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 utmost 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), 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$_3$ is considered less toxic than 1,25D$_2$, and thus has therapeutic potential. The studies on the mechanism underlying biological effects of 1,25D$_3$ analogues provide important information that allows us to determine what structural modifications of 1,25D$_3$ molecule are responsible for their changed biological properties.

### Objectives

We analysed the biological profiles of 6 new Vitamin D$_3$ analogues and compared them to that of 1,25D, PRI-1906, and PRI-1907 (Figure 1).

### Methods & References

Relative binding affinity of 1,25D$_3$, 1,25D$_2$, and 1,25D$_1$ analogues for human VDR

<table>
<thead>
<tr>
<th>Analogues</th>
<th>1,25D$_3$</th>
<th>1,25D$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRI-1906</td>
<td>3.22±0.10</td>
<td>5.59±0.10</td>
</tr>
<tr>
<td>PRI-1916</td>
<td>6.06±0.08</td>
<td>8.46±0.08</td>
</tr>
<tr>
<td>PRI-1907</td>
<td>1.00±0.03</td>
<td>1.00±0.03</td>
</tr>
<tr>
<td>PRI-5100</td>
<td>1.93±0.03</td>
<td>1.93±0.03</td>
</tr>
<tr>
<td>PRI-5101</td>
<td>3.59±0.10</td>
<td>3.59±0.10</td>
</tr>
</tbody>
</table>

**Table 1: Vitamin D receptor binding.** (RBA: Relative Binding Affinity).

Analogues PRI-5201 and PRI-5202 induce differentiation of HL60 cells at a lower concentration than 1,25D$_3$ or PRI-1907.

**Figure 3.** $E_{50}$ dose response curves depicting differentiation of AML cells in response to either 1,25D$_3$, or analogues.

CYP24A1, VDR’s most highly regulated gene, is greatly upregulated in the leukemia 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 of 1,25D$_3$, or analogues for 48 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.

CYP24A1 is the enzyme that regulates the metabolism of 1,25D$_3$ and is important in the homeostasis of calcium and phosphate levels.

**Figure 5.** Expression of CYP24A1 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 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.

### Results

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

**Table 2:** Vitamin D receptor binding.

<table>
<thead>
<tr>
<th>Analogues</th>
<th>Control</th>
<th>1,25D</th>
<th>PRI-1906</th>
<th>PRI-1916</th>
<th>PRI-1907</th>
<th>PRI-5100</th>
<th>PRI-5101</th>
<th>PRI-5201</th>
<th>PRI-5202</th>
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</thead>
<tbody>
<tr>
<td>RBA</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**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 of 1nM, 10nM, and 100nM for 96 hours. The expression of CD14 was detected using flow cytometry. Mean values (±SEM) of positive cells are shown in the Y-axis.

**Figure 3:** $E_{50}$ dose response curves depicting differentiation of HL60 cells in response to either 1,25D$_3$, or analogues. Cells were exposed to a range of concentrations (1nM, 10nM, 100nM) for 3 days. Cell differentiation was determined by the percentage of cells with a red color in the flow cytometry.

**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 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.

**Figure 5:** Expression of CYP24A1 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 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.

**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 vehicle every other day for 3 weeks, and calcium levels were measured on day 21. Error bars represent standard deviation (±SEM).

### 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 the 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$_3$, and it correlated with their potential to increase the expression of the master regulator of monocytic differentiation, C/EBPβ transcription factor.
- Analogues were shown to have lower calcemic activities than that of 1,25D$_3$, and it was evaluated in mice.
- Affinity of analogues to VDR did not correlate with their biological activity.

**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$_3$ 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 a loading control. Bands were visualized using chemiluminescence. The values were normalized to β-actin expression.

**Figure 8:** Expression of VDR protein in AML cells in response to either 1,25D$_3$, or analogues. H60 cells were exposed to 1nM 1,25D$_3$ or analogues 3, 24, 48, and 72 hours. Nuclear (N) fractions were isolated and analysed in western blots using VDR- and anti-actin antibodies.

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