The effect of 1,25-dihydroxyvitamin D₃ on lymphoma cell lines and expression of vitamin D receptor in lymphoma

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Summary 1,25(OH)₂D₃ promotes differentiation and has an antiproliferative effect in a variety of cell lines derived from the immunohaematopoietic system. α-Calciolod which is metabolised to 1,25(OH)₂D₃ has been shown to produce tumour regression in follicular low grade non-Hodgkin's lymphoma (NHL) and the dose limiting toxicity is hypercalcaemia. The cellular action of 1,25(OH)₂D₃ is mediated by binding to an intracelluar protein, the vitamin D receptor (VDR). We have evaluated the activity of 1,25(OH)₂D₃, and its non-calcemic analogue MC903 in the SU-DHL4 and SU-DUL5 B cell lines which carry the 14;18 translocation characteristic of follicular NHL, and also the expression of the VDR in a range of B cell NHLs. Both agents induced differentiation and had an antiproliferative effect on the SU-DHL4 and SU-DUL5 cell lines. However this occurred at a relatively high concentration (10⁻¹⁷M) which exceeds the physiological concentration of 1,25(OH)₂D₃ by approximately 10⁶-fold. Expression of the VDR was low in each cell line and in the low grade lymphoma tumour samples. To account for the observed clinical response to 1αOH-D₃ (α-calcio) in follicular NHL a network is suggested whereby 1,25(OH)₂D₃ modulates the activity of CD4+ T cells which have previously been shown to promote follicle centre cell proliferation. Vitamin D₃ analogues may enable serum levels to be achieved which produce a direct action on follicular lymphoma cells without disturbing calcium metabolism.

1,25(OH)₂D₃ is the active metabolite of vitamin D₃ and its action is mediated by an intracellular receptor (VDR) which binds to DNA (Mangelsdorf et al., 1984; Reichel et al., 1989). The presence of the VDR in cells of the immunohaematopoietic system first indicated 1,25(OH)₂D₃ may have a role in regulating their activity. 1,25(OH)₂D₃ has been shown in vitro to have an antiproliferative effect and promote differentiation in monoblastic and promyelocytic cell lines, to inhibit differentiation in K562 leukaemia cells, to inhibit IL-1 production, to suppress CD4⁺T cell proliferation and IL2 production and inhibit immunoglobulin production by B cells (Jordan et al., 1990; Rebel et al., 1992; Tsoukas et al., 1989; Rigby et al., 1987; Moore et al., 1991; Ito et al., 1986; Tsoukas et al., 1984; Bhalla et al., 1989). In a number of cell lines the degree of response to 1,25(OH)₂D₃ appears to be dependent on the level of ligand binding (Chen et al., 1986) however the precise relationship is unknown. Furthermore 1,25(OH)₂D₃ has been shown to modulate expression of its own receptor (Lee et al., 1989; Strom et al., 1989). In actively proliferating lymphocytes VDR expression appears to be high and correlates with proliferative status except in B-cells in which expression is low (Kizaki et al., 1991).

B cell lymphomas have been shown to be sensitive to vitamin D₃. In a clinical trial, 1 μg of 1α,25(OH)₂D₃ (α-calcio) daily (metabolised to 1,25(OH)₂D₃ in the liver) produced an overall response rate of 30% in advanced follicular B cell non-Hodgkin's lymphoma (NHL) (Raina et al., 1991). The clinical importance of this finding is that it indicates vitamin D₃ may have a role as a maintenance therapy in the setting of minimal residual disease in follicular NHL. Vitamin D₃ analogues which have the antiproliferative effect of 1,25(OH)₂D₃, but lack its effect on calcium metabolism have been evaluated in a number of cell lines (Norman et al., 1990; Zhou et al., 1989; Binderup & Bramm, 1988; Colston et al., 1992). These could be of clinical value as they may enable higher dosages without calcium toxicity.

The basis of the antilymphoma effect is uncertain. A problem in studying the biology of low grade lymphoma is the lack of suitable in vitro and in vivo models. Low grade lymphoma cells do not proliferate in vitro without aduleration for example by immortalisation with the Epstein Barr virus or culture with CD4⁺ T cells (Umemoto et al., 1990) and such interventions clearly will change the nature of the cells studied. An in vitro model which may represent some aspects of follicular lymphoma cell biology are those cell lines which have a t(14;18) chromosomal translocation since this abnormality is found in at least 85% of cases of follicular NHL in association with rearrangement of the bcl2 gene (Yunis et al., 1987; Weiss et al., 1987).

The aim of this study was to investigate the antiproliferative effects and the in vitro effects of 1,25(OH)₂D₃ on t(14;18) lymphoma cell lines and relate this to expression of the VDR in lymphoma tumour biopsy material. 1,25(OH)₂D₃ and an analogue, MC903 (calcipotriol) which has equivalent VDR binding affinity but 100 fold less effect on Ca²⁺ metabolism were assessed.

