

# Novel vitamin D analogues; cytotoxic and anti-proliferative activity against a diffuse large B-cell lymphoma cell line and B-cells from healthy donors

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**Novel vitamin D analogues; cytotoxic and anti-proliferative activity against a diffuse large B-cell lymphoma cell line and B-cells from healthy donors**

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**Highlights**

- We have examined the action of 1,25D<sub>3</sub> and vitamin D analogues (VDAs) on malignant and healthy B cells.
- 1,25D<sub>3</sub> and VDAs displayed moderate cytotoxic and pro-apoptotic actions upon DLBCL cells.
- 1,25D<sub>3</sub> and VDAs displayed concentration and time-dependent anti-proliferative actions upon stimulated B-cells from healthy donors.
- VDAs may offer therapeutic potential for the treatment of DLBCL or conditions benefitted by B-cell depletion.

**Abstract**

Calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>, 1,25D<sub>3</sub>) and vitamin D side-chain modified analogs (VDAs) have gained considerable attention as potential drugs in the treatment of acute myeloid leukemia (AML), yet studies of the impact of 1,25D<sub>3</sub> and VDAs upon other haematological malignancies are more limited. To address this gap in knowledge, we have examined the action of 1,25D<sub>3</sub> and VDAs on a human cell line (DOHH2, K422) typifying diffuse large B-cell lymphoma (DLBCL) and also peripheral blood B-cells isolated from healthy donors.

1,25D<sub>3</sub> and certain VDAs displayed moderate cytotoxic and pro-apoptotic actions upon DLBCL cells. 1,25D<sub>3</sub> and VDAs (100 nM) caused the death of approximately 40% DOHH2 cells after 24 hours stimulation, similar to their impact on HL-60 cells (acute myeloid leukaemia cell line). In addition, 1,25D<sub>3</sub> and VDAs displayed concentration and time-dependent anti-proliferative actions upon stimulated B-cells from healthy donors. The VDAs inhibited proliferation by approximately 30%. Hence VDAs may offer therapeutic potential for the treatment of DLBCL or conditions benefitted by B-cell depletion.

Keywords: vitamin D analogues; B-cells; Diffuse Large B-cell lymphoma; proliferation; drug synergy

## 1. Introduction

Calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>; 1,25D<sub>3</sub>) impacts physiological processes in the human body, with perhaps the most notable being the regulation of Ca<sup>2+</sup> and phosphate (P<sub>i</sub>) transport and bone mineralization [1]. Other actions include control of the growth, differentiation and functional activity of numerous cell types including those of the skin, immune system and pancreas [2].

Numerous studies demonstrate that the active form of vitamin D promotes the differentiation of HL-60 cells (a cell line that typifies acute myeloid leukaemia AML) to a phenotype resembling differentiated monocytes in bone marrow [3], such that majority of cells are now CD14<sup>+</sup> cells [4]. Similar results have been obtained for cell lines of biologically related origin, e.g. THP-1 and U-937 cells [5, 6]. In addition to the pro-differentiative effects of 1,25D<sub>3</sub>, considerable attention has been paid to its cytotoxic and anti-proliferative actions. This has been studied in various mouse models, human cultured breast and colon cancers and also in HL-60 cells, which growth arrest in the G1 phase of cell cycle [7-9]. It should be noted however that the antitumor activity of 1,25D<sub>3</sub> is evident at supra-physiological concentrations, which also promote hypercalcemia and hypercalciuria [10], which are likely to limit therapeutic potential. This has motivated the search for vitamin D analogues (VDAs) with less propensity to increase Ca<sup>2+</sup> levels. Some identified compounds retain interaction with the VDR and the anti-proliferative/pro-differentiative capacity but display more limited effect on calcium levels in comparison to 1,25D<sub>3</sub> [11].

Lymphoma is a cancer of lymphocytes presenting as either Hodgkin's (HL) or non-Hodgkin's lymphoma (NHL); over 90% of all NHL are of B-cell origin. According to the

Cancer Research UK statistics over 12,000 people are diagnosed with NHL every year in the UK which makes it the 6th most common cancer in this country and the 11th most common cause of cancer death worldwide. As many as 40% of people diagnosed with this disease are predicted to survive less than 10 years.

It is estimated that 50% of NHL cases in the UK and 25-35% worldwide can be categorised as diffuse large B-cell lymphoma (DLBCL) [12]. DLBCL is an aggressive malignancy of B lymphocytes of germinal centre origin and is characterised by the presence of rapidly proliferating large cells with basophilic cytoplasm and vesicular nuclei [13]. Current treatments for DLBCL include the anti-CD20 monoclonal antibody, rituximab, and cycles of a combination of cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP). Unfortunately, rituximab-CHOP treatment (R-CHOP) can be associated with severe toxicity and only displays failure-free survival of 65% after 3 years [14].

There is little data concerning the therapeutic potential of vitamin D analogues for the treatment of NHL. To address further therapeutic potential from 1,25D<sub>3</sub> and VDAs, we investigate in the present study the impact of 1,25D<sub>3</sub> and novel VDAs upon DOHH2 and K422, cell lines representative of DLBCL, as well as B-cells from healthy donors.

## 2. *Experimental*

### 2.1 *Vitamin D analogues*

1,25D<sub>3</sub> and all analogues (PRI-1890, PRI-1906, PRI-2191, PRI-2205; Fig. 1) were synthesized in the Pharmaceutical Research Institute (Warsaw, Poland). The compounds were dissolved in absolute ethanol, the concentration adjusted to 100 µM and the aliquots stored at -20°C prior to experiments.

### 2.2 *Cell culture*

HL-60 and DOHH-2 cells were cultured in RPMI-1640 medium (Sigma Aldrich, UK) supplemented with 1 % L-glutamine (Life Technologies), 1 % penicillin/streptomycin (Sigma Aldrich, 10000 units penicillin and 10 mg streptomycin per ml) and 10 % heat-inactivated fetal bovine serum (later referred as: FBS HI, Sigma Aldrich). Cells were subcultured at a density of 1 – 1.5 x 10<sup>6</sup> cells/ml.

