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METABOLISM AND BIOLOGICAL ACTIVITY OF $1,25(OH)_2D_2$ AND ITS METABOLITES IN A CHRONIC MYELOGENOUS LEUKEMIA CELL LINE, RWLEU-4

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Abstract: We previously described the metabolism of $1,25(OH)_2D_2$ into various side chain hydroxylated metabolites formed as a result of C-24, C-26 and C-28 hydroxylations in rat kidney[24]. We now demonstrate C-24 hydroxylation of $1,25(OH)_2D_2$ in human leukemic cells and also present evidence to show that C-24 and C-26 hydroxylations either alone or in combination do not significantly alter the effect of $1,25(OH)_2D_2$ on these cells, while C-28 hydroxylation reduces its activity.

Introduction

Vitamin D related compounds are a group of seco-sterol hormones which play an important role in the maintenance of calcium and phosphate homeostasis in vivo. The biological effects of these seco-sterol hormones are felt to be primarily mediated through their binding to and activating nuclear vitamin D receptors (VDR), although non-genomic effects have also been described [1,2]. Hormone-activated receptors modulate gene expression by binding to regulatory regions of target genes. In addition to the classical vitamin D target tissues (bone, intestinal mucosa, and kidney), vitamin D receptors are present in a number of other tissues including hematopoietic cells. Studies suggest that $1,25(OH)_2D_3$, the hormonal form of vitamin D, plays a role in regulating hematopoietic cell growth, differentiation, and function [4,5]. Therefore, vitamin D compounds (especially ones which are less calcemic) might be useful both as immunomodulators and as differentiating/antiproliferative agents in the treatment of hematopoietic malignancies. Factors which impact on the biological effects of vitamin D-related compounds include their stability, affinity for the VDR and serum vitamin D-binding protein (DBP), uptake into cells, and metabolism by those cells [6,7]. Of these, the best characterized have been compound stability and affinity for the VDR[8] and DBP [9,10]. **Relatively less is known about the effect of metabolism of vitamin D related compounds on their biological activity in hematopoietic cells.**

Most of the metabolism studies have been performed with the natural hormone $1,25(OH)_2D_3$. Studies of $1,25(OH)_2D_3$ metabolism in leukemic cells suggest that it occurs by similar pathways as in the kidney [11-16]. Pretreatment with $1,25(OH)_2D_3$ significantly enhances its own metabolism in HL-60 cells and affects the amounts of various natural metabolites produced [12,15]. Studies in the U937 myelomonocytic precursor leukemic cell line suggest that the induction of 24-hydroxylase by $1,25(OH)_2D_3$ and its subsequent metabolism through the 24-oxidation pathway may play a role in autocrine inhibition of mononuclear cell proliferation [16]. **Thus, metabolism, due to its effect on the regulation of the intracellular concentration of the hormone, appears to be important in controlling the biological effects of vitamin D related compounds[11-16].** Very little is known about the metabolism of other naturally occurring or synthetic analogs of vitamin D in hematopoietic cells. Vitamin D_2 is the naturally occurring vitamin in plants and is less toxic in vivo when compared to vitamin D_3. [17-19]. Previous studies have shown that $1,25(OH)_2D_2$ (the hormonal form of vitamin D_2) also

inhibits proliferation and induces the differentiation of a number of tumor cell lines (including leukemic cells) [20-22]. As the metabolism of $1,25(OH)_2D_2$ in kidney occurs through the 24 hydroxylation pathway [24] and as the 24-hydroxylase of the chronic myelogenous leukemia cell line RWLeu-4 is rapidly induced by $1,25(OH)_2D_3$ [23], we asked the following question: Is $1,25(OH)_2D_2$ metabolized in these cells and if it is metabolized, do any of its natural metabolites have biological effects on these cells? **We found not only that $1,25(OH)_2D_2$ is metabolized efficiently in RWLeu-4 cells through the 24-hydroxylation pathway but also that a number of its natural C-24 and C-26 hydroxylated metabolites as well as the closely related synthetic analogs have effects comparable to $1,25(OH)_2D_2$ itself.**

Materials and Methods

Reagents: $1,25(OH)_2D_3$, $1,24,25(OH)_3D_3$, $1,25(OH)_2D_2$, $1,25(S),26(OH)_3D_2$ and $1,25,28(OH)_3D_2$ were synthesized at Hoffmann-La Roche. All three natural metabolites of $1,25(OH)_2D_2$ namely $1,24,25(OH)_3D_2$, $1,24,25,26(OH)_4D_2$ and $1,24,25,28(OH)_4D_2$ were produced utilizing a kidney perfusion system as previously described [24]. [5-Methyl-^{3}H]-thymidine was purchased from ICN. Monoclonal antibodies to CD4, CD11b (Mo-1), and CD45 were purchased from commercial sources.