Materials and methods

Cells

The cell lines studied for their response to 1,25(OH)₂D₃ and MC903 were as follows: SU-DHL4 and SU-DUL5 (both derived from high grade B cell lymphomas): each carries a t(14;18) with an associated rearrangement of the bcl2 gene; in SU-DHL4 rearrangement through the major breakpoint region (Cleary et al., 1986a) and SU-DUL5 rearrangement through the minor cluster region (Cleary et al., 1986b). U937 is a monoblastic cell line for which both 1,25(OH)₂D₃ and MC903 have been shown to inhibit proliferation and promote differentiation along the monocytic/macrophage pathway. SU-DHL4 cells were a gift from Dr A. Epstein (UCLA, Los Angeles, CA), SU-DUL5 cells were a gift from Dr M. Cleary (Stanford, CA). Cells were cultured in medium supplemented with 10% foetal bovine serum in humidified atmosphere with 5% CO₂.

1,25(OH)₂D₃ and MC903

1,25(OH)₂D₃ and MC903 (provided by Dr L. Binderup, Leo Laboratories, Denmark) were dissolved in 100% ethanol to

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a stock concentration of $10^{-3}$ M and stored at $-20^\circ$C and protected from light. Dilutions of the stock solutions were made in ethanol and then medium. The maximum concentration of ethanol in culture (0.1%) did not influence cell growth.

Modulation of cell proliferation and analysis of differentiation

Cells in log phase growth were seeded at $2 \times 10^5$ cell ml$^{-1}$ and each agent (or vehicle = control) were added at the required concentration. Cells were counted daily using a Coulter counter and assayed for viability with Trypan blue. Each experiment lasted 5 days. On days 0 and 4 cells were examined using a panel of monoclonal antibodies to assay for differentiation. The experiment was performed in duplicate and repeated once.

1,25(OH)$_2$D$_3$ receptor binding assay

Cells were harvested by centrifugation and washed twice in ice cold phosphate buffered saline. Cells were then homogenised in KTMED (KCl 22.4 g l$^{-1}$, Tris Cl 1.21 g l$^{-1}$, sodium molybdate 2.06 g l$^{-1}$, EDTA 0.336 g l$^{-1}$, Dithiothreitol 0.62 g l$^{-1}$) $10^6$ cells ml$^{-1}$ were sonicated and then centrifuged 100,000g for 1 h. One hundred and ninety μl of cytosol is then added to 10 μl [3H]-1,25(OH)$_2$D$_3$ (2.6 $\times$ 10$^{-8}$ M) either with or without excess radioinert 1,25(OH)$_2$D$_3$. After 4 h incubation receptor-bound $[3]$-1,25(OH)$_2$D$_3$ was separated from free $[3H]$.1,25(OH)$_2$D$_3$ with hydroxylapatite (Colston et al., 1980). Cytosol protein concentration was determined by the method of Bradford (Bradford, 1976). The experiment was performed in duplicate and repeated once.

Tumour samples and assay for VDR expression

Tumour biopsy specimens were snap frozen and 5 μm cryostat sections were mounted onto poly-l-lysine coated slides and fixed in 4% formaldehyde and methanol and washed in phosphate buffered saline. (Fixed sections were stored at $-20^\circ$C in glycerol/sucrose storage medium before staining.) Sections were analysed for the presence of the VDR using a Vectastain ABC anti-rat alkaline phosphatase kit with the monoclonal antibody 9A7a (provided by Dr J. Wesley Pike). The alkaline phosphatase substrate contained a levamisole block. Sections were processed in duplicate. The MCF-7 cell line which has a high level of VDR expression served as the positive control.

Results

Effect of 1,25(OH)$_2$D$_3$ and MC903 on cell proliferation

These agents inhibited the proliferation of SU-DHL4 and SU-DUL5 with no effect on cell viability (Figure 1) at $10^{-7}$ M. There was no effect on proliferation at lower concentrations. U937 was inhibited at lower concentrations of each as recorded elsewhere (Binderup & Bramm, 1988). At $10^{-5}$ M there was an approximately 50% reduction of proliferation of SU-DHL4 and SU-DUL5 on day 4.

Effect of 1,25(OH)$_2$D$_3$ and MC903 on induction of differentiation (Table 1)

SU-DHL4 acquired markers of mature B-cell differentiation (B1, B4 and CD38) whilst SU-DUL5 lost a marker of immaturity (CD14) when cultured with each agent at $10^{-7}$ M. U937 has been demonstrated to differentiate along the monocyte/macrophage pathway when cultured in the presence of 1,25(OH)$_2$D$_3$ and MC903 (Dodd et al., 1983; Binderup & Bramm, 1988; Bhalla et al., 1989) and this was confirmed in these experiments.

Effect of 1,25(OH)$_2$D$_3$ and MC903 and VDR expression (competitive binding assay) (Table 1)

Expression of VDR was low in SU-DHL4 and SU-DUL5 both before and after treatment with each agent over 5 days at $10^{-7}$ M in comparison to the U937 cell line. One way analysis of variance was applied to the data. Dunnett’s test (Zar, J.H.) demonstrated that incubation with 1,25(OH)$_2$D$_3$ over 96 h produced a reduction in VDR expression in the SU-DHL4 cells ($P<0.001$, 2 tailed test) and an increase in VDR expression in the SU-DUL5 cells ($P<0.05$, 2 tailed). MC903 had no effect on VDR expression in either cell line.
Neither agent significantly altered expression of the VDR at 96 h in the U937 cell line.

