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CYP24 inhibition preserves 1 α ,25-dihydroxyvitamin D₃ anti-proliferative signaling in lung cancer cells

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Abstract

Human lung tumors aberrantly express the 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)-catabolizing enzyme, CYP24. We hypothesized that CYP24 reduces 1,25(OH)₂D₃-mediated transcription and allows lung cancer cells to escape its growth-inhibitory action. To test this, H292 lung cancer cells and the CYP24-selective inhibitor CTA091 were utilized. In H292 cells, CTA091 reduces 1,25(OH)₂D₃ catabolism, significantly increases 1,25(OH)₂D₃-mediated growth inhibition, and increases 1,25(OH)₂D₃ effects on induced and repressed genes in gene expression profiling studies. Pathway mapping of repressed genes uncovered cell cycle as a predominant 1,25(OH)₂D₃ target. In H292 cells, 1,25(OH)₂D₃ significantly decreases cyclin E2 levels and induces G0/G1 arrest. A broader set of cyclins is down-regulated when 1,25(OH)₂D₃ is combined with CTA091, and cell cycle arrest further increases. Effects of CTA091 on 1,25(OH)₂D₃ signaling are vitamin D receptor-dependent. These data provide evidence that CYP24 limits 1,25(OH)₂D₃ anti-proliferative signaling in cancer cells, and suggest that CTA091 may be beneficial in preserving 1,25(OH)₂D₃ action in lung cancer.

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Keywords

lung cancer; vitamin D receptor; CYP24; 1, 25-dihydroxyvitamin D₃; CTA091

1. Introduction

The active metabolite of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)¹ inhibits carcinogenesis and suppresses the growth of established tumors (Eisman et al., 1987; McElwain et al., 1995; Banach-Petrosky et al., 2006; Mernitz et al., 2007; Welsh, 2007). The effects of 1,25(OH)₂D₃ are mediated by binding to the vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor superfamily (Evans, 1998). Upon binding 1,25(OH)₂D₃, VDR forms a heterodimer with the retinoid-X-receptor (RXR) and modulates the expression of genes whose promoters contain a vitamin D response element (VDRE). The VDREs of genes that are positively regulated by 1,25(OH)₂D₃ are comprised of direct repeats of two hexameric-core-binding motifs separated by 3 or 4 nucleotides. Known inducible 1,25(OH)₂D₃ targets in tumor cells include genes implicated in G₀/G₁ cell cycle arrest (such as the cyclin-dependent kinase inhibitor, p21^{Waf1/Cip1}), differentiation, and apoptosis (Swami et al., 2003; Krishnan et al., 2004; White, 2004). It is possible that mechanisms interfering with 1,25(OH)₂D₃ signaling in tumor cells could enhance properties of tumor growth and progression.

1 α ,25-dihydroxyvitamin D₃ 24-hydroxylase (CYP24) is the primary enzyme responsible for the catabolic inactivation of 1,25(OH)₂D₃ and has been implicated as an oncogene (Prosser and Jones, 2004; Albertson et al., 2000). When bound by ligand, the VDR induces *CYP24* transcription through a series of VDREs located in the *CYP24* promoter and by binding to a distal enhancer region (Chen and DeLuca, 1995; Ohyama et al., 1996; Meyer et al., 2010). This auto-regulatory loop is thought to limit 1,25(OH)₂D₃ signaling and the potential for hypercalcemia toxicity. We and others have demonstrated that *CYP24* expression is dysregulated in lung cancer: It is frequently expressed in non-small cell lung cancer (NSCLC) cell lines under basal growth conditions, and *CYP24* mRNA and protein are over-expressed in primary human lung tumors (Beer et al., 2002; Anderson et al., 2006; Parise et al., 2006). In patients diagnosed with adenocarcinoma of the lung, *CYP24* expression is independently prognostic of survival: Five-year survival rates were 81% in individuals whose tumors expressed low levels of *CYP24* mRNA and 42% for those with high *CYP24* mRNA expression (Chen et al., 2011).

We hypothesized that CYP24 promotes tumor growth by restricting 1,25(OH)₂D₃-mediated transcriptional regulation, thus allowing lung cancer cells to escape its inhibitory action. The studies outlined in this report were designed to test this hypothesis. In the experiments described, CYP24 inhibition was achieved using the highly selective inhibitor CTA091. CTA091 is a 24-sulfoximine analogue of 1,25(OH)₂D₃ which binds to the substrate binding pocket of CYP24 (Kahraman et al., 2004). It inhibits CYP24 at low nanomolar concentrations (IC₅₀, 7.4 nM), in comparison with the high nanomolar concentrations which are required to inhibit enzymes involved in the biosynthesis of 1,25(OH)₂D₃ (CYP27A1 (IC₅₀ > 1000 nM) or CYP27B (IC₅₀, 554 nM) (Kahraman et al., 2004). CTA091 has low calcemic activity and does not itself regulate gene expression through the VDR (Kahraman et al., 2004; Posner et al., 2010). The ability of 1,25(OH)₂D₃ to regulate gene expression and

¹**Abbreviations:** 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; CSS, Charcoal-stripped serum; CYP24, 1 α ,25-dihydroxyvitamin D₃ 24-hydroxylase; CTA091, MK-24(S)-S(O)(NH)-Ph-1; PBS, Phosphate buffered saline; VDR, Vitamin D Receptor; VDRE, Vitamin D Response Element

induce cell cycle arrest was significantly increased in the presence of CTA091, indicating that CYP24 restricts anti-proliferative $1,25(\text{OH})_2\text{D}_3$ signaling in lung cancer cells.

