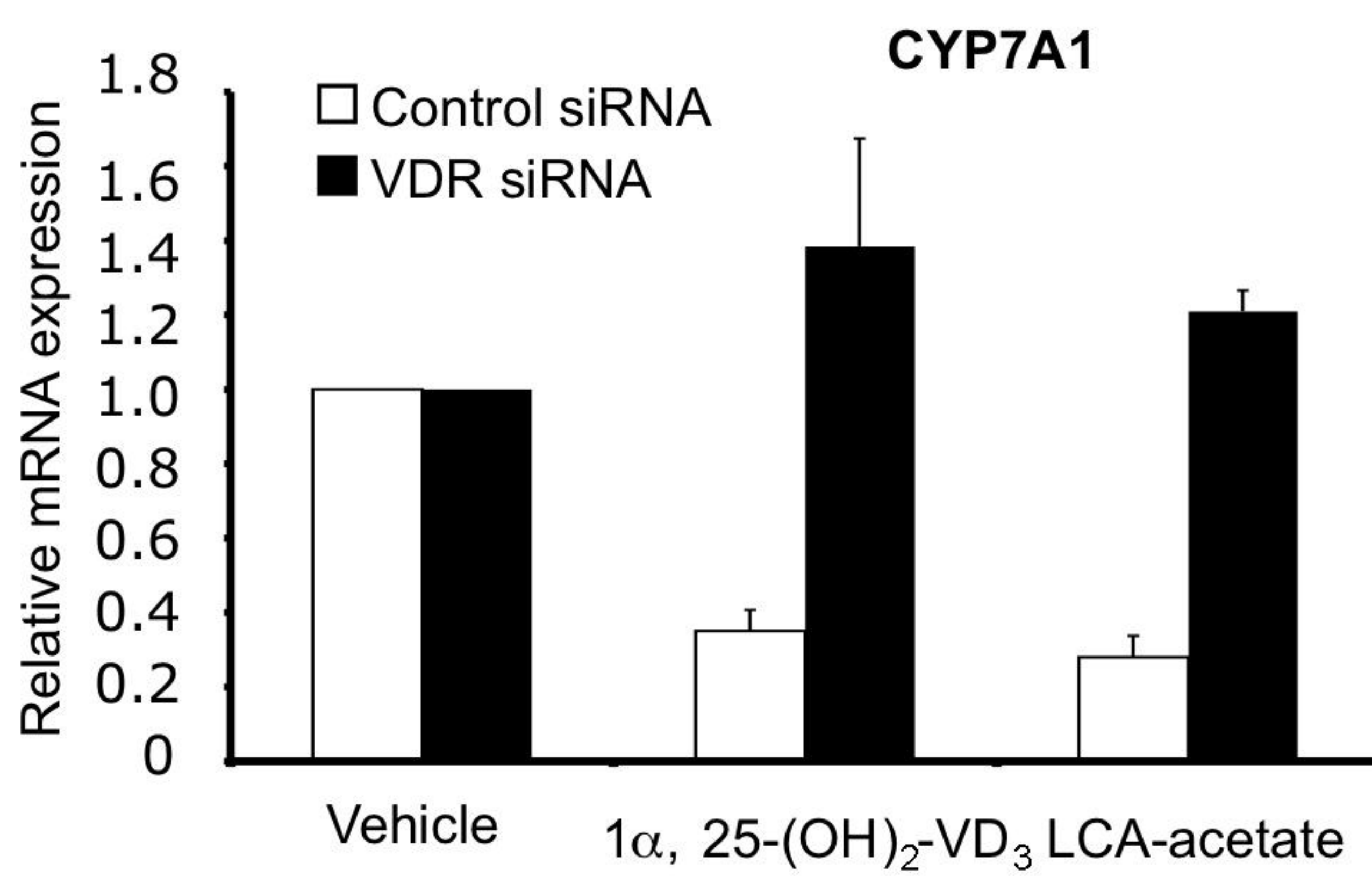
A



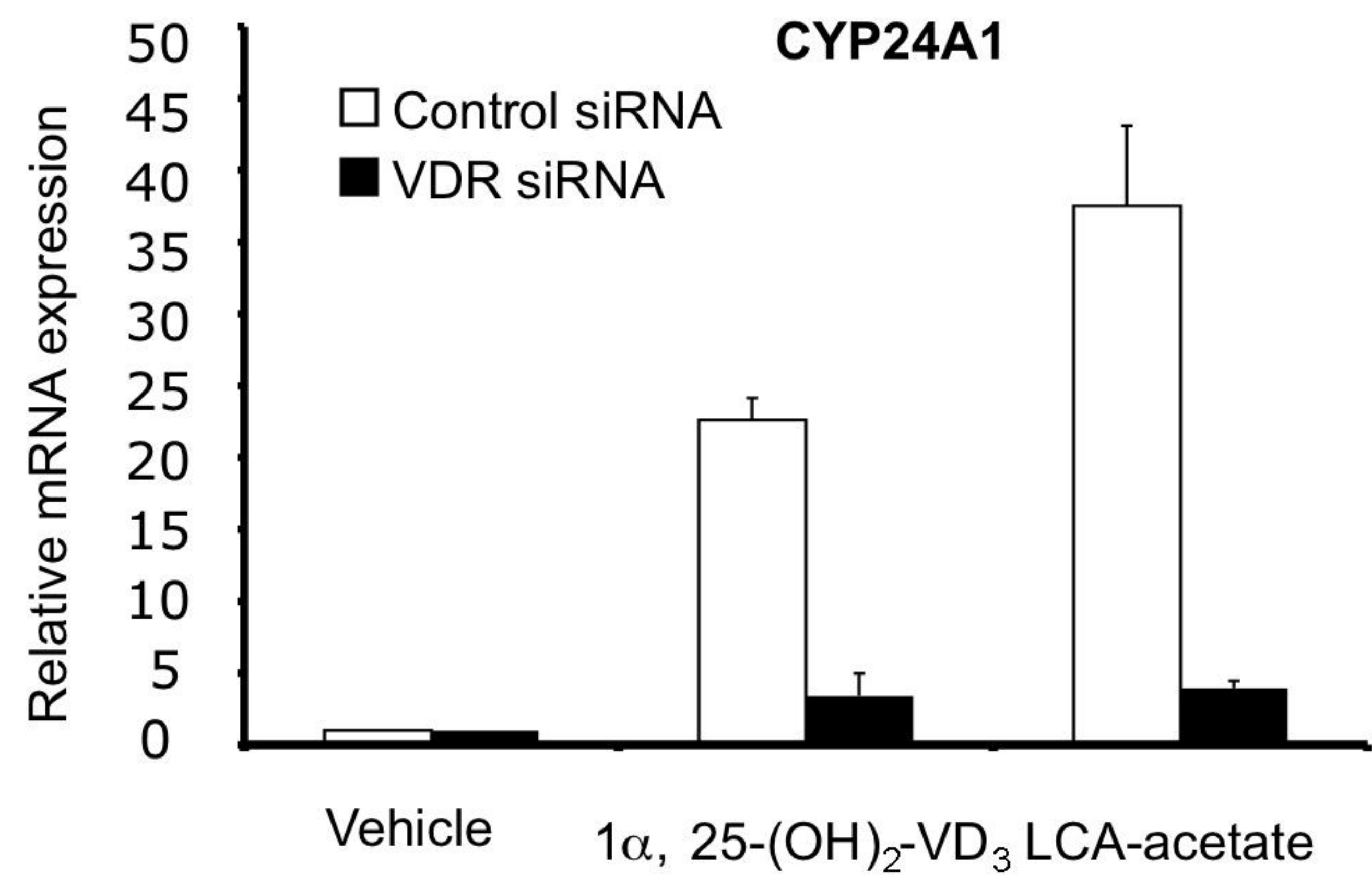
B



C



D



E

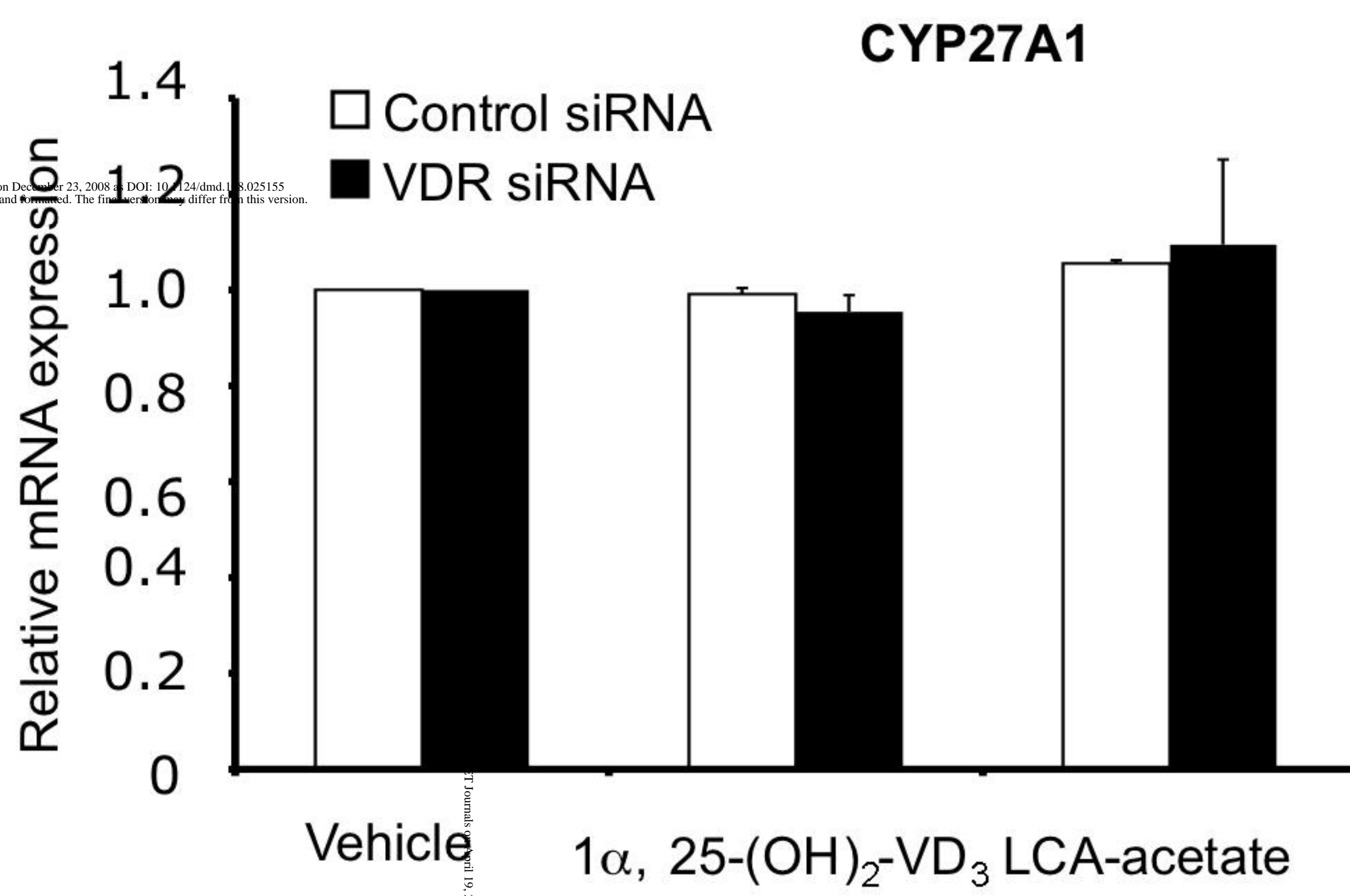