**VDR expression; immunocytochemistry of lymph node biopsy specimens**

Results are summarised in Table II (and see Figure 2). Biopsy samples were analysed for VDR expression from 13 patients with various categories of NHL as defined by the Working Formulation. VDR expression was detectable in 11/13 samples. Only in a case of high grade lymphoblastic lymphoma was there strong staining comparable to the MCF-7 cell line. In the two negative cases, the macrophages stained positively providing an internal positive control. In all cases macrophages stained strongly positive as did cells in the paracortex which by morphology and location were considered to be T cells.

![Image](image_url)

**Figure 2** Immunostaining with 9A7a for the VDR. a, The MCF7 cell line – expresses the VDR abundantly. b, A lymph node biopsy from a case of follicular mixed small cleaved and large cell lymphoma (working formulation category C). Note weak positive staining and occasional intensely staining cells which in the section shown are macrophages. c, A lymph node biopsy from a case of lymphoblastic lymphoma. The tumour cells are strongly positive.

**Table II** Intensity of staining for the VDR in relation to histological classification according to the working formulation

| Patient | Histology | Intensity of staining |
|---------|-----------|----------------------|
| M.B.    | WF: B     | Weak +ve             |
| L.S.    | C         | Not detected         |
| M.L.    | C         | Not detected         |
| T.F.    | C         | Weak +ve             |
| G.H.    | C         | Weak +ve             |
| K.K.    | C         | Weak +ve             |
| J.P.    | G         | Weak +ve             |
| A.M.    | Tranformed F to H | Weak +ve |
| R.M.    | G         | Moderate +ve         |
| J.L.    | H         | Weak +ve             |
| M.G.    | Richters  | Weak +ve             |
| J.L.    | J         | Strong +ve           |
| M.S.    | J         | Moderate +ve         |
| MCF-7   | Breast cancer cell line | Strong +ve |
Discussion

1,25(OH)_{2}D_{3} and its analogue, MC903, had an equivalent antiproliferative effect on the SU-DHL4 and SU-DUL5 cell lines and this was associated with the induction of differentiation. The expression of the VDR was low in both cell lines in accord with other studies (Kizaki et al., 1991) but was slightly altered (±20%) by incubation with 1,25(OH)_{2}D_{3} and not MC903 over 96 h. The reason for the apparent discrepancy between the two drugs in impact on VDR expression, particularly as they had a similar effect on proliferation, is unclear. The time-course of VDR modulation in response to each drug may differ and hence the response to MC903 may have been missed. The observation that 1,25(OH)_{2}D_{3} produced a reduction in VDR expression in SU-DHL4 and an increase in SU-DUL5 on day 4 may also reflect different time-course functions of VDR modulation in each cell line.

However these effects occurred at a relatively high concentration (10^{-7} M) of 1,25(OH)_{2}D_{3} which exceeds the physiological concentration by approximately 10^3-10^4 fold. Furthermore expression of VDR in the follicular NHL tumour samples was low. These data suggest that the observed clinical response of advanced follicular NHL to 1,25(OH)_{2}D_{3} may not be due to a direct action of the agent on the lymphoma cells. CD4^{+} T helper cells which recognise allot antigens expressed by follicular lymphoma cells induce the lymphoma cells to proliferate (Umetu et al., 1990) indicating a possible role for CD4^{+} T cells in promoting follicular NHL. 1,25(OH)_{2}D_{3} inhibits CD4^{+} T cell proliferation over a concentration range 10^{-9}-10^{-11} M and this effect on CD4^{+} T cells appears to be both direct and also indirect through suppression of IL1 production by monocytes (Jordan et al., 1990; Tsoukas et al., 1989; Rigby, 1988; Binderup, 1992). Therefore the antifollicular NHL effect of 1,25(OH)_{2}D_{3} may be mediated (at least in part) by an inhibitory effect on CD4^{+} T cells. The development of the bcl2 transgenic mouse (McDonnell et al., 1989) provides a novel model of follicular NHL and may enable an in vivo analysis of the interplay between B and T cells under the influence of 1,25(OH)_{2}D_{3} and its analogues. Certainly the t(14;18) cell lines are limited as a model of follicular NHL since they are derived from high grade B cell lymphomas. Nevertheless, if the antiproliferative and differentiation-promoting effect produced by 1,25(OH)_{2}D_{3} on the SU-DHL4 and SU-DUL5 cell lines extends to lymphoma of follicle centre cell type, then using 1,25(OH)_{2}D_{3} analogues it may be possible to achieve serum levels that act directly on the centrocytes, in addition to the postulated indirect mechanism of T cell inhibition, without perturbing calcium metabolism.

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