2. Materials and Methods

2.1. Chemicals, Reagents, and Antibodies

$1, 25 (\text{OH})_2 \text{D}_3$ was purchased from Sigma-Aldrich (St. Louis, MO, USA). The CYP24 inhibitor CTA091 was generously provided by Cytochroma, Inc. (Markham, Ontario, Canada). Mouse anti-CYP24 antibody (Clone 1F8) was purchased from Sigma-Aldrich. Rat anti-VDR antibody (MA1-710) was from Thermofisher (Pittsburgh, PA, USA). Mouse anti-Cyclin A (4656), mouse anti-Cyclin D1 (2978) and rabbit anti-Cyclin E2 (4132) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-rabbit IgG (NA934) and sheep anti-mouse IgG (NA931) horseradish peroxidase (HRP)-linked species-specific antibodies were purchased from Amersham Pharmacia Biotech (Little Chalfort, UK). HRP-conjugated donkey anti-rat IgG (712-035-150) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

2.2. Cells

H292, HCC827, and A549 human lung cancer cells were obtained from the American Type Culture Collection (Manassas, VA). H292 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 100 U/ml penicillin-streptomycin. HCC827 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. A549 cells were maintained in BME supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 . The cell lines were authenticated by RADIL (St. Louis, MO) and determined to be mycoplasma-free using the VenorGeM mycoplasma detection kit (Sigma Aldrich).

2.3. Analysis of $1,25(\text{OH})_2\text{D}_3$ catabolism by LC-MS/MS

H292 cells were seeded at a density of 6×10^5 cells per T25 flask in growth medium containing 10% charcoal-stripped serum (CSS). The next day, the medium was removed, the cells were washed with PBS, and 5.0 mL of fresh medium containing 100 nM $1,25(\text{OH})_2\text{D}_3$ or 100 nM $1,25(\text{OH})_2\text{D}_3$ plus CTA091 was added. At various times post-treatment (0h or 24h), cell homogenates were prepared by scraping the cells up into the tissue culture medium. Homogenates were flash frozen in liquid nitrogen and stored at -80°C until analysis by LC-MS/MS.

A standard curve for the $1,25(\text{OH})_2\text{D}_3$ assay was prepared by placing duplicate, 1 ml samples of tissue culture medium containing 1, 3, 5, 10, 30, 50 and 100 ng/ml $1,25(\text{OH})_2\text{D}_3$ into 13×100 mm glass screw top tubes. Ten microliters of a 5 $\mu\text{g}/\text{mL}$ solution of internal standard D_6 - $1,25(\text{OH})_2\text{D}_3$ in methanol were added to each tube, followed by 1 ml of methyl-*tert*-butyl ether. The samples were vortexed at a setting of 8 on the vortex genie for 1 minute. The samples were then centrifuged at $2,000 \times g$ for 5 minutes. The tubes were then placed into a dry ice cold methanol bath for 10 minutes. The resulting supernatants were evaporated to dryness under a stream of nitrogen at 27°C. Each dried residue was dissolved in 100 μL of mobile phase, vortexed briefly, transferred to HPLC autosampler vials, and injected into the LC-MS/MS system. Experimental samples (1.0 mL each homogenate) were processed similarly.

An Agilent (Palo Alto, CA) 1100 thermostated autosampler and binary pump were used as the LC system. The column was a Phenomenex (Torrance, CA, USA) SYNERGI 4 micron Hydro-RP 80 2×100 mm. The HPLC mobile phase was isocratic consisting of methanol-water (85:15) with 2 mM ammonium acetate (by volume). The flow rate was 0.3 ml/min. Sample injection was 50 µL. Mass detection was carried out using a Waters (Milford, MA, USA) Quattro micro triple stage bench top mass spectrometer with electrospray ionization in positive ion mode and multiple reaction mode (MRM). The settings of the mass spectrometer were as follows: The Capillary voltage was 4.0 kV. The Cone voltage was 15 V. The source temperature and desolvation temperatures were 120°C and 400°C, respectively. The cone and desolvation gas flow were 110 and 550 L/h, respectively. The collision voltage was set at 12 V. The MRM m/z for 1,25(OH)₂D₃ was 434>399 and 440>405 m/z for [D₆]-1,25(OH)₂D₃ (internal standard). Waters (Milford, MA, USA) Masslynx version 4.0 software was used to control the LC system and mass spectrometer and collect data. The IS ratio was calculated for each standard by dividing the analyte peak area by the peak area of the internal standard. Standard curves of 1,25(OH)₂D₃ were constructed by plotting the IS ratio versus the known concentration of analyte in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, followed by back calculation of concentrations.

2.4. Clonogenic Assay

Cells were seeded in 6-well plates at a density of 250 cells/well in complete growth medium. The next day, cells were treated with fresh medium (controls) or medium containing the indicated concentrations of 1,25(OH)₂D₃ ± CTA091. Treatments were repeated every 3 days. After 7d, colonies were fixed with methanol and stained with crystal violet, as described by us previously (Owonikoko et al. 2010). To enumerate colonies, grids were scored onto the back of each plate. Colonies in each section of the grid were inspected using a microscope, and those containing ≥ 50 cells were counted. The percent remaining colonies was calculated using the equation: % Remaining Colonies = $100 \times [\text{number colonies for treatment group} / \text{average number colonies for control group}]$.

2.5. RNA extraction and gene expression analysis

H292 cells (5×10^5 /well) were seeded into 6-well plates in complete growth medium. When still sub-confluent, the cells were treated with vehicle (control), CTA091 (50 nM), 1, 25(OH)₂D₃ (10 nM or 100 nM) or 1,25(OH)₂D₃ plus CTA091 for 24 h. RNA was extracted using the PerfectPure RNA Cultured Cell Kit (5 Prime, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. The RNA concentrations were determined by NanoDrop, and RNA quality was assessed by RNA Integrity Number (RIN) using the Agilent Bioanalyzer. Only RNA preparations having a RIN greater than 7.0 were used for gene expression profiling. Whole genome gene expression analysis was carried out using the Illumina HT-12 Expression BeadChip which targets more than 25,000 annotated genes with more than 48,000 probes derived from the RefSeq (Build 36.2, Rel 22) and UniGene databases. Image processing and raw data extraction were performed using Illumina GenomeStudio software. The data were normalized for background correction and plate scaling using GenomeStudio, and differential expression analyses were carried out using the Mann-Whitney error model. Differential expression scores (diff scores) were used to identify genes that were significantly induced (diff score ≥ +13) or repressed (≤ -13) by each treatment compared to vehicle control. To identify candidate cellular pathways that were modulated by treatment, lists of significantly regulated genes were imported into MetaCore software (St. Joseph, MI).

