Biological Evaluation of new Vitamin D_2 analogues

Aoife Corcoran[1], Maria A. Bermudez[2], Samuel Seoane[2], Roman Perez-Fernandez[2*], Andrzej Kutner[3], Ewa Marcinkowska[1*]

[1]Faculty of Biotechnology, University of Wroclaw, 14a Joliot-Curie, 50-383 Wroclaw, Poland
[2]Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), University of Santiago de Compostela, Barcelona Avenue, 15782 Santiago De Compostela, A Coruña, Spain
[3]Pharmaceutical Research Institute, 8 Rydygiera, 01-793 Warsaw, Poland

Introduction

The active form of vitamin D_3, the hormone 1α,25-dihydroxyvitamin D_3 (1,25D) regulates cellular differentiation and proliferation in addition to its classical role in mineral homeostasis and bone mineralization. The cellular and physiological activities of 1,25D are mediated through a specific receptor known as VDR (a member of the nuclear receptor superfamily), and acts as a ligand dependent transcription factor. VDR forms a heterodimer with the retinoid X receptor (RXR) and binds to the gene promoter region of target genes. When 1,25D or other agonists bind to VDR, the receptor undergoes a conformational change. This conformational shift enables the release of co-repressor proteins and recruitment of co-activator proteins, thus leading to activation of gene transcription. Because of its ability to play a role in various biological processes, 1,25D has attracted considerable interest in the development of potential drugs for the treatment of hyperproliferative diseases and immune disorders. However, the clinical application of 1,25D is severely restricted due to its side effects such as potent hypercalcemia and increased bone resorption. Therefore 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 order to develop such analogues it is necessary to investigate the mechanisms underlying the biological effects of 1,25D analogues. These studies may provide vital information in determining which structural modifications of 1,25D molecule are responsible for their changed biological properties.

Aim

We recently synthesized new active analogues of 1,25D, which were designed based on the previously characterised analog PRI-1907, and will be denoted as PRI-5100, PRI-5101, PRI-5104, PRI-5201, PRI-5202 throughout this poster. In this study we wanted to analyse the biological profiles of these 5 new Vitamin D2 analogues and compare them to that of 1,25D and PRI-1907.

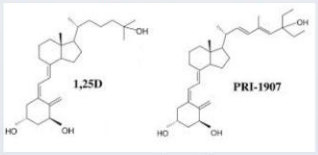


Figure 1: Structures of 1,25D and PRI-1907.

Materials and 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.

Serum Calcium Quantification and weight measure
The analogues were dissolved in sesame oiland 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 cell lysates (derived from 1.25×10^6 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

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

Figure 2: 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

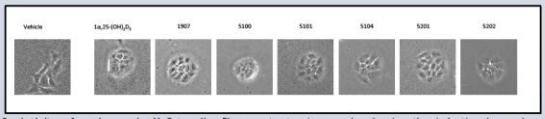


Figure 3: Activity of analogues in HaCat cells. Phase-contrast micropgraphs showing the induction by analogues of a differentiated adhesive in human keratinocytes. The cells were treated with analogues or 1,25D at a concenration of 10^{-7}M for 48h.

Analogues induce differentiation of the leukemic cell line HL60 into monocytic like cells

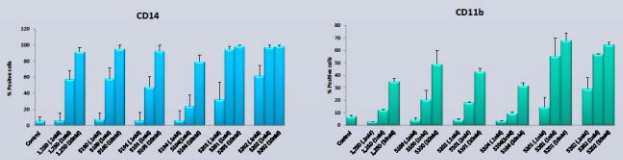
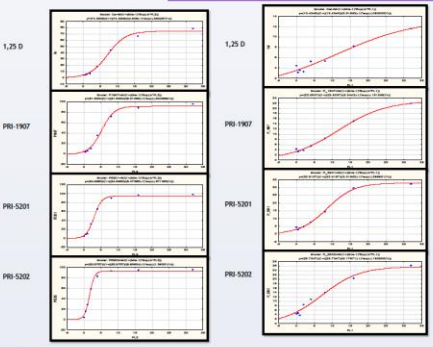


Figure 4: Differentiation of AML cells in response to either 1,25D or analogues. Cells were exposed to either 0.1nM, 1nM or 10nM for 96 hours and expression of differentiation markers CD14 anf CD11b was detected using flow cytometry. Mean values of percentages of Positive cells are presented in Y-axis.

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



Compound	CD14 EC_{50} (nM)
1,25D	9.09
PRI-1907	5.5
PRI-5201	3.1
PRI-5202	1.7

Figure 5: EC_{50} dose response curves depicting differentiation of AML cells in response to either 1,25D or analogues PRI-1907, 5201, 5202. Cells were exposed to a range of concenrations for 96 hours and expression of differentiation markers CD14(FI-2) and CD11b (FI-1) 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.

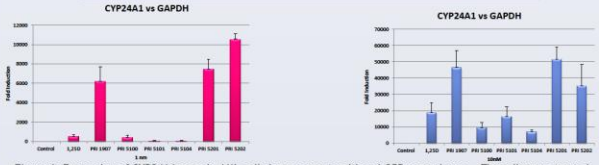


Figure 6: Expression of CYP24A1 gene in AML cells in response to either 1,25D or analogues. The cells were treated with either 1nM or 10nM 1,25D or analogues for 96 hours and expression levels were tested via real time PCR. The bar charts show mean values (±SEM) of fold changes in mRNA levels relative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

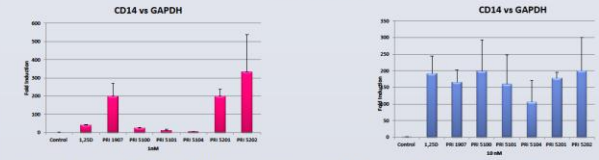


Figure 7: Expression of CD14 gene in AML cells in response to either 1,25D or analogues. The cells were treated with either 1nM or 10nM 1,25D or analogues for 48hours and expression levels were tested via real time PCR. The bar charts show mean values (±SEM) of fold changes in mRNA levels relative to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

VDR protein expression is upregulated in HL60 cells following treatment with analogues at various time-points

Figure 8: Expression of VDR protein in AML cells in response to either 1,25D or analogues. Hl6o 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

- All analogues have lower calcemic activities than that of 1,25D.
- Analogues PRI-5201 and PRI-5202 induce differentiation of AML into monocytic cells at a higher rate than 1,25D
- Analogues PRI-5201 and PRI-5202 seem to be more active than that of 1,25D in inducing of CYP24A1 and CD14
- Nuclear levels of VDR vary at different time-points for each of the analogues.

References

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Contact: aoifecorkie@hotmail.com