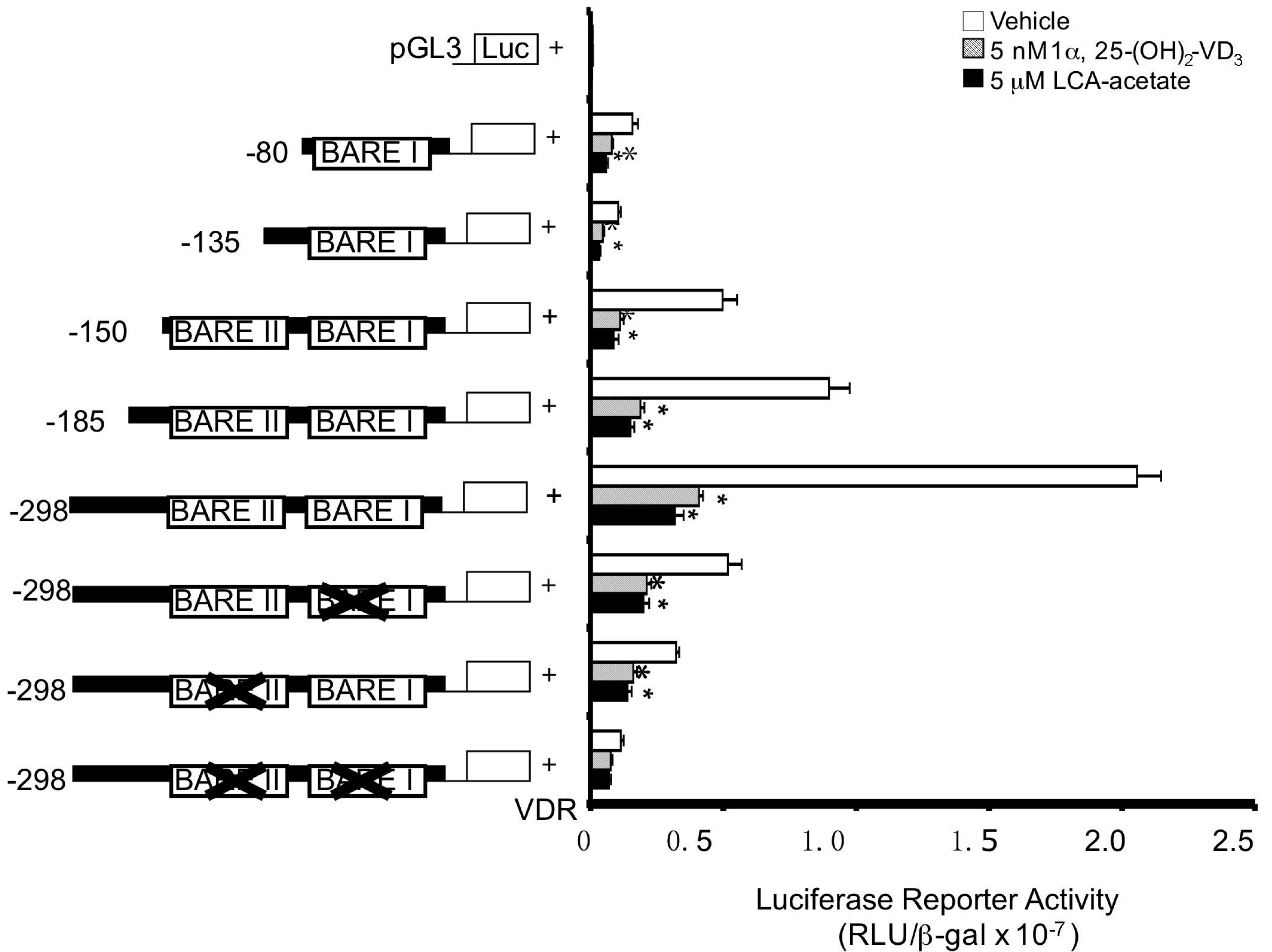


Fig. 4



WT BARE I: TTGTAACCAAGCTCAAG

Mutant BARE I: TTGTAACCAA *aaa* AAG

WT BARE II: TGGACTTAGTTCAAGGCCA

Mutant BARE II: TGG*ggg* TA*aa*TCAAGGCCA

Fig. 5

A

Human CYP7A1 BARE-I: gatcTTTGTCAACCAAGCTCAAGTT  
 Human CYP7A1 M-I: TTTGTCAACCAaaaaAAGTTAATGG