2.6. RT-PCR

RNA was prepared as outlined above (for gene expression analysis). RNA (500 ng) was converted into cDNA using the First Strand cDNA Synthesis kit from Origene (Rockville, MD), according to manufacturer's instructions. A fixed volume of cDNA (1.3 μ L) was used for each PCR reaction. The reactions were run on a BioRadCycler (initial incubation at 95°C for 2 min followed by 30 cycles of 95°C for 30 sec, 60°C for 45 sec, 72°C for 1 min). Primer sequences were: *CD14* Forward-5'-GCCGCTGTGTAGGAAAGAAG-3'; *CD14* Reverse-5'-TAGGTCCTCGAGCGTCAGTT-3'; *LL37* Forward-5'-CCAGGTCCTCAGCTACAAGG-3'; *LL37* Reverse-5'-GGGTAGGGCACACACTAGGA-3'; *CDH5* Forward-5'-ACCGGATGACCAAGTACAGC-3'; *CDH5* Reverse-5'-CGGATGGAGTATCCAATGCT-3'. PCR products were resolved on 1.2% agarose gels, visualized by staining with ethidium bromide, and photographed. Densitometry was used to quantify each PCR product, and the resulting intensity values were normalized to *GAPDH*. *GAPDH* primers were purchased from Clontech (Mountainview, CA).

2.7. Cell Transfections and Treatments

H292 cells (3×10^5 /well) were seeded into 6- well plates in RPMI 1640 complete growth medium without antibiotics. When cells reached approximately 50% confluence, they were transfected with VDR siRNA (L003448-00, Dharmacon, Lafayette, CO) or control non-targeting siRNA (Dharmacon, D-001810-10-05) using the DharmaFECT Duo Transfection reagent in accordance with the manufacturer's instructions. At 5h after transfection, the cells were rinsed with PBS, and then treated with vehicle, CTA091, or 1,25(OH) $_2$ D $_3$ $\pm$ CTA091 in medium containing CSS. Whole cell extracts were prepared 3d post-treatment.

2.8. Preparation of whole cell extracts and immunoblot analysis

Procedures were conducted essentially as described previously (Parise et al., 2006). Briefly, cells were lysed in TX-100/SDS lysis buffer supplemented with protease and phosphatase inhibitors at 4°C for 30 min. Protein extracts were clarified by centrifugation at 4°C at $12,000 \times g$ for 10 min. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Proteins (20 to 30 μ g) were separated on 10% Precast SDS gels (BioRad, Hercules, CA) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA). Blots were incubated with primary antibodies at 4°C overnight. The blots were washed in TTBS (0.05 % Tween 20, 150 mMNaCl, 10 mMTris-HCl, pH 7.6) and then incubated with a HRP-conjugated secondary antibody for 1 h. Blots were developed using ECL or supersignalchemiluminescent reagents. Densitometry was performed on resulting films using a hpscanjet 3970 scanner and UN-SCAN-IT 6.1 software (Silk Scientific Corporation, Orem, Utah).

2.9. Cell Cycle Analysis

H292 cells were seeded in 6-well plate at a density of 2×10^5 /well. The next day, the cells were treated with vehicle, CTA091, or 1,25(OH) $_2$ D $_3$ $\pm$ CTA091 in medium containing CSS. Forty-eight h post-treatment, cells were harvested for cell cycle analysis. To do this, cells were collected after trypsinization and washed twice with phosphate-buffered saline (PBS) containing 0.1% BSA. The cells were then fixed for at least 1 h with 70% ethanol at 4°C, washed twice with PBS, centrifuged, and resuspended in 1 ml PBS containing 50 μ g propidium iodide (PI) (Invitrogen, Carlsbad, CA) and 3.8 mM sodium citrate. RNase A(Sigma-Aldrich) was added to a final concentration of 20 μ g/mL, and the cell suspensions were incubated 3 h at 4°C. Immediately after RNase digestion, the cells were analyzed on anAccuri C6 flow cytometer (Ann Arbor, MI). A minimum of 5,000 events was collected for each sample. Data were analyzed using CFlowPlus Analysis software.

2.10. Statistical Analysis

All statistical analyses were conducted using SAS (version 9.2; SAS Institute Inc., Cary, NC). For the catabolism assay, a t-test was used to compare the control group separately with each treatment group. For all other assays, analysis of variance (ANOVA; GLM procedure in SAS) was used to compare the means of the different treatment groups. This was followed by pairwise comparisons, specifically: between vehicle (control) and each treatment group, between 1,25(OH)₂D₃ treatment dose levels, and between 1,25(OH)₂D₃ and 1,25(OH)₂D₃ + CTA091 treatment groups. We controlled for batch/experiment effect when appropriate by adding batch/experiment as a categorical variable to the model. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. CYP24 inhibition reduces 1,25(OH)₂D₃ catabolism and increases 1,25(OH)₂D₃ anti-proliferative activity in human lung cancer cells

Our prior data indicate that, when expressed in lung cancer cells, CYP24 catabolizes 1,25(OH)₂D₃ and significantly reduces its anti-proliferative activity (Parise et al., 2006). We hypothesized that this loss of activity is due to a CYP24-dependent diminution in 1,25(OH)₂D₃ signaling. The following studies using the human lung cancer cell line, H292, were undertaken to test this hypothesis. H292 cells express the VDR and low levels of CYP24 under basal growth conditions. When treated with 1,25(OH)₂D₃, H292 cells are induced to express CYP24 (Fig. 1A). This is expected as *CYP24* is a known transcriptional target of 1,25(OH)₂D₃ (Chen 1995). CYP24 induction is associated with a significant decrease in 1,25(OH)₂D₃ levels in H292 tissue culture homogenates (Fig. 1B, compare the 1,25(OH)₂D₃ concentration in the t=0 sample with the 1,25(OH)₂D₃ concentration in the 24h/0 nM CTA091 sample). In contrast, when H292 cells are exposed to 1,25(OH)₂D₃ in the presence of the CYP24-selective inhibitor CTA091 (at concentrations ≥ 10nM), there is no significant loss of 1,25(OH)₂D₃ from the culture system (Fig. 1B). 1,25(OH)₂D₃ stabilization occurs despite the fact that CYP24 protein levels are consistently higher in cells treated with 1,25(OH)₂D₃ plus CTA091 compared to 1,25(OH)₂D₃ alone (Fig. 1A). This latter observation is attributed to the fact that when 1,25(OH)₂D₃ catabolism is inhibited by CTA091, the level of VDR-dependent transcription of *CYP24* exceeds that which is induced by 1,25(OH)₂D₃ alone (data not shown). The effects of CTA091 on 1,25(OH)₂D₃ catabolism are not specific to H292 cells. CTA091 also inhibits catabolism of 1,25(OH)₂D₃ in 128.88T lung cancer cells that express high basal levels of CYP24 (Parise et al., 2006).