### 2.3 *Isolation of human peripheral blood mononuclear cells (PBMCs)*

Peripheral blood mononuclear cells were isolated from whole blood from healthy donors or from Leukocyte Reduced System (LRS) cones using Ficoll–Paque (GE Healthcare). 20 ml of blood was layered on 25 ml of Ficoll-Paque and centrifuged at 900 x g for 30 minutes. The PBMC layer was mixed with RPMI in a new tube and centrifuged an additional three times, at 400 x g for 10 minutes, 8 minutes and 5 minutes respectively.



#### *2.4 Preparation of cell nuclear fractions*

Cells were washed twice with PBS and resuspended in an ice-cold lysis buffer (10 mM Tris, pH 7.4, 1 mM EGTA, 200 mM sucrose) with protease inhibitors (Sigma Aldrich). The lysates were centrifuged for 5 min at 18000 x g at 4°C and disrupted by sonication on ice 3 x 3 seconds. The protein concentration in the lysates was measured using a Bradford protein assay.

#### *2.5 SDS-PAGE and Western blot*

The lysates were treated with Radioimmunoprecipitation assay buffer (RIPA) (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin, 0.5 mM PMSF) and gel sample buffer containing 12% sodium dodecyl sulfate and 10% β-mercapto-ethanol. Samples were placed in boiling water for 10 minutes and cooled down on ice before loading onto a gel. Typically 15 µg of proteins were loaded onto 12% Tris-Glycine gel and run at 35 mA for about 2 hours. Proteins were then transferred to PVDF membranes which were then blocked in 10% non-fat milk for 2 hours at room temperature. Membranes were then incubated in primary mouse anti-human VDR antibody (SantaCruz, clone D6, used at 1:200 dilution) in 5% non-fat milk overnight at 4°C. Membranes were washed and incubated subsequently in secondary antibody (Cell Signalling, horse anti-mouse IgG HRP linked, used at 1:3,000 dilution) in 5% non-fat milk at room temperature for 2 hours. The protein bands were visualized with chemiluminescence.

### 2.6 Determination of cell apoptosis and necrosis using PhiPhiLux and propidium iodide

Cells were stimulated for the indicated periods of time and following the incubation were washed with PBS by centrifugation at  $400 \times g$  for 5 minutes. The supernatant was discarded and cells were resuspended in 60  $\mu$ l of PhiPhiLux solution and incubated for 30 minutes at  $37^{\circ}\text{C}$ . PBS was added to each well and the plate centrifuged at  $400 \times g$  for 5 minutes. Cells were resuspended using flow cytometry buffer (supplied in the PhiPhiLux kit) and 2 minutes before running the samples on the cytometer, propidium iodide was added to a final concentration of 5  $\mu\text{g}/\text{ml}$ .

### 2.7 Determination of cell proliferation using eFluor450 proliferation dye

PBMCs were resuspended in PBS to a concentration of  $20 \times 10^6$  cells/ml and labelled with eFluor450 (20  $\mu\text{M}$ ). The cell suspension was incubated at  $37^{\circ}\text{C}$  (protected from light) for 20 minutes. To stop the reaction 10 ml cold complete media was added to the cells and left for 5 minutes. The cells were centrifuged 3 times ( $400 \times g$  for 5 minutes) in 30 ml of ice-cold complete medium. After the final wash the cells were plated in 96-well plate along with test compounds or vehicle. Cells were incubated for 3, 5 and 7 days in the presence of Protein A from *Staphylococcus aureus* (SAC, 1:10,000) and Interleukin-2 (IL-2, 20 units/ml). The cells were washed twice with PBS and stained with mouse anti-human CD19 antibody PE conjugated (BD) for 20 minutes at  $4^{\circ}\text{C}$ . The cells were again washed and resuspended in 1% FBS in PBS buffer, before assay by flow cytometry (with the addition of a known number of counting beads).

### 2.8 Statistical analysis

Where applicable multiple comparisons statistical tests were performed in GraphPad Prism 6 software using one-way ANOVA which was followed by the Bonferroni post-test.

## 3. Results and discussion

There are already a few reports describing the impact of vitamin D on B-cells. It is known that normal B-cells express VDR at very low levels though the protein is up-regulated following the stimulation of the cells [15]. Malignant B-cells express VDR and both Hodgkin's and Non-Hodgkin's lymphoma cells have relatively high levels of this protein [16]. It has been shown that 1,25D<sub>3</sub> inhibits proliferation of anti-CD40, anti-IgM, and IL-21 stimulated B-cells [17]. 1,25D<sub>3</sub> has also been shown to up-regulate the production of IL-10 in stimulated B-cells [18]. Of relevance, it has been shown recently that vitamin D deficiency has been associated with significantly poorer outcome in DLBCL patients treated with rituximab [19].

### 3.1 VDR is expressed by DOHH2 cells and its nuclear translocation is induced after application of VDAs

Expression of VDR in nuclear fractions of DOHH2 cells was assessed after stimulation with 1,25D<sub>3</sub> or various VDAs (all at 10 nM) for 48 hours. HL-60 cells were included as a positive control (Fig. 2). 1,25D<sub>3</sub> and the VDAs all increased the expression of VDR protein. Similarly VDR up-regulation in the nuclear fraction by 1,25D<sub>3</sub> was evident in HL-60 cells (Fig. 2).

It has to be noted that there is a discrepancy in the literature between binding affinities of the VDAs to VDR and their biological profile. Other analogues of vitamin D have been reported to bind to the receptor with much lower affinities than the active form of vitamin D, yet some of them had more pronounced biological activities (e.g. anti-proliferative properties, lower calcemic effects) than the parent compound [20, 21]. One of the possible explanations for this phenomenon is that upon binding to the VDR downstream signaling actions differ, which manifests differences [22]. Alternatively the discrepancy between the binding affinities and the biological activities of the VDAs may be explained by their differential susceptibility to metabolism by CYP24A1. Indeed it may also be relevant that the expression of this enzyme is differentially induced in cell lines by structurally distinct VDAs [23].

