# BIOLOGICAL EVALUATION OF NEW VITAMIN $D_2$ ANALOGUES.

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## Introduction

1,25-dihydroxyvitamin $D_3$ (1,25D) a well known anti-rachitic agent has also been shown to regulate cellular differentiation, proliferation and immunomodulation. The biological responses to 1,25D are mediated by binding to the vitamin D receptor (VDR), which then forms a heterodimer with retinoid X receptor (RXR). Together this complex binds to vitamin D response elements (VDREs) in the promoter region of target genes. CYP24A1, an enzyme responsible for degradation of 1,25D, is the most strongly regulated gene by VDR. Another VDR-target gene is a monocytic cell differentiation marker CD14, a co-receptor for bacterial lipopolysaccharide characteristic for monocytes and macrophages. Due to the expanse magnitude of 1,25D activity, it therefore suggests its potential therapeutic applications for the treatment of several diseases and disorders, including hyperproliferative diseases, immune dysfunction, endocrine disorders, and metabolic bone diseases. However, therapy using natural vitamin D hormone, 1,25D has been impeded in most cases due to its potent calcemic activity. Current research is focused on developing analogues with selective properties including improved anti-proliferative and pro-differentiating activities, as well as lower calcemic effects. In this study, we described the activities of a new set of analogues, compared to the one of PRI-1907 and to the 19-*nor* analogs of 1,25-dihydroxyvitamin $D_2$ with the natural or 24-*epi* side-chain, PRI-5100 (paricalcitol) and PRI-5101, respectively.

## Objectives

We analysed the biological profiles of 4 new Vitamin D2 analogues and compared them to that of 1,25D and PRI-1907 (Figure 1).

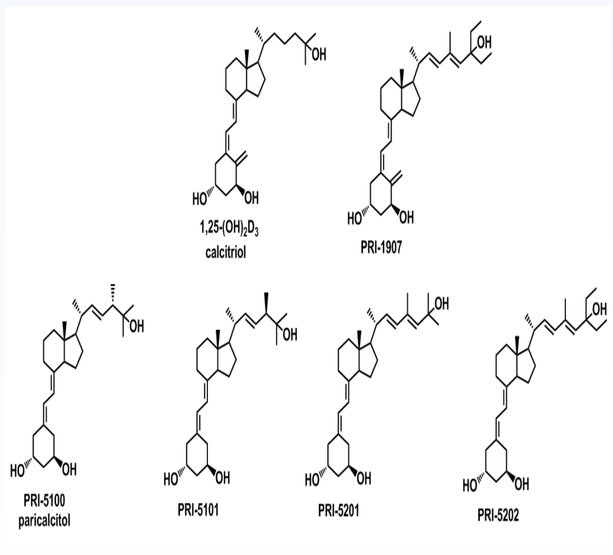



**Figure 1: Structures of 1,25D, PRI-1907, PRI-5100, PRI-5101, PRI-5201, PRI-5202**

## Methods

**Cell Lines:**
HL60 and HaCat cells were cultured in RPMI 1640 medium and DMEM respectively, supplemented with 10% fetal calf serum,100 units/ml penicillin and 100µg/ml streptomycin and grown in standard cell culture conditions, i.e. humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

**Human VDR Binding Assay:**
Binding affinity to VDR was evaluated using a 1,25D assay kit under manufacturer conditions (Polarscreen Vitamin D receptor competitor assay, Red, catalogue no. A15907; Life Technologies). The polarized fluorescence was measured using Envision, Perkin-Elmer.

**Serum Calcium Quantification and weight measure**
The analogues were dissolved in sesame oil and administered intraperitoneally (0.3µg/kg) every other day for three weeks. Calcium measurement was determined a day after the last dose using QuantiChom calcium Assay Kit. Weight was checked once a week.

**Determination of cell differentiation by flow cytometry**
Monocytic differentiation was determined using the expression of cell surface markers CD11b and CD14 and analysed using the FACs calibur flow cytometer (Becton Dickson, San Jose, CA). Data analysis was performed using flowing software.