To examine the effects of CYP24 inhibition on cell growth, clonogenic assays were conducted. When used as a single agent, CTA091 had no significant effect on the growth of H292 cells at any of the concentrations tested (Fig. 1C). In contrast, 1,25(OH)₂D₃ inhibited growth in a dose-dependent manner (Fig. 1D; compare cells treated with 10 nM 1,25(OH)₂D₃ vs. 100 nM 1,25(OH)₂D₃). The anti-proliferative activity of 1,25(OH)₂D₃ in H292 cells was significantly increased by CTA091 (Fig. 1D; compare cells treated with 10 nM 1,25(OH)₂D₃ plus CTA091 vs. 10 nM 1,25(OH)₂D₃ alone). The CYP24 inhibitor also significantly increased the anti-proliferative effects of 1,25(OH)₂D₃ in HCC827 cells, which express high levels of CYP24 upon 1,25(OH)₂D₃ treatment, and in A549 cells, which express high basal levels of CYP24 (Fig. 1D). Therefore, the effects of CTA091 on 1,25(OH)₂D₃ anti-proliferative activity are not cell line specific.

3.2. CYP24 restricts 1,25(OH)₂D₃-mediated transcription in H292 cells

To determine whether CYP24 impacts transcriptional regulation by 1,25(OH)₂D₃ in H292 cells, a whole-genome, gene expression profiling study was undertaken. This approach was selected (instead of using a VDRE-reporter construct to monitor 1,25(OH)₂D₃-mediated

gene regulation) because it afforded the opportunity to examine effects on a broad array of genes and to gain insights into the anti-proliferative actions of 1,25(OH)₂D₃ in lung cancer cells. H292 cells were treated for 24 h with vehicle (control), CTA091 alone, 10 nM 1,25(OH)₂D₃ ± CTA091, or 100 nM 1,25(OH)₂D₃. CTA091 was used at a final concentration of 50 nM, which was the lowest concentration at which we observed consistent inhibition of 1,25(OH)₂D₃ catabolism (Fig 1B). RNA was extracted and profiled using the IlluminaHT-12BeadChip. Compared to vehicle control, CTA091 alone significantly increased the expression of 161 genes (maximal induction 2-fold) and decreased the expression of 120 genes (maximal repression, 2-fold). However, these genes did not map strongly to specific gene ontology pathways (data not shown). The highest ranking induced pathway was apoptosis and survival ($P = 4.19 \times 10^{-4}$), and the highest ranking repressed pathway was galactose metabolism ($P = 1.76 \times 10^{-3}$). 1,25(OH)₂D₃ (100 nM) significantly increased the expression of 378 genes (1.5-fold to 105-fold induction, compared to control) and decreased the expression of 431 genes (1.4-fold to 5-fold suppression, compared to control). Genes that were significantly induced by 1,25(OH)₂D₃ included those whose promoters are known to contain VDREs such as *CYP24* (105-fold increase), *CD14* (12-fold increase), *LL37* (4.6-fold increase), cytidinedeaminase (2.9-fold increase), and *TRPV6* (2-fold increase) (Wang et al., 2005). To validate the array data, 2 genes that were induced by 100 nM 1,25(OH)₂D₃ (*CD14* and *LL37*) and 1 gene that was repressed (*CDH5*) were selected for independent verification by RT-PCR. As shown in Fig. 2, the 3 genes were regulated by 1,25(OH)₂D₃ in the manner predicted by the array results.

Consistent with prior reports showing that 1,25(OH)₂D₃ inhibits progression through the cell cycle in many tumor cell types (reviewed in (Samuel and Sitrin, 2008)), we found that cell cycle pathways were featured prominently among the top 5 gene ontology pathways that were repressed by 1,25(OH)₂D₃ (100 nM) and by 1,25(OH)₂D₃ (10 nM) + CTA091 in H292 cells (Table 1). Specific effects of 1,25(OH)₂D₃ on cell cycle regulatory proteins will be discussed in more detail below.

To establish the effect of *CYP24* on 1,25(OH)₂D₃-mediated gene regulation in H292 cells, we compared the potency for gene regulation by 10 nM 1,25(OH)₂D₃ (where *CYP24* is active) with that of 10 nM 1,25(OH)₂D₃ plus CTA091 (where *CYP24* is inactive). We selected as our data set for this analysis only those genes that were significantly induced or repressed by 100 nM 1,25(OH)₂D₃ (identified above). The majority of 1,25(OH)₂D₃-induced genes (92%) were expressed at a greater level when *CYP24* was inhibited with CTA091 (see *CD14* and *LL37* as representative examples, Fig. 2). This proportion is significantly higher than random chance ($P < 0.0001$). Conversely, most of the 1,25(OH)₂D₃-repressed genes (93%) were expressed at a lower level in the presence of CTA091 (see *CDH5* as a representative example, Fig. 2). This proportion is also significantly higher than random chance ($P < 0.0001$). We conclude from these data that the capacity of 1,25(OH)₂D₃ to induce or repress the expression of specific genes is increased when its *CYP24*-mediated catabolism is inhibited.