### *3.2 VDAs are cytotoxic to DOHH2 and HL-60 cells*

The impact of 1,25D<sub>3</sub> and VDAs upon the viability of the DOHH2 and HL-60 cells was assessed at different time points (24, 48 and 72 hours). 1,25D<sub>3</sub> and VDAs (100 nM) were toxic to cells with cell death primarily associated with necrosis rather than apoptosis. DOHH2 cells were most prone to the VDAs following 24 hours treatment however no clear differences were evident between different analogues (Fig. 3 A). At the two later time points we assume that the compounds have lost their activity (perhaps due to metabolism) and that the cells affected at the early stages of culture have died and hence do not form part of the analyses at 48 and 72h. On the contrary, the viability of HL-60 cells decreased with time and was the lowest after 72 hours (Fig. 3 B). In K422 cells cytotoxicity of vitamin D and some analogues – albeit less

pronounced that in DOHH-2 cells - has been retained as was the impact of staurosporine, suggesting the greater resistance of the cell line (Fig. 3 C)

### *3.3 VDAs inhibit the proliferation of stimulated CD19+ B-cells*

Next, we examined the effects of 1,25D<sub>3</sub> and VDAs on anti-CD19 labelled B-cells from PBMCs obtained from healthy donors. B-cells within the PBMC population were induced to proliferate in response to SAC/IL-2 [24]. The cells were kept in culture for 7 days to assess the impact of VDAs (1, 10 and 100 nM; Fig. 4)

1,25D<sub>3</sub> and VDAs decreased the number of proliferated CD19+ after 7 days in a concentration-dependent manner indicating that VDAs inhibit the proliferation on SAC/IL-2 stimulated normal B-cells, which potentially may serve as beneficial in diseases diagnosed with over proliferating B-cells, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) [25, 26].

### *3.3 VDAs and clomipramine cooperate in their anti-proliferative effects*

Many studies have demonstrated that 1,25D<sub>3</sub> and some analogues can enhance the anti-proliferative efficacy of various chemotherapeutics in different cancer cells [9, 27]. In addition, with the realisation that a single drug modality – a single ‘magic bullet’ – is unlikely to offer complete molecular remission, we have also investigated potential synergies of VDAs with other drugs. In the present study we report the VDA combination with the anti-depressant drug clomipramine. Clomipramine has been shown to be cytotoxic and pro-apoptotic against glioma cells [28] and anti-proliferative against malignant, and to a lesser extent, normal B-cells [29, 30]. In previous studies [30, 31] 5 - 10 µM clomipramine was used to demonstrate activity in proliferation assays. The goal of the polytherapy is to obtain similar or higher potential therapeutic outcome with lower adverse effects by combining 2 or more

active compounds in lower concentrations. Therefore clomipramine was used here at 1  $\mu$ M. In the present study we applied clomipramine with a range of 1,25D<sub>3</sub> and VDAs concentrations (1, 10 and 100 nM) to SAC/IL-2 stimulated PBMCs for 7 days and demonstrated an increase in the action of the drug combination in comparison with either drug alone (Fig. 5), however these increases did not reach statistical significance.

#### **4. Conclusions**

In summary, this study demonstrates that relatively high concentrations of 1,25D<sub>3</sub> and vitamin D analogues are cytotoxic to DOHH2 cells (with less pronounced effect in K422 cells) with the extent of killing similar to that observed against HL-60 cells. We have also shown that DOHH2 cells express VDR and that ligand engagement increases expression. From this, we conclude that DOHH2 is a good model to study the effects of 1,25D<sub>3</sub> and derived compounds in DLBCL. VDAs also inhibited the proliferation of SAC/IL-2 stimulated normal B-cells in a concentration-dependent manner. This effect was enhanced when the analogues were used in combination with the tricyclic anti-depressant clomipramine. Together, this work identifies the potential of using VDAs in a variety of malignant and non-malignant B-cell diseases.