Metabolism Studies: 4 X 10^8 RWLeu-4 cells were grown in roller bottles in 300 mL of α-MEM medium, supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 37°C and 50 nM $1,25(OH)_2D_2$ (to maximally stimulate metabolic enzymes for 72 hours). $1,25(OH)_2D_2$ (300 nmoles) was added to the 300 mL culture for an additional 24 hours. The reaction was stopped with methanol and lipid extraction was performed using Bligh and dyer method.[31] The lipid extract was analyzed by HPLC using the chromatographic conditions described in the legend from figure 1.

Antiproliferation and Differentiation studies: For antiproliferation studies, triplicate cultures of 1 X 10^5 RWLeu-4 cells/ml were seeded into 96 well microtiter plates in a final volume of 200 μL of α-MEM (supplemented as above) containing diluent alone or serial three fold dilutions of $1,25(OH)_2D_3$, $1,25(OH)_2D_2$, or their related metabolites and analogs. Concentrations tested usually ranged from 0.1 nM to 500 nM. After 72 hrs, 0.5 μCi of [5-methyl-^{3}H]-thymidine were added per well. After an additional four hour incubation, cells were harvested onto glass fiber filters using a Cambridge PHD cell harvester and the amount of ^{3}H-thymidine incorporated into cells was determined. Results are expressed as the percent of ^{3}H-thymidine counts incorporated by control cells. For differentiation studies, morphological characteristics were determined by examining Wright-Geimsa stained cells.[23] The ability of cells to undergo oxidative metabolism, a characteristic of differentiation into more mature monocytic cells, was determined by Nitroblue tetrazolium (NBT) reduction. $2x10^4$ cells were resuspended in 100 ml of α-MEM medium with 10% FCS to which was added 50 ml of PBS containing NBT (2 mg/ml) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (1.2 mg/ml). Cells were incubated in 5% CO_2 at 37°C for 30 min and evaluated by light microscopy for formation of dark blue crystals. The extent of monocytic differentiation was further analyzed by changes in cell surface antigens which occur during maturation of these cells into more mature monocyte-like cells using monoclonal antibodies reactive with CD4, CD11b (Mo1), and CD45 (a tyrosine phosphatase). Indirect immuno-fluorescent detection of cell surface antigens was performed as previously described [21,23]. For these studies, cultures (2 X 10^3 cells/ml) were treated with diluent alone or 500 nM $1,25(OH)_2D_2$ or $1,25(S),26(OH)_3D_2$ for 72 hours. Cells were stored up to 96 hours at 4°C until analysis was performed on a Becton-Dickenson cell sorter.

Results and Discussion

In order to use vitamin D compounds as therapeutic agents, an understanding of the mechanisms by which they mediate their biological effects is necessary. One of the mechanisms which has not been extensively evaluated is the role of the intracellular metabolism of vitamin D-related compounds in the final expression of their biological activity. In our present study, we not only evaluated the metabolic fate of $1,25(OH)_2D_2$, a naturally occurring vitamin D analog, but also the biological activity of its natural as well as synthetic metabolites in terms of their ability to induce differentiation or inhibit proliferation of a CML cell line, RWLeu-4.

Metabolism of $1,25(OH)_2D_2$ into $1,24,25(OH)_3D_2$ by RWLeu-4 cells

From Figure 1 it becomes obvious that $1,25(OH)_2D_2$ (retention time 17.02 min) is metabolized in RWLeu-4 cells into a single major metabolite (retention type 41.60 min) as represented by its typical vitamin D UV spectrum. This metabolite comigrated with $1,24,25(OH)_3D_2$ and to further confirm its structure mass spectrum (70 eV) of the metabolite was obtained on a Hewlett-Packard 5985 mass spectrometer by introducing the purified metabolite (1 μg) into the ion source maintained at 200°C via a direct-insertion probe. The mass spectrum of the matabolite exhibited peaks at M/Z 287, 269, 251, 152, and 134 which indicate that the seco-steroid nucleus of its parent $1,25(OH)_2D_2$ has remained unchanged. The molecular ion at M/Z 444 indicated that an additional hydroxyl group has been added to the side chain of $1,25(OH)_2D_2$ and furthermore, this metabolite's susceptibility to chemical oxidative cleavage by sodium periodate establishes that the additional hydroxyl group is vicinal to the original 25-hydroxyl group. With these findings we identified the major metabolite of $1,25(OH)_2D_2$ produced in RWLeu-4 cell as $1,24,25(OH)_3D_2$.