3.3. *CYP24* restricts 1,25(OH)₂D₃-mediated cell cycle arrest

Little is known about the mechanisms by which 1,25(OH)₂D₃ regulates the growth of lung cancer cells. Our array data suggest that a predominant effect of 1,25(OH)₂D₃ is to block cell cycle progression. To test this, we examined the effects of 1,25(OH)₂D₃ ± CTA091 on cyclin D1, cyclin A, and cyclin E2 protein levels by immunoblot (Fig. 3). We focused on the expression of these cyclins initially because they promote G1/S progression, and the G1/S transition was significantly repressed by 1,25(OH)₂D₃ treatment in H292 cells (Table 1). CTA091 alone did not modulate cyclin expression. Compared to vehicle, treatment with 100 nM 1,25(OH)₂D₃ was only associated with a significant reduction in cyclin E2 expression, while treatment with 1,25(OH)₂D₃ plus CTA091 resulted in a significant decrease in each of

the cyclins examined. Thus, a broader set of cyclins is affected when $1,25(\text{OH})_2\text{D}_3$ catabolism is inhibited with CTA091.

To determine whether the effects of CTA091 on cyclin expression were dependent upon $1,25(\text{OH})_2\text{D}_3$ signaling through the VDR, we suppressed receptor expression using siRNA. Cells were transfected with siRNA, and 5 h later cells were treated as indicated in Fig. 4. Whole cell extracts were prepared 3 d post-treatment and analyzed for expression of VDR, CYP24, and cyclins by immunoblot. This time point was chosen based on manufacturer's recommendations for achieving maximum siRNA-mediated down-modulation of protein expression. The VDR siRNA effectively reduced VDR protein levels and resulted in a functional loss of signaling (Fig. 4). This was evidenced by our finding that CYP24 was induced by $1,25(\text{OH})_2\text{D}_3$ + CTA091 in cells transfected with control siRNA but not in VDR-deficient cells. CTA091 alone had no significant effect on cyclin expression in cells transfected with either the control or VDR siRNAs. Compared to vehicle, cyclins A and E2 were significantly decreased in control siRNA-transfected cells treated with $1,25(\text{OH})_2\text{D}_3$ + CTA091, as expected. In contrast, no significant decreases in cyclin expression were observed in the VDR-deficient cells treated with either $1,25(\text{OH})_2\text{D}_3$ or $1,25(\text{OH})_2\text{D}_3$ + CTA091. These results support the conclusion that $1,25(\text{OH})_2\text{D}_3$ down-modulates cyclin expression via a VDR-dependent process, and that cyclin down-modulation is compromised by CYP24 activity.

A $1,25(\text{OH})_2\text{D}_3$ -mediated decrease in expression of cyclins required for S-phase entry is predicted to result in accumulation of cells in the G1 phase of the cell cycle. To determine if $1,25(\text{OH})_2\text{D}_3$ mediated cell cycle arrest, and whether such arrest was affected by CYP24, we treated cells for 48 h, stained them with propidium iodide (PI), and examined them by flow cytometry (Fig. 5). CTA091 alone had no effect on the cell cycle distribution. $1,25(\text{OH})_2\text{D}_3$ treatment resulted in a dose-dependent increase in cells in G0/G1 and a decrease in cells in G2/M compared to vehicle controls. The percentage of cells in G0/G1 increased significantly following treatment with 10 nM $1,25(\text{OH})_2\text{D}_3$ plus CTA091 compared to 10 nM $1,25(\text{OH})_2\text{D}_3$ alone, consistent with the conclusion that CYP24 activity restricts the induction of cell cycle arrest by $1,25(\text{OH})_2\text{D}_3$.

4. Discussion

The vitamin D₃ catabolizing enzyme CYP24 is frequently over-expressed in lung cancer (Beer et al., 2002; Anderson et al., 2006; Parise et al., 2006), and it is independently associated with poor survival in patients diagnosed with adenocarcinoma of the lung (Chen et al., 2011). We hypothesized that CYP24 contributes to the growth or progression of lung cancer by diminishing $1,25(\text{OH})_2\text{D}_3$ signaling. In support of this hypothesis, we now provide direct evidence that transcriptional regulation by $1,25(\text{OH})_2\text{D}_3$ is compromised in lung cancer cells in which CYP24 is active. Moreover, we show that $1,25(\text{OH})_2\text{D}_3$ mediates G0/G1 arrest in lung cancer cells, and that arrest can be achieved at ten-fold lower $1,25(\text{OH})_2\text{D}_3$ concentrations when CYP24 is inactivated. These data provide further support for the idea that, when over-expressed, CYP24 allows tumor cells to bypass the anti-proliferative effects of endogenous $1,25(\text{OH})_2\text{D}_3$.

Consistent with the notion that $1,25(\text{OH})_2\text{D}_3$ signaling suppresses lung cancer growth, we recently demonstrated that high nuclear VDR expression is associated with better overall survival in NSCLC. The 5 year OS rates were 59% (95% CI: 39–80%) for patients with high nuclear VDR expression versus 27% (95% CI: 12–41%) for low nuclear VDR expression (Srinivasan et al., 2011). Data presented in this manuscript provide a mechanistic explanation for these prior findings: $1,25(\text{OH})_2\text{D}_3$ signaling through the VDR results in down-modulation of cyclins which promote S phase entry (Fig. 4). Although cyclin down-

modulation was VDR-dependent in H292 cells, we cannot conclude from our studies that cyclins are direct transcriptional targets of $1,25(\text{OH})_2\text{D}_3$. Rather, cyclin suppression (observed at 24 h post- $1,25(\text{OH})_2\text{D}_3$ treatment) may have occurred as a downstream consequence of the prior modulation of direct target genes. For example, as recently reported in ovarian cancer cells (Shen et al. 2011), $1,25(\text{OH})_2\text{D}_3$ may directly repress transcription of a growth factor receptor, which results in the subsequent cyclin down-modulation.