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## References

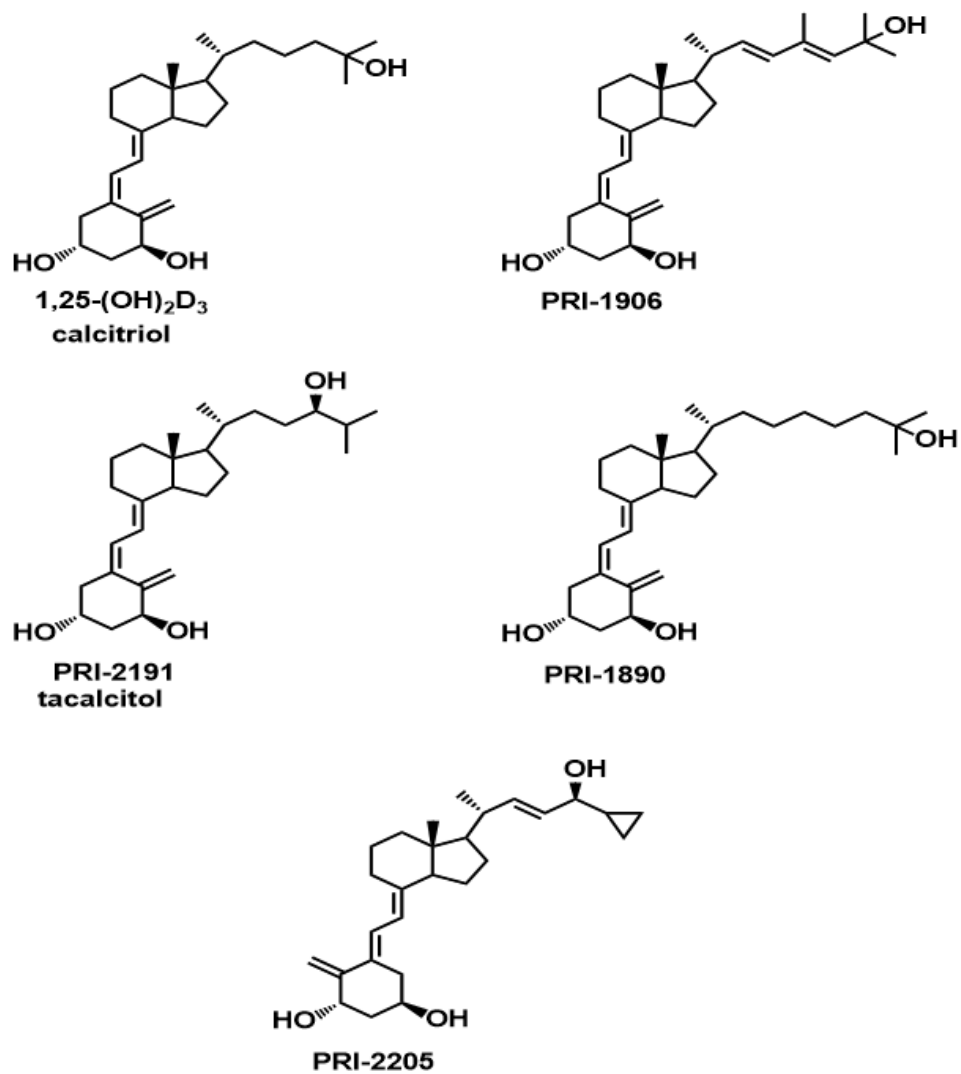
1. Chen, M.L., et al., *Induction of vitamin D receptor mRNA expression in psoriatic plaques correlates with clinical response to 1,25-dihydroxyvitamin D3*. J Invest Dermatol, 1996. **106**(4): p. 637-41.
2. Pike, J.W. and M.B. Meyer, *The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3)*. Endocrinol Metab Clin North Am, 2010. **39**(2): p. 255-69, table of contents.
3. Brackman, D., F. Lund-Johansen, and D. Aarskog, *Expression of leukocyte differentiation antigens during the differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D3: comparison with the maturation of normal monocytic and granulocytic bone marrow cells*. J Leukoc Biol, 1995. **58**(5): p. 547-55.
4. Marcinkowska, E., A. Kutner, and C. Radzikowski, *Cell differentiating and anti-proliferative activity of side-chain modified analogues of 1,25-dihydroxyvitamin D3*. J Steroid Biochem Mol Biol, 1998. **67**(1): p. 71-8.
5. Daigneault, M., et al., *The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages*. PLoS One, 2010. **5**(1): p. e8668.
6. Olsson, I., et al., *Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1 alpha,25-dihydroxycholecalciferol*. Cancer Res, 1983. **43**(12 Pt 1): p. 5862-7.
7. Jung, C.W., et al., *Antiproliferative effect of a vitamin D3 analog, EB1089, on HL-60 cells by the induction of TGF-beta receptor*. Leuk Res, 1999. **23**(12): p. 1105-12.

8. Milczarek, M., et al., *Synthesis and Biological Activity of Diastereomeric and Geometric Analogs of Calcipotriol, PRI-2202 and PRI-2205, Against Human HL-60 Leukemia and MCF-7 Breast Cancer Cells*. *Cancers* (Basel), 2013. **5**(4): p. 1355-78.
9. Milczarek, M., et al., *Vitamin D analogs enhance the anticancer activity of 5-fluorouracil in an in vivo mouse colon cancer model*. *BMC Cancer*, 2013. **13**: p. 294.
10. Mocellin, S., *Vitamin D and cancer: deciphering the truth*. *Biochim Biophys Acta*, 2011. **1816**(2): p. 172-8.
11. Nadkarni, S., et al., *Double point modified analogs of vitamin d as potent activators of vitamin d receptor*. *Curr Pharm Des*, 2015. **21**(13): p. 1741-63.
12. Cultrera, J.L. and S.M. Dalia, *Diffuse large B-cell lymphoma: current strategies and future directions*. *Cancer Control*, 2012. **19**(3): p. 204-13.
13. Martelli, M., et al., *Diffuse large B-cell lymphoma*. *Crit Rev Oncol Hematol*, 2013. **87**(2): p. 146-71.
14. Holte, H., et al., *Dose-densified chemoimmunotherapy followed by systemic central nervous system prophylaxis for younger high-risk diffuse large B-cell/follicular grade 3 lymphoma patients: results of a phase II Nordic Lymphoma Group study*. *Ann Oncol*, 2013. **24**(5): p. 1385-92.
15. Provvedini, D.M., et al., *1,25-dihydroxyvitamin D<sub>3</sub> receptors in human leukocytes*. *Science*, 1983. **221**(4616): p. 1181-3.
16. Renne, C., A.H. Benz, and M.L. Hansmann, *Vitamin D<sub>3</sub> receptor is highly expressed in Hodgkin's lymphoma*. *BMC Cancer*, 2012. **12**: p. 215.
17. Chen, S., et al., *Modulatory effects of 1,25-dihydroxyvitamin D<sub>3</sub> on human B cell differentiation*. *J Immunol*, 2007. **179**(3): p. 1634-47.