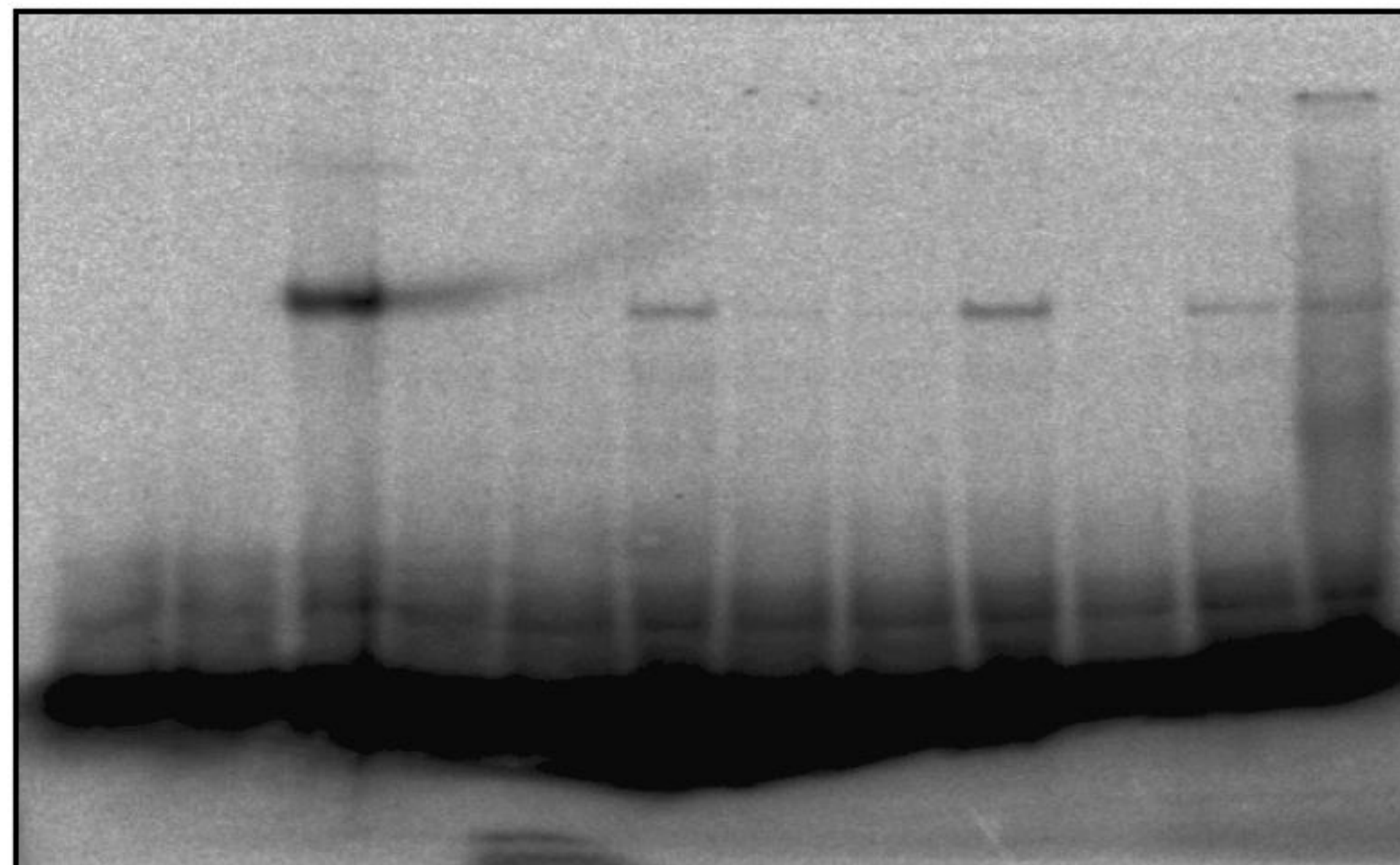
Human CYP7A1 BARE-II: gatcCTGTGGACTTAGTTCAAGGCCA  
 Human CYP7A1 M-II: CTGTGGACTTAaaaaAAGGCCA

CYP3A4: gatcAATATGAACTCAAAGGAGGTCAGTCA



B

TNT lysate	VDR RXR $\alpha$ PXR	- + + + - - - + + + + +
Probe	ER6 M-I BARE-I	+ + + - - - - - - - - - - - + - - - - - - - - - - - - + + + + + + + +
Competitor		- - - - - - - - - B-I M-I -
Anti-VDR		- - - - - - - - - - +



C

TNT lysate	VDR RXR $\alpha$ HNF4 $\alpha$	+ + - - - - + + +
Probe	ER6 M-II BARE-II	+ - - - - - - - - - + + - - - - - - - - - - + + + + + + +
Competitor		- - - - - - - - ER6