A corollary to our hypothesis that CYP24 allows tumor cells to bypass the anti-proliferative effects of endogenous $1,25(\text{OH})_2\text{D}_3$ is that therapeutic approaches to block CYP24 will restore anti-proliferative $1,25(\text{OH})_2\text{D}_3$ signaling in lung cancer. The CYP24-selective inhibitor employed in this study (CTA091) increased $1,25(\text{OH})_2\text{D}_3$ stability, transcriptional regulation, and growth inhibition in H292 cells. Thus, CTA091, represents one possible agent that could be used to increase $1,25(\text{OH})_2\text{D}_3$ signaling therapeutically. CTA091 has no effects on the growth of H292 cells when administered as a single agent (Fig.1). However, it was not devoid of biological activity in H292 cells as it did induce the expression of 161 genes and decrease the expression of 120 genes upon 24 h exposure. Of these genes, 7 were also induced and 16 were also repressed by $1,25(\text{OH})_2\text{D}_3$ (100 nM). One possible explanation for these findings is that single agent CTA091 regulated gene expression by stabilizing low levels of endogenous vitamin D_3 metabolites that were contained in our tissue culture medium, and the genes that were regulated at these hormone concentrations differed from those that were modulated at the nanomolar concentrations of $1,25(\text{OH})_2\text{D}_3$ used in our gene expression profiling experiments. Alternatively, CTA091 may regulate gene expression by a VDR-independent mechanism, which remains to be elucidated. Future experiments will be required to distinguish between these possibilities.

With regard to its potential *in vivo* application, CTA091 has been delivered to normal rats, where it had the expected effect of increasing $1,25(\text{OH})_2\text{D}_3$ exposure (Posner et al., 2010). No acute toxicities were reported in these short term studies. We have also administered CTA091 to mice in combination with $1,25(\text{OH})_2\text{D}_3$ on a once weekly schedule for 3 weeks and observed no toxicity (as assessed by weight loss or increase in serum calcium) (data not shown). Cumulatively, these data suggest that CTA091 administration may represent a feasible approach to increase transcriptional regulation by $1,25(\text{OH})_2\text{D}_3$ in cancer.

Anti-proliferative effects of $1,25(\text{OH})_2\text{D}_3$ in lung cancer models have been previously reported by us and others (Parise et al. 2006; Mernitz et al. 2007; Chen et al. 2011). However, little information exists as to the basis for this activity. Our gene expression profiling studies revealed a predominant effect of $1,25(\text{OH})_2\text{D}_3$ on cell cycle regulation in H292 cells (Table 1). Consistent with this result, we were able to confirm in independent assays that $1,25(\text{OH})_2\text{D}_3$ decreases expression of cyclins that govern S phase entry and mediates G0/G1 arrest in H292 cells. While a limitation of our functional studies is the use of single cell line, G1 arrest is commonly observed upon $1,25(\text{OH})_2\text{D}_3$ treatment in other epithelial tumor cell lines (i.e. MCF-7, LNCaP, and SCCVII SF) (McElwain et al., 1995; Zhuang and Burnstein, 1998; Jensen et al., 2001). Extensive biochemical studies in MCF-7 breast cancer cells led to a model in which (1) $1,25(\text{OH})_2\text{D}_3$ inhibits cyclin D-dependent kinases; (2) This leads to Rb hyperphosphorylation and sequestration/inhibition of the transcription factor, E2F; and (3) Continued E2F inhibition results in suppression of cyclin A and cyclin E protein expression and a G1 phase block (Jensen et al., 2001). The observed $1,25(\text{OH})_2\text{D}_3$ -mediated changes in cyclin expression in H292 cells would support the existence of a similar mechanism for growth inhibition.

We focused in this report on those genes/pathways that were repressed in response to $1,25(\text{OH})_2\text{D}_3$ treatment. It is important to note that $1,25(\text{OH})_2\text{D}_3$ also induced the

expression of more than 400 genes, and that these could positively or negatively influence its anti-tumor activity. A detailed functional investigation of 1,25(OH)₂D₃-induced genes in lung cancer growth control, and the influence of CYP24 on these genes, is ongoing in our laboratory.

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References

- Abertson DG, Yistra B, Segreaves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW, Pinkel D. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nature Genetics*. 2000; 25:144–146. [PubMed: 10835626]
- Anderson MG, Nakane M, Ruan X, Kroeger PE, Wu-Wong JR. Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemother Pharmacol*. 2006; 57:234–240. [PubMed: 16180015]
- Banach-Petrosky W, Ouyang X, Gao H, Nader K, Ji Y, Suh N, DiPaola RS, Abate-Shen C. Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3.1; Pten mutant mice. *Clin Cancer Res*. 2006; 12(19):5895–5901. [PubMed: 17020998]
- Beer DG, Kardias SL, Huang CC, Giordano TJ, Levin AM, Misek DE, Lin L, Chen G, Gharib TG, Thomas DG, Lizyness ML, Kuick R, Hayasaka S, Taylor JM, Iannettoni MD, Orringer MB, Hanash S. Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med*. 2002; 8(8):816–824. [PubMed: 12118244]
- Chen G, Kim SH, King AN, Zhao L, Simpson RU, Christensen PJ, Wang Z, Thomas DG, Giordano TJ, Lin L, Brenner DE, Beer DG, Ramnath N. CYP24A1 is an independent prognostic marker of survival in patients with lung adenocarcinoma. *Clin Cancer Res*. 2011; 17(4):817–826. [PubMed: 21169243]
- Chen K-S, DeLuca HF. Cloning of the human 1 α ,25-dihydroxyvitamin D₃ 24-hydroxylase gene promoter and identification of two vitamin D-responsive elements. *Biochim Biophys Acta*. 1995; 1263:1–9. [PubMed: 7632726]
- Eisman JA, Barkla DH, Tutton PJ. Suppression of in vivo growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D₃. *Cancer Res*. 1987; 47(1):21–25. [PubMed: 3024816]
- Evans RM. The steroid and thyroid hormone receptor superfamily. *Science*. 1998; 240:889–895. [PubMed: 3283939]
- Jensen SS, Madsen MW, Lukas J, Binderup L, Bartek J. Inhibitory effects of 1 α ,25-dihydroxyvitamin D₃ on the G(1)-S phase-controlling machinery. *Mol Endocrinol*. 2001; 15(8):1370–1380. [PubMed: 11463860]
- Kahraman M, Sinishtaj S, Dolan PM, Kensler TW, Peleg S, Saha U, Chuang SS, Bernstein G, Korczak B, Posner GH. Potent, selective and low-calcemic inhibitors of CYP24 hydroxylase: 24-sulfoximine analogues of the hormone 1 α ,25-dihydroxyvitamin D₃. *J Med Chem*. 2004; 47(27):6854–6863. [PubMed: 15615534]
- Krishnan AV, Shinghal R, Raghavachari N, Brooks JD, Peehl DM, Feldman D. Analysis of Vitamin D-Regulated Genes in LNCaP Human Prostate Cancer Cells Using cDNA Microarrays. *The Prostate*. 2004; 59:243–251. [PubMed: 15042599]
- McElwain MC, Dettelbach MA, Modzelewski RA, Russell DM, Uskokovic MR, Smith DC, Trump DL, Johnson CS. Antiproliferative effects in vitro and in vivo of 1,25-dihydroxyvitamin D₃ and a vitamin D₃ analog in a squamous cell carcinoma model system. *Mol Cell Differentiation*. 1995; 3(1):31–50.
- Mernitz H, Smith DE, Wood RJ, Russell RM, Wang XD. Inhibition of lung carcinogenesis by 1 α ,25-dihydroxyvitamin D₃ and 9-cis retinoic acid in the A/J mouse model: evidence of retinoid mitigation of vitamin D toxicity. *Int J Cancer*. 2007; 120(7):1402–1409. [PubMed: 17205520]