18. Heine, G., et al., *1,25-dihydroxyvitamin D(3) promotes IL-10 production in human B cells*. Eur J Immunol, 2008. **38**(8): p. 2210-8.
19. Bittenbring, J.T., et al., *Vitamin D deficiency impairs rituximab-mediated cellular cytotoxicity and outcome of patients with diffuse large B-cell lymphoma treated with but not without rituximab*. J Clin Oncol, 2014. **32**(29): p. 3242-8.
20. Carballa, D.M., et al., *Synthesis and biological evaluation of 1 $\alpha$ ,25-dihydroxyvitamin D(3) analogues with a long side chain at C12 and short C17 side chains*. J Med Chem, 2012. **55**(20): p. 8642-56.
21. Sokolowska, K., et al., *Synthesis and biological activity of two C-7 methyl analogues of vitamin d*. J Org Chem, 2015. **80**(1): p. 165-73.
22. Slatopolsky, E., J. Finch, and A. Brown, *New vitamin D analogs*. Kidney Int Suppl, 2003(85): p. S83-7.
23. Bauraska, H., et al., *Structure-function analysis of vitamin D(2) analogs as potential inducers of leukemia differentiation and inhibitors of prostate cancer proliferation*. J Steroid Biochem Mol Biol, 2011. **126**(1-2): p. 46-54.
24. Li, L., et al., *The apoptosis and proliferation of SAC-activated B cells by IL-10 are associated with changes in Bcl-2, Bcl-xL, and Mcl-1 expression*. Cell Immunol, 1997. **178**(1): p. 33-41.
25. Harvey, P.R. and C. Gordon, *B-cell targeted therapies in systemic lupus erythematosus: successes and challenges*. BioDrugs, 2013. **27**(2): p. 85-95.
26. Clark, E.A. and J.A. Ledbetter, *How does B cell depletion therapy work, and how can it be improved?* Ann Rheum Dis, 2005. **64 Suppl 4**: p. iv77-80.

27. Siwinska, A., et al., *Potentiation of the antiproliferative effect in vitro of doxorubicin, cisplatin and genistein by new analogues of vitamin D*. *Anticancer Res*, 2001. **21**(3B): p. 1925-9.
28. Daley, E., et al., *Chlorimipramine: a novel anticancer agent with a mitochondrial target*. *Biochem Biophys Res Commun*, 2005. **328**(2): p. 623-32.
29. Meredith, E.J., et al., *The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential anti-tumor target for psychotropics*. *FASEB J*, 2005. **19**(9): p. 1187-9.
30. Chamba, A., et al., *SLC6A4 expression and anti-proliferative responses to serotonin transporter ligands chlomipramine and fluoxetine in primary B-cell malignancies*. *Leuk Res*, 2010. **34**(8): p. 1103-6.
31. Wasik, A.M., *Mining dop aminergic pathways for novel therapeutic approaches to Non-Hodgkin's Lymphoma*, in *School of Immunity and Infection, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences*. 2009: University of Birmingham. p. 269.



*Figure 1. Structures of calcitriol (1,25D<sub>3</sub>) and vitamin D<sub>2</sub> analogues that were used in the present study.*