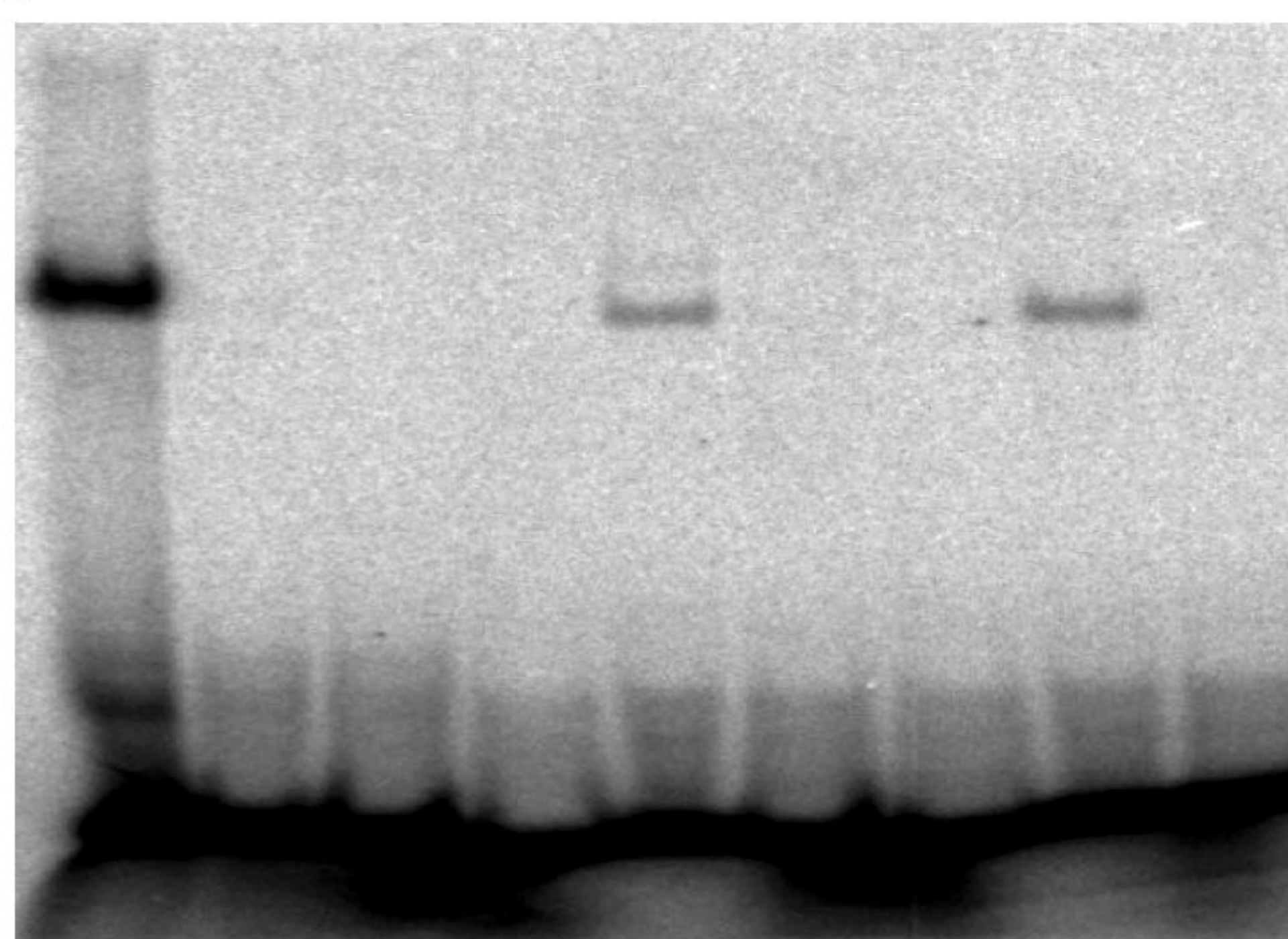
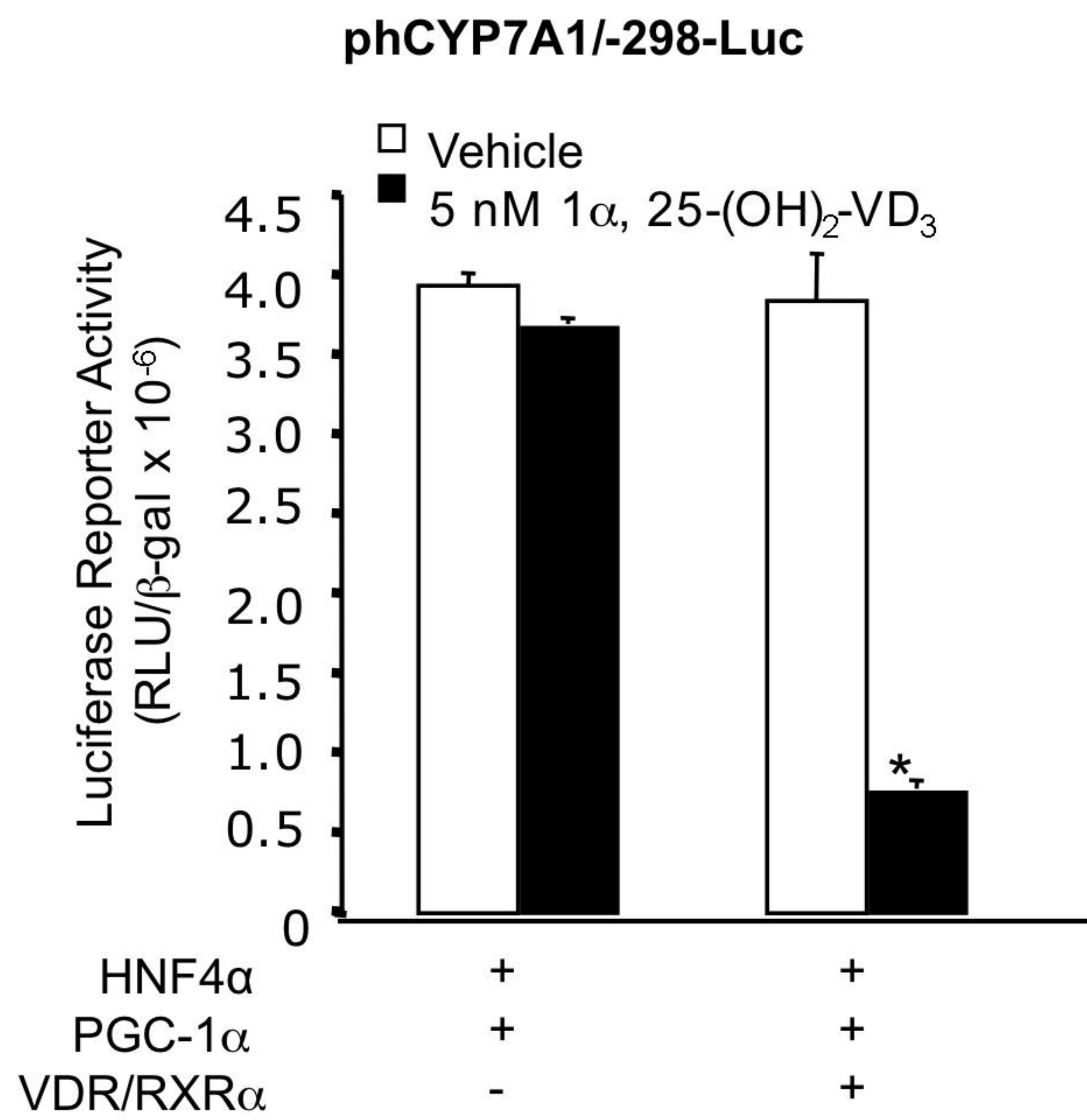




