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PEOPLE MARIE CURIE ACTIONS DECIDE

Structure-function analysis of the analogues of 1,25-dihydroxyvitamin D_3 as potential candidates for cell differentiation therapy for AML.

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Acute Myeloid Leukaemia (AML), cancer of the myeloid cell line, is characterised by the inability of myeloid cells to undergo differentiation and thus resulting in a rapid accumulation of immature white blood cells. Current therapies to treat AML such as chemotherapy have not been successful, considering the increasing amount of cases being diagnosed each year, it is of the upmost importance to consider alternative therapies to treat patients. Studies have shown that this block in cell differentiation can be potentially mitigated by various agents, termed differentiation therapy. One such agent is that of 1,25-dihydroxyvitamin D_3 ($1,25D_3$), however, its clinical application is severely restricted due to the dose-side effects: potent hypercalcemia and increased bone resorption, making it necessary to develop analogues with selective properties. There are two main forms of 1,25D, $1,25D_2$ and $1,25D_3$. However, $1,25D_2$ is considered less toxic than $1,25D_3$ and thus has therapeutic potential. The studies on the mechanism underlying biological effects of 1,25D analogues provide important information that allow us to determine what structural modifications of 1,25D molecule are responsible for their changed biological properties.

Objectives

We analysed the biological profiles of 6 new Vitamin D_2 analogues and compared them to that of 1,25D, PRI-1906, and PRI-1907 (Figure 1).

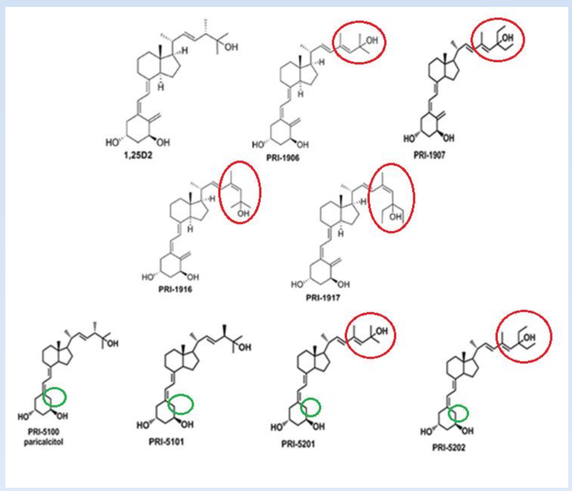

Figure 1: Structures of $1,25D_3$, PRI-1906, PRI-1907, PRI-1916, PRI-1917, PRI-5100, PRI-5101, PRI-5201, PRI-5202

Methods & References

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.

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Baurska H *et al* (2011) *Journal of Steroid Biochemistry& Molecular Biology* **126**:46-54
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Eelen G *et al* (2007) *Current Medicinal Chemistry* **14**:1893-1910 3-1014

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.

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Bouillon R *et al* (1995) *Endocrine Reviews* **16**:200-216
Ryynänen J *et al* (2013) *Cancers* **5**:1221-1241
Simmons D *et al* (1989) *Blood* **72**: 283-289
Pietraszek A *et al* (2013) *Steroids* **78**:1003-1014

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 $1,25D_3$, $1,25D_2$, and analogues for human VDR

	$1,25D_3$	$1,25D_2$	PRI-1906	PRI-1916	PRI-1907	PRI-1917	PRI-5100	PRI-5101	PRI-5201	PRI-5202
IC_{50}	$2.32x10^{-9}$	$1.466x10^{-8}$	$5.561x10^{-8}$	$6.048x10^{-9}$	$6.172x10^{-9}$	$6.848x10^{-8}$	$5.599x10^{-10}$	$4.921x10^{-10}$	$1.193x10^{-9}$	$3.598x10^{-9}$
RBA[a]	100	30.66	4.17	37	38	3	414.35	471.4	94.46	64.4

Table 1: Vitamin D receptor binding. ([a]RBA: Relative Binding Affinity)

Table 1. Vitamin D receptor binding. Competitive binding of $1,25D_3$ 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.