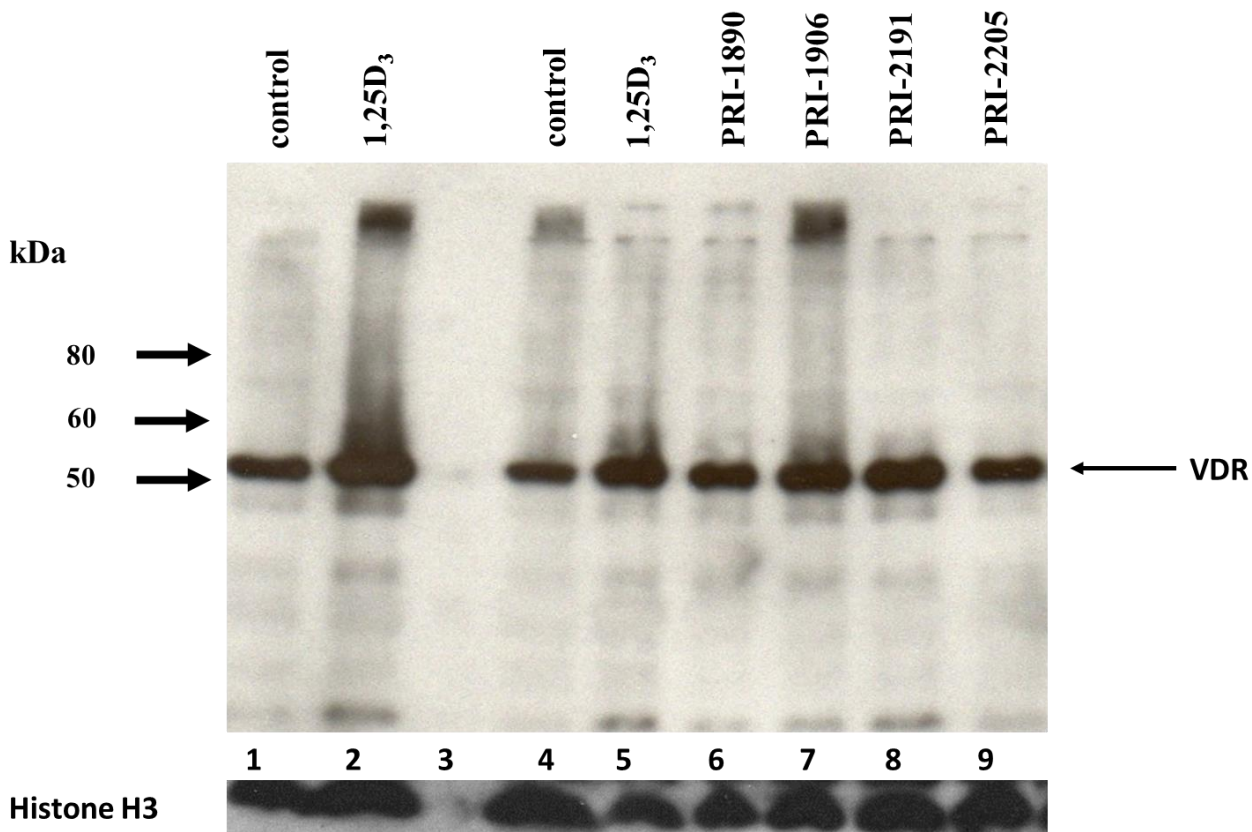
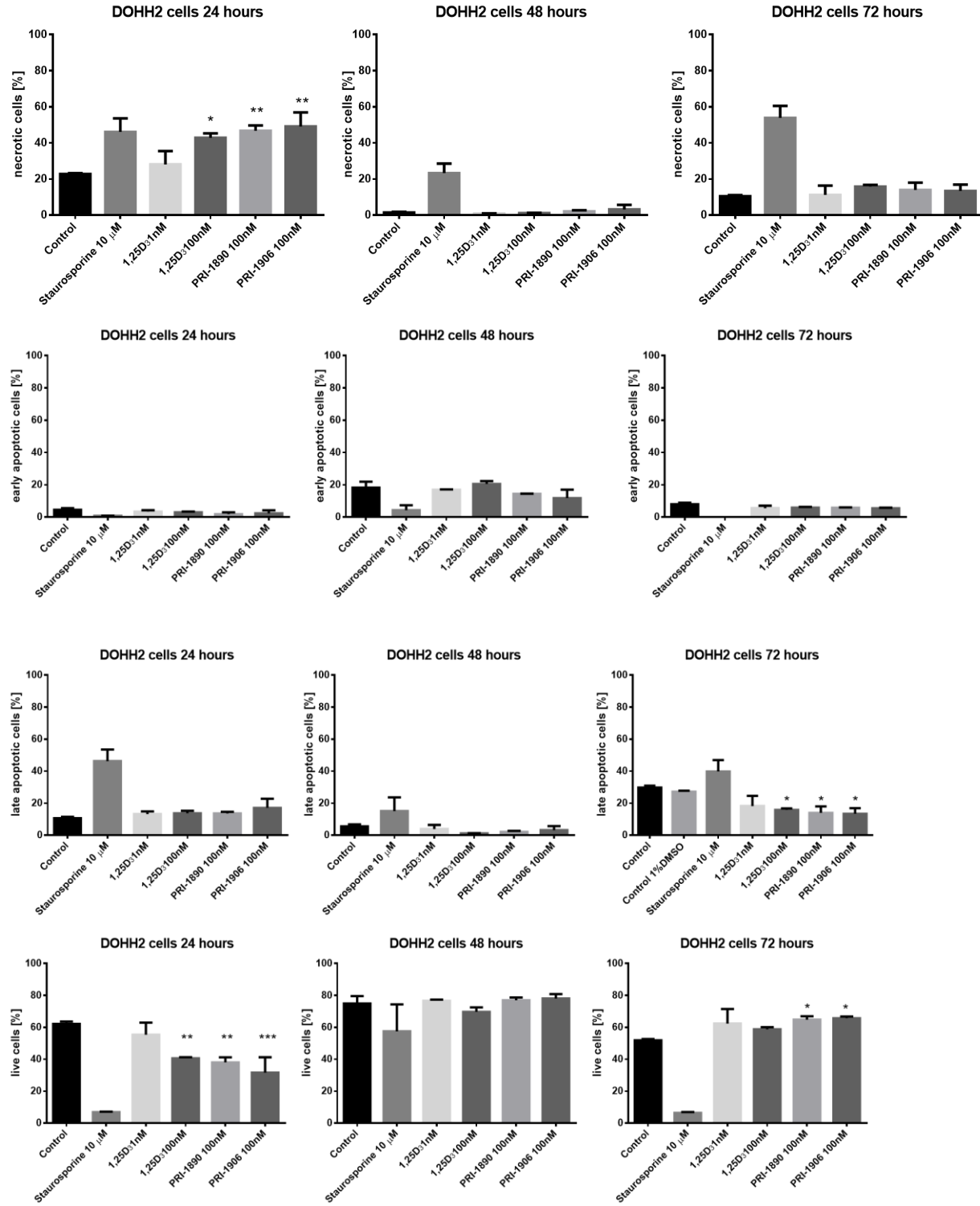
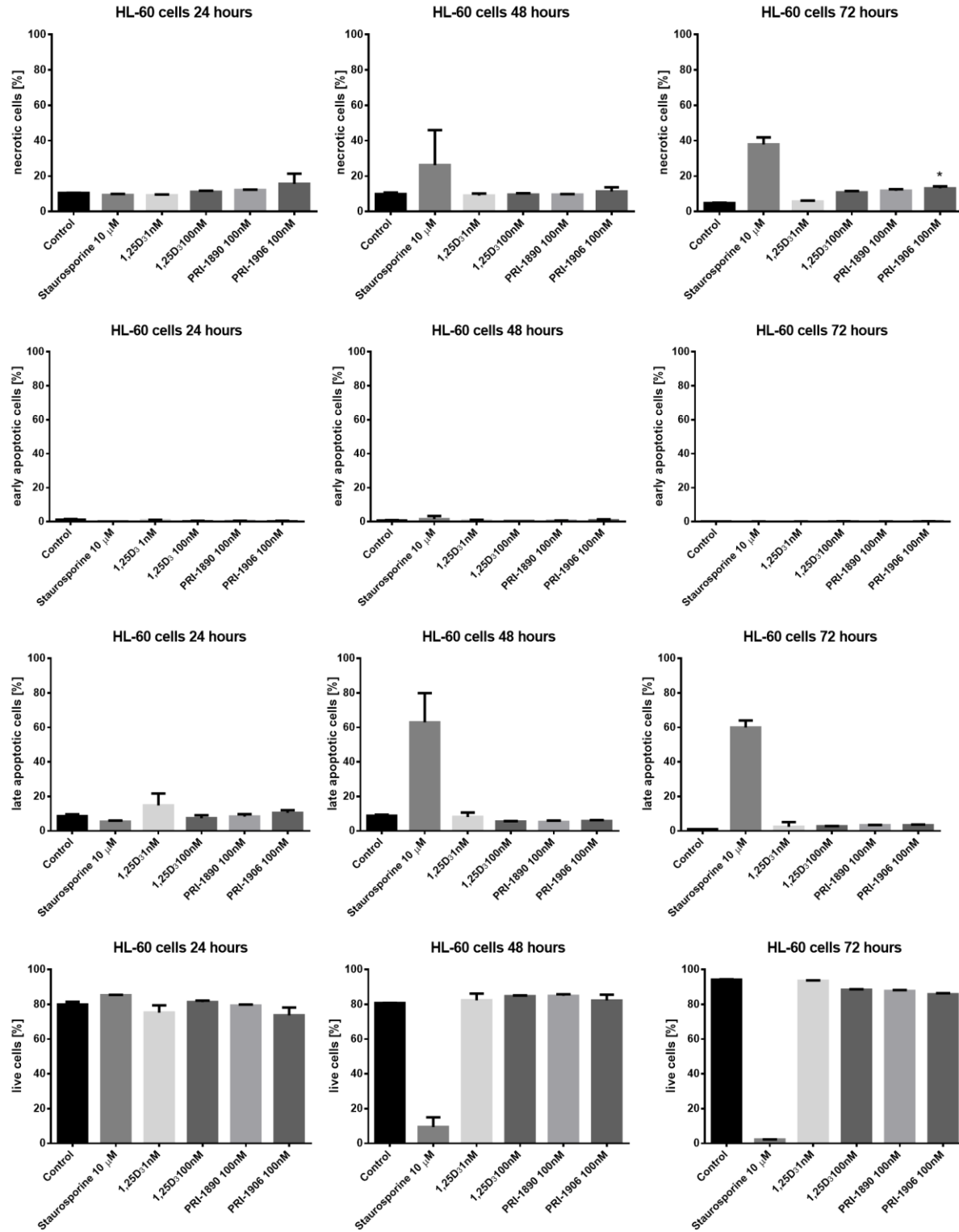