Figure 6

A



B

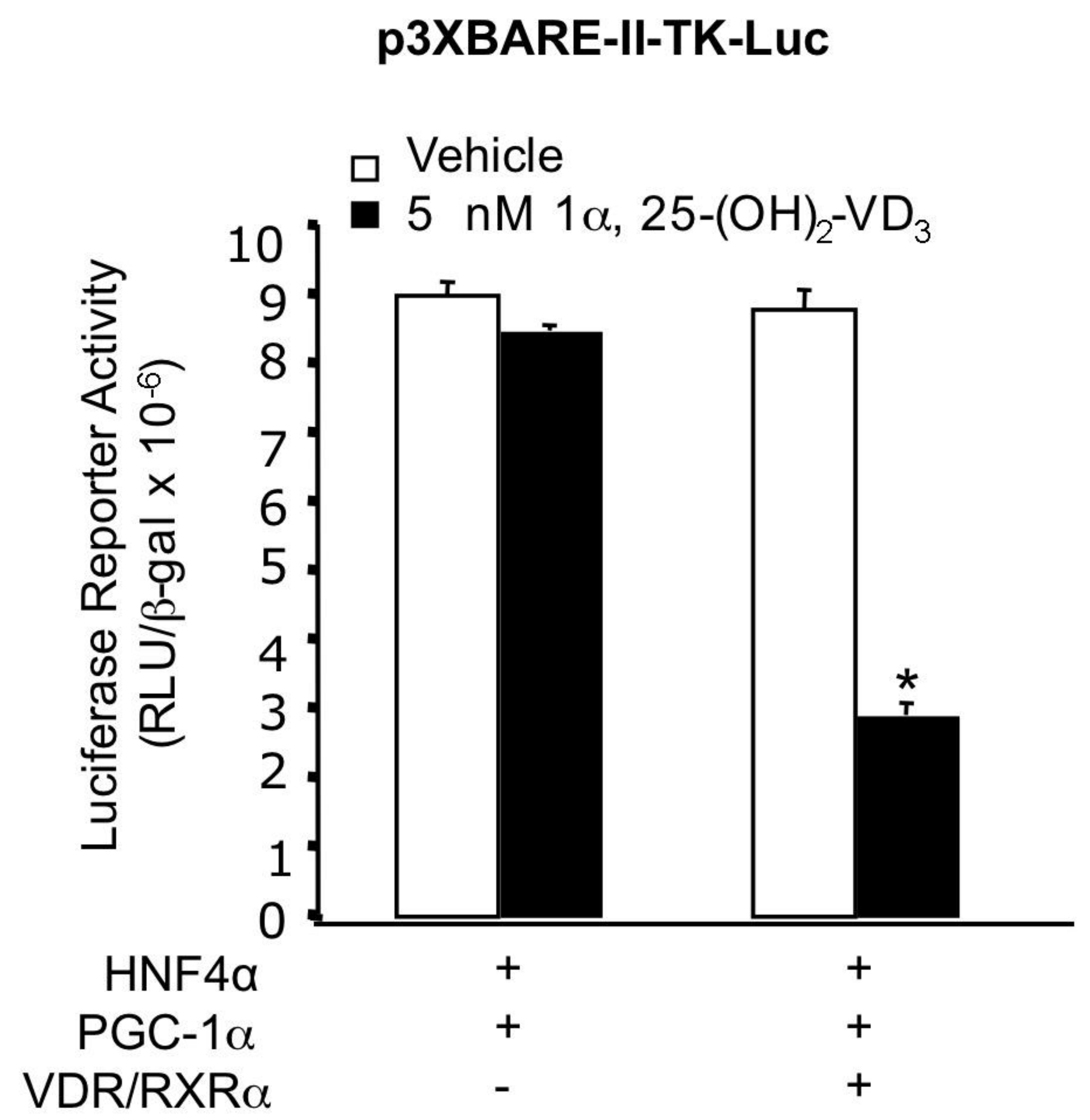