Results

Geometric Isomers of PRI-1906 and PRI-1907 have diverse pro-differentiating activities

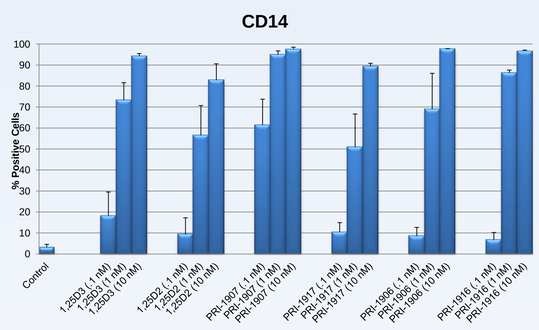
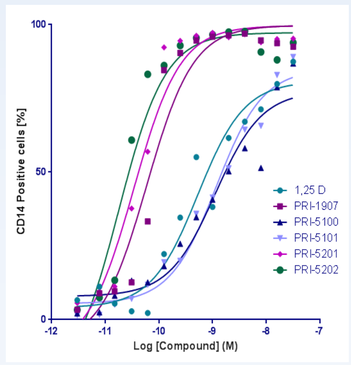


Figure 2. Expression of CD14 in HL60 cells exposed to $1,25D_3$, $1,25D_2$, and Analogues. The cells were exposed to compounds at the concentrations 0.1 nM, 1 nM and 10 nM for 96 hours and expression of CD14 was detected using flow cytometry. Mean values (±SEM) of percentages of positive cells are presented in the Y-axis.

Analogues PRI-5201 and PRI-5202 induce differentiation of HL60 cells at a lower concentration than $1,25D_3$ or PRI-1907



	EC_{50}
$1,25D_3$	$5.347x10^{-10}$
PRI-1907	$5.952 x10^{-11}$
PRI-5100	$1.128x10^{-9}$
PRI-5101	$1.179 x10^{-9}$
PRI-5201	$3.397 x10^{-11}$
PRI-5202	$1.788 x10^{-11}$

Figure 3. EC_{50} dose response curves depicting differentiation of AML cells in response to either $1,25D_3$ 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.

All analogues have lower calcemic activities in mice compared to $1,25D_3$

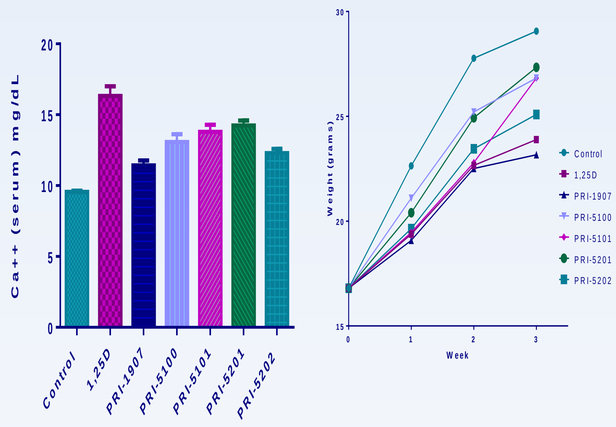


Figure 6. Calcium levels in mice treated with the natural hormone $1,25D_3$ and analogues. Five mice per group were treated with 0.3µg/kg of compounds, $1,25D_3$ or vehichle every other day for 3 weeks, and calcium levels were measured on day 21. Error bars represent standard deviation (± SEM).

C/EBPβ isoforms in HL60 cells treated with 1nM $1,25D_3$ and analogues

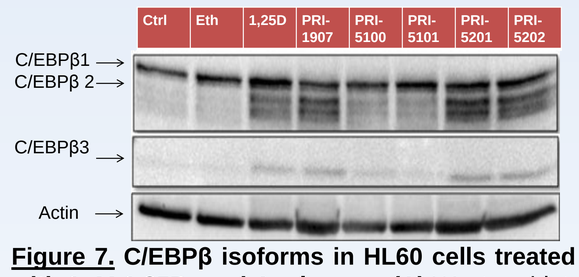


Figure 7. C/EBPβ isoforms in HL60 cells treated with 1nM $1,25D_3$ and Analogues. (A) Western blot. HL60 cells were treated for 3 days with 1nM 1,25D and analogues. The nuclear fractions were separated by electrophoresis and transferred onto PVDF membrane, and probed with antibodies against C/EBPβ, and 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