Figure 2. Expression of VDR immunoreactivity in nuclear fractions of HL-60 (lanes 1 and 2) and DOHH2 (lanes 4-9). Cells were treated for 48 hours with 10 nM of each compound or vehicle control. 15  $\mu$ g of protein were loaded for each well. Histone H3 immunoreactivity was used as a loading control. A representative example of two independent experiments with similar results is shown.

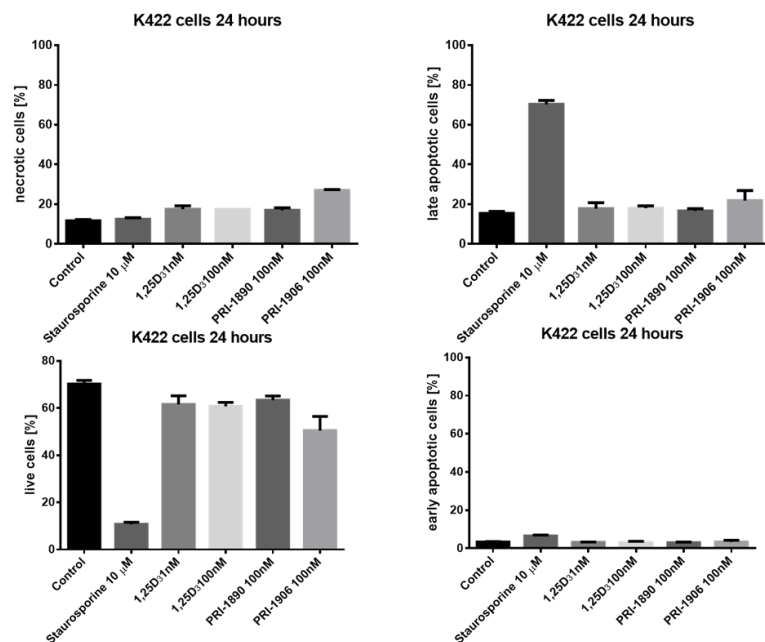
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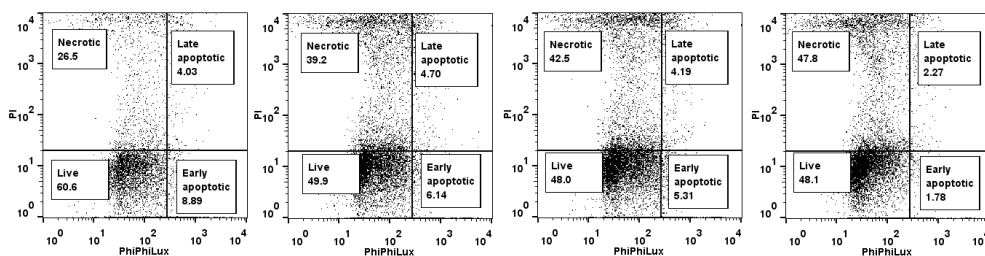


Figure 3. Impact of 1,25D<sub>3</sub> and some VDAs upon viability of (A) DOHH2, (B) HL-60 cells and (C) K422 cells following different treatment for 3 different time periods assayed by PhiPhiLux assay. PRI-1906 markedly increased the number of necrotic HL-60 cells after 72 hours. Representative flow cytometry plots (D) for DOHH2 cells (from left to right: control, 1,25D<sub>3</sub>, PRI-1890 and PRI-1906 at 100 nM). Data represent mean  $\pm$  SD, n=3. \*\*\* ( $P < 0.01$ ), \*\* ( $P < 0.05$ ), \* ( $P < 0.1$ ) compared with Control.