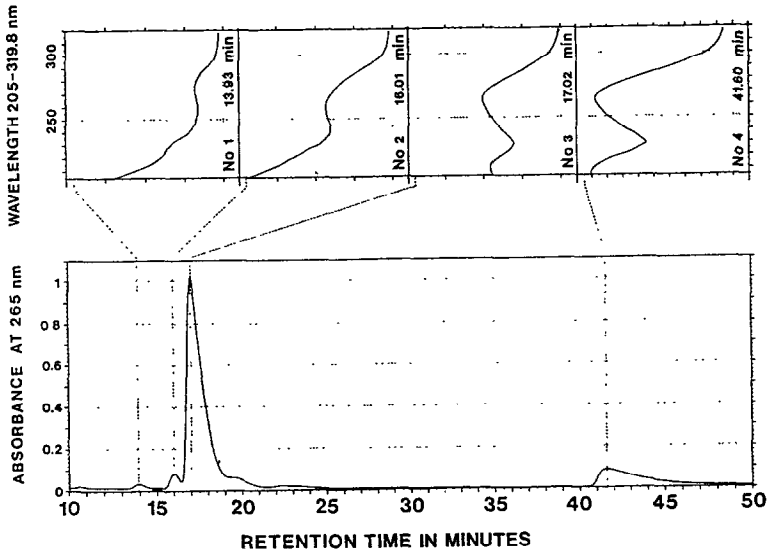


Figure 1: HPLC and UV analysis of the lipid extract of both cells and media obtained by incubating $4x10^8$ RWLeu-4 cells for 24 hours with 300 nmoles of $1,25(OH)_2D_2$ in 300 mL of media (10^{-6}M). Chromatographic conditions:Zorbax-SIL column (25cm x 4.6 mm), hexane:2-propanol (94:6), Flow rate of 2 mL/min.

Effects of $1,25(OH)_2D_2$ and its related metabolites on cellular DNA synthesis and differentiation

As can be seen (Table 1), metabolites of $1,25(OH)_2D_2$ [$1,24,25(OH)_3D_2$, $1,25(S),26(OH)_3D_2$, and $1,24,25,26\ (OH)_4D_2$] which have not undergone 28 hydroxylation are essentially equally active to $1,25(OH)_2D_2$ itself in inducing differentiation and inhibiting proliferation of RWLeu-4, whereas there was a significant decrease in activity after 28 hydroxylation (although $1,25,28(OH)_3D_2$ was still active at very high concentrations).

Table 1. $1,25(OH)_2D_2$ and its natural metabolites and synthetic analogs were tested for their antiproliferative and differentiative effects on the RWleu4 CML cell line. ($1,25(OH)_2D_3$ and $1,24(R),25(OH)_3D_3$ served as controls).

Analog	IC_{50}[a]
$1,25(OH)_2D_3$	6
$1,24(R),25(OH)_3D_3$	11
$1,25(OH)_2D_2$	13
$1,24,25(OH)_3D_2$	11
$1,25(S),26(OH)_3D_2$	8
$1,24,25,26(OH)_4D_2$	11
$1,25,28(OH)_3D_2$	96
$1,24,25,28(OH)_4D_2$	> 100

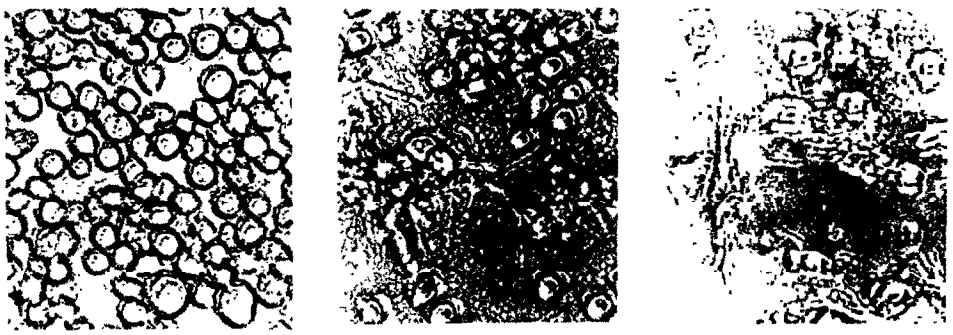
[a]The IC_{50} is the nanomolar (nM) dose of the compound which decreases [^{3}H]-thymidine incroporation by 50 percent as compared to untreated cells. Each IC_{50} represents the mean of at least three separate experiments except for compounds $1,24,25,28(OH)_4D_2$ and $1,24,25,26(OH)_4D_2$ which were tested in two experiments each.