- Meyer MB, Goetsch PD, Pike WJ. A downstream intergenic cluster of regulatory enhancers contributes to the induction of CYP24A1 expression by 1 α ,25-dihydroxyvitamin D₃. *J Biol Chem*. 2010; 285(20):15599–15610. [PubMed: 20236932]
- Ohyama Y, Ozono K, Uchida M, Yoshimura M, Shinki T, Suda T, Yamamoto O. Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D₃ 24-hydroxylase gene. *J Biol Chem*. 1996; 271:30381–30385. [PubMed: 8940000]
- Owonikoko TK, Ramalingam SS, Kanterewicz B, Balus TE, Belani CP, Hersherberger PA. Vorinostat increases carboplatin and paclitaxel activity in non-small-cell lung cancer cells. *Int J Cancer*. 2010; 126(3):743–755. [PubMed: 19621389]
- Parise RA, Egorin MJ, Kanterewicz B, Taimi M, Petkovich M, Lew AM, Chuang SS, Nichols M, El-Hefnawy T, Hersherberger P. CYP24, the enzyme that catabolizes the antiproliferative agent vitamin D, is increased in lung cancer. *Int J Cancer*. 2006; 119(8):1819–1828. [PubMed: 16708384]
- Posner GH, Helvig C, Cuerrier D, Collop D, Kharebov A, Ryder K, Epps T, Petkovich M. Vitamin D analogues targeting CYP24 in chronic kidney disease. *J Steroid Biochem Mol Biol*. 2010; 121(1–2):13–19. [PubMed: 20347976]
- Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem Sci*. 2004; 29(12):664–673. [PubMed: 15544953]
- Samuel S, Sitrin MD. Vitamin D's role in cell proliferation and differentiation. *Nutr Rev*. 2008; 66(10 Suppl 2):S116–124. [PubMed: 18844838]
- Shen Z, Zhang X, Tang J, Kasiappan R, Jinwal U, Li P, Hann S, Nicosia SV, Wu J, Bai W. The coupling of epidermal growth factor receptor down regulation by 1 α ,25-dihydroxyvitamin D₃ to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells. *Mol Cell Endocrinol*. 2011; 338(1–2):58–67. [PubMed: 21458521]
- Srinivasan M, Parwani AV, Hersherberger PA, Lenzner DE, Weissfeld JL. Nuclear vitamin D receptor expression is associated with improved survival in non-small cell lung cancer. *J Steroid Biochem Mol Biol*. 2011; 123(1–2):30–36. [PubMed: 20955794]
- Swami S, Raghavachari N, Muller UR, Bao YP, Feldman D. Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. *Breast Cancer Res Treat*. 2003; 80:49–62. [PubMed: 12889598]
- Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, Bourdeau V, Konstorum A, Lallemant B, Zhang R, Mader S, White JH. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D₃ target genes. *Mol Endocrinol*. 2005; 19(11):2685–2695. [PubMed: 16002434]
- Welsh J. Vitamin D and prevention of breast cancer. *Acta Pharmacol Sin*. 2007; 28(9):1373–1382. [PubMed: 17723171]
- White JH. Profiling 1,25-dihydroxyvitamin D₃-regulated gene expression by microarray analysis. *Steroid Biochem Mol Biol*. 2004; 89–90:239–244.
- Zhuang SH, Burnstein KL. Antiproliferative effect of 1,25-Dihydroxyvitamin D₃ in Human Prostate Cancer Cell Line LNCaP Involves Reduction of Cyclin-Dependent Kinase 2 Activity and Persistent G1 Accumulation. *Endocrinology*. 1998; 139:1197–1207. [PubMed: 9492054]

Manuscript Highlights

The vitamin D₃ catabolizing enzyme, CYP24, is aberrantly expressed in lung cancer.

The effect of CYP24 inhibitor CTA091 on vitamin D₃ (1,25D₃) activity was examined.

CTA091 significantly increased effects of 1,25D₃ on endogenous gene expression.

Expression profiling revealed cell cycle as a 1,25D₃ target in lung cancer cells.

Inhibitory effects of 1,25D₃ on cell cycle and cell growth were reinforced by CTA091.

Figure 1. CYP24 catabolizes 1,25(OH)₂D₃ and reduces its anti-proliferative activity in lung cancer cells

(A) H292 cells were treated with 100 nM 1,25(OH)₂D₃ ± 50 nM CTA091. At the times indicated, whole cell extracts were prepared. Equivalent amounts of protein from each extract were analyzed by immunoblot for CYP24 expression. (B) H292 cells were exposed to 100 nM 1,25(OH)₂D₃ in the absence or presence of CTA091 (0–100 nM). After 0h or 24h of treatment, cell homogenates were prepared and 1,25(OH)₂D₃ concentrations determined by LC-MS/MS, as described in Methods. Each data point represents a separate 1,25(OH)₂D₃ determination. Horizontal bars represent the mean of 3 determinations (**, *P* < 0.001 treatment vs. control). The lower limit of detection for 1,25(OH)₂D₃ using this assay is 1 ng/mL. The ability of 50 nM CTA091 to inhibit 1,25(OH)₂D₃ elimination from H292 cultures was confirmed in 3 additional experiments. (C) H292 cells were treated with vehicle (control) or the indicated concentrations of CTA091. Treatments were repeated every 3 days. Crystal violet staining was used to assess colony formation after 7 days of treatment. Bars represent means ± SD for triplicate determinations within a single experiment. No significant differences in growth were observed. (D) Cells were treated with vehicle (control) or the indicated concentrations of 1,25(OH)₂D₃ in the absence or presence of CTA091 (50 nM). As above, crystal violet staining was used to assess colony formation after 7 days of treatment. Bars represent means ± SD for triplicate determinations within a single experiment. *, *P* < 0.05, treatment vs. vehicle (control). Horizontal lines are used to indicate significant differences in mean % colonies remaining between 1,25(OH)₂D₃ treatment dose levels, and between 1,25(OH)₂D₃ and 1,25(OH)₂D₃ + CTA091 treatment groups. Clonogenic assays were conducted twice, and similar results were obtained.