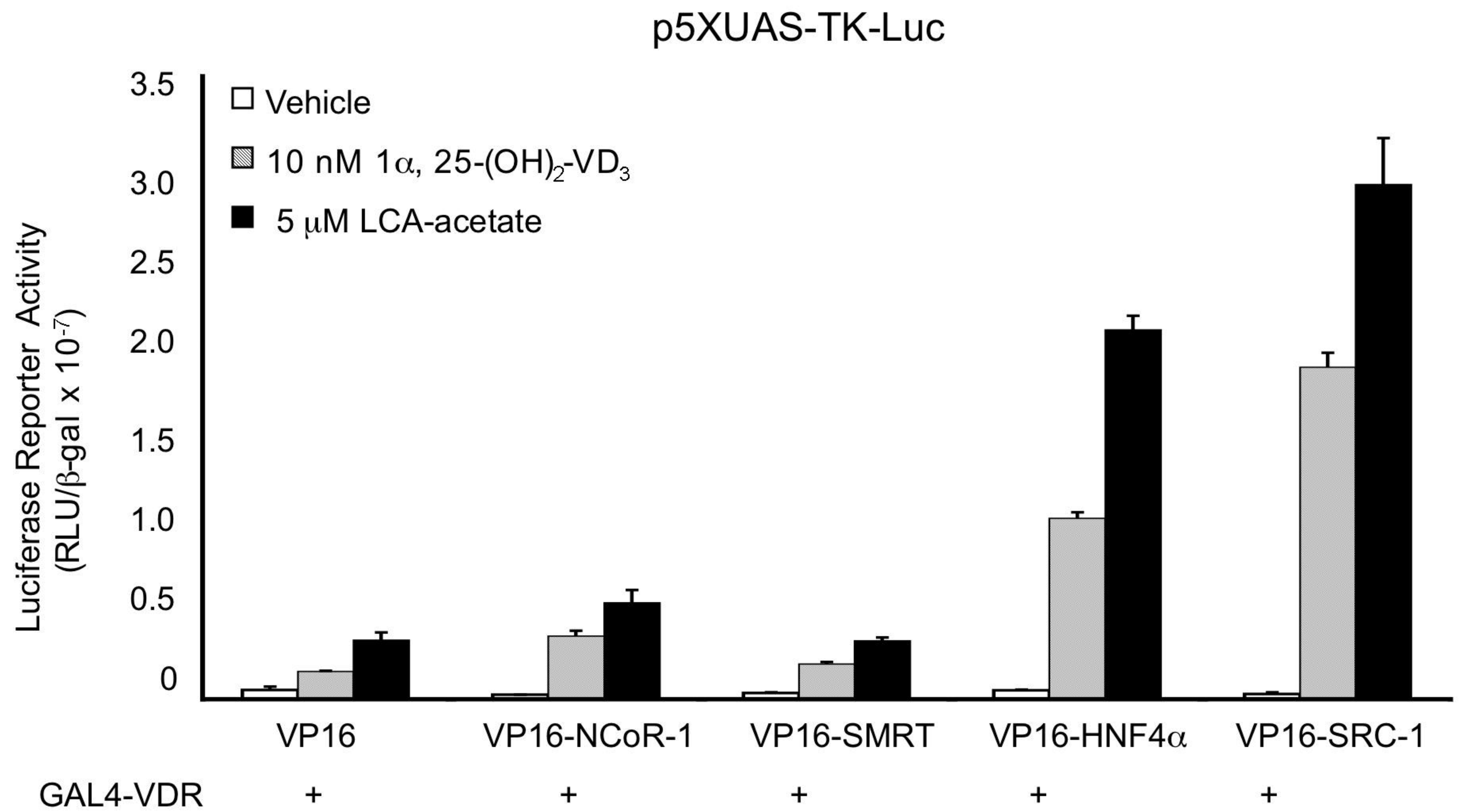
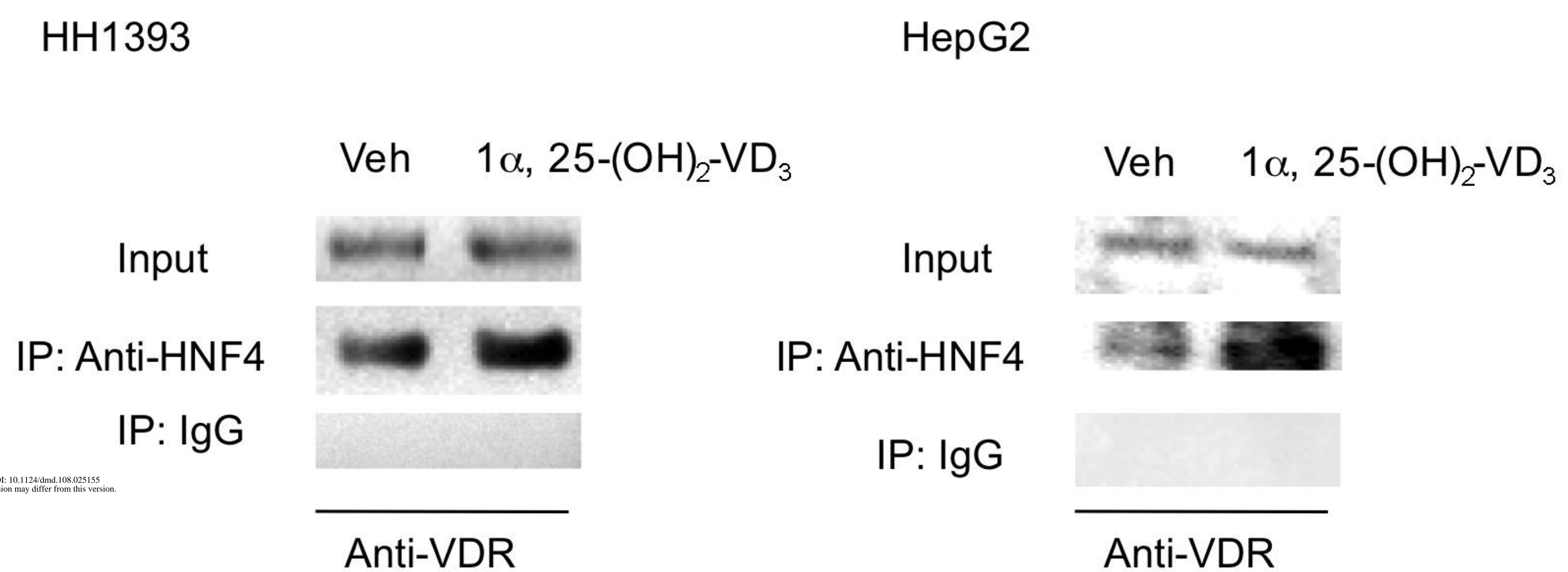