**Western Blotting:**
10% SDS-PAGE gels were used to seperate proteins (derived from 5 x $10^6$ cells) and transferred to PVDF membranes. The membranes were dried and incubated with a primary antibody, and a horseradish peroxidase-conjugated secondary antibody. The protein bands were visualised with chemiluminescence.

**cDNA synthesis and PCR**
Total RNA was isolated using TriPure reagent according to manufacturer's recommendations. RNA quantity was determined using Nanodrop and quality of RNA was determined by gel electrophoresis. RNA was transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit. Initially, CYP24A1 and CD14 gene expression was assessed using semi-quantitative RT-PCR. Fold changes of mRNA levels of the genes CD14 and CYP24A1 relative to the GAPDH gene were calculated by relative quantification analysis.

## Results

### Relative binding affinity of 1,25D and analogues for human VDR

| | 1,25D | PRI-1907 | PRI-5100 | PRI-5101 | PRI-5201 | PRI-5202 |
|---|---|---|---|---|---|---|
| $IC_{50}$ | 2.320 e-009 | 6.172 e-009 | 5.599 e-010 | 4.921 e-010 | 1.193 e-009 | 3.598 e-009 |
| Relative Binding Affinity[a] | 100 | 38.6 | 414.35 | 471.4 | 194.46 | 64.4 |

**Figure 2: Vitamin D receptor binding.** Competitive binding of 1,25D and analogues to the full-length human VDR. The experiments were carried out in duplicate on three different occasions. $IC_{50}$ values were derived from dose-response curves and represent the measure of 50% inhibition of polarization of 1,25D and analogues. [a]The potency of 1,25D is normalized to 100.

### All analogues have lower calcemic activities in mice compared to 1,25D

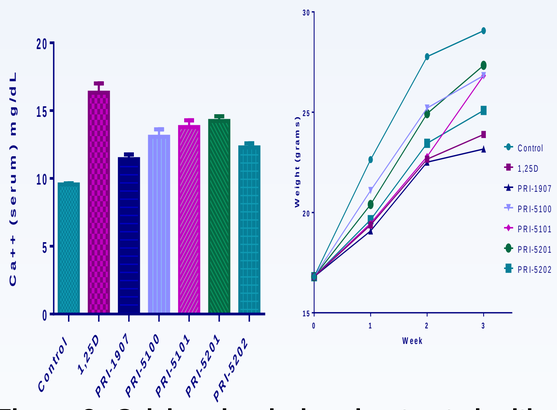



**Figure 3: Calcium levels in mice treated with the natural hormone 1,25D and analogues**. Five mice per group were treated with 0.3µg/kg of compounds, 1,25D or vehichle every other day for 3 weeks, and calcium levels were measured on day 21. Error bars represent standard deviation (SD).

### Analogues induce differentiation in human keratinocyte HaCat cells

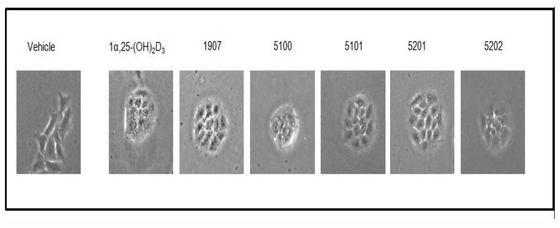
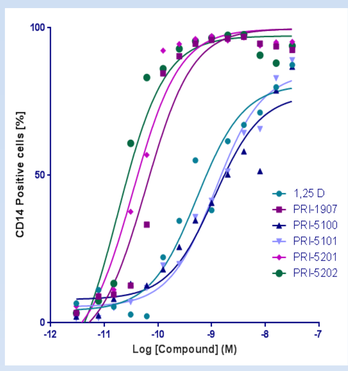



**Figure 4: Activity of analogues in HaCat cells**. Phase-contrast micrographs showing the induction by analogues of a differentiated adhesive in human keratinocytes. The cells were treated with analogues or 1,25D at a concentration of $10^{-7}$M for 48h.