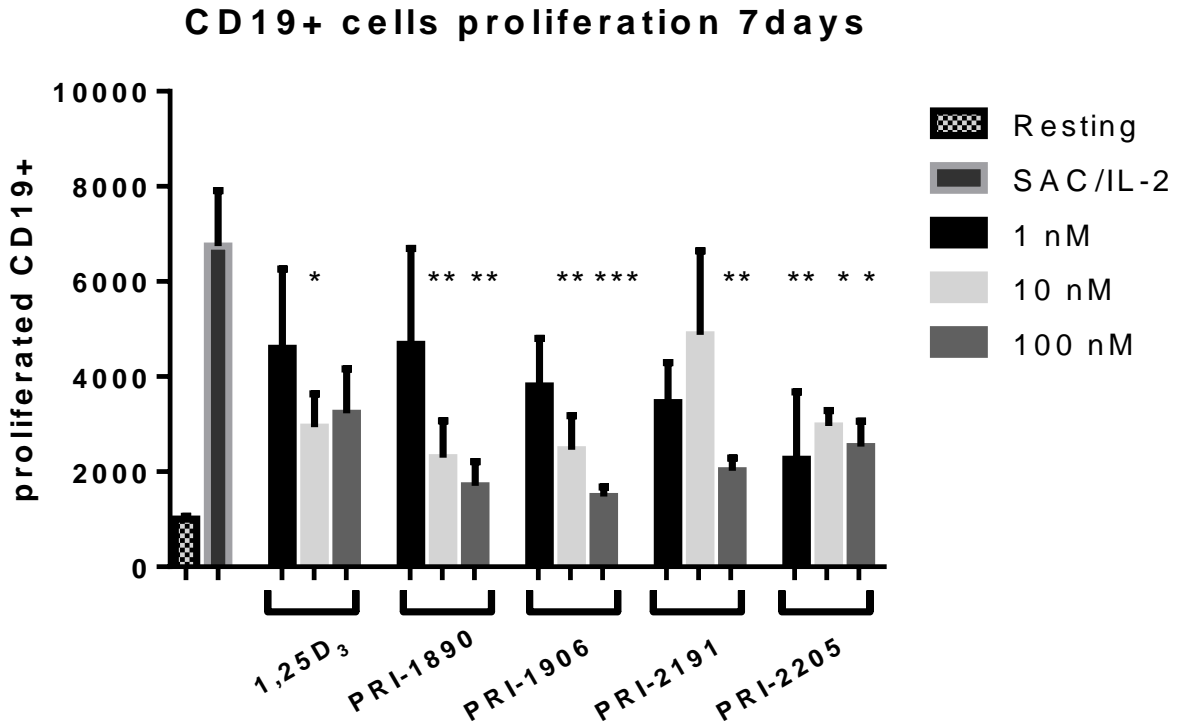


Figure 4. VDAs have anti-proliferative effects on SAC/IL-2 stimulated CD19+ B- cell (7 days). Data represent mean  $\pm$  SD from 3 independent experiments.\*\*\* ( $P < 0.01$ ), \*\* ( $P < 0.05$ ), \* ( $P < 0.1$ ) compared with SAC/IL-2 stimulated cells.



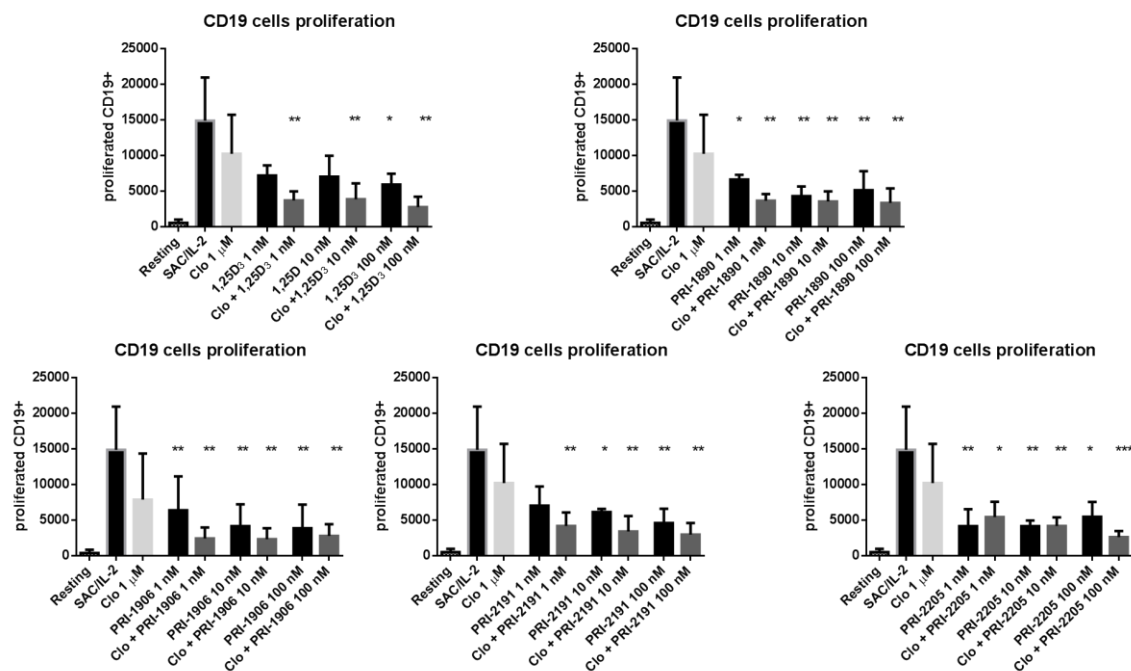


Figure 5. Additive anti-proliferative effect of the combination of 1,25D<sub>3</sub> and VDAs (indicated concentrations) with clomipramine (Clo; 1 μM) on SAC/IL-2 stimulated CD19<sup>+</sup> B-cells. Cells were stimulated for 7 days and labelled with CD19 antibody. Data represent mean ± SD from 3 independent experiments. \*\*\* ( $P < 0.01$ ), \*\* ( $P < 0.05$ ), \* ( $P < 0.1$ ) compared with SAC/IL-2 stimulated cells. No statistically significant differences were found when the combinations of VDAs with Clo were compared to VDAs used alone.