Figure 2. CYP24 impairs transcriptional regulation by 1,25(OH)₂D₃

H292 cells were treated with vehicle (control), CTA091 alone (50 nM), 10 nM 1,25(OH)₂D₃ ± CTA091 (50 nM) or 100 nM 1,25(OH)₂D₃. RNA was extracted 24 h post-treatment, and expression of *CD14*, *LL37*, *CDH5*, and *GAPDH* was analyzed by RT-PCR. PCR products were resolved on 1.2% agarose gels, visualized with ethidium bromide, and photographed. Gel images from three separate experiments are presented in the left panels. Densitometry results for these three experiments are summarized in the right panels. Band intensities of the RT-PCR products were quantified by densitometry and normalized to the intensity of the band for *GAPDH*. Bars represent the mean normalized intensity ± SD. *, $P < 0.05$, treatment vs. vehicle (control). No significant differences between 10 nM 1,25(OH)₂D₃ and 100 nM 1,25(OH)₂D₃ were observed. Brackets are used to indicate significant differences in mean normalized intensity between 10 nM 1,25(OH)₂D₃ and 10 nM 1,25(OH)₂D₃ + CTA091 treatment groups.

Figure 3. CYP24 restricts 1,25(OH)₂D₃-mediated repression of cyclin expression in lung cancer cells

H292 cells were treated with vehicle or the indicated concentrations of 1,25(OH)₂D₃ in the absence or presence of CTA091 (50 nM). Whole cell extracts were prepared after 24 h and analyzed by immunoblot for cyclin expression. Blots were reprobbed for actin as a control for protein quantitation and loading. Densitometry was used to quantify the expression of each protein, and composite actin-normalized expression data from 4 independent experiments are presented. Bars represent the mean actin-normalized expression $\pm$ SD. *, $P < 0.05$, treatment vs. vehicle (control). Horizontal lines are used to indicate significant differences in mean actin-normalized expression between 1,25(OH)₂D₃ treatment dose levels, and between 1,25(OH)₂D₃ and 1,25(OH)₂D₃ + CTA091 treatment groups.

Figure 4. The VDR is required for 1, 25(OH)₂D₃-mediated down-modulation of cyclin expression in lung cancer cells

H292 cells were cultured in complete medium for 24 h followed by transfection with human VDR siRNA or control siRNA. After 5 h, cells were treated with vehicle, CTA091 (50nM) 1,25(OH)₂D₃ (10 nM or 100 nM), or 1,25(OH)₂D₃ + CTA091. Cell extracts were prepared 3 d post-treatment and subjected to western blot analysis to evaluate protein expression. Blots were re-probed for actin as a control. Densitometry was used to quantify the expression of each protein, and protein expression was normalized to the intensity of the band for actin. Bars represent the mean normalized intensity $\pm$ SD from 3 independent experiments. Vehicle (control) was compared to each treatment. * $P < 0.05$, treatment vs. control.

Figure 5. CTA091 increases 1,25(OH)₂D₃-mediated G0/G1 arrest in H292 cells

H292 cells were seeded into 6-well dishes. After attachment, cells were treated with the indicated agents for 48 h. The cells were harvested and processed for cell cycle analysis. The cell cycle distribution was determined by flow cytometric analysis of PI-stained cells, as described in the Methods. Results, shown by phase, from 3 independent experiments are summarized. Bars represent mean % cells in that particular phase $\pm$ SD. *, $P < 0.05$, treatment vs vehicle (control); ^, $P < 0.05$, treatment vs 10 nM and 100 nM 1,25(OH)₂D₃ + CTA091. ^, $P < 0.05$, treatment vs 100 nM 1,25(OH)₂D₃ + CTA091.

Table 1

Pathway analysis

Treatment	Pathway	P-value	Network Objects (%)
100 nM 1,25(OH)₂D₃	1. Cell cycle (generic schema)	3.5×10^{-8}	7/21 (33)
	2. Cell cycle: Role of SCF in cell cycle regulation	4.2×10^{-7}	7/29 (24)
	3. Cell cycle: Nucleocytoplasmic transport of CDK/Cyclins	2.4×10^{-6}	5/14 (36)
	4. Cell cycle: Regulation of G1/S transition	3.9×10^{-6}	6/26 (23)
	5. Cell cycle: Transition and termination of DNA replication	6.2×10^{-6}	6/28 (21)
10 nM 1,25(OH)₂D₃ + CTA091	1. Cell cycle: Start of DNA replication in early S phase	1.3×10^{-11}	14/32 (44)
	2. Cell cycle (generic schema)	8.3×10^{-8}	9/21 (43)
	3. Cell cycle: Nucleocytoplasmic transport of CDK/Cyclins	6.7×10^{-7}	7/14 (50)
	4. Cell cycle: Sister chromatid cohesion	2.0×10^{-6}	8/22 (36)
	5. Immune response: MIF-JAB1	4.3×10^{-6}	8/24 (33)

MetaCoresoftware was used to assign functional pathways to genes that were significantly repressed by treatment with either 100 nM 1,25(OH)₂D₃ or 10 nM 1,25(OH)₂D₃ + CTA091 (compared to vehicle control). The top 5 regulated gene ontology pathways for each treatment, and the number of objects within each network that were modulated by treatment, are indicated.