Figure 7

A



B



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C

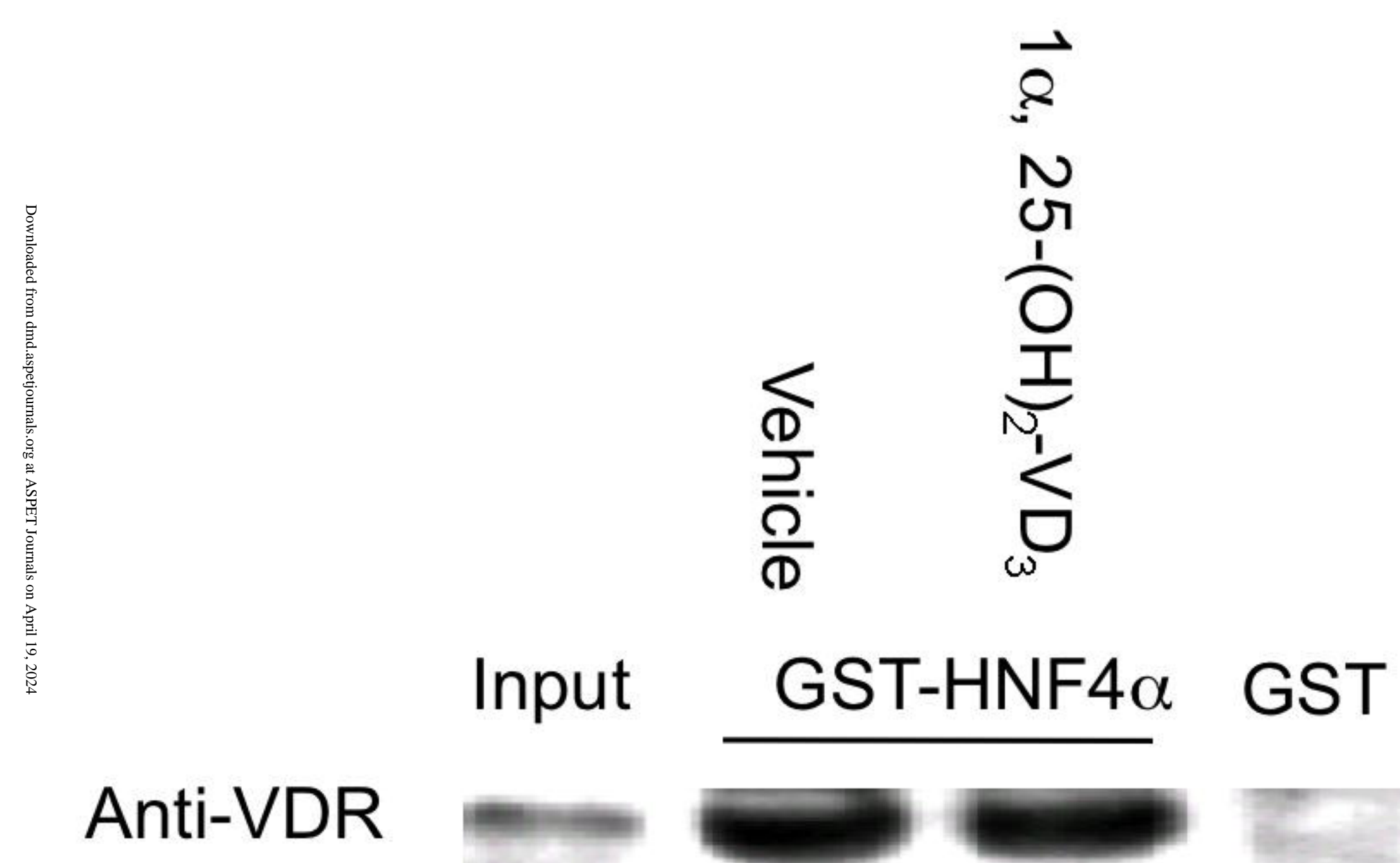


Figure 8