## Results

### Analogues PRI 5201 and PRI 5202 induce differentiation of HL60 cells at a lower concentration than 1,25 D or PRI 1907




| | EC50 | EMR |
|---|---|---|
| 1,25D | 5.347 e-10 | 1 |
| PRI-1907 | 5.952 e-011 | .11 |
| PRI-5100 | 1.128 e-009 | 2.12 |
| PRI-5101 | 1.179 e-009 | 2.22 |
| PRI-5201 | 3.397 e-011 | 0.063 |
| PRI-5202 | 1.788 e-011 | 0.033 |

**Figure 5: $EC_{50}$ dose response curves depicting differentiation of AML cells in response to either 1,25D or analogues.** Cells were exposed to a range of concenrations for 96 hours and expression of differentiation marker CD14 was detected using flow cytometry. From this the $EC_{50}$ values were calculated, represented in the table above.

### CYP24A1, VDR's most highly regulated gene, is greatly upregulated in the leukemic HL60 cell line following treatment with analogues.

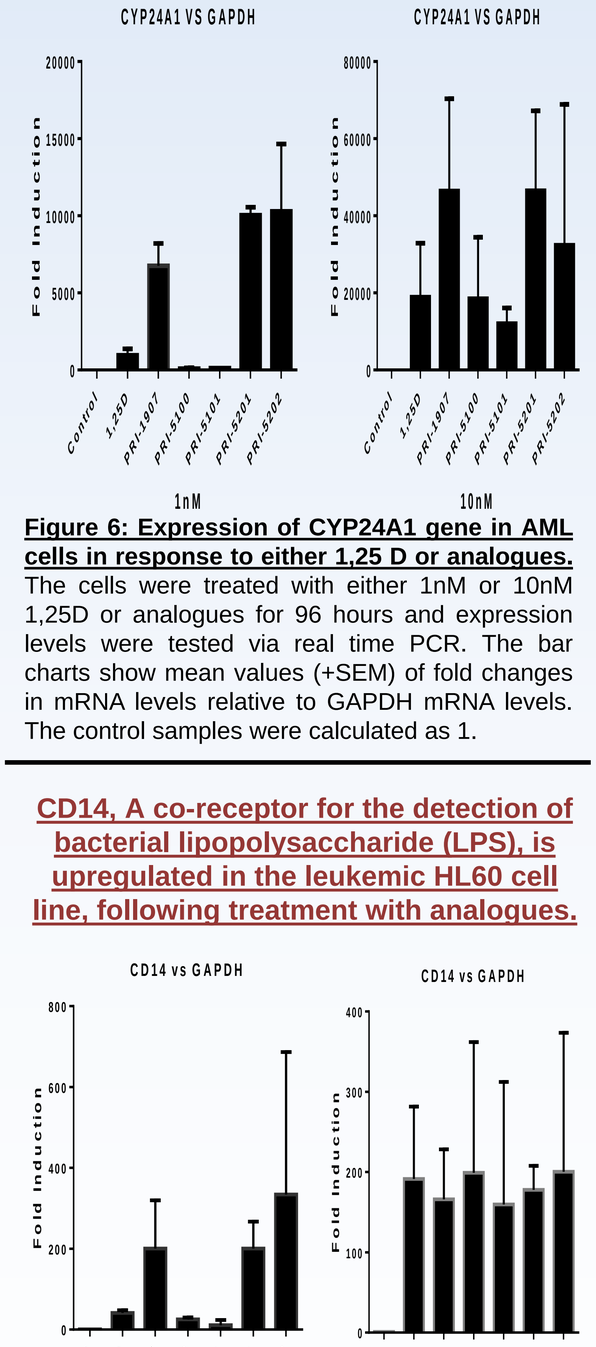



**Figure 6: Expression of CYP24A1 gene in AML cells in response to either 1,25 D or analogues.** The cells were treated with either 1nM or 10nM 1,25D or analogues for 96 hours and expression levels were tested via real time PCR. The bar charts show mean values (+SEM) of fold changes in mRNA levels relative to GAPDH mRNA levels. The control samples were calculated as 1.