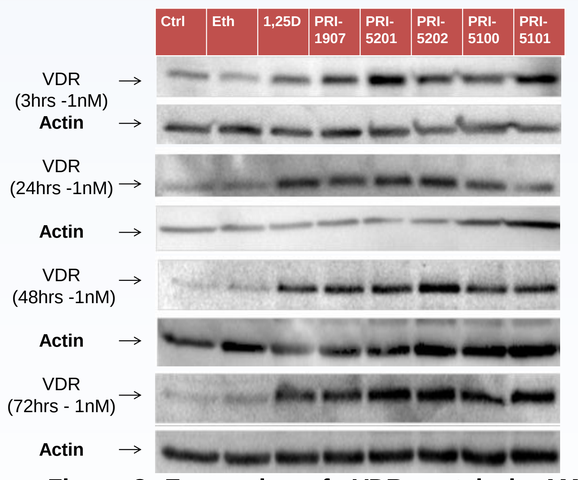


Figure 8. Expression of VDR protein in AML cells in response to either $1,25D_3$ or analogues Hl60 cells were exposed to 1nM $1,25D_3$ or analogue for 3, 24, 48 and 72 hours. Nuclear (N) fractions were isolated and analysed in western blots using anti-VDR and anti-actin antibodies.

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

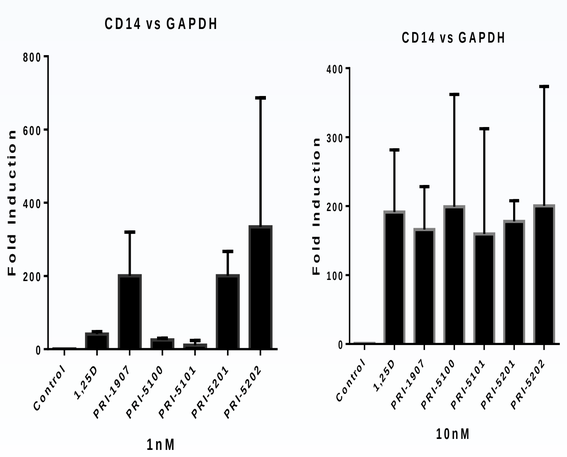


Figure 4. Expression of CD14 gene in HL60 cells in response to either $1,25D_3$ or analogues. The cells were treated with either 1nM or 10nM $1,25D_3$ or analogues for 48h and expression levels were tested via real time PCR. The bar charts show mean values (± SEM) of fold changes in mRNA levels relative to GAPDH mRNA levels. The control samples were calculated as 1.

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

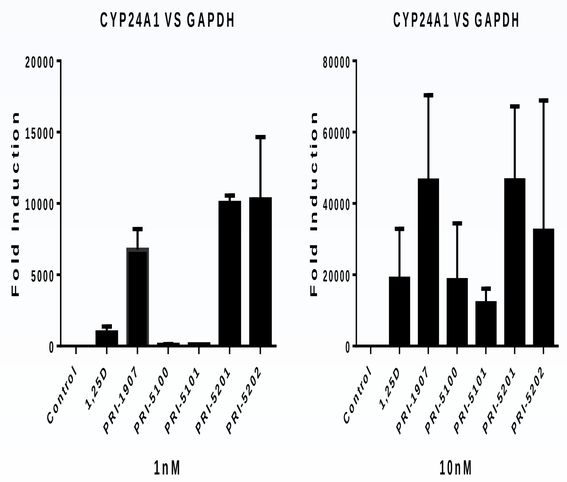


Figure 5. Expression of CYP24A1 gene in HL60 cells in response to either 1,25 D or analogues. The cells were treated with either 1nM or 10nM 1,25D or analogues for 96 hours and expression levels were tested via real time PCR. The bar charts show mean values (± SEM) of fold changes in mRNA levels relative to GAPDH mRNA levels. The control samples were calculated as 1.

Conclusions

- **Double point modified analogues are significantly more active than the analogues containing a single point modification.**
- **Double point modified analogues were shown to have significantly increased biological activities including; pro-differentiating activities, transcriptional activities, and up-regulation of proteins such as VDR and C/EBPβ.**
- **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 were shown to have lower calcemic activities then that of $1,25D_3$ when evaluated in mice.**
- **Affinity of analogues to VDR did not correlate with their biological activity.**

Acknowledgements

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