Table 2 shows that the $1,25(S),26\ (OH)_3D_2$ compound is as effective as $1,25(OH)_2D_2$ at enhancing Mo-1 and CD45 tyrosine phosphatase and decreasing CD4 expression which are all characteristics of the differentiation of these cells into a more mature monocytic/macrophage-like state [23,25,26]. Figure 2 shows the morphological differentiation induced in these cells treated with $1,25(OH)_2D_2$ or $1,25(S),26(OH)_3D_2$. Similar results were obtained with the $1,24,25(OH)_3D_2$ and $1,24,25,26\ (OH)_4D_2$. Three metabolites ($1,24,25(OH)_3D_2$, $1,25(S),26(OH)_3D_2$, and $1,24,25,26\ (OH)_4D_2$) had ED_{50}'s

Table 2. Changes in cell surface markers consistent with differentiation along monocyte-macrophage pathway on RWLeu-4 cells and % of cells positive for NBT reduction after 72 hours of treatment with $1,25(OH)_2D_2$ or $1,25(S),26(OH)_3D_2$. Control cells were treated with diluent alone. Values represent the percentage of total cells positive for the indicated cell surface marker (CD11b,CD4) or in the case of CD45, the mean fluorescent channel normalized to cells cultured in diluent alone.

		VITAMIN D_2 COMPOUND (500 nM)	
	CONTROL	$1,25(OH)_2D_2$	$1,25(S),26(OH)_3D_2$
CD11b	10	88	89
CD4	44	15	21
CD45	100	184	177
NBT+ (%)	3	86	73

comparable to $1,25(OH)_2D_2$ in terms of inhibiting proliferation and inducing differentiation. Clearly, both 24 and 26 hydroxylated metabolites of $1,25(OH)_2D_2$ are able to induce differentiation and inhibit proliferation when added directly to these cells in the absence of $1,25(OH)_2D_2$ itself, so they could potentially have a role in mediating the biological effects of vitamin D_2. In comparing these results to those previously reported for $1,25(OH)_2D_3$ related compounds hydroxylated on the same positions, the slightly greater potency of $1,25(S),26(OH)_3D_2$ as compared to $1,25(OH)_2D_2$ is consistent with previous reports in other leukemic cell lines and the enhanced activity of 26 hydroxylated $1,25(OH)_2D_3$ [27,28]. $1,24R,25(OH)_3D_3$ was found to be slightly less potent than $1,25(OH)_2D_3$ in inducing monocytic differentiation of HL-60 cells [28-30] and this was probably due to its decreased affinity for the VDR [28,29] As seen in Table 1, we also found $1,24(R),25(OH)_3D_3$ to be less potent than $1,25(OH)_2D_3$, whereas $1,24,25(OH)_3D_2$ is essentially equipotent to $1,25(OH)_2D_2$. This suggests that additional hydroxylation at the 24 position on $1,25(OH)_2D_2$ does not have the same impact on activity as it does for $1,25(OH)_2D_3$, possibly because it does not have as much of an effect in altering receptor binding although further studies are needed to explain this finding. **Thus our results indicate that 24 and 26 hydroxylations, either alone or in combination do not appear to significantly decrease the antiproliferative or differentiating activity of $1,25(OH)_2D_2$ during its intracellular metabolism in myeloid leukemic cells.** In contrast to this, 28 hydroxylation markedly decreases the activity of $1,25(OH)_2D_2$. $1,25,28(OH)_3D_2$ was only active at significantly higher concentrations ($\geq 10^{-7}$ Molar). This is similar to the findings previously reported for this compound using a number of other myeloid leukemic cell lines as targets [28]. The mechanism for the decreased activity of 28 hydroxylated vitamin D_2 compounds is uncertain, although the addition of the 28 hydroxyl group may modify the structure of $1,25(OH)_2D_2$ in a manner which decreases its interaction with the nuclear receptor.



A. B. C.

Fig. 2. Morphological differentiation of RWLeu-4 CML cells into more mature monocyte-macrophage like cells. A. Untreated cells; B. Cells treated with 50 nm $1,25(OH)_2D_2$; C. Cells treated with 50 nm $1,25(S),26(OH)_3D_2$.

From our current study, it is not possible to be certain whether the natural intermediary metabolites of $1,25(OH)_2D_2$ formed during its intracellular metabolism might play a role in mediating some of the unique biological effects of $1,25(OH)_2D_2$. However, **as some intermediary metabolites have significant biologic activity, it is tempting to hypothesize that they may be responsible for some of the cellular responses induced by $1,25(OH)_2D_2$.** To address this issue, studies are in progress in our laboratory evaluating the potential role of the intermediary metabolites of

$1,25(OH)_2D_2$ in the regulation of cell growth and differentiation. In addition, **the increased activity of the 26-hydroxylated compounds and the decreased activity of the 28-hydroxylated compounds have implications for the design of future synthetic vitamin D analogs.**

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