### CD14, A co-receptor for the detection of bacterial lipopolysaccharide (LPS), is upregulated in the leukemic HL60 cell line, following treatment with analogues.




**Figure 7: Expression of CD14 gene in AML cells in response to either 1,25 D or analogues.** The cells were treated with either 1nM or 10nM 1,25D or analogues for 48hours and expression levels were tested via real time PCR. The bar charts show mean values (+SEM) of fold changes in mRNA levels relative to GAPDH mRNA levels. The control samples were calculated as 1.

### C/EBPβ isoforms in HL-60 cells treated with 1nM 1,25D and analogues.

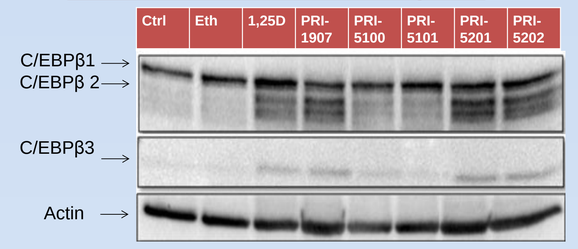



**Figure 8: C/EBPβ isoforms in HL-60 cells treated with 1nM 1,25D and Analogues. (A)** Western blot. HL60 cells were treated for 3 days with 1nM 1,25D and analogues. The nuclear fraction was separated by electrophoresis and transferred onto PVDF membrane, and probed with antibodies against C/EBPβ, and β-actin as fractionation/loading controls. In addition to the three C/EBPβ isoforms, unidentified bands, possibly cleavage products of C/EBPβ, are present.

### VDR expression is upregulated in HL60 cells following treatment with analogues at various timepoints

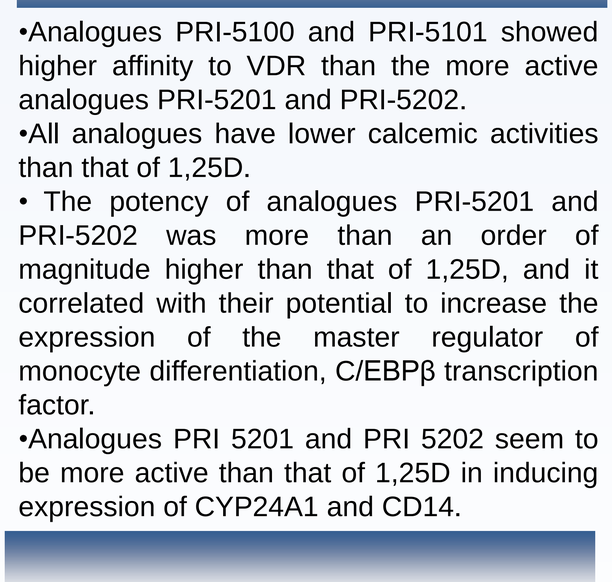



**Figure 9: Expression of VDR protein in AML cells in response to either 1,25D or analogues .** HI60 cells were exposed to 1nM 1,25D or analogue for 3, 24, 48 and 72 hours. The cells were fractioned into nuclear (N) fractions and analysed in western blots using anti-VDR and anti-actin antibodies.

## Conclusions

- Analogues PRI-5100 and PRI-5101 showed higher affinity to VDR than the more active analogues PRI-5201 and PRI-5202.
- All analogues have lower calcemic activities than that of 1,25D.
- The potency of analogues PRI-5201 and PRI-5202 was more than an order of magnitude higher than that of 1,25D, and it correlated with their potential to increase the expression of the master regulator of monocyte differentiation, C/EBPβ transcription factor.
- Analogues PRI 5201 and PRI 5202 seem to be more active than that of 1,25D in inducing expression of CYP24A1 and CD14